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Critical review

Fungicidal assemblies and their mode of action

AM Carmona-Ribeiro[1]*, LDM Carrasco[1,2]

Abstract

Introduction

Fungicidal assemblies can be built from lipids, polymers and/or drugs to yield optimal activity against fungus in virtual absence of haemolysis. *Candida albicans* has often been used as a model for testing novel fungicidal assemblies both *in vitro* and *in vivo*. Fungicidal drugs require appropriate formulations to improve their therapeutic index at low cost and toxicity. Inexpensive synthetic lipids or surfactants and biocompatible, water soluble polymers can be assembled to provide novel vehicles for carrying the fungicidal drugs or eventually being the fungicidal agent themselves. In this critical review, perspectives of some important fungicidal assemblies of low toxicity and cost are disclosed and major factors such as mobility of the quaternary ammonium moiety, charge of the fungus cell, size and shape of the fungicidal nanostructures determining their mode of action are presented.

Conclusion

The major factors determining the activity of fungicidal assemblies are the mobility of the antimicrobial moiety, the charge on the fungus cell and the size and shape of the antifungal assembly.

Introduction

The development of novel fungicidal assemblies is important to circumvent the generally high toxicity of antifungal drugs and the problem of fungus resistance derived from the widespread use of such drugs in clinics. Drugs of choice for treating fungal infections belong to different classes such as the azoles which inhibit the synthesis of ergosterol, the echinocandins which inhibit the cell wall synthesis, the polienic antibiotics (e.g. amphotericin B and nystatin) which combine with ergosterol in the fungus membrane to form pores thereby altering the membrane permeability, the nucleoside analogues which inhibit the synthesis of nucleic acids, some antibiotics (e.g. griseofulvin) which inhibit cell division by hampering the synthesis of the microtubules and others.[1] Alternative and less toxic antifungal formulations may include natural products, synthetic agents and polymeric materials such as some saponins, alkaloids, peptides, essential

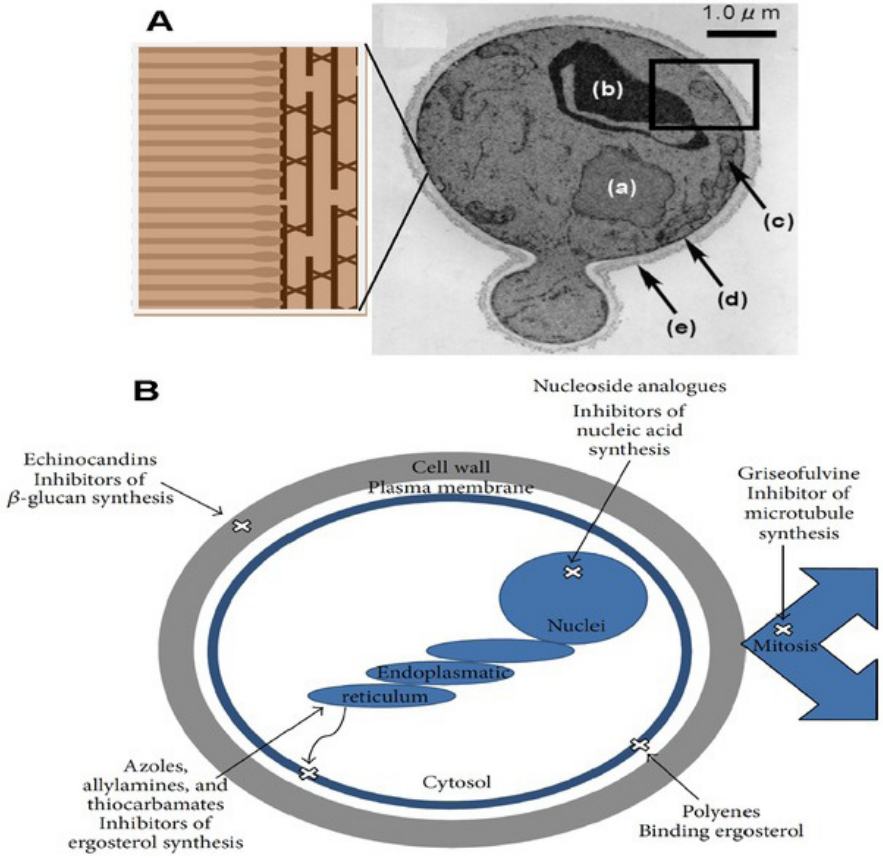

Figure 1: (A) Transmission electron micrograph of a *Saccharomycces cerevisiae* cell where some subcellular structures such as the nucleus (a), a vacuole (b), a mitochondria (c), the cytoplasmic membrane (d) and the cell wall (e) can be seen. Adapted from ref. 4. The enlarged cell wall of the yeast cell shows an inner skeletal layer composed of cross-linked and stress-bearing polysaccharides and chitin running parallel to the cell surface and acting as a scaffold for a dense outer layer of glycoproteins (A). The primary targets of several antifungal agents in the yeast cell are in (B). Reprinted from ref. 1.

* Corresponding author
Email: amcr@usp.br

[1] Departamento de Bioquímica, Instituto de Química, Universidade de São Paulo, Caixa Postal 26077, 05513-970, São Paulo, Brazil
[2] Departamento de Análises Clínicas e Toxicológicas, Faculdade de CiênciasFarmacêuticas, Universidade de São Paulo, São Paulo, Brazil

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oils, polymers with quaternary nitrogen atoms, synthetic amphiphiles, lipids or polymers loaded or not with antifungal compounds[1–3].

Candida albicans is the most important fungal opportunistic pathogen that can invade the bloodstream and disseminate to internal organs causing life-threatening invasive candidiasis. Figure 1(A) illustrates the cell wall structure of a *Saccharomycces cerevisiae* cell[4] and Figure 1(B) shows the site of action for different antifungal drugs[1]. The aim of this review was to discuss fungicidal assemblies and their mode of action.

Discussion

The authors have referenced some of their own studies in this review. The protocols of these studies have been approved by the relevant ethics committees related to the institution in which they were performed.

Fungicidal supramolecular assemblies

The therapeutic index of toxic drugs can be considerably increased by appropriate formulation. Among the classical fungicides, amphotericin B (AB) is a good example of this statement with several improved formulations available such as liposomal AB, AB lipid complex, AB colloidal dispersion, AB in microspheres and nanoparticles. However, the formulation cost is an important issue mainly for neglected infectious diseases in developing countries (e.g. leishmaniasis). In this regard, a low-cost AB lipid formulation using an inexpensive and synthetic cationic lipid (dioctadecyldimethylammonium bromide, DOD) at low drug to lipid molar ratios was developed based on the high affinity of AB for the borders of cationic bilayer fragments (BFs)[5]. Despite the relatively high dose of the pro-inflammatory and toxic cationic lipid, the DOD/AB formulation displayed a high therapeutic index *in vivo*[6], low nephrotoxicity[7] and low general toxicity[8]. Based on the coating of AB aggregates with the DOD cationic bilayer, DOD concentrations could be reduced in AB/DOD formulations prepared at high drug to lipid molar ratios[9]. In order to improve the colloidal stability of AB/DOD or DOD/AB, the cationic assemblies were added with biocompatible consecutive layers of polyelectrolytes such as the negatively charged carboxymethyl cellulose (CMC) and the positively charged poly (diallyldimethylammonium chloride) (PDDA) yielding robust fungicidal activity both at high and low drug to lipid molar ratios[10]. Later on, unloaded assemblies of DOD/CMC/PDDA and the PDDA polyelectrolyte itself were described as potent bactericides[11] or fungicides[12] in complete absence of any classical antifungal drug. Figure 2 shows some schematic representations of the quoted fungicidal assemblies: DOD/AB[5,10], AB/DOD[9], DOD/AB/CMC/PDDA[10], AB/DOD/CMC/PDDA[10] and DOD/CMC/PDDA[11,12]. Among these assemblies with high fungicidal

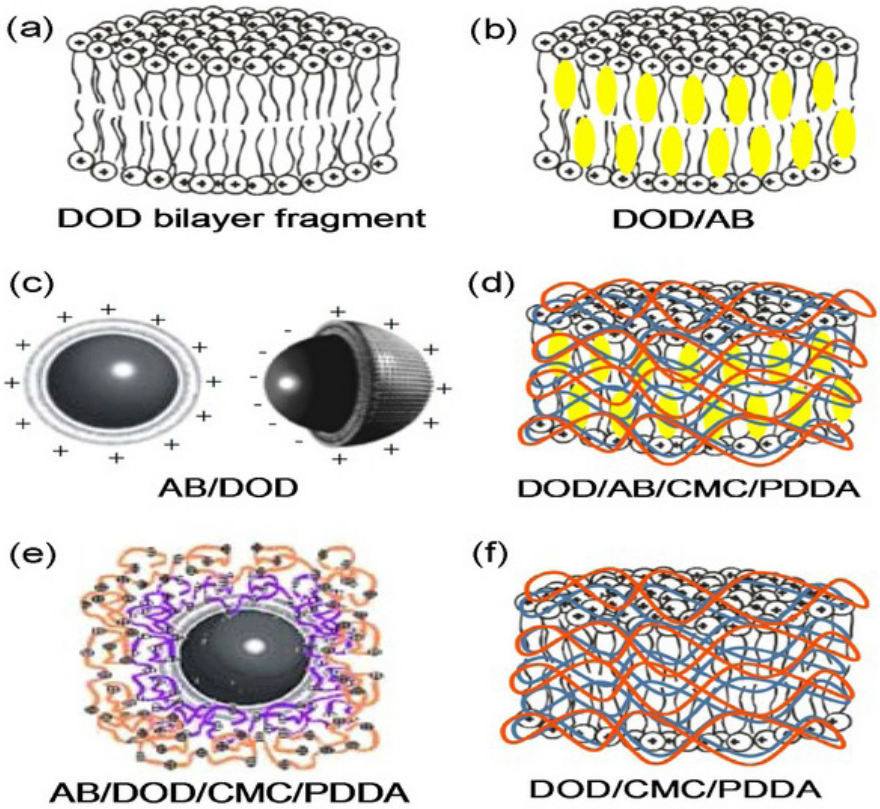

Figure 2: Schematic representations of some fungicidal assemblies based on bilayer fragments (BF) of the cationic lipid DOD prepared from sonication with tip in aqueous solution. DOD BF are unloaded (a), loaded with amphotericin B yielding DOD/AB (b) or assembled as a bilayer coating on amphotericin B aggregates yielding AB/DOD(c). (a) is adapted from ref. 5. Copyright (2001), with permission from Elsevier; (b) is adapted from ref. 10; (c) is adapted from ref. 9, by permission of Oxford University Press. DOD/AB, AB/DOD or unloaded DOD BF further covered by consecutive layers of carboxymethyl cellulose (CMC) and PDDA yielded the DOD/AB/CMC/PDDA (d), AB/DOD/CMC/PDDA (e) and DOD/CMC/PDDA fungicidal assemblies (f), respectively. (d) and (e) are adapted from ref. 10; (f) is adapted with permission from ref. 11. Copyright (2010) American Chemical Society.

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activity *in vitro* only the DOD/AB assembly was evaluated *in vivo* against systemic candidiasis in mice[6]. All other fungicidal assemblies still need to be tested *in vivo*. Figure 3 shows the potent fungicidal activity of PDDA by itself or as the outermost layer of nanostructured particles against *C. albicans* plus its interesting non-haemolytic property[12]. PDDA has been considered safe for human health and is widely used in paper manufacturing, water treatment, mining industries and food processing. Furthermore, this polyelectrolyte can be easily combined with other molecules such as lipids and proteins or their assemblies such as bilayers, nanoparticles and drugs both in the form of dispersions or nanostructured coatings protecting different materials against fungus attack and biodegradation.

Among the azoles, miconazole (MCZ) was used as a model drug to obtain DOD/MCZ assemblies at low drug to lipid molar ratios based on MCZ solubilisation in DOD bilayers[13].

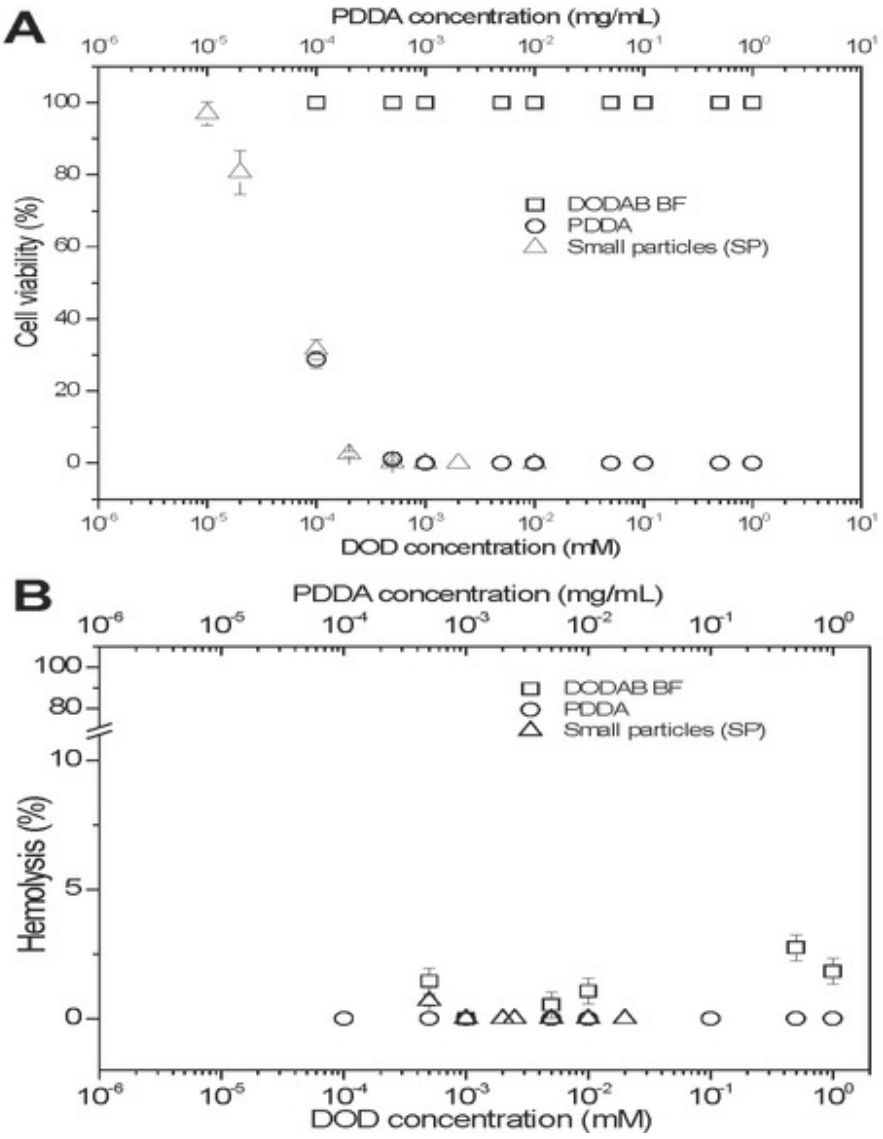

Figure 3: Remarkable fungicidal activity (A) in virtual absence of haemolysis (B) for the unloaded DOD/CMC/PDDA discoidal nanoparticles. Adapted with permission from ref. 12.

At high drug to lipid molar ratios, MCZ cationic aggregates in water solution were covered by anionic sodium dihexadecyl phosphate (DHP) BF[13]. The minimal fungicidal concentrations (MFC) for MCZ in ethanol and in formulations with DOD BF (DOD/MCZ) or DHP BFs (DHP/MCZ) were determined against *C. albicans* ATCC90028 yielding improved drug efficiency in comparison with Zoltec™ (St. Louis, MO), the classical fluconazole formulation[14]. DOD BF-loading capacity relative to miconazole was determined from size distributions as 0.5 mM MCZ in 5 mM DOD dispersed as DOD BF[14]. BF-loading capacity relative to amphotericin B was 0.1 mM AB in 2 mM DOD assembled as BF[9]. DOD by itself kills *Cryptococcus neoformans* and *Candida* sp. at 2 and 2– >250 mg/L of MFC, respectively[9]. Both AB/DOD and MCZ/DOD assemblies (drug particles surrounded by lipid) interacted with *C. albicans* for 48 h yielding lower MFCs than those for the respective drug acting alone[9]. At high drug to lipid molar ratio, only the DOD-covered MCZ particles (MCZ/DOD) exhibited synergistic action against *C. albicans*[9]. It was observed that the lipid capsule retarded drug action but the formulation was very effective given enough time[9].

Fungicidal activity of the quaternary ammonium moiety: mode of action

The antifungal activity of agents bearing the quaternary ammonium moiety largely depends on molecular structure[15]. While substantial fungicidal activity was described for the micelle-forming quaternary ammonium surfactants, the bilayer forming, double-chained DOD lipid with long C18 hydrocarbon chains did not show the ability to move from the bilayer assembly to the fungus cell membrane; there is a poor fungicidal activity of DOD BF[12] or large vesicles (LV) against *C. albicans*[16]. Adsorption isotherms on *C. albicans* for

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cetyltrimethylammonium bromide (CTAB), DOD from LV and DOD from BF revealed the competitive adsorption of DOD LV onto *C. albicans* cells: increasing DOD concentration reduced DOD adsorption onto the cells due to the preferential vesicle–vesicle association instead of the vesicle–cell association[17]. In contrast to other neutral or anionic surfactants such as sodium dodecyl sulphate, CTAB did not cause the disruption of the fungus cell membrane as depicted from the absence of leakage of phosphorylated compounds after interaction of *C. albicans* with CTAB over a range of concentrations[18]. Fungus death could be associated with a change of the cell surface charge from negative to positive as determined from measurements of the cell electrophoretic mobility[18]. In order to cause fungus death, the cationic quaternary ammonium moiety has to adsorb onto the cell, change its charge and penetrate through the cell wall reaching the fungus cell membrane. The rigid gel state of the DOD bilayer hampers its penetration into the fungus cell wall and cytoplasmic membrane explaining the poor fungicidal activity of the DOD cationic lipid[16,18] in comparison with CTAB[18] or other micelle-forming cationic surfactants[19]. Positively charged and highly fluid bilayers of dimyristoylphosphatidylcholine (DMPC)/CTAB over a range of CTAB molar ratios can incorporate chlorin e6 photosensitiser substantially improving its adsorption and uptake by *C. albicans* and facilitating the photodynamic inactivation of the fungus[20]. However, these liposomes containing CTAB were not able to kill *C. albicans* suggesting the larger affinity of CTAB for the DMPC fluid bilayer than the one for the fungus membrane. CTAB monomers coming from CTAB micelles, on the other hand, are excellent fungicides due to their high mobility in solution. The quaternary ammonium moiety can only interact with the fungus cells if its host molecule is mobile. A good example can be found in the dentistry field where resins have been modified with pendant and permanent quaternary ammonium groups[21]. Grafting of a quaternary ammonium antimicrobial component in a polymer network can be achieved through copolymerisation of the antimicrobial monomers with the conventional methacrylate monomers[21]. While free, non-polymerised quaternary ammonium monomers can rapidly kill oral pathogens, the antimicrobial component immobilised by polymerisation does not exhibit equally strong inhibitory effects[21]. The weakened effects after polymerisation are understandable from the restricted motility of the quaternary ammonium. The high affinity between the biocompatible poly(methyl methacrylate) (PMMA) polymer and CTAB or DOD allowed the preparation of antimicrobial films[22,23] or antimicrobial particles with the quaternary ammonium compounds embedded in the polymeric network[24]. For these hybrid materials, CTAB mobility caused bacterial death both on bacteria contact and in solution, whereas DOD killed only on contact due to its low mobility in the polymer matrix and high affinity for the PMMA polymer[22–24]. Novel hybrid materials easily assembled from weak intermolecular interactions such as electrostatic attraction, hydrogen bonds, van der Waals forces, hydrophobic effects, π/π* stacking, ion/ion, ion/dipole and dipole/dipole interactions can be very effective to build controllable nanostructures as films or nanoparticles[22–24]. Biocompatible polymers and their non-covalent assemblies with quaternary ammonium compounds deserve further investigation regarding their antifungal activity.

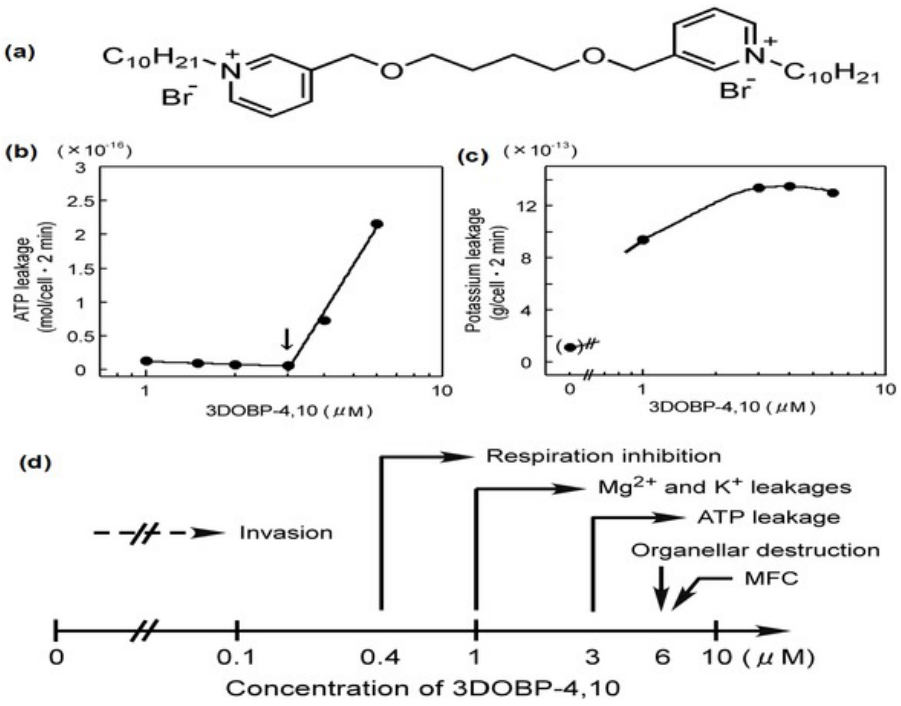

Figure 4: Chemical structure of the gemini quaternary salt, 3,3'-(2,7-dioxaoctane) bis (1-decylpyridinium bromide) (3DOBP-4,10) (a) and its induction of ATP (b) and potassium ion leakage (c) from *Saccharomycces cerevisiae* cells after just 2 minutes of interaction with the cells. The sequence of cellular events taking place on increasing the gemini surfactant concentration are shown in (d). Adapted from ref. 4.

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The mode of action of gemini surfactants bearing the quaternary ammonium moiety seems to involve lysis of the cell membrane and organelles with no apparent damage to the fungus cell wall[4,25]. The gemini quaternary salt (gemini-QUAT) containing two pyridinium residues per molecule, 3,3'-(2,7-dioxaoctane)bis (1-decylpyridiniumbromide) (3DOBP-4,10), exerts fungicidal activity against *S. cerevisiae* and causes respiration inhibition and the cytoplasmic leakage of ATP, magnesium and potassium ions[4]. The gemini surfactant was more effective than the mono-QUAT N-cetylpyridinium chloride (CPC)[4]. The production of reactive oxygen species was significantly elevated under aerobic conditions and associated with the activity of the gemini surfactant against *S. cerevisiae* and *C. albicans* with addition of scavengers of free radicals or anaerobic conditions reducing the fungicidal activity[25]. Figure 4 shows the sequence of events culminating in cell death of *S. cerevisiae* in the presence of the gemini-QUAT salt.

The evaluation of the antifungal activity of gemini quaternary ammonium salts over a range of hydrocarbon chain lengths showed that the compound with double 10 carbons chains was the most active one but exhibited significant toxicity against mammalian red blood cells around the minimal inhibitory concentrations (MIC) effective against the fungus[26]. Since *C. albicans* may grow in different forms such as yeast, pseudohyphal and hyphal and its virulence and biofilm formation is related to the switch from yeast to hyphae, inhibiting this switch hampers the biofilm formation. Figure 5 shows the chemical structures of the gemini compounds tested, their MIC and their impact on the filamentous growth of *C. albicans*[26].

Recently, C12 single and gemini cationic surfactants derived from arginine with spacers from 6-12 C were combined with a phospholipid (L-dilauroylphosphatidylcholine, DLPC) or cholesterol (CHOL)-forming cationic vesicles or aggregates with variable sizes for which the haemolytic activity and antimicrobial and antifungal activity were affected by the same parameters[27]. Their haemolytic activity is lower than one of the bis(QUATS) gemini surfactants as their antimicrobial activity[27]. For triblock polymers with the quaternary ammonium as the outermost block, simple changes to the core served to direct the self-assembly into distinct morphologies: spheres and rods[28]. Despite the vast majority of material consisting of poly(lactide) (interior block) and cationic polycarbonates (exterior block) testing the spherical and rod-like morphologies for antimicrobial properties showed that both possessed broad-spectrum activity (Gram-negative and Gram-positive bacteria as well as fungi) with minimal haemolysis, although only the rod-like assemblies were effective against *C. albicans*[28]. These triblock assemblies acted similarly to PDDA and its discoidal assemblies (Figure 2).The activity of the triblock assemblies with different shapes is illustrated in Figure 6.

For the quaternary ammonium hybrid assemblies with water soluble polymers, optimal fungicidal and non-haemolytic activities have been achieved using the self-assembly of BFs, CMC and the PDDA antimicrobial polymer[10–12]. Table 1 shows the

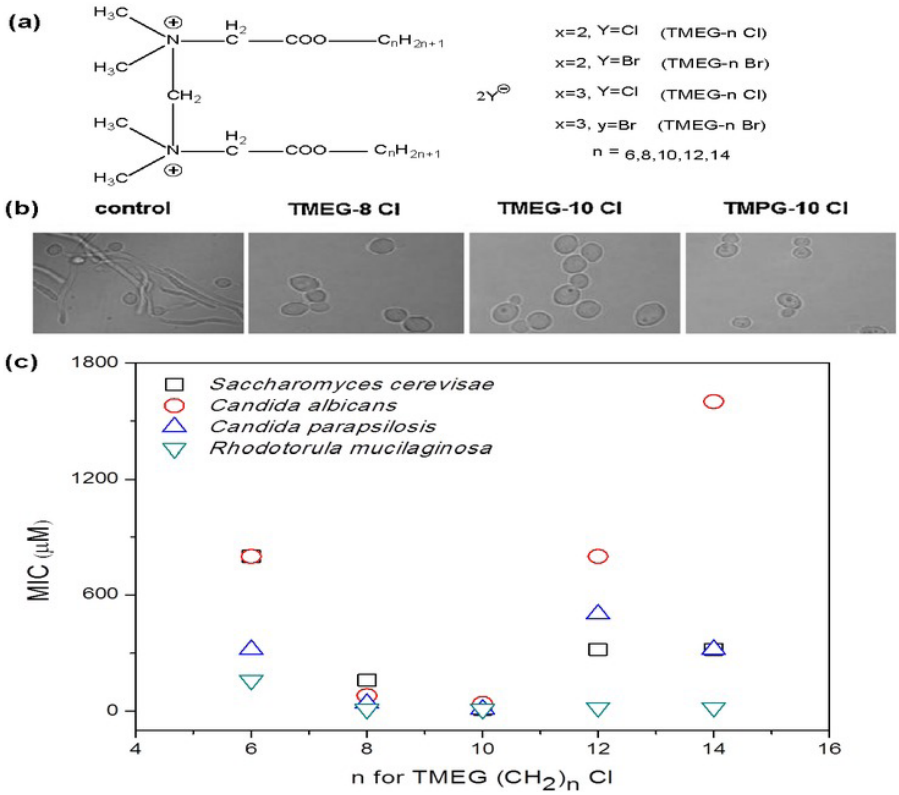

Figure 5: Chemical structure of gemini quaternary ammonium chloride and bromide, with various alkyl chain and spacer lengths, derivatives of N,N,N′,N′-tetramethylethylenediamine (TMEG) or N,N,N′,N′-tetramethyl-1,3-propanediamine (TMPG) (a), their inhibitory effect on filamentous growth of *Candida albicans* after 6 h incubation at 37°C, at 0.25 of their minimal inhibitory concentrations (MIC) (b) and their MIC as a function of n for TMEG $(CH_2)n$ Cl against four different fungus (c).Reprinted from ref. 26, Copyright (2013), with permission from Elsevier.

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Table 1 Comparison between fungicidal and haemolytic activity for some cationic assemblies against *C. albicans*

Ref.	Assembly	MIC		MFC	Haemolysis (H)	
		(μg/mL)	(mM)	(μg/mL)	C for 50%H (μM)	%H at (C)
28	C9 1	>180[a]			205	
	C9 2	112[a]			155	
	C9/CHOL 1	90[a]			215	
	C9/CHOL 2	>112[a]			135	
	C9/DLPC 1	>180[a]			88	
	C9/DLPC 2	56[a]			-	
27	TMEG-8 Cl		0.08[b]			9 (0.08 mM)
	TMEG-10 Cl		0.04[b]			13 (0.04 mM)
12	PDDA			0.4[b]		0(PDDA 10^3 μg/mL)
	DOD/ CMC/PDDA			0.32 DOD 0.5 CMC 0.5 PDDA[b]		0 (12 μg/mL DOD, 20 μg/mL CMC, 20 μg/mL PDDA)
18	CTAB		1[b]		0.7	
29	Triblock 5b-L	75[c]				< 4 (5 ´ 10^3μg/mL)
	Triblock 5b-D	75[c]				< 4 (5 ´ 10^3μg/mL)
	Triblock 5b-R	100[c]				< 4 (5 ´ 10^3μg/mL)

MIC: minimal inhibitory concentrations; MFC, minimal fungicidal concentrations; CHOL, cholesterol; DLPC, dilauroylphosphatidylcholine; TMEG, tetramethylethylenediamine; PDDA, poly(diallyldimethylammonium chloride); DOD, dioctadecyldimethylammonium bromide; CMC, carboxymethyl cellulose; CTAB, cetyltrimethylammonium bromide. [a]ATCC 10231, [b]ATCC 90028, [c]clinical samples.

antifungal and haemolytic activity for several fungicides and their assemblies. PDDA as a fungicidal agent has an MFC equal to 0.4 μg/mL and is effective in complete absence of haemolysis, contrasting with the poor activity of DOD against the fungus. Furthermore, PDDA is easily assembled with proteins, for example, albumin to yield non-haemolytic nanoparticles[29]. Important assemblies described for AB were non-hemolytic cross-linked albumin microspheres (5 μ of mean diameter)[30] or hybrid nanoparticles (0.08 μ of mean diameter), self-assembled from lipid and consecutive layers of water soluble hydrophilic polymers, yielding effective AB formulations[10,11].

Conclusion

Fungicidal activity and low toxicity of antifungal assemblies requires the controllable self-assembly of toxic and biocompatible materials. This critical review emphasises how readily available materials including the antimicrobial quaternary ammonium moiety and water soluble hydrophilic polymers can be assembled to yield non-haemolytic and efficient fungicidal nanostructures. Based on the absence of haemolysis only, some promising combinations have been described that should be further tested for antifungal activity. Based on the fungicidal activity only, some promising combinations have been described that should be further tested for haemolytic activity. Among the most promising fungicidal agents is PDDA which is basically a non-haemolytic, water soluble hydrophilic polymer with excellent antimicrobial activity easily assembled with proteins, for example, albumin to yieldnon-haemolytic nanoparticles, although the haemolysis test was not yet performed. Amphotericin B can be incorporated in cross-linked albumin microspheres (5 μ of mean diameter) or in hybrid nanoparticles (0.08 μ of mean diameter), self-assembled from lipid and consecutive layers of water soluble hydrophilic polymers, yielding effective AB formulations. PDDA itself or in nanostructured, hybrid lipid-polymer particles efficiently kills *C. albicans* in the absence of haemolysis. The gemini assemblies seldom satisfy the low toxicity and high fungicidal activity requirements for use *in vivo* and need to be formulated with less toxic vehicles.

Abbreviations list

AB, amphotericin B; BF, bilayer fragment; CMC, carboxymethyl cellulose; CTAB, cetyltrimethylammonium bromide; DHP, dihexadecyl phosphate; DOD, dioctadecyldimethylam-

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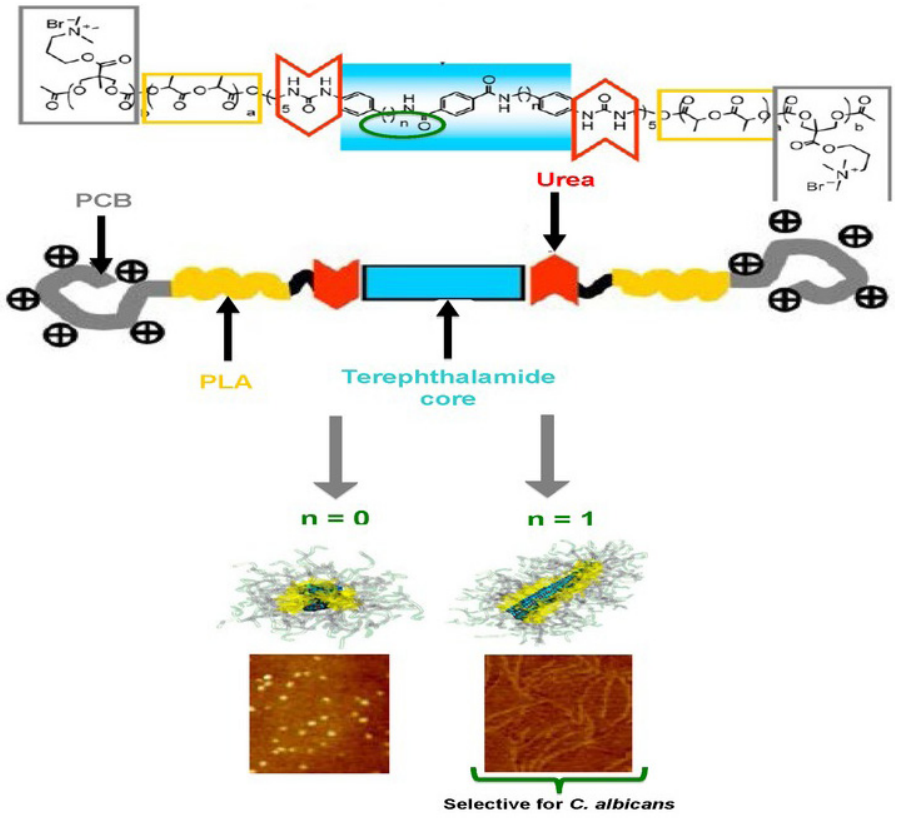

Figure 6: Chemical structure and schematic representation of triblock copolymers that were polymerised from an assembly directing a rigid hydrophobic terephthalamide-bisurea core, flanked by hydrophobic poly (lactide) blocks (PLA) along with peripheric hydrophilic polycarbonate blocks having cationic propyl trimethylammonium bromide (PCPAB) as the antimicrobial block. Two different bisurea cores were used: one with and the other without a flexible methylene spacer between terephthalamide and aryl urea groups, in order to direct the self-assembly into the rod (n = 1) or sphere morphology (n = 0). The two shapes were evidenced by molecular modelling and atomic force microscopy. Adapted with permission from ref. 28. Copyright (2012) American Chemical Society.

monium bromide; LV, large vesicle; MFC, minimal fungicidal concentrations; MCZ, miconazole; MIC, minimal inhibitory concentration; PDDA, poly (diallyldimethy lammonium chloride).

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