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The Intervention of Nanotechnology Against Epithelial Fungal Diseases

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REVIEW

Fungal infections can attack epithelial tissues and, according to the immunological state of the patient, some of them invade deeper organs, becoming seriously life compromising. Besides, bloodstream and local infections associated with intravascular devices constitute a significant problem associated with increased mortality. Topical therapy is desirable since, in addition to targeting the site of infection, it reduces the risk of systemic side effects and increases patient compliance. In this review we describe the pros and cons of using nano-objects that being toxic in nature could be used to cover surfaces of medical devices, or can act as carriers for targeted delivery of antifungals to skin. Non-toxic nano-objects were also included because they improve the ocular delivery of antifungals, classically suffering from ineffective topical administration, difficult access for systemic medication or local invasive administration. The new preclinical developments of nanoparticulate agents against cutaneous and ocular mycosis are grouped in three main sections: (1) *In vitro* antifungal activity of metallic nanoparticles, (2) *In vitro* and *in vivo* antifungal activity of non metallic nanoparticles (3) Ocular delivery of non metallic nanoparticles.

Keywords: Nano-Objects, Metallic Nanoparticles, Epithelia, Fungi.

CONTENTS

1. INTRODUCTION

Fungal infections can attack epithelial tissues and, according to the immunological state of the patient, some of them invade deeper organs, becoming seriously life compromising. In particular, the invasive fungal infections (IFIs)[1] are difficult to diagnose, prevent and treat. During the last two decades their incidence, prevalence and mortality increased dramatically worldwide.[2,3] IFI mainly affects patients from developed countries with large population of hospitalized patients suffering serious underlying diseases,[4,5] inmunodepressed due to chemotherapy, bone marrow, stem cells or other organ transplantation,[6–9] or submitted to intensive treatment including broad-spectrum antibiotic therapy.[8,10] Additionally, host immunity can be impaired during infancy, in old age, by pregnancy, by disease, e.g., diabetes mellitus, or through the administration of antibiotics and glucocorticoids.[11] The most common fungal pathogens causing IFI are the species of *Candida* and *Aspergillus*.[12,13] *Candida spp*. represent one of the most common pathogens often causing hospital-acquired sepsis with an associated mortality rate of up to 40%.[14] Due in part to effective control of *C. albicans* with azole prophylaxis, particularly with fluconazole (FLZ), the

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aetiology of IFI has shown a shift from *C. albicans* to *Aspergillus* and other moulds.[15, 16] Latin American countries suffer from a significant burden of IFI, which need to be addressed in terms of public health policies.[17]

Skin mycosis on the other hand, are divided according to the level of tissue involvement into: superficial (which include diseases that generally do not provoke a significant histopathological inflammatory response in the host); cutaneous (which induce pathological changes in the host, although the fungus is confined to the *stratum corneum*, include: dermatophytosis, candidasis and other non-dermatophyte infections) and deep or subcutaneous, which involve the dermis and subcutaneous tissues e.g., *sporotrichosis, coccidoiodomycosis, actinomycosis.*

Superficial mycosis does not usually threaten life but can be disfiguring and their unsightly appearance cause social stigmatization. The incidence of superficial mycoses is increasing and according to a recent report more than 25% of the world's population is affected;[18, 19] disease progression is more rapid and its severity increased in patients with compromised immune function.[20] In low- and middle-income countries, skin diseases are dominated by bacterial and fungal infections that may be modified by HIV induced immunosuppression.[21, 22]

Cutaneous candidiasis most often reoccurs and is rarely cured; hence patients receive therapy over a long time. Cutaneous candidiasis is an opportunistic infection that arises, in most cases, from endogenous, saprophytic candidal blastospores that selectively colonize oral, gastrointestinal, vaginal, and cutaneous epithelium. Additionally, in people with weakened immune systems, *Candida spp.* invades deeper tissues as well as the blood, causing life-threatening systemic candidiasis. Topical therapy is desirable since, in addition to targeting the site of infection, it reduces the risk of systemic side effects.

Besides, bloodstream and local infections associated with intravascular devices are a significant problem associated with increased mortality, length of hospitalization and healthcare costs. *Candida spp.*, together with coagulase-negative staphylococci, *Staphylococcus aureus*, *Enterococcus spp*. and Gram-negative bacilli are the main pathogens associated with catheter-related infections. These organisms usually enter the bloodstream from the skin insertion site or the catheter hub, whereas haematogenous seeding and contamination of the infused fluid are rare. Accordingly, the primary strategies used to prevent catheter-related infections focus on reduction of colonization at the insertion site and hubs, thus preventing microbial spread to the catheter tip lying in the bloodstream. Given the importance of cutaneous microorganisms in the pathogenesis of intravascular device-related infections, measures to reduce colonization at the insertion site are of the highest priority. Reducing skin and catheter colonization has long been associated with a reduced incidence of local and systemic infections. However, currently used topical antiseptics suffer from a short duration of killing activity. Topical antibiotics may offer a longer period of protection but they are associated with an increased frequency of fungal infections or the emergence of bacterial resistance.[23, 24]

The first line treatments against epithelial and subcutaneous fungal infections are the polyene amphotericin B (AmB) and azole antifungals such as clotrimazole (CLZ), econazole nitrate (ECZ) and FLZ.[25] However, AmB is water-insoluble macrolide, consisting of seven double bonds along the hydrophobic moieties of the ring, multiple hydroxyl groups along the hydrophilic moieties and a mycosamine residue. Azole antifungals on the other hand, are highly lipophilic (although there are exceptions (e.g., FLZ) and they can readily partition into the lipid-rich intracellular space in the stratum corneum. In these cases, the challenge is to develop a simple stable formulation that facilitates drug delivery to the epidermis and dermis.[26] Undesirable systemic absorption is reported for AmB and FLZ. The existent topical formulations (e.g., Fungizone cream, lotion, gels, ointments) generally produce local reactions (including irritation, burning sensation, erythema, stinging, pruritic rash, and tenderness) in patients treated topically and therefore failed to achieve mycological eradication.[27] Other problems associated with creams include failed stability test, either chemical instability or physical separation of emulsion caused by the salting out effect of the imidazole salt when used at a concentration of about 1% or more. The toxic effects of conventional medication combined with the growing yeast resistance to antifungal therapy[28, 29] generate a pressing need for the search of new antimicrobial agents from natural and inorganic sources.[30–34]

1.1. The Intervention of Nanotechnology Against Epithelial Fungal Diseases

Prophylaxis and antifungal therapy require of new strategies capable of eliminating extracellular eukariotic microorganism, that both colonize living beings and inert surfaces as biofilms. From the point of view of the classical medicinal chemistry, the filogenetic similitude between fungi and host demands the careful search for leading compounds acting on selected therapeutic targets. Apart from this, the search for antifungals could be enriched by new alternative approaches. For instance, the growing resistance against conventional chemotherapy has led to the use of agents that because of their non specific damage make difficult the assembling of survival mechanisms. Another way of improving therapeutic strategies is to control the delivery of classical drugs to accurately selected tissues or places within these tissues. It is here where Nanotechnology, being a source of structures with new quantum, mechanical, thermal and superficial properties, alone or in combination with classical drugs, can offer new therapeutic/prophylactic options. Nanotechnology encompasses a broad conjunct of techniques aimed to engineer, characterize and make use of structures of 1 (nanoplates),

2 (nanotubes) or 3 dimensions (nanoparticles) in the nanoscale, known as nano-objects. The upper limit of the nanoscale was fixed at 100 nm,[35] but in the nanopharmaceutical field the nano-scale is accepted to rise up to 200–300 nm. Biosynthesized molecules (such as hormones, proteins, nucleic acids) and drugs, whose activity depends on a primary structure and not on new phenomena derived from its size in the nano-scale, do not fit into the definition of nano-object.[36] Also the lower limit of the nanoscale was fixed in 1 nm in order to exclude atoms.[36] Beyond these constraints, there is no restriction in the chemical nature of nano-objects.

In this review we have focused on describing the nature and action mode of nano-objects that can be toxic in nature or that act as carriers for targeted delivery of classical antifungals. The covered targets will be the skin and the ocular epithelia, a place of difficult access for systemic medication or that requires local invasive administration and in general is ineffective. In this context, developing new antifungal agents, especially against the opportunistic *Candida,* a cutaneous mycosis that can become invasive and that can both infect skin epithelium and eye tissue, is of particular interest. The new preclinical developments of nano-objects against cutaneous and ocular mycosis are grouped in three main sections:

(1) *In vitro* antifungal activity of metallic nanoparticles,
(2) *In vitro* and *in vivo* antifungal activity of non metallic nanoparticles
(3) Ocular delivery of non metallic nanoparticles.

2. *IN VITRO* ANTIFUNGAL ACTIVITY OF METALLIC NANOPARTICLES

2.1. Silver Nanoparticles

The use of silver compounds as antimicrobials is well known from ancient times. Currently, silver compounds are used against bacterial infections in wounds.[37,38] and in preventing bacterial colonization of prostheses and catheters.[39,40] In this context, developing and characterizing different types of silver nanoparticles has became a task of paramount importance because of the singular responses induced in prokaryote and eukaryote cells as compared to silver cations (Ag^+).

Silver nanoparticles (AgNp) has emerged as a promising new type of antimicrobial agent. The action mechanism of AgNp is non specific and presumably broadly similar to that of Ag^+.[41] The weak acid Ag^+ has a great tendency to react with sulfur- or phosphorus-containing weak bases, such as R-S-R, R-SH, RS- or PR3. AgNp in biological media may act as a constant source of Ag^+ on particles' slow oxidation. Ag^+ released from the AgNp even at very low concentrations can bind to and thereby damage cells at multiple sites (Fig. 1). It has been observed that 10–20 nm AgNp are taken up by living cells.[42,43] Sulfur-containing proteins in the membrane or inside the cells

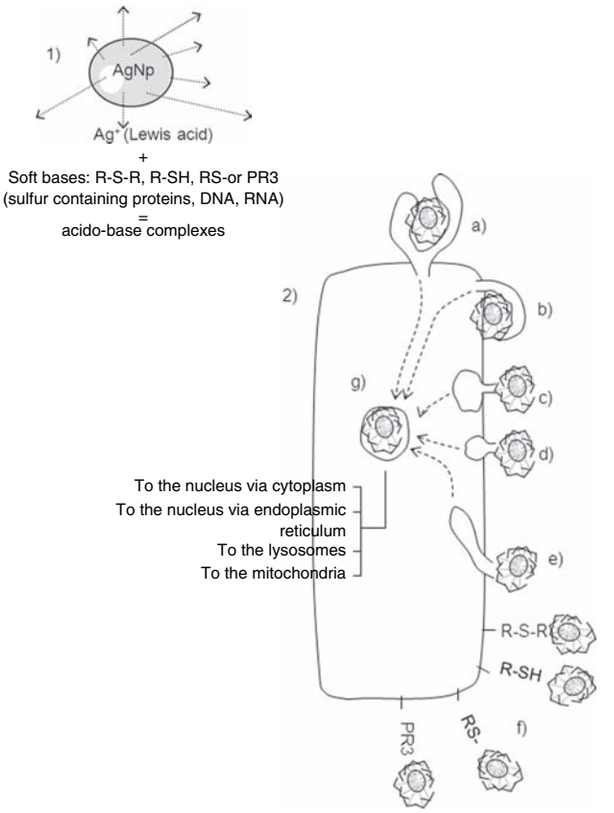

Fig. 1. (1) NpAg as a source of soluble Ag^+; (2) Interaction of AgNp with cells: (a)–(e): Internalization: according to the size, shape and surface characteristics of AgNp and on the cell type, different endocytic mechanisms will be involved in the uptake of AgNp: (a) phagocytosis, (b) macropynocytosis, (c) clathrin mediated endocytosis, (d) caveolin mediated endocytosis, (e) non clathrin, non caveolin mediated mechanisms; (g) endocytosed AgNp are not free but trapped within different types of cytoplasmic vesicles from where will be finally released to different targets; (f) in the absence of internalization AgNp can interact with weak basis at the cell surface.

and phosphorus—containing elements like DNA are likely to be the preferential sites for Ag^+ binding. The inhibition of respiratory enzyme(s), facilitate the generation of reactive oxygen species (ROS) and consequently damage the cell.

The bactericidal effect against both gram-positive and gram-negative bacteria including multiresistant strains of AgNp as well as silver nanocomposites or AgNp based materials has been intensively studied.[42,44–48] Whereas most antibiotics only attack one specific structure of the microbial cell, Ag^+ interferes with the bacterial replication process and kills bacteria by binding to proteins of the cell wall, to thiol groups present in enzymes as well as to DNA and RNA.[49–52] AgNp were reported to kill bacteria at concentrations in the order of units of μg/ml.[45,53] which do not produce acute toxic effects on human cells.[54,55] In addition, AgNp have not been shown to cause bacterial resistance, currently complicating antibiotic therapy of bacterial infections.

The study of antifungal action of AgNp started by 2008, to find out that AgNp also affect yeast cells by attaching to the sulfur containing proteins of cell membranes, thus disrupting membrane potential. After exposure to AgNp, important changes in the membranes of *C. albicans* are observed, such as the formation of "pits" on the membrane surfaces that lead to pores and subsequent cell death.[56] Recently it was found that AgNp induce increased ROS and hydroxyl radical production in *C. albicans*, together with mitochondrial dysfunction and apoptotic features.[57] The activity of 3 nm diameter AgNp was comparable to that of AmB (IC80 1–5 μg/ml) and superior to that of FLZ (IC80 10–30 μg/ml) on ATCC strains of *Trichophyton mentagrophytes* and *Candida spp*. (Table I).[56, 58] AgNp was also found to exert activity on the mycelia. The antifungal activity of AgNp was also proved in biostabilization of footwear materials.[59] In this application, AgNp at 1% solution inhibited the growth of the majority of yeast-like fungal and mold strains. AgNp at 100 ppm totally inhibited bacterial growth, but its activity against molds and dermatophytes was observed to be lower, being reported that molds and bacteria were resistant to 50 ppm of AgNp.

Considering spherical particles of uniform size, a reduction in size from $\sim$10 μm to 10 nm will increase the contact surface area by 10^9. This explains why the activity of AgNp is inversely related to its size: a large contact surface is expected to enhance the extent of microbicidal activity. On the other hand, recent reports emphasized that shape dependent interactions of AgNp played a crucial role in their microbicidal properties.[47] Spherical AgNp (generally with a cubo-octahedral or multiple-twinned decahedral or quasi-spherical morphology) predominantly have (100) facets along with small percentage of (111) facets, while in case of the rod-like AgNp (e.g., pentagonal rods), side surfaces are bound by (100) and the ends by (111) facets. It has been demonstrated that the reactivity of silver is favored by high-atom-density facets such as truncated triangular nanoplate in comparison to other particles that contain fewer than (111) facets, like spherical or rod-shaped particles.[42]

Interestingly, the bactericidal activity of AgNp is higher than its fungistatic (minimum inhibitory concentrations, MIC) and fungicidal (minimum fungicidal concentration, MFC) activities. The MIC and MFC of 25 nm mean size AgNp either plain or stabilized by sodium dodecyl sulfate (SDS), sodium polyoxyethylene sorbitan monooleate (Tween 80), Brij (35; 58, 97 and 98) surfactants, or the polymers polyvinylpyrrolidone (PVP; average molecular weights (MW) of 10,000; 40,000 and 360,000) and polyethylene glycol (PEG; MW of 1500; 4,000; 10,000 and 35,000) against *C. albicans* was recently determined.[60] At first sight the results were far from being promising. The MIC of plain AgNp against *C albicans II* showed no significant differences with the MIC of Ag^+ (Table I). It was observed that surfactant and polymer stabilization of AgNp (used at concentrations between 0.5 and 5×10^4% w/w) decreased the MIC. And even though the fungicidal activity (measured by development of MIC in time as well as by MFC) of Ag^+ against *C albicans* was higher (MFC of 135 μg/ml) than that of plain AgNp (MFC of 27 μg/ml), the MFC of SDS stabilized AgNp was 338 μg/ml. Remarkably, the main differences between Ag^+ and AgNp were found in their cytotoxicity against fibroblast BJ cells, that resulted in an absolute lethal concentration (LC100) value of 1 μg/ml for Ag^+ but ascended to 30 μg/ml for plain or PEG/PVP stabilized AgNp. The high cytotoxicity of surfactants solutions alone impaired further cytotoxicity test of surfactant stabilized AgNp. Both fungi and host cells are eukaryotes and this would explain the close values of MFC and LC100 of AgNp. The low concentrations of AgNp (below than 10 μg/ml) needed to exert bactericidal activity on the other hand, would obey to the fact that yeast cell type can resist higher concentrations of silver thanks to their improved cell organization and structure, and more efficient detoxification system than evolutionarily older prokaryotic types of bacteria. Overall, these studies showed that AgNp, in spite of their close MFC and LC100 values (27 vs. 30 μg/ml), are still safer antifungal than Ag^+. This is underscored by the far higher MFC than LC100 value (13.5 vs. 1 μg/ml) of Ag^+

Recently, 32.5 nm average size spherical AgNp obtained by extracellular biosynthesis by the fungus *Alternaria alternate,* were tested in combination with FLZ against *C. albicans*, among other skin fungi (Table I).[61] Topical treatment of severe life threatening skin fungal infections with FLZ is an efficient therapy and occupies a prominent position among the alternatives of treatment.[62] However, topical delivery of FLZ results in systemic absorption, skin irritation and therefore failing to achieve mycological eradication.[63] These problems affect patient compliance and compromise the efficacy of the therapy. Nonetheless AgNp significantly increases the antifungal activity of FLZ (measured as diameter of inhibition zone and increased fold area). The combination between FLZ and AgNp showed the maximum inhibition against *C. albicans*, followed by *Phoma glomerata* and *Trichoderma spp.,* whereas no significant enhancement of activity was found against *Pleospora herbarum* and *F. semitectum*.

In another approach, 7–20 nm AgNp synthesized by a proprietary biostabilization process, were found to exhibit good antifungal activity (Table I).[64] Interestingly, AgNp exhibited good anti-inflammatory properties as indicated by concentration-dependent inhibition of marker enzymes (matrix metalloproteinase 2 and 9). The post agent effect (a parameter measuring the length of time for which bacterial growth remains suppressed following brief exposure to the antimicrobial agent) varied with the type of organism (e.g., 10.5 h for *P. aeruginosa*, 1.3 h for *Staphylococcus spp*. and 1.6 h for *C. albicans*) indicating that dose regimen of the AgNp formulation should ensure sustained release of the antifungal. To meet this requirement, a gel formulation containing AgNp was prepared. As part of toxicity

Table I. Metallic nanoparticles used as *in vitro* antifungal agents.

Nanoparticle type	Fungus	Nanoparticles features and antifungal activity	Reference
AgNp	*C. albicans*	Formation of "pits" on the membrane surfaces	[56]
	C. albicans	Increased ROS and hydroxyl radical production	[57]
	Clinical isolates and ATCC strains of *Trichophyton mentagrophytes* and *Candida spp.*	3 nm AgNp; IC80 1–4 μg/ml	[56, 58]
	C. albicans II	Ag^+; MIC 0.21–1.69 μg/ml; MFC 13.5 μg/ml; LD 100 on fibroblast BJ cells 1 μg/ml Plain 25 nm AgNp; MIC 0.21–1.69 μg/ml; MFC 27 μg/ml; LD 100 30 μg/ml 25 nm SDS-AgNp; MIC 0.052 μg/ml; MFC 3.38 μg/ml	[60]
	C. albicans	20–60 nm AgNp significantly increases the antifungal activity of FLZ	[61]
	C. albicans	7–20 nm AgNp MIC 25 μg/ml 50% inhibition at 75 μg/ml with antifungal index 55.5% against *A. niger* IC50 on Hep G2 cell line 251 μg/ml	[64]
	C. albicans	500 nm $\times$ 50 $\times$ 50 nm square bases *salep* capped Ag nano-wedges MIC 5 μg/ml, MFC300 μg/ml	[65]
	C. albicans and C. glabrata adhered cells and biofilms	5 nm AgNp stabilized with ammonia MIC 0.4 and 3.3 μg/ml	[66]
	C. albicans, C. glabrata and *M. sympodialis*	1560 nm AgNp loaded within an inorganic matrix showed complete or nearly complete growth inhibition	[74]
	Aspergillus glaucus	14 nm Ag@Fe_3O_4; MIC 2 mg/ml	[76]
	Candida spp.	Ag@Fe_3O_4 with $\sim$70 nm Fe_3O_4 magnetic cores covered by a shell of $\sim$5 nm AgNp γ-Fe_2O_3@Ag with 20–40 nm AgNp cores covered by a shell of $\sim$5 nm γ-Fe_2O_3 Both nanocomposites MIC 1.9–31.3 μg/ml 26 nm AgNp; MIC 0.2 μg/ml	[77]
ZnONp	*Fusarium spp.*	2–28 nm ZnONp less active than $CuSO_4 \cdot 5H_2O$	[98]
TiO_2Np	*C. albicans* biofilms	TiO_2Np were deposited on 50–100 nm diameter ZnO nanowires. Viability of cells significantly decreased nearly 4.3 times after 5 h exposure visible light	[101]
	C. albicans biofilms	Branched carbon nanotube arrays covered with TiO_2Np Highly photocatalytic antifungal activity	[102]
	Aspergillus niger, *C. albicans,* *C. neoformans*	250–300 nm TiO_2AgNp combined nanoparticles; MIC 3–25 μg/ml; LD50 on THP-1 monocytes 55.9 μg/ml AgNp; MIC 20–25 nm; LD50 10 μg/ml	

studies, localization of AgNp in Hep G2 cell line, cell viability, biochemical effects and apoptotic/necrotic potential were assessed. It was found that AgNp localized in the mitochondria and had an IC50 value of 250 μg/ml. Even though they elicit an oxidative stress, cellular antioxidant systems (reduced glutathione content, superoxide dismutase, catalase) get triggered and prevent oxidative damage. Further, AgNp induce apoptosis at concentrations up to 250 μg/ml, which could favor scarless wound healing. Acute dermal toxicity studies on gel containing AgNp in Sprague-Dawley rats, showed complete safety for topical application. These results suggest that AgNp could provide a safer alternative to conventional antimicrobial agents in the form of a topical antimicrobial formulation.

On the other hand, highly sized *salep* capped Ag nano-wedges, were prepared by photochemical facile green synthesis. *Salep* (a palmate-tuber, multi-component polysaccharide with a high content in glucomannan possessing natural, neutral and watersoluble fibers) caused creation of flower-like self-assembled structures of the Ag nano-wedges. The MIC value of *salep*-Ag nano-wedges against *C. albicans* was similar to that of AmB (2.5–5 μg/ml). The MFC however, resulted to be excessively high (Table I).[65] These results, indicating poor fungicidal efficiency, could be owed to the large size of the poniards. Nonetheless, in order to assess the poniards safety, their cytotoxicity against host cells remains to be tested.

Recently the effect of 5 nm diameter AgNp stabilized with ammonia against *C. albicans* and *C. glabrata* adhered cells and biofilms, was tested. AgNp were applied to adhered cells (2 h) or biofilms (48 h) (Table I). It was

also determined that AgNp were more effective in reducing biofilm biomass when applied to adhered cells (2 h) than to pre-formed biofilms (48 h), with the exception of *C. glabrata* ATCC, which in both cases showed a reduction of ~90%. AgNp were highly effective on adhered *C. glabrata* and respective biofilms. On *C. albicans* the effect was not so evident but there was also a reduction in the number of viable biofilm cells. These results suggest that AgNp could be an effective alternative to conventional antifungal agents for future therapies in Candida-associated denture stomatitis.[66]

A well known problem of silver is that it is toxic to human at high concentration.[47] The topical application of Ag compounds that can be significantly percutaneously absorbed can cause argyrosis and argyria, leading to a local or systemic tissue deposition of Ag in skin, nerve tissues and inner organs—particularly liver, spleen and kidney—with the attendant risk of organ dysfunction.[67–71] Therefore, maximum contamination levels for silver in drinking water (100 ppb).[72] and the occupational exposure limit to the various forms of silver (0.01 mg/m^3)[73] have been established in order to avoid the accumulation of silver in the human body. In this context, recently highly sized (1560 nm mean diameter) AgNp were loaded within an inorganic matrix, to impair its systemic absorption after topical application in mice. The silver contents of AgNp and silver sulphadiazine used as control at concentrations of 1% were calculated to be 880 ppm (880 μg/g) and 3020 ppm (3020 μg/g), respectively. Strikingly, AgNp at a 0.1% concentration exhibited comparable antibacterial and antifungal potencies as silver sulphadiazine at 0.1%, although its absorption was considerable lower. AgNp at 0.1% proved to be a potent antifungal agent, exhibiting complete or nearly complete growth inhibition of the dermatophytes *M. canis* and *T. rubrum.* Moreover, AgNp at ultra low doses of 0.001% was effective against *C. albicans C. glabrata* and *M. sympodialis*, yeast species often found in atopic dermatitis patients. Despite its 3.4-times lower silver content, the AgNp preparation exhibits an antimicrobial activity against the bacteria yeasts and dermatophytes tested, comparable to that of silver sulfadiazine at concentrations of 0.1%.[74]

2.2. Strategies Used to Impair Uncontrolled Dispersion of AgNp in the Environment: Magnetic AgNp

Extensive use and increasing demand for AgNp will lead to their accumulation in the environment, especially in landfills and water effluents. Non-targeted effects of AgNp on the population of microbes that play beneficial roles in the environment could have negative consequences.[75] Magnetic nanoparticles of iron oxides (Fe_3O_4 and/or γ-Fe_2O_3-maghemite-) represent one family of the most suitable candidates for the preparation of magnetic nanocomposites owing to their convenient magnetic application (e.g., superparamagnetism), biochemical properties (e.g., non-toxicity, biocompatibility, biodegradability) and low price.

An example of nanoparticles designed to prevent their uncontrolled release into the environment are the spherical 14 nm average size core–shell nanoparticles made of Fe_3O_4 (magnetite) core coated with AgNp ($Ag@Fe_3O_4$) at Ag and Fe concentration of 702 and 215.6 mg/l, respectively. The diamagnetic Ag shell prevents the agglomeration of the Fe_3O_4 during the formation of core–shell nanoparticles. The resulting core–shell nanoparticles were superparamagnetic in nature, although a 71% decrease in the magnetization of $Ag@Fe_3O_4$ with respect to Fe_3O_4 was observed. The $Ag@Fe_3O_4$ can be recovered using a steel wool filter and recycled from the site of action by means of an external magnetic field, being detected by their absorption at 399 nm (the Surface Plasmon Resonance band of $Ag@Fe_3O_4$). However, although the recycling efficiency is > 80% over four cycles, the MIC values of $Ag@Fe_3O_4$ and AgNp against *Aspergillus glaucus* isolates were high (Table I). *A. glaucus* is the potential cause of fatal brain infections and hypersensitivity pneumonitis in immunocompromised patients and leads to death despite aggressive multidrug antifungal therapy. The elevated MIC values of this approach make mandatory the further investigation of the biocompatibility for the host, once the route of administration is decided.[76]

In a similar fashion, the antifungal activity of two types of nanocomposites including molecules of polyacrylate serving as a spacer among iron oxide and AgNp was recently tested.[77] In one hand, $Ag@Fe_3O_4$ nanoparticles made of Fe_3O_4 magnetic cores (~70 nm) covered by a shell of AgNp (~5 nm, 5.8% weight content) were prepared. On the other hand, γ-Fe_2O_3@Ag nanoparticles with a higher content (10.5%) of larger (20–40 nm) AgNp cores, covered by a shell of (~5 nm) γ-Fe_2O_3 were also prepared. Both nanocomposites possess eminent magnetic properties (e.g., high value of magnetization achievable at relatively low applied fields, superparamagnetic and soft magnetic behavior at room temperature from the viewpoint of superconducting quantum interference device measurements, suppression of inter-particle magnetic interactions due to the molecules of polyacrylate) since they were very easily controlled by a low external magnetic field in the order of 1 Tesla. Both nanocomposites exhibited very significant antifungal activities against four *Candida* species (Table I) although these values were higher than the corresponding to 26 nm AgNp. Moreover, acute nanocomposite cytotoxicity against mice embryonal fibroblasts was observed at concentrations higher than 430 μg/ml ($Ag@Fe_3O_4$) and 292 μg/ml (γ-Fe_2O_3@Ag). Considering the non-cytotoxic nature of the polyacrylate linker, both kinds of Ag nanocomposites are well applicable for a targeted magnetic delivery of AgNp in medicinal and disinfection applications.

2.3. Nanoparticles of Semi-Conducting Oxides

Currently inorganic metal oxides (TiO_2, MgO, CaO and ZnO) have attracted interest as antimicrobial agents because of their safety and stability. Compared to organic materials, inorganic materials such as ZnO possess superior durability, greater selectivity and heat resistance.[78, 79]

In particular TiO_2 and ZnO semiconductors have been extensively studied as antimicrobial agents due to their photocatalytic activity under UV light.[80, 81] Photocatalysis is a light induced catalytic process whereby photogenerated electron–hole pairs (e^-h^+) in a semiconductor undergo redox reactions with molecules adsorbed onto the surface, thereby breaking them into smaller fragments. The electronic structure of semiconductors such as TiO_2, ZnO, Fe_2O_3, CdS and ZnS is characterized by a filled valence band and an empty conduction band and can act as sensitizers for light-induced redox processes. When a photon with energy of $h\nu$ matches or exceeds the bandgap energy of the semiconductor, an electron (e_{cb-}) is promoted from the valence band into the conduction band, leaving a hole (h_{vb+}) behind. The (e^-h^+) migrate to the nanoparticle surface and can recombine and dissipate the input energy as heat, get trapped in metastable surface states or react with electron donors and electron acceptors adsorbed on the semiconductor surface. In the absence of suitable (e^-h^+) scavengers, the stored energy is dissipated within a few nanoseconds by recombination. If a suitable scavenger or surface defect state is available to trap the electron or hole, recombination is prevented and subsequent redox reactions may occur. The h_{vb+} are powerful oxidants while the e_{cb-} are good reductants.[82] In general terms, the photocatalytic activity in aqueous oxygenated media produces ROS such as radical hydroxyle ($OH^\bullet$) and H_2O_2 (Fig. 2). Surface area and surface defects play an important role in the photocatalytic activity of metal oxide nanostructures, as the molecules need to be adsorbed on to the photocatalytic surface for the redox reactions to occur. The higher the effective surface area, the higher will be the adsorption of molecules leading to better photocatalytic activity. One dimensional nanostructures, such as nanowires and nanorods, offer higher surface to volume ratio compared to nanoparticulate coatings on a flat plate.[83]

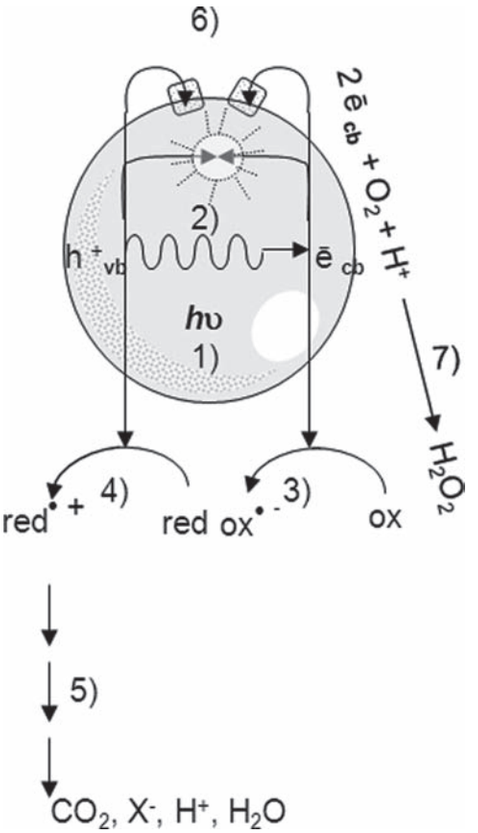

Fig. 2. Primary steps in the photoelectrochemical mechanism of photoactivated semiconductors (ZnO, TiO_2): (1) formation of charge carriers: conduction band electron (e_{cb}^-) and valence-band hole (h_{vb}^+) by a photon $h\nu$; (2) charge carrier recombination to liberate heat; (3) initiation of an oxidative pathway by a h_{vb}^+; (4) initiation of a reductive pathway by a e_{cb}^-; (5) further thermal (e.g., hydrolysis or reaction with active oxygen species) and photocatalytic reactions to yield mineralization products; (6) trapping of a e_{cb}^- in a dangling surficial bond to yield a trapped conduction-band electron e_{tr}^- or trapping of a h_{vb}^+ at a surficial group. Red is an electron donor (reductant) and Ox is an electron acceptor (oxidant). Dangling bond is a chemical bond associated with an atom in the surface layer of a solid that does not join the atom with a second atom but extends in the direction of the solid's exterior; (7) an example of oxigen reactive species generation (H_2O_2) in aerated solutions occurring via the reduction of adsorbed oxygen by e_{cb}^-.

2.3.1. Zinc Oxide Nanoparticles

Zinc is a mineral element essential to human health and ZnO is a form in the daily supplement for zinc. The antibacterial and antifungal activity of bulk ZnO powders has been demonstrated already.[84, 85] ZnO is an *n*-type semiconductor with wide direct band gap (3.37 eV) and high exciton energy (60 meV) at room temperature which allows it to act as an efficient semiconducting and piezoelectric material. As its size is decreased, the band gap of ZnO (ZnONp) increases, as well as its surface area. ZnO is a polar crystal of positive zeta potential at the surface, Zn^{2+} lies within a tetrahedral group of four oxygen ions. Zinc and oxygen atoms are arranged alternatively along the *c*-axis and the top surfaces are Zn terminated while the bottom surfaces are oxygen terminated. The high surface reactivity of ZnONp owes to a large number of native defect sites arising from oxygen nonstoichiometry. Because of this ZnONp exhibits comparatively higher reaction and mineralization rates and can generate hydroxyl ions more efficiently than TiO_2.[86]

After contact with ZnONp disruption of cell membrane activity has been observed and disorganization of the triple membrane was formerly reported in the Gram-negative *E. coli*.[87] The resulting increase of membrane permeability leads to accumulation of ZnONp in the bacterial membrane

followed by their cellular internalization, as also reported against *Pseudomona aeruginosa*.[88] The microbicidal activity of ZnONp is owed to the generation of ROS such as $OH^{\bullet}$, H_2O_2, and O_2^{2-}, which is directly proportional to the exposed surface area.[89] ZnONp with defects can be activated by both UV and visible light. In the ZnONp surface the holes split H_2O molecules into OH and H^+. Dissolved oxygen molecules are transformed to superoxide radical anions ($^{\bullet}O_2^-$), which in turn react with H^+ to generate ($HO_2^{\bullet}$) radicals, which upon subsequent collision with electrons produce hydrogen peroxide anions (HO_2^-). They then react with hydrogen ions to produce molecules of H_2O_2. Since, the hydroxyl radicals and superoxides are negatively charged particles, they cannot penetrate into the cell membrane and remain in direct contact with the outer surface of the bacteria; however, H_2O_2 can penetrate into the cell and kill bacteria.

Similar to AgNp, an inverse relationship between bactericidal activity and size of ZnONp was shown. At a fixed concentration (1 mM), the bactericidal activity of 12 nm ZnONp is higher than that of 45 nm and 2 μm ZnONp. These differences are explained from the fact that a single isolated colony of 2 μm diameter bacteria can accommodate larger number of 12 nm than 45 nm and 2 μm ZnONp. ZnONp presumably remain tightly adsorbed on the surface of the leftover/dead bacteria, but continue to release peroxides into the medium. Besides, since size and specific surface area are inversely related: bulk ZnO exposes 5.11 m^2/g, but 47 nm ZnONp ascends to 68 m^2/g and 12 nm ZnONp to 115 m^2/g. Therefore, the smaller is the nanoparticle, the higher its production of ROS on the surface and its antibacterial activity. The abrasive surface texture of ZnONp is another possible explanation of its antibacterial effect. ZnONp have been found to be abrasive due to surface defects, revealed by a broad visible emission band in the region of 450–550 nm in a photoluminescence spectrum of ZnONp. The abrasiveness of ZnONp compared with bulk ZnO is caused by the uneven surface texture due to rough edges and corners. Opposite to the deleterious action of ZnONp, trace concentrations of Zn^{+2} ions are a supplement promoting the metabolic action of bacteria.[90] On the other hand, the release of soluble Zn^{+2} from the ZnONp are responsible for toxicity in lung cell lines,[91] while under realistic environmental conditions, similar results on algae have been reported.[92] Overall, ZnONp proved to be toxic against prokaryotic organisms at the concentrations of units of mM Zn. Nonetheless, ZnONp had minimal effects on eukaryote human T-lymphocytes cell viability at concentrations toxic to bacteria.[93]

The advantages of using ZnONp as antifungal agent have recently started to raise attention. For instance in agriculture the use of ZnONp does not affect the soil fertility in comparison to traditional antifungals.[94] Similar to what occurs with bacteria, the cytotoxic effect of ZnONp against fungi is mediated by bilayer rupture resulting in the drainage of the cytoplasmic contents.[95–97] Recently antifungal activity of 2–28 nm ZnONp (hexagonal wurtzite) stabilized with surfactants isolated from *Acacia concinna* seeds, was determined.[98] It was observed that the size of the ZnONp drastically decreases from 28 nm to 2.5–5 nm with the surfactant stabilization. Surprisingly, this reduction in size is accompanied by a reduced activity of ZnONp against *Fusarium spp.*, when compared to that of standard antifungal 0.1 M $CuSO_4 \cdot 5H_2O$. Recently, anti *Candida* activity of glass supported ZnO nanrods were shown.[99]

2.3.2. Titanium Oxide Nanoparticles

The optical absorption in the ultraviolet region (peaking around 220 nm and covering only $\sim 5\%$ of the solar spectrum) and low photoefficiency of TiO_2Np are factors that deter its wide scale use for photocatalytic activities under sunlight. In spite of sharing similar band gap with ZnONp (3.2 eV), TiO_2Np can only be exited under UV light irradiation and its photocatalytic activity under solar and visible light is not efficient. TiO_2 exists in three main crystallographic structures e.g., anatase, rutile and brookite. It is also well-known that it is more difficult to obtain TiO_2Np with good crystallinity and high surface area from rutile than anatase. The photoexcited TiO_2 catalyst produces ($e^- h^+$) that migrate to the TiO_2 surface; ($\mathbf{h}_{vb}^+$) can react with adsorbed H_2O or OH^- at the catalyst/water interface to produce the highly reactive $OH^{\bullet}$ and the ($\mathbf{e}_{cb}^-$) can react with oxygen vacancies to form radical superoxide (O_2^{-1}); finally, the various generated ROS can oxidize organic compounds or cells adsorbed on the TiO_2 surface, resulting in the death of the microorganisms.[100] In an approach aimed to enhance the visible light photocatalytic activity of TiO_2Np and improve its antifungal activity, anatase and rutile crystal TiO_2Np were deposited on 50–100 nm diameter ZnO nanowires. The resulting TiO_2Np/ZnO nanocomposite exhibited a low band gap and high visible light activity against *C. albicans* biofilms. The antifungal activity of the ZnO nanowires was higher than the TiO_2Np in dark. Also, the antifungal activity of the ZnO nanowires in dark was decreased by the TiO_2Np coating. But, viability of cells significantly decreased nearly 4.3 times due to photocatalytic activity of TiO_2Np/ZnO nanocomposite under after 5 h exposure visible light. The alloy structure at the interface of the TiO_2Np/ZnO is said to decrease the band gap that can be exited under visible light. Again, the excitation of the nanocomposite using the light exposure leads to generate ($e^- h^+$) and the ($\mathbf{h}_{vb^+}$) lead to generate OH on the surface of the microorganism.[101]

Similarly, branched carbon nanotube (CNT) arrays covered with TiO_2Np presented antifungal effect on *C. albicans* biofilms under visible light. The TiO_2Np/branched CNTs showed a highly improved photocatalytic antifungal activity in comparison with the TiO_2Np/non branched CNTs and TiO_2Np film. The excellent visible

REVIEW

light-induced photocatalytic antifungal activity of the TiO_2Np/branched CNTs was attributed to the generation of ($e^- h^+$) by visible light excitation with a low recombination rate, in addition to the high surface area provided for the interaction between cells and nanostructures.[102]

As an example of an non photocatalytic use of TiO_2, recently 250–300 nm TiO_2AgNp combined nanoparticles (prepared by reaction between anatase TiO_2Np with Ag at 50:1 molar ratio) showed significant antifungal activity (Table I), although the activity resulted comparable to that of 20–25 nm AgNp used as control. The LD50 on THP-1 monocytes of the TiO_2AgNp was higher than that of AgNp. In spite of their higher size, TiO_2AgNp increased by nearly tenfolds the therapeutic index when used as antifungal agents as compared to AgNp.[103]

3. *IN VITRO* AND *IN VIVO* ANTIFUNGAL ACTIVITY OF NON METALLIC NANOPARTICLES

The following is a survey on preliminary improvements in delivery of conventional antifungals loaded in different types of nanovesicles (liposomes, niosomes, ethosomes and ultradeformable liposomes), solid lipid nanoparticles (SLN), nanostructured lipid carriers (NLC), polymeric micelles and microemulsions. The modified delivery after topical application led to better antifungal properties.

3.1. Nanovesicles

The skin delivery of AmB loaded in liposomes, ethosomes and ultradeformable liposomes was tested in a recent comparative assay (Table II).[104] The vesicles were further incorporated into Carbopol 934 hydrogel (1% w/w). Overall, 50–75% of AmB was released from gels after 24 h. *In vitro* assay on the AmB permeation across hairless rat skin showed that transdermal flux was maximal for ultradeformable liposomes and minimal for conventional liposomes. There were no differences between the transdermal flux of AmB in the dispersion of vesicles or within the gel. Skin retention of AmB was maximal for ultradeformable liposomes (81 μg) as compared to ethosomes (64 μg) and liposomes (45 μg). Confocal laser scanning microscopy study using rhodamine 123-loaded vesicles confirmed the penetration profile of the vesicles *in vivo*. Skin irritation test revealed negligible irritation scores for all the vesicular formulations. *In vitro* antifungal activity against fungal strain *Trychophytone rubrum* showed that AmB loaded in ultradeformable liposomes induced the largest zone of inhibition area.

The skin delivery of FLZ loaded in liposomes and niosomes incorporated into Carbopol gel (1% w/w) was tested for sustained release after localized application (Table II).[62] *In vitro* and *in vivo* skin penetration experiments showed a higher accumulation of FLZ when FLZ was loaded in liposomes. The *in vivo* localization studies in viable skin showed that liposomal gel produce 14.2- and 3.3-fold higher drug accumulation compared with plain gel and niosomal gel, respectively. Antifungal activity carried out on experimentally induced cutaneous candidiasis in immunosuppressed albino rats showed maximum therapeutic efficacy of liposomal gel, as the lowest number of colony forming units/ml was recorded following liposomal FLZ application.

The skin delivery of ECZ loaded in ethosomes incorporated into carbopol gel (1% w/w) with 2% w/w propylene glycol and 2% w/w *N*-methyl-2-pyrolidone as permeation enhancers was recently tested for therapeutic efficacy and storage stability (Table II).[105] Drug flux and permeation through albino rats skin were significantly higher in ethosomal formulation (0.46 μg/cm^2/hr and 91%, respectively) than for liposomes (soy lecithin), hydroethanolic gel and liposomal gel; this could indicate that ethanol enhances drug permeation across rat skin. The stability measurements of ethosomes revealed very low aggregation and insignificant growth in vesicular size for 180 days. The antifungal activity evaluated by cup plate method with strains of *C. albicans* showed that ethosomal formulation induced the largest zone of inhibition.

3.2. Polymeric Micelles

The skin delivery of azoles (CLZ, logP o/w 5.9, aqueous solubility 0.03 g/l; ECZ logP o/w 5.2, aqueous solubility 0.8 g/l; and FLZ logP o/w 0.4, aqueous solubility 0.001 g/l) loaded in polymeric micelles was recently determined (Table II).[106] Micelles differed in core hydrofobicity, from the more hydrofobic core mono-hexyl-substituted, followed by di-hexyl-substituted to the less hydrofobic core-polylactide. In spite of the expected ability of micelles to incorporate significant amounts of hydrophobic drugs in their core, CLZ showed poor incorporation efficiency (11–36%) in all the micellar systems. FLZ and ECZ on the other hand, were successfully encapsulated at 250 and 268 mg/g of drug contents and 83 to 98% incorporation efficiencies. Further assays were carried out with ECZ loaded in MPEG-dihexlPLA micelles. *In vitro* skin retention studies in porcine and human skin showed that ECZ deposition following 6 h application was $\sim$13-fold and 7.5-fold higher respectively, than that from ECZ commercial cream (Pevaryl® cream, 1% w/w ECZ). The amounts of ECZ deposited were 11 and 1.5 μg/cm^2, for porcine and human skin respectively. Confocal laser scanning microscopy studies using similarly-sized fluorescein loaded micelles showed that micells penetrate the skin mainly thought the follicular pathway.

3.3. Dendrimers

The effects of generation number and surface groups of poly(amidoamine) (PAMAM) dendrimers on aqueous

Table II. Non-metallic nanoparticles as antifungal agents.

Nanoparticle type	Fungus	Drug	Nanoparticles features	Reference
Nanovesicles	*Trychophytone rubrum*	AmB	1. Conventional liposomes:soybean phosphatidylcholine (soyPC)/cholesterol 7:3 molar ratio; 580 nm; 63% entrapment efficiency 2. Ethosomes:soyPC/ethanol 300:2 w/w; 220 nm; 84 entrapment efficiency 3. Ultradeformable liposomes:soyPC/sodium deoxycholate 85:15 w/w; 420 nm; 71% entrapment efficiency	[104]
	Experimentally induced cutaneous candidiasis	FLZ	350 nm liposomes and niosomes	[62]
	C. albicans	ECZ	Ethosomes:soy lecithin 3% w/w, ethanol 30% v/v; 200 nm; −75 mV Z potential; 80% entrapment efficiency	[105]
Polymeric micelles	Not tested	CLZ, FLZ and ECZ	30 to 40 nm micelles of amphiphilic methoxy-poly(ethylene glycol)-hexyl substituted polylactide (MPEG-hexPLA) block copolymers	[106]
Dendrimers	*C. parapsilosis* ATCC 22019 and susceptible and drug resistant clinical strains of *C. albicans*, *C. glabrata*, *C. krusei*, *C. dubliniensis* and *C. tropicalis*	CLZ	Poly(amidoamine) (PAMAM) dendrimers generation 2 (G2) and generation 3 (G3) with amine (PAMAM-NH_2) or hydroxyl surface groups (PAMAM-OH)	[107]
Lipid nanoparticles	Experimentally induced cutaneous candidiasis	FLZ	1. SLN:glyceryl behenate as core and 2:1 ratio of egg PC and pluronic F-68 as emulsifiers; 180 nm; 25 mV Z potential; 75% entrapment efficiency 2. NLC:glyceryl behenate and oleic acid as core and egg PC and pluronic F-68 as emulsifiers; 130 nm; −29 mV Z potential; 81% entrapment efficiency	[108]
Microemulsions	*C. albicans and* skin fungal infections (tinea corporis, tinea circinata and tinea pedis).	CLZ	1. Lemon oil/Tween 80/*n*-butanol/water 2. Isopropyl myristate/Tween 80/*n*-butanol/water	[109]

solubility and antifungal activity of CLZ was determined in 2009 (Table II).[107] It was found that PAMAM-NH_2 dendrimers improved CLZ solubility in a higher extent than the other polymers. PAMAM-NH_2 G3 and G2 exhibited the highest solubilising potential for CLZ (around 6.7-fold, from 0.38 to 2.55 μg/ml). Antifungal activity was evaluated using broth microdilution method. MIC and MFC values significantly indicate that PAMAM-NH_2 dendrimers increased the antifungal activity of CLZ against all the *Candida* cultures. CLZ in solution of PAMAM-NH_2 G2 was 4-32-fold more potent than pure CLZ, at a dendrimer concentration of 10 mg/ml, that the authors postulate as non-toxic.

3.4. Lipid Nanoparticles

Recently, SLN and NLC were developed with the aim of improving the skin delivery of FLZ (Table II).[108] *In vitro* skin-permeation and retention studies in hairless rat skin showed that the amount of permeated drug was higher for plain drug solution (41 $\mu g/cm^2$). The SLN and NLC based formulations decreased the amount of permeated FLZ to 14 and 12 $\mu g/cm^2$, respectively. Besides, NLC and SLN based formulations induced 2.12-fold and 1.73-fold higher amounts of FLZ accumulation in the skin respectively, as compared to the plain solution. The lipid nanoparticles induced a maximal accumulation of FLZ within the *stratum corneum*, with 16% and 14% drug retention from NLC and SLN respectively, as compared to 10% from plain solution. After topical application of the lipid nanoparticles to hairless rats, NLC and SLN induced 1.7-fold and 1.5-fold higher retention of FLZ within the *stratum corneum* as compared to plain solution. On the other hand, the amount of drug recovered from viable skin followed the same order as recorded in *stratum corneum*; but its accumulation was significantly low. Antifungal activity of FLZ loaded in SLN and NLC was carried out on experimentally induced cutaneous candidiasis on

REVIEW

immunosuppressed Sprague-Dawley rats infected with *C. albicans*. The topical treatments were applied once daily for 3 consecutive days 24 h after the infection was induced. The animals treated with both FLZ loaded in NLC and SLN demonstrated a low fungal burden in skin, with a colony count significantly less abundant than those treated with plain solution.

3.5. Microemulsions

Microemulsions have recently been used to improve the clinical outcome of topical CLZ (Table II).[109] 60% of the CLZ loaded in lemon oil and isopropyl myristate based microemulsion was released within 8 h. The *in vitro* permeation studies on mice skin of the CLZ loaded microemulsion (formulated as liquid or incorporated into 1% carbopol gel), showed significantly higher skin retention of CLZ over the marketed CLZ cream. Moreover, the gel provided two- to three-fold increased skin retention compared to their corresponding liquid microemulsion. The isopropyl myristate based microemulsion was more stable than those based on lemon oil. *In vitro* antifungal activity of CLZ loaded in microemulsion as liquid and gel against *C. albicans* showed that the mean diameter of the inhibition zone for microemulsion was significant higher than that of CLZ cream. Moreover, clinical evaluation proved that 1- and 2-weeks after treatment, 1% CLZ microemulsion gel showed a significant reduction in the scores of symptoms of the evaluated skin fungal infections. The overall evaluation of the clinical efficacy of microemulsion gel was good to excellent in 92.31% of the patients. Moreover, the preparation was well tolerated by all patients with no discontinuation of treatment due to any side effects.

REVIEW

4. OCULAR DELIVERY OF NON METALLIC NANOPARTICLES

Ocular fungal infections may involve the cornea (keratitis), the interior of the eye (endophthalmitis), the retina (retinitis) or the orbit and may occur following trauma (including surgery) or upon systemic disseminated infection. Fungal infections of the retina are among the most devastating ocular infections.[110] Fungal infections with *Candida*, *Fusarium*, *Curvularia* and *Aspergillus* can lead to serious ulceration of the cornea and must be treated rapidly. The most common among these infections is candidal chorioretinitis, usually caused by *C. albicans*.[111] Aspergillus species is the second most common fungal group that infects the choroid and the retina.[112]

In general, common routes of administration for the anterior-segment of the eye (cornea, conjunctiva, sclera, anterior uvea) are topical instillation and subconjunctival injection, whereas for the posterior-segment (retina, vitreous, choroid) common routes include systemic dosing, periocular and intravitreal (IVT) injections, and topical dosing.[113]

Upon systemic administration, the tight junctions of blood-ocular barrier in the retinal capillary and the iris/ciliary endothelial cells, keep most drugs out of the eye.[114] On the other hand, IVT administration is able to maximize the intraocular level of drug in the vitreous and the retina while avoiding toxicities associated with systemic treatment.[115] However, to reach and to maintain effective therapy, repeated injections are necessary. Frequent administration of drugs via this route can lead to endophthalmitis, damage to lens, retinal detachment and hemorrhage. Moreover, high acute intraocular drug concentrations may induce severe local toxicity and increase intraocular pressure.

Topical route is the preferred route of drug administration, primarily for reasons of better patient compliance and cost affordability. However, topical drug delivery to the eye is often impaired by removal mechanisms (blinking, tears, and nasolacrymal drainage) and by the relatively impermeable corneal barriers. The three membranes of the cornea (epithelium, inner stroma and endothelium) act as lipophilic selective barrier for small molecules, preventing the paracellular diffusion of macromolecules and maintaining normal corneal hydration.[116, 117] Usually, less than 5% of topically administered drug penetrates the cornea and reaches intraocular tissues.[118] The conjunctiva and sclera are more permeable than the cornea for drugs topically applied into the eye, but the circulation removes the drugs before it can be absorbed by inner ocular tissues. Both trans-conjunctival penetration and trans-nasal absorption after drainage are generally undesirable, not only because of the loss of active ingredient but also because of possible severe systemic side effects.

Topical AmB (0.1–0.3%) is the standard treatment for ocular infections due to *Candida* and related fungi while other polyene macrolide natamycin (5%) is the usual treatment of filamentous fungi such as *Fusarium.*[119] The current formulation of AmB eye drops (Fungizone®) contains deoxycholate, necessary to solubilize the poorly water soluble AmB,[120] which renders their instillation painful and leads to poor compliance and aggravation of symptoms, especially when direct IVT injection of AmB deoxycholate is used to treat fungal endophtalmitis. Although doses of 5 to 10 μg of IVT AmB are recommended and generally well tolerated, doses as low as 1.0 μg have caused marked retinal damage.[121]

In this context, nanoparticles could advantage the conventional ocular dosage forms, by offering increased residence time of drugs on the corneal surface, reduction in the amount of dose, reduction in systemic toxicity of drug, increased drug concentrations in the infected tissue and suitability for poorly water-soluble drugs.[122, 123] Other potential advantages are the possibility of self-administration by patients as eyedrops; no impairment of sight because of small dimensions of the delivery systems; protection against metabolic enzymes (such as peptidases and nucleases); possible uptake by corneal cells;

Table III. Non-metallic nanoparticles in ocular delivery.

Nanoparticle type	Fungus	Drug	Nanoparticles features	Reference
Nanovesicles	Not tested	AmB	Liposomes:egg PC/cholesterol/tocopherol succinate at 3:5:1 molar ratio	[124, 125]
	Experimentally induced *C. albicans* endophthalmitis	AmB	Liposomes:PC/phosphatidylglycerol/cholesterol at 4:1:3 molar ratio	[126]
	Not tested	AmB	1. AmB lipid complex:dimyristoyl phosphatidylcholine and dimyristoyl phosphatidyl-glycerol in a molar ratio of 3:10:7; micron sized ribbon-like structure 1–6 μm 2. AmBisome:unilamellar liposomes:hydrogenated soy PC/cholesterol/disteroylphosphatidylglycerol and AmB in a molar ratio of 2:1:0.8:0.4; $<$ 100 nm	[129]
	Not tested	AmB	Extemporaneous lipid emulsion of AmB, prepared by mixing concentrated alkaline solution of AmB with Intralipid $\sim$20% followed by neutralization and buffered	[120]
	Not tested	KTZ	1. Ultradeformable niosomes:span 60/tween 80; 80:20 w/w; 120 nm 2. non-ultradeformable niosomes:span 60/cholesterol; 80:20; w/w	[133]
Polymeric nanoparticles	*Fusarium solani*	AmB	Eudragit, 130 to 300 nm; +19 to +42 mV zeta potential	[143, 144]
	C. albicans	ECZ	Chitosan nanoparticles:sulfobutylether-β-cyclodextrin (SBE-β-CD) cross linker; 185 nm, +25 mV Z potential; 45% entrapment efficiency; 50% of drug release over 8 h	[148]

and possible reduction of the number of instillation or injection.

4.1. Nanovesicles

Liposomal AmB were formerly employed for IVT administration. The toxicity upon IVT administration of liposome-intercalated AmB was compared to AmB deoxycholate in rabbits[124] and rhesus monkeys (Table III).[125] It was found that liposomes markedly reduce the ocular toxicity of AmB in terms of vitreal band formation, focal retinal damage and retinal atrophy or necrosis. Liposomes also reduce the toxicity of AmB by at least four fold compared to AmB deoxycholate and as much as 30 μg of AmB may be tolerated by the IVT route in rhesus monkey.

In a further study, a rabbit model was used to compare the safety and efficacy of liposomal AmB with that of AmB deoxycholate up to 40 μg in experimentally induced *C. albicans* endophthalmitis (Table III).[126] Similar to the earlier study, it was concluded that reduced toxicity occurred at higher doses of liposomal AmB than AmB deoxycholate. However, higher doses of the lipid formulation were associated with decreased efficacy.

The first comparative toxicity study of three commercial AmB formulations (AmB deoxycholate, AmB lipid complex[127] and AmBisome,[128] Table III) on IVT administration on rabbits was carried out in 2003.[129] Although cataract formation was observed in the majority of the animals (75%), this was a result of the injection technique. From 10 μg for ABCL and 30 μg for AmB deoxycholate the appearance of vitreal opacities or bands was observed, but were absent in AmBisome-treated eyes. All the IVT administrations caused however, vitreal inflammation and retinal necrosis or atrophy. Retinal ganglion cell loss was found to be similar among the various treatment groups (81%–97%). In general, cell loss was mild to moderate with severity increasing with increasing doses. In this work however, the antifungal efficacy of the formulations was not assessed.

The first topical application of AmB formulations was reported by 1996. The tolerability of 400 nm extemporaneous lipid emulsion of AmB (Table III).[120] The results showed that the tolerance to AmB deoxycholate decreased with the number of instillations. On the contrary, AmB emulsion showed a very good tolerance, even after the fifth instillation on rabbits. The intraocular penetration of the AmB in the emulsion was not improved in comparison with AmB deoxycholate. The average concentration for the AmB deoxycholate group was indeed higher than the emulsion group, not significant in the aqueous humour (0.49 μg/ml vs. 0.3 μg/ml, respectively) and slightly significant in the cornea (500 μg/ml vs. 275 μg/ml, respectively). The presence of sodium deoxycholate might explain this difference. This substance, which is an absorption promoter, caused lesions in the cornea whose seriousness could increase with the number of instillations, thus facilitating the passage of the AmB into the ocular tissues. The AmB concentration both in the aqueous humour and in the cornea resulted in average higher than the MIC of fungi (around 0.2 μg/ml); while the plasmatic concentrations remained lower than 20 ng/ml.

Simultaneously, the ocular bioavailability of AmBisome was equal or better than that of AmB deoxycholate in the cornea of rabbits, but caused lower ocular toxicity.[130] After the first 15 min, the corneal AmB levels were significantly higher for animals receiving AmB deoxycholate than those treated with AmBisome. Later, AmB corneal drug levels showed no differences and remained stable following the. Drug levels in the aqueous humor did not differ between the two AmB preparations and remained below therapeutically effective concentrations.

A problem associated with the commercial formulation of AmBisome is its low shelf life once reconstituted. According to the manufacturer's instructions, AmBisome can only be kept refrigerated for 1 week after reconstitution. A longer shelf-life at ambient temperature would be preferable for a preparation made in a hospital pharmacy and delivered to patients. In this context, a study in 2007,[131] showed that the hydrodynamic diameter remained constant at 108 ± 30 nm with a polydispersity index lower than 0.15, after 6 months at room temperature or at +2–8 °C. AmB content was maintained between 94 and 107%. AmB and soy PC proportions remained constant, indicating that the liposomes remained intact and retained the drug. These results show the feasibility of an ophthalmic preparation based on liposomal AmB developed in hospital pharmacies

Elastic (ultradeformable) liposomes were used to increase the ocular bioavailability of the poorly water soluble antiviral ganciclovir after topical application.[132] Ganciclovir elastic liposomes (PC/cholesterol/sodium deoxycholate, 2:1.7:1 w/w, 200 nm), showed a 3.9-fold higher *ex vivo* transcorneal permeability than ganciclovir solution and 1.7-fold higher ocular bioavailability in rabbits than that of ganciclovir solution with similar precorneal clearance. The results were attributed to the small particle size (200 nm) and the elasticity of liposomes. The authors proposed that ultradeformable liposomes can enter the corneal structure which is similar to the *stratum corneum*.

A similar approach was employed to increase the corneal permeability of the highly lipophilic ketoconazole (KTZ), which posses a short ocular half life (19 min in aqueous humour and 43 min in cornea) by loading KTZ in ultradeformable niosomes.[133] Even though KTZ lipophilicity may help in its permeation, its large molecular weight (531.44 Da) impedes its transport across biological membranes. Further, while its high lipid solubility can ensure its passage across corneal epithelium, its further passage through the hydrophilic corneal stroma is hampered.[134] Moreover, the limiting water solubility (0.04 mg/ml) makes difficult to present KTZ in a solubilised form on the corneal surface, being a prerequisite for an ocular formulation. Ultradeformable niosomes (spanlastics) were tested for *ex vivo* corneal permeability, *in vivo* safety and *in vivo* ocular distribution. It was found that the corneal permeation of KTZ loaded in ultradeformable and non-ultradeformable niosomes were increased above that of KTZ suspension. The flux and total amount of permeated KTZ from ultradeformable niosomes was significantly higher than that from niosomes and KTZ aqueous suspension (2.6 μg/min/cm^2; 1153 μg) > (2.0 μg/min/cm^2; 860 μg) > (1.5 μg/min/cm^2; 625 μg) respectively. The amount of KTZ permeated was highest for ultradeformable niosomes (23.1%) and lowest for suspension (12.5%). The non-irritant/corrosive nature of KTZ encapsulated in ultradeformable niosomes when applied to dermal tissues and upon acute or chronic use to ocular tissues was confirmed. Both empty and KTZ loaded ultradeformable niosomes did not show any significant toxic effect on cell proliferation of normal human gingival fibroblast cell line and were non-genotoxic. The *in vivo* studies of local biodistribution were encouraging. Fluorescent vesicles were found within the aqueous humor and vitreous 4 h post instillation in rabbit eyes of 6-carboxyfluorescein loaded in ultradeformable niosomes, but not after instillation of carboxyfluorescein solution. Moreover, after repeated instillations of 6-carboxyfluorescein loaded in ultradeformable niosomes, fluorescence was found in both the aqueous and vitreous samples from 2 h onwards. Cryo-sections of rabbit eye, 2 h post single drop instillation, showed fluorescence and fluorescent vesicles in different eye tissues including the retinal layer. These results confirm that spanlastics can be used to deliver drugs to the posterior segment of the eye, although the mechanism is unknown.

An interesting study has recently showed that liposomes can target the retina when administered topically as eye drops.[135] It was shown that liposomes (distearoylphosphatidylcholine-DSPC, 105 nm, −66 mV Z potential) are able to deliver hydrophobic molecules into the retina. The fluorescence emission of the hydrophobic dye coumarin-6, was found in the posterior segment of the eye after submicron-sized liposomes containing coumarin-6 were topically administered as eye drops. The magnitude of fluorescence in the retina was closely related to the particle size (MLV vs. LUV-6000 nm vs. 105 nm) and rigidity of the liposomes (EggPC vs. DSPC). Submicron-sized liposomes with rigid structures could be potential carriers for targeting the posterior segment of the eye. Absorption of liposomes after topical administration to the surface of the eye seemed to occur mainly via three routes: the systemic, corneal and non-corneal pathways.[136] Epifluorescence microscopy of the entire eye revealed that the delivery route of liposomes to the posterior segment of the eye may not occur via corneal penetration or systemic delivery caused by nasolachrymal drainage.

4.2. Polymeric Nanoparticles

Polymers used to make nanospheres or nanocapsules aimed to ocular delivery must be biodegradable and transparent. In general, the active molecules are confined to polymeric matrices by relatively strong noncovalent

interactions such as ionic, hydrogen bonding, hydrophobic or dipole. Upon administration onto the eye, the particles are maintained at the delivery site and the drug is released from their matrix through diffusion, chemical reaction, polymer degradation or ion exchange mechanisms. Without bioadhesion, nanoparticles are eliminated from the precorneal site almost as quickly as aqueous solutions, so cationic polymers are used to make nanoparticles, in order to maximized interaction with negatively charged cornea. These nanoparticles would act as depots and it is speculated that would not be absorbed as occurs with liposomes.

Polyalkylcyanoacrylate (PACA) nanospheres and nanocapsules have been shown to improve and prolong the corneal penetration of anti-inflammatory hydrophilic and lipophilic drugs (triamcinolone[137] and dexamethasone[138]). Despite these positive results, the potential of PACA nanoparticles is limited because they cause disruption of the corneal epithelium cell membrane.[139]

Eudragit is cationic a copolymer of poly (ethylacrylate, methyl-methacrylate, and chloro trimethyl-ammonioethyl methacrylate) containing 8.8–12% for Eudragit RL and 4.5–7.0% for Eudragit RS of quaternary ammonium groups. Eudragit is insoluble at physiologic pH values and capable of limited swelling, thus representing a good material for the dispersion of drugs. Upon topical administration of Eudragit RL and RS nanoparticles in rabbits eyes, sustained release and increased absorption of the incorporated nonsteroidal anti-inflammatory drugs (ibuprofen and flurbiprofen) were observed.[140] Furthermore, no signs of inflammation or discomfort were detected in the rabbits' eyes, suggesting a local tolerance of these nanoparticles.[141, 142] The higher content of quaternary ammonium groups of Eudragit RL 100 increases its water permeability and provides a faster drug release than Eudragit RS 100.

AmB was loaded in nanoparticles (Np) made of Eudragit RL 100[143] and Eudragit RS 100,[144] prepared by a solvent displacement process avoiding the use of toxic chlorinated organic solvents (Table III). PVA, a highly aqueous soluble surfactant, was needed for physical stability of the Np suspension and also for maintaining desired viscosity. The size of the Np remained within 130 to 300 nm with positive zeta potential even after 6 months. *In vitro* release studies in simulated tear fluid (pH 7.4) revealed that nearly 60% AmB was fastly released from the two types of Np within 30 minutes. The antimicrobial activity against *Fusarium solani* by paper disk diffusion method showed that the antifungal activity of AmB loaded in the two types of Np was equal to or slightly lower than that of free-AmB solution. *In vivo* eye irritation study by a modified Draize test showed that, following topical instillation of nanoparticles to a rabbit's eye there was no irritation. The ocular penetration of AmB loaded Eudragit Np was not assayed, so the advantages of these formulations over other AmB formulations can not be addressed. Moreover as Nps were administered only once in the irritation study, toxicity of these Np can not be discarded.

Other cationic mucoadhesive and biodegradable polymer that has demonstrated excellent ocular compatibility[145, 146] and prolonged contact time with rabbit's ocular surface is chitosan.[147] Chitosan nanoparticles can be spontaneously formed through ionic gelation using negatively charged compounds such as the precipitating agent sodium tripolyphosphate. Chitosan nanoparticles were prepared using sulfobutylether-β-cyclodextrin (SBE-β-CD) containing ECZ as polyanionic crosslinker and their potential as ocular drug delivery systems was studied (Table III).[148] The unique properties of SBE-β-CD (being polyanionic and a solubilizing agent) makes it a versatile substance, which can form nanoparticles with chitosan by ionic gelation and in addition solubilize poorly water soluble drugs. To test their use as ocular drug delivery, sterile 6 mm diameter filter paper discs were placed under the eyelid of albino rabbit for 1 min at specific time intervals following a single instillation of the investigated formulae in the conjunctival sac of the right eyes of rabbits. The discs were then placed in *C. albicans* inoculated tubes and the growth inhibition of yeast was evaluated by measuring the cultures' optical density at 600 nm. Results showed that the ECZ loaded chitosan/SBE-β-CD nanoparticles provided to the eye surface greater antifungal effect than that of ECZ solution. The differences in ECZ effect between the nanoparticles and the solution were significantly higher at all times assayed with the exception of time 1 h. The ECZ antifungal effect associated with the application of nanoparticles increased gradually with time showing a maximum at 4 h post-administration and decreased gradually afterwards. Since the controls with chitosan and CD alone are lacking, it is not possible to owe the obtained effect to the combination of both.

5. CONCLUSIONS

The idea of testing most of the above described nano-objects as antifungal agents has emerged nearly five years ago. The inherent structural complexity of each nano-object makes necessary the inclusion of substantial quantitative data, since structure of nanoparticles and biological activity (or function) is highly related. Even though the toxic effects of nano-objects deserve to be carefully highlighted, it is of equal importance to avoid toxicological issues that could spoil the development of potentially benefit nanotechnologies for human health. A key to solve this challenge is to increase our knowledge on the interactions between nano-objects and biological systems. This is required to build regulatory guidelines for the use of nanomaterials in consumer products and in health, as repeatedly stressed for instance by the *Scientific Committee on Emerging and Newly Identified Health Risks* (SCENIHR, European Commission). Vesicles reviewed here for ocular delivery have shown their relative safety when administered by

parenteral routes and already entered the clinics against other pathologies nearly twenty years ago. However, especially for metallic nanoparticles, only *in vitro* tests of their antifungal activity have been reported. Thus, there is still an important lack of critical information on pre-clinical efficacy and safety in animal models of fungal infection. Data on biocompatibility in human beings is even more scarce, a fact that envisions a difficult pathway to the translation. Besides, validated methods for large scale production are still absent. Although results on antifungal metallic nanoparticles looks promising, there remain the challenges of demonstrating that (a) the same mechanisms mediating oxidative stress and responsible for fungi elimination are innocuous for human beings, and (b) the absence of ecotoxicity. Nonetheless, different to other therapeutic strategies employing nanoparticles, the applications reviewed here, are or should be intended for the topical route. This is advantageous in terms of avoiding the expectance of acute infusion related toxicity (such as the complement activation related pseudo allergy). Given the nature of the oxidative damage, determining the toxicity after repeated doses, in longitudinal studies, will be required. Because of this, the development of antifungal metallic nano-objects with high therapeutic indices will be of critical importance.

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