CHLORHEXIDINE:BETA-CYCLODEXTRIN INHIBITS YEAST GROWTH BY EXTRACTION OF ERGOSTEROL

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ABSTRACT

Chlorhexidine (Cx) augmented with beta-cyclodextrin (β-cd) inclusion compounds, termed Cx:β-cd complexes, have been developed for use as antiseptic agents. The aim of this study was to examine the interactions of Cx:β-cd complexes, prepared at different molecular ratios, with sterol and yeast membranes. The Minimal Inhibitory Concentration (MIC) against the yeast *Candida albicans* (*C.a.*) was determined for each complex; the MICs were found to range from 0.5 to 2 μg/mL. To confirm the MIC data, quantitative analysis of viable cells was performed using trypan blue staining. Mechanistic characterization of the interactions that the Cx:β-cd complexes have with the yeast membrane and assessment of membrane morphology following exposure to Cx:β-cd complexes were performed using Sterol Quantification Method analysis (SQM) and scanning electron microscopy (SEM). SQM revealed that sterol extraction increased with increasing β-cd concentrations (1.71×10^3; 1.4×10^3; 3.45×10^3, and 3.74×10^3 CFU for 1:1, 1:2, 1:3, and 1:4, respectively), likely as a consequence of membrane ergosterol solubilization. SEM images demonstrated that cell membrane damage is a visible and significant mechanism that contributes to the antimicrobial effects of Cx:β-cd complexes. Cell disorganization increased significantly as the proportion of β-cyclodextrin present in the complex increased. Morphology of cells exposed to complexes with 1:3 and 1:4 molar ratios of Cx:β-cd were observed to have large aggregates mixed with yeast remains, representing more membrane disruption than that observed in cells treated with Cx alone. In conclusion, nanoaggregates of Cx:β-cd complexes block yeast growth via ergosterol extraction, permeabilizing the membrane by creating cluster-like structures within the cell membrane, possibly due to high amounts of hydrogen bonding.

Key words: Chlorhexidine, β-cyclodextrin, membrane-drug interactions

INTRODUCTION

Chlorhexidine (Cx) is an effective chemical antiseptic that has a bactericidal effect at high concentrations (2-5%) and a bacteriostatic effect at low concentrations (~0.6%). The antimicrobial activity of Cx is thought to be due to bacterial membrane disruption, which leads to increased cell permeability and leakage of intracellular ions (Na$^+$, K)(17, 26,

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36, 37). Agents containing high concentrations of Cx have been used in periodontic and endodontic clinical practice. Cx has also been safely used at very low concentrations in several commercial products for dental hygiene, including mouthwashes, gels, and sprays. However the transitory effects of Cx have been shown to have deleterious effects on cells *in vitro* (15, 17, 40).

Cyclodextrins have been used as tools to manipulate the lipid composition of biological and model membranes (29). Their hydrophilic outer surface and hydrophobic inner core enable them to form inclusion complexes by trapping small amphiphilic molecules in their core. Beta-cyclodextrins (β-cds) are particularly efficient sterol acceptors, apparently because the size of their inner hydrophobic cavity matches the size of the sterol molecule (18, 28, 30, 34). Indeed, Cholesterol can be removed from cells by extraction into small unilamellar vesicles of β-cds (5, 6, 7).

Hence, in order to reduce the toxicity caused by high concentrations of Cx, chlorhexidine:beta-cyclodextrin (Cx:β-cd) complexes have been developed for use as antiseptic agents. These complexes offer advantages in effectiveness, long-term activity, and antimicrobial efficacy at low concentrations (10). Their key component, β-cd, creates regions of hydrophobic cavity that surround Cx. Gels containing Cx:β-cd complexes were shown to exhibit higher antimicrobial activity against oral microorganismal pathogens than non-augmented Cx in a long-term *in vitro* study (10). However, previous studies characterizing Cx activity provide little information regarding the mechanisms underlying microorganism-drug interactions on a nanometer scale. Understanding the structure and properties of microbial surfaces at the nanometer level is of great importance for the development of novel antimicrobial compounds.

Candida species, including *Candida albicans* (*C.a.*), are commensal organisms that proliferate when the body is out of homeostasis, leading to the development of disease. Although it is almost universally described as dimorphic, *C.a.*'s cell morphology actually lies on a pleomorphic continuum, ranging from ovoid yeast cells to filamentous hyphae (1, 2, 12, 13, 27, 42). This yeast has developed specific mechanisms of virulence that allow it to colonize epithelial cells of the host for the purpose of invading deeper layers or influencing the body's defenses. Interestingly, exposure of *C.a.* to 0.1% Cx has been shown to result in cell wall changes, leading to a loss of intracellular components and aggregation of nuclear proteins (2, 13, 14,16 19, 21, 22, 23).

Loftsson and Brewster (24) have shown that molecular envelopment of Cx with β-cd increases the gradient concentration at the membrane site. This increase most likely occurs due to Cx:β-cd developing a stronger interaction the cell membrane than that which forms between Cx and the membrane. Therefore, complexes of Cx with β-cd inclusion compounds may represent more effective microbicidal agents than pure Cx. Although data regarding the interactions between Cx inclusion complexes and biological membranes are limited, these complexes have attracted interest in many fields, as they could be used to release biologically-active drugs through membrane contact in diverse tissues (35).

The aim of this study was to investigate the anti-fungal activity of Cx inclusion complexes comprised of different Cx:β-cd molar ratios and to characterize the interactions of these complexes with yeast membranes using a *C.a.* strain as an experimental model.

MATERIALS AND METHODS

Chemicals and yeast stocks

Chlorhydrate of Cx was obtained from Ecadil® (SP, Brazil), and β-cd from Cerestar®, Co. (Milwaukee, WI, USA). Fluconazole was obtained from Pfizer® Pharmaceutics Group (New York, NY, USA). Sabouraud dextrose agar (SDA) and broth (SDB) were purchased from Biobras S.A® (MG, Brazil). *C.a.* (ATCC 28804) was obtained from the American Type Culture Collection (Manassas, VA, USA). All other materials

and solvents were of analytical grade.

Preparation of supramolecular complexes

Cx:β-cd inclusion complexes were prepared by freeze-drying aqueous solutions of β-cd and Cx at different relative molar ratios (Cx:β-cd: 1:1, 1:2, 1:3, and 1:4) as described elsewhere (9).

Antimicrobial assay

Yeast cultures were grown and maintained in Sabouraud dextrose broth (SDB) and Sabouraud dextrose agar. Prior to the assay, *C.a.* cells were cultured for 24 h at 34 °C under aerobic conditions in SDB. Yeast cultures were then further diluted to obtain a density of 1.5×10^8 CFU/mL for inoculums. Minimal Inhibitory Concentration (MIC_{90}) is the lowest concentration of drug that inhibits more than 90% of a microorganism population and MIC_{90} is the lowest concentration of drug that inhibits 90% of the microorganism population. MIC_{90} was determined using the macrodilution method (8). Briefly, aliquots containing 500 µL of 128 µg/mL Cx or 1:1; 1:2; 1:3, 1:4 Cx:β-cd solutions were serially diluted in 3.4 mL of SDB. Normalized Cx or Cx:β-cd dilutions were then inoculated with 100 µL of *C.a.* inoculums. Cx alone and fluconazole (FLZ) were used as standard antimicrobial positive controls and β-cd was used as a negative control (8).

Sample absorbance was measured using a standard spectrophotometer (Spectrumlamb 22PC®) set to 580 nm, and sample data were analyzed and compared to controls. Six replicates were analyzed for each condition, and MIC values were recorded and evaluated using the non-parametric Kruskal-Wallis test. *P*-values less than 0.05 were considered significant.

Yeast viability assay

The concentration of cells in a 0.5 µg/mL suspension was determined using a standard hemocytometer. The yeast suspension was diluted 100-fold with sterile water. Cells were then diluted in an equal volume of 0.4% trypan blue to assess the number of viable cells. Total cell counts were also performed with a hemocytometer. Calculations involving cell populations were based on viable cell counts (33).

Sterol Quantification Method (SQM)

Total intracellular sterols were extracted using the SQM described by Arthington-Skaggs et al. (3) with slight modifications. Briefly, a single *C.a.* colony from an SBD agar plate incubated overnight was used to inoculate SBD broth containing 0, 1, 2, 4, or 8 µg/mL of Cx or Cx:β-cd inclusion compounds (1:1, 1:2, 1:3, 1:4). The cultures were incubated with agitation for 16 h at 34 °C. Stationary-phase cells were harvested by centrifugation at 2700 rpm in a centrifuge for 5 min and washed once with sterile distilled water. The net wet weight of the cell pellet was determined. To each pellet was added 3 mL of a 25% alcoholic potassium hydroxide solution, followed by vortexing for 1 min. Cell suspensions were incubated at 85 °C in a water bath for 1 h. Following this incubation, the tubes were allowed to cool to room temperature. Sterols were then extracted by the addition of a mixture of 1 mL sterile distilled water and 3 mL *n*-heptane, followed by vigorous vortexing for 3 min. The heptane layer was transferred to a clean tube and stored at 20 °C for 24 h. Prior to analysis, a 20-µl aliquot of sterol extract was diluted five times in 100% ethanol and analyzed spectroscopically over a range of wavelengths from 240 to 300 nm. The presence of ergosterol and the late sterol intermediate 24(28)DHE in the extracted sample resulted in a characteristic four-peaked curve. A dose-dependent decrease in the height of the absorbance peaks was evident, corresponding to decreased ergosterol concentration. Ergosterol content was calculated as a percentage of the wet weight of the cells using the following equations:

% ergosterol % + 24(28) DHE = [(*A*281.5/ 290) *F*] / pellet weight

% 24(28) DHE = [(*A*230/518) x *F*] / pellet weight

% ergosterol = [% ergosterol +%24(28) DHE] - %24(28)DHE

Where F is the factor for dilution in ethanol and 290 and 518 are the E values (%/cm) determined for crystalline ergosterol and 24(28)DHE, respectively.

Scanning electron microscopy (SEM)

SEM was used to assess cellular membrane integrity and morphologic changes caused by the drug treatments. Yeast suspensions (1 mL) treated with inclusion complexes were centrifuged at 3000 rpm for 5 min, and the resulting pellets were pre-fixed at 4 °C for 3 h in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2). After fixation, 5-µl aliquots of this dilution were added to a solid substrate made of mica. A contact tapping mode fluid cell was sealed against a freshly cleaved muscovite mica substrate using a silicone O-ring. Samples were dried for 7 d at 37 °C. Samples were then coated with gold in a Penning sputter system in a high-vacuum chamber and observed using a field emission scanning electron microscope (model Zeiss-DSM 950). Micrographs were obtained using secondary electrons.

RESULTS

Antifungal activity

The MIC_{90} data are summarized in Table 1. Cx alone exhibited robust fungicidal activity against C.a., with a MIC_{90} value of 2 µg/mL. The MIC_{90} value of the 1:4 Cx:β-cd complex, 0.5 µg/mL, differed significantly from that of Cx . Similar patterns were found with the 1:2 and 1:3 Cx:β-cd complexes, which exhibited MIC values of ~1 µg/mL.

Results from cell counts of viable cells obtained using a hemocytometer were in agreement with the MIC_{90} values (Table 1). For cells treated with 0.5 µg/mL Cx, the number of viable cells after treatment was 3.55×10^3. Increasing concentrations of β-cd in Cx:β-cd complexes (1:1, 1:2, 1:3, and 1:4) led to concomitant decreases in cell viability. . Indeed, as can be seen in Table 1, the 1:4 complex resulted in a 4-fold reduction in C.a growth (0.5 µg/ mL), relative to Cx alone.

Table 1. Inhibition concentration of Cx and Cx:β-cd inclusion compounds (1:1; 1:2; 1:3; 1:4) against *Candida albicans* and viable cell counting.

Groups	MIC (µg/mL)	MIC (mol/L)	Cell viability
Chlorhexidine chlorhydrate	2 µg/mL	$(3.46 \times 10^{-6}$ mol/L)	3.55×10^3
Chlorhexidine:β-cd 1:1	2 µg/mL	$(3.46 \times 10^{-6}$ mol/L)	0.87×10^3
Chlorhexidine:β-cd 1:2	1µg/mL	$(1.73 \times 10^{-6}$ mol/L)	0.32×10^3
Chlorhexidine:β-cd 1:3	1 µg/mL	$(1.73 \times 10^{-6}$ mol/L)	0.32×10^3
Chlorhexidine:β-cd 1:4	0.5 µg/mL[*]	$(8.65 \times 10^{-7}$ mol/L)	0.07×10^3
Fluconazole	19 µg/mL	$(3.28 \times 10^{-7}$ mol/L)	4.25×10^3
β-cd			8.88×10^3
C.a			9.30×10^3

P<0.01 significance level

Effects of Cx:β-cd complexes on sterols as shown by SQM analysis

Yeast was treated for 24 h with the complexes analyzed at concentrations equivalent to the MIC_{90} values. Spectrophotometric analysis (wavelength range, 240–330 nm) showed that treatment with Cx alone or with the inclusion compounds, particularly Cx:β-cd 1:3 and 1:4, led to significant reductions in ergosterol levels (Fig. 1). Membrane leakage increased progressively as cells were exposed to greater concentrations of β-cd (1:1, 1:2 vs. 1:3 or 1:4, P <0.05). Furthermore, the 1:3 and 1:4 exposed cells showed significant increases in ergosterol levels in the SQM test, 100% (3.15 to 3.74×10^3) is the ergosterol content of the *C.a* cells without exposition to the drugs.
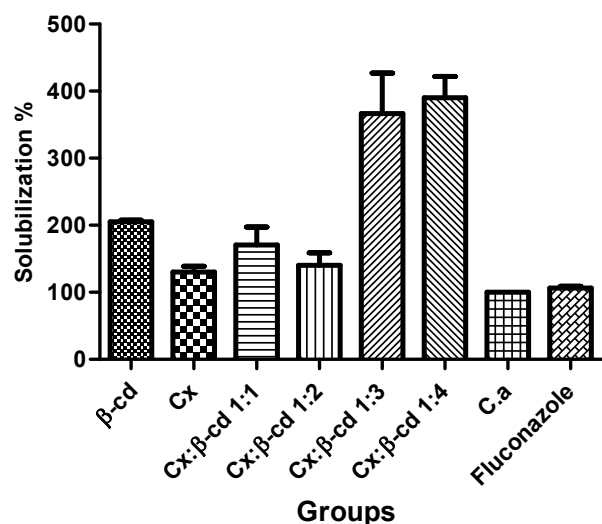
Figure 1. Percentage of solubilization of *Candida albicans* membrane treated with Chlorhydrate of Chlorhexidine, Chlorhexidine:β-cyclodextrin 1:1, 1:2, 1:3, 1:4; β-cyclodextrin and Fluconazole.

SEM analysis of Cx:β-cd complex interactions with membranes

SEM revealed crystalline structures in some regions of the membranes that were exposed to Cx (see micrograph in Fig. 2A). The β-cd alone control molecules (Fig. 2B) produced a semi-crystalline structure. However, the addition of β-cd molecules appeared to disrupt the crystalline character of the Cx structures. That is, with increasing ratios of β-cd in the Cx:β-cd complexes from 1:1 to 1:4 (Fig. 2C-F) —and therefore increasing concentration of β-cd being delivered to the cellular membranes—the membrane-associated Cx molecules appeared to lose their regular crystalline appearance, becoming increasingly amorphous (Fig. 3C-D).

C.a. cells treated with Cx and Cx:β-cd complexes were also analyzed by SEM; untreated *C.a.* cells served as controls to assess normal yeast membrane morphology (Fig. 3A). In the Cx alone treatment group, Cx molecules surrounded the yeast cells, binding the membranes only at singular points (Fig. 3B). Structures resembling Cx were visualized around the yeast

cellular membrane, generally near one or two break points in the membrane. Initial morphological changes in Cx-treated cells included the appearance of indentations on the surface of some cells as well as the presence of micelle-like structures and membrane residues near the cells (Fig. 3C-D).

The membranes of *C.a.* cells treated with Cx:β-cd complexes (1:1, 1:2, 1:3, and 1:4 ratios) showed slight differences in the size and shape of the liquid-ordered domain relative to membranes exposed to Cx alone (Fig. 3C-F), and showed various leakage points in the membrane structure that were not seen in Cx-exposed membranes (Fig. 3B). Exposure of *C.a.* to Cx:β-cd complexes at a concentration of 1 μg/mL led to dramatic changes in membrane structure. In these samples, the *C.a.* membrane domains and inclusion compounds coalesced into larger, less rounded domains (Fig. 3E-F). Additionally, the cells appeared to lose their characteristic shape and to allow leakage of intracellular substances (Fig. 3 C-F) (11, 20, 38).
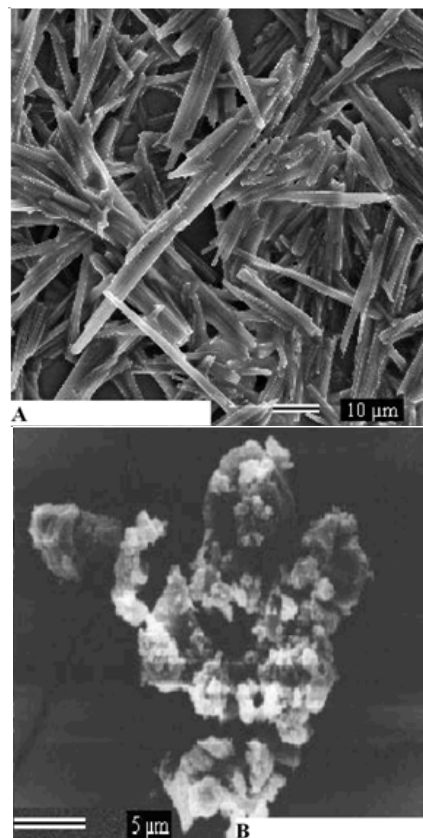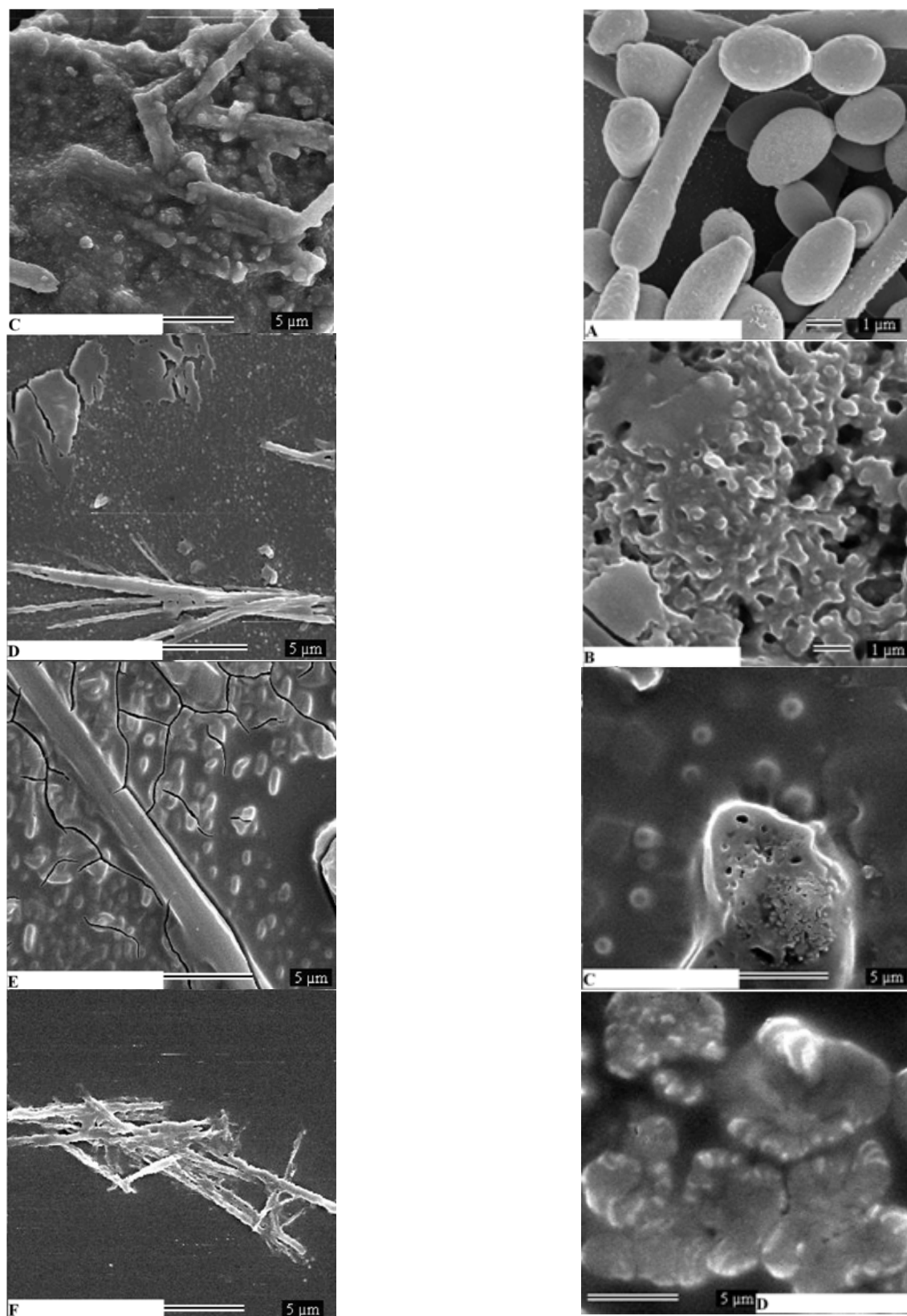
Figure 2. SEM micrographs of Chlorhexidine Chlorhydrate (A), Beta-cyclodextrin (B), Chlorhexidine:beta-cyclodextrin 1:1(C), 1:2 (D), 1:3(E), 1:4(F).
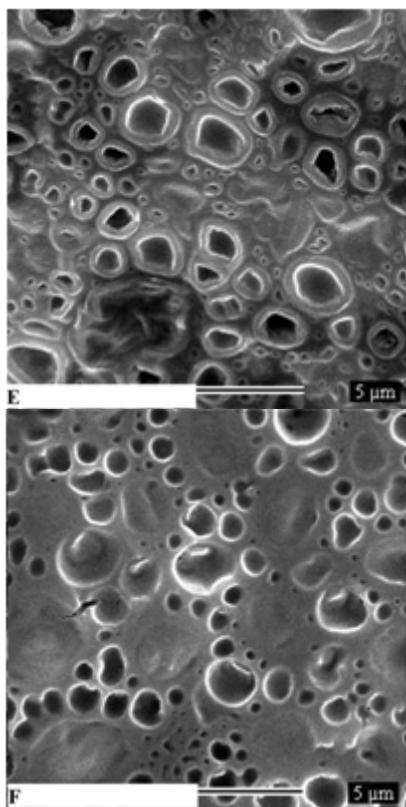
Figure 3. SEM micrographs of *Candida albicans* untreated (A) and C.a cells treated with Chlorhydrate Chlorhexidine (B), Chlorhexidine:β-cyclodextrin 1:1(C), 1:2 (D), 1:3 (E), 1:4 (F).

DISCUSSION

In this study, enveloping Cx in β-cd inclusion compounds was shown to significantly alter the inhibitory effect of Cx exposure on *C.a*., and greater molar ratios of β-cd were associated with enhanced antifungal activity. The heightened antifungal activity of the Cx:β-cd complexes, especially the 1:4 complex, can be explained by the observed greater adhesion efficiency of these complexes with the membrane versus free Cx or β-cd. Indeed, as can be seen in Table 1, the 1:4 complex resulted in a 4-fold reduction in *C.a* growth (0.5 µg/ mL), relative to Cx alone.

Importantly, our cell counts of viable cells and MIC value data were in agreement with one another. The heightened

antifungal activity that is produced by enveloping Cx in β-cd inclusion molecules should allow Cx to be efficacious at lower concentrations. The antifungal activity on *C.a.* of Cx alone observed here was consistent with previous research, which reported that Cx inhibited *C.a* at a concentration of 2.19 µg/ mL (16).

Our SQM findings showing that the 1:4 ratio Cx:β-cd complex produced greater ergosterol extraction than the other lower ratio complexes indicate that the complex was more potent at the 1:4 ratio than at the lower ratio solutions analyzed. On the other hand, the toxicity of antibiotics, such as Cx, toward human cells may be reduced in the presence of β-cd inclusion compound molecules since the antibiotic molecules must then compete with phospholipids in order to interact with cholesterol and form pores (25, 32).

Our SEM images showed that exposure of cells to Cx:β-cd complexes with increasing amounts of β-cd led to significant increases in *C.a.* membrane leakage. These observations indicate that addition of β-cd can increase leakage of ergosterol from *C.a.*, a phenomenon which may lead to depletion of cellular amino acids and render the *C.a.* membranes unstable (32, 41). This leak-inducing effect is likely due to the intrinsic chemical affinity of β-cd molecules, wherein hydrophobic interactions and electrostatic contributions favor binding of β-cd with cellular membrane molecules (23, 25, 38, 39).

In general, membrane surfaces are strongly affected by the ability of the aqueous structures to form hydrogen bonds (24, 38). Hydrophilic membrane structure-disrupting cyclodextrins can enhance drug delivery by increasing the concentration gradient of a drug and favoring more rapid drug delivery to the membrane surface.

In this study, we found that sterol extraction increased with rising concentrations of β-cd in the Cx:β-cd complex solutions. The cholesterol content of the host cell membrane plays a particularly important role in the potential of agents to perturb membranes. The eukaryotic membrane cannot be permeabilized by Cx:β-cd because it contains cholesterol,

though drugs can bind lipoprotein rafts in the eukaryotic membrane, and then be taken up via endocytosis (3, 4). The cholesterol-free composition of the prokaryotic membrane makes prokaryotes susceptible to permeabilization. Hence, data describing the ability of molecules to extract sterol provide important information for understanding the function and mechanism of action of positively-charged antimicrobial agents and how they interact with eukaryotic and prokaryotic cell membranes.

Modifications of the plasma membrane lipid composition can affect the capacity of cells to fuse with model membranes. Hence, since membrane lipid composition has the potential to modulate membrane fusogenic capacity, it may also affect susceptibility to pathogens (31).

In conclusion, the experiments reported here demonstrated that β-cd has an important effect on the interaction between Cx and *C.a.* cells. We showed for the first time that β-cd facilitates the interaction between Cx and the yeast cell membrane by enhancing the molecular affinity of Cx for the membrane, wherein Cx:β-cd complexes form nanoaggregate clusters within the yeast cell membrane. We further showed that these Cx:β-cd nanoaggregates inhibit yeast growth by extracting ergosterol and thereby permeabilizing the yeast cell membrane. Finally, this permeabilization effect appears to enhance the antifungal efficacy of Cx.

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