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Antifungal Properties of Cationic Phenylene Ethynylenes and Their Impact on β-Glucan Exposure

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***Candida* species are the cause of many bloodstream infections through contamination of indwelling medical devices. These infections account for a 40% mortality rate, posing a significant risk to immunocompromised patients. Traditional treatments against *Candida* infections include amphotericin B and various azole treatments. Unfortunately, these treatments are associated with high toxicity, and resistant strains have become more prevalent. As a new frontier, light-activated phenylene ethynylenes have shown promising biocidal activity against Gram-positive and -negative bacterial pathogens, as well as the environmental yeast *Saccharomyces cerevisiae*. In this study, we monitored the viability of *Candida* species after treatment with a cationic conjugated polymer [poly(*p*-phenylene ethynylene); PPE] or oligomer ["end-only" oligo(*p*-phenylene ethynylene); EO-OPE] by flow cytometry in order to explore the antifungal properties of these compounds. The oligomer was found to disrupt *Candida albicans* yeast membrane integrity independent of light activation, while PPE is able to do so only in the presence of light, allowing for some control as to the manner in which cytotoxic effects are induced. The contrast in killing efficacy between the two compounds is likely related to their size difference and their intrinsic abilities to penetrate the fungal cell wall. Unlike EO-OPE-DABCO (where DABCO is quaternized diazabicyclo[2,2,2]octane), PPE-DABCO displayed a strong propensity to associate with soluble β-glucan, which is expected to inhibit its ability to access and perturb the inner cell membrane of *Candida* yeast. Furthermore, treatment with PPE-DABCO unmasked *Candida albicans* β-glucan and increased phagocytosis by Dectin-1-expressing HEK-293 cells. In summary, cationic phenylene ethynylenes show promising biocidal activity against pathogenic *Candida* yeast cells while also exhibiting immunostimulatory effects.**

Bloodstream infections affect a huge patient population in the United States, with more than 250,000 cases reported each year (1). Patients with indwelling medical devices, such as central venous catheters (CVCs), are most at risk for these infections (2). Frequently, various microorganisms from the skin of the patient, or the respective health care professional, can gain access through the catheter wound as a result of nonsterile conditions (3–6). Of these resulting bloodstream infections, *Candida* species account for 9% of all instances and are associated with an ~40% mortality rate (2, 7). The most commonly isolated fungal pathogen from bloodstream infections is *Candida albicans*, but the prevalence of other species, such as *Candida parapsilosis*, *Candida glabrata*, and *Candida tropicalis*, is increasing (8, 9).

An important determinant of pathogenicity among *Candida* spp. is the outer cell wall. The cell wall is composed primarily of carbohydrates and, structurally, is separated into two layers. The outer layer is composed mostly of cell wall glycoproteins with N- and O-linked mannans, and the inner layer is composed of β-glucan and chitin. The complexity of the cell wall contributes to various pathogenic factors, including adherence of the fungus and establishment of cross talk with the host, which is known as "glycan code" (10–12). Cell wall components, such as β-glucan and other polysaccharides, are also found in the extracellular matrix secreted by *Candida* species biofilms, which can contaminate the synthetic material surfaces of indwelling medical devices. β-Glucan in the extracellular matrix of *Candida* biofilms has been shown to sequester antifungal drugs, which contributes to the decreased drug susceptibility of biofilms (13–15).

Various antimicrobial impregnation approaches have been devised to prevent catheter infections. Catheter materials coated with chlorhexidine-silver sulfadiazine and minocycline/rifampin have shown trends in reduced infection rates, but their clinical effectiveness remains questionable (16, 17). Other treatments, including the use of silver-impregnated subcutaneous collagen cuffs, have also shown to be ineffective in recent trials (18, 19). CVC contamination generally requires device removal and replacement, in addition to a prolonged course of antifungal drug therapy, which raises concerns regarding drug toxicity and the development of antifungal resistance. Antifungal chemotherapy is also problematic, as many antifungal drugs are either toxic to the host (amphotericin B) or result in drug-resistant strains (triazoles) (20). Due to the high morbidity and mortality rates of catheter-related *Candida* species bloodstream infections, strategies for preventing medical device contamination by fungal pathogens remain a top priority for infection control.

In this study, we sought to elucidate the antimicrobial effectiveness of two *p*-phenylene ethynylene (PE) compounds against

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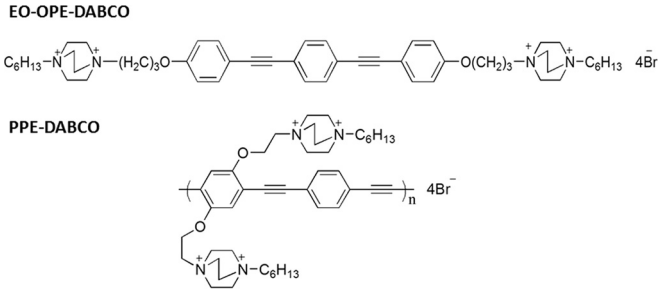

FIG 1 Molecular structures of oligomeric EO-OPE-DABCO and polymeric PPE-DABCO.

Candida species. A subset of conjugated polyelectrolytes, phenylene ethynylenes, have shown promising biocidal activity against Gram-positive and -negative bacterial pathogens, as well as the environmental yeast *Saccharomyces cerevisiae* (21–23). The chemical structure of these compounds renders them capable of inducing broad-spectrum cell damage. The phenylene ethynylenes studied consist of alternating phenyl and acetylenic groups with appended cationic groups (Fig. 1). The interaction of the cationic quaternary ammonium groups with net-anionic membranes and cell walls facilitates interactions with microbes, leading to membrane perturbation, pore formation, and the leakage of cell contents (24). In addition, when PEs are irradiated by the appropriate wavelength, the backbone produces reactive oxygen species (ROS) that induce rapid cell death (25, 26).

In previous studies, the oligomeric and polymeric molecular sizes of PE compounds have played an important role in their mechanisms of killing. The antimicrobial activity of these compounds is dependent on various factors, including molecular conformation, size, functional groups, and, of course, membrane composition of the target pathogen (22). The studies described here serve as a preliminary investigation into the utility of a cationic conjugated polymer, poly(*p*-phenylene ethynylene) (PPE), and an oligomer, "end-only" oligo(*p*-phenylene ethynylene) (EO-OPE), as antifungals. After treatment with the compounds—here referred to, respectively, as PPE-DABCO and EO-OPE-DABCO (where DABCO is quaternized diazabicyclo[2,2,2]octane)—the viability of *Candida* spp. was monitored using flow cytometry. This novel class of compounds may provide an innovative approach to the prevention of catheter-related bloodstream infections caused by *C. albicans* and other *Candida* species.

MATERIALS AND METHODS

Fungal culture. *Candida albicans* (ATCC MYA-2876), *C. parapsilosis* (ATCC 22019), and *C. glabrata* (ATCC 2001) were grown from glycerol stocks and stored at −80°C. This stock was transferred to 5 ml filter-sterilized yeast extract-peptone-dextrose (YPD) medium (Becton Dickinson) and grown for 16 h at 30°C, with a shaking speed of 300 rpm. Such growth conditions yielded yeast cells at the late log phase (27).

Following a 10-min centrifugation at 2,900 relative centrifugal force (RCF), the supernatant was replaced with sterile phosphate-buffered saline (PBS) and subsequently vortexed. This washing step was repeated a second time to mitigate cell debris. The cell concentration was then determined using a disposable hemocytometer (C-Chip; Bulldog Bio catalog no. DHC-N01).

Derivation of clinical isolate strains of *C. albicans*. Patient specimens (peripheral blood or catheter tips) were processed by Tricore Reference Laboratories (Albuquerque, NM) and identified as *C. albicans* by use of a Bruker Biotyper MALDI-TOF (matrix-assisted laser desorption ionization–time of flight mass spectrometry) system (MS ID score > 2.0). Clonal isolates so identified were subcultured on Sabouraud agar slants and provided to the investigators as unique strains. Isolate strains were provided in completely deidentified form according to procedures approved by the University of New Mexico School of Medicine Human Research Protections Office. For biocidal assays, clinical isolates were grown in YPD broth as described above.

Biocidal testing. All biocidal experiments were carried out in either translucent or opaque 1.5-ml microcentrifuge tubes, at cell concentrations of 5×10^6/ml in PBS. Two cationic compounds were tested, both based on the *p*-phenylene ethynylene backbone, with quaternized diazabicyclo[2,2,2]octane (DABCO) functionalities. EO-OPE-DABCO and PPE-DABCO stocks were prepared in sterile deionized water (18.2 MΩ · cm at 25°C) and contained 0.47% dimethyl sulfoxide (by volume) to improve solubility and minimize aggregate formation. Negative controls contained equal amounts of dimethyl sulfoxide.

Samples were exposed to controlled amounts of light by use of a 14-lamp photoreactor (LZC-4V; Luzchem Research, Ottawa, Ontario, Canada). A rotating carousel ensured that all samples receive equivalent levels of light exposure; ventilation kept the photoreactor below 30°C. EO-OPE-DABCO absorbs in the UV region (28), warranting the use of UVA lamps (350-nm emission peak; 4.46 ± 2.41 mW/cm^2) to optimize singlet oxygen yields. Conversely, 420-nm blue-light lamps (6.62 ± 2.93 mW/cm^2) were used in PPE-DABCO tests; unlike its oligomeric counterpart, polymeric PPE-DABCO absorbs in the near-visible range (29). Power density output was measured at the peak excitation wavelength for both lighting configurations. Data shown are an aggregation of the results of two independent replicate experiments.

Samples were then stained with 5 μM membrane-permeable SYTO 9 (Invitrogen catalog no. S34854) and 1 μM membrane-impermeable TO-PRO-3 (Invitrogen catalog no. T3605), both of which are nucleic acid stains. After 30 min, samples were evaluated by flow cytometry (FACSCalibur; Becton Dickinson). At least 10,000 events were evaluated in each trial. A heat-killed sample (70°C for 30 min) was used to identify the fluorescence characteristics of dead cells.

Fifteen-minute assays of activity in the dark were carried out in a somewhat different manner. Samples were prepared and stained with SYTO 9 and TO-PRO-3, albeit in the absence of any biocide. After a 30-min staining duration, EO-OPE-DABCO was added (10 μg/ml final concentration); the sample was then vortexed and analyzed by flow cytometry. Every minute, viability data were collected (again, 10,000 events/sample), for a total of 15 min. It is important to note that EO-OPE-DABCO was added one sample at a time, so that, in each case, flow cytometry readings could begin within 1 min of the introduction of the biocide.

Spectroscopy of β-glucan interactions. Stocks of *S. cerevisiae* β-(1,3)-glucan (high, medium, or low molecular weight [MW]; gift of Biothera, Eagan, MN), PPE-DABCO, and EO-OPE-DABCO were mixed with 10 mM phosphate buffer at pH 7.4 to a final concentration of 2 μg/ml for PPE-DABCO or EO-OPE-DABCO and 100 μg/ml for β-glucan. Solutions of 200 μl were transferred to a 160-μl-nominal-volume fused quartz fluorimetry cuvette and read on a PTI QuantaMax 40 steady-state fluorescence spectrophotometer (HORIBA Scientific, Edison, NJ) with photomultiplier tube detection. Emission spectra were obtained using excitation wavelengths of 350 nm for EO-OPE-DABCO and 420 nm for PPE-DABCO, and excitation spectra were obtained with the corresponding maximum emission wavelength.

Interactions of PPE-DABCO with glucan microparticles. Glucan microparticles were prepared from *C. albicans* SC5314 yeast cells using the extraction techniques described by Lowman et al. (30). Glucan microparticles were then suspended in PBS at a concentration of 1×10^5 ml^{-1}. Microparticles were sonicated for 10 min and subsequently vortexed for another 5 min. PPE-DABCO was then added at a concentration of 2 μg/ml and incubated at room temperature for 1 h. No PPE-DABCO was

added to the negative control. Five hundred microliters of each sample was transferred to petri dishes for examination by confocal microscopy. Excitation at 405 nm was used to induce fluorescence of PPE-DABCO.

Surface exposure of β-glucan. *C. albicans* yeast cells were treated in a manner similar to that of the previously described biocidal experiments. In an effort to maintain a consistent degree of cell death across samples, OPE-DABCO exposure to UVA light was limited to just 10 min. A thermal positive control was also implemented, which entailed heating samples to 100°C for 30 min. Following the appropriate treatment and removal from the photoreactor, samples were blocked with 1% (wt/vol) bovine serum albumin (BSA) for 30 min at room temperature. The samples were then treated with a primary antibody, anti-β-glucan IgG (catalog no. 400-2; Biosupplies, Victoria, Australia), at a final concentration of 10 μg/ml for an additional 30 min. Negative controls contained 10 μg/ml isotype-matched murine IgG in place of anti-β-glucan IgG. An Alexa Fluor 647-conjugated secondary antibody (Invitrogen catalog no. A21235) (1 μg/ml) and SYTO 9 (5 μM) in PBS plus 1% BSA were simultaneously added and allowed to stain cells for 30 min at 25°C prior to analysis by flow cytometry. Data shown are an aggregation of the results of two independent replicate experiments.

Tissue culture and transfection. HEK-293 cells (ATCC CRL-1573) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum (CS), 1% penicillin-streptomycin, 2 mM L-glutamine, and 1 mM sodium pyruvate at 37°C, 5% CO_2. The cells were then plated in 6-well plates at 1×10^5 cells per well. mApple-human Dectin-1A-C-10 (gift of Michael Davidson; Addgene plasmid no. 54883) was transfected into cells by following standard protocols using Fugene 6 (Promega product no. E2691). Cell cultures were used for further experimentation at 24 h posttransfection with growth in normal medium, as described above.

Phagocytosis assay. *C. albicans* yeast cells were subjected to the same treatment conditions described above for the β-glucan exposure study, before being spun down and washed in PBS. Following the last washing step, the *C. albicans* yeast cells were stained with 7.5 μM SYTO 9 and 75 μM CypHer5E NHS ester (GE Healthcare; PA 15401) for 1 h at 25°C. After the *C. albicans* cells were stained, they were added to live, Dectin-1A-C-10-transfected HEK-293 cells for 1 h. Next, ice-cold PBS was used to lift the HEK-293 cells off the plate. The controls with *C. albicans* or HEK-293 cells alone or the above samples with a mixture of *C. albicans* and HEK-293 cells were analyzed using an LSR Fortessa flow cytometer (Becton Dickinson) and FlowJo software (FlowJo, Ashland, OR). At least 10,000 side scatter (SSC)-positive events are evaluated in each trial. CypHer5E, SYTO 9, and mApple fluorescence was excited using the following laser lines: CypHer5E, 640 nm; SYTO 9, 488 nm; mApple, 561 nm. Emission from the same dyes was observed using the following optics: CypHer5E, band-pass 670/14 nm filter (center/full-width half maximum); SYTO 9, long pass 505 nm dichroic mirror and band-pass 530/30 nm filter; mApple, band-pass 582/15 nm filter. Temporal separation of excitation from independent laser lines and satisfactory spectral separation of emissions ensured negligible cross-talk between these three fluorescent probes. Data shown are an aggregation of the results two independent replicate experiments.

RESULTS

A series of biocidal studies were carried out to gain insight into the light-activated effects of EO-OPE-DABCO and PPE-DABCO on *Candida* species pathogens. Phenylene ethynylenes are unique in that their mechanism of action differs depending on the presence of light, in particular with respect to light intensity, emission wavelength, and duration (31). In the studies described here, duration of light exposure was the primary variable being studied. Light intensity was kept constant using a photoreactor with 14 interchangeable lamps. Lamps were chosen to have an emission wavelength overlapping the excitation spectrum of the phenylene ethynylene being used. UVA lamps that were centered on 350 nm were implemented for OPE testing, while 420-nm-centered lamps were used in PPE tests. With the light intensity and the spectrum being held constant for a given phenylene ethynylene, we focused on light exposure duration as the major focal point in an effort to discern the susceptibility of *C. albicans* to phenylene ethynylenes in the light versus that in the dark. Even though all samples were exposed to one of the two compounds for a total of 60 min, the duration of light exposure was varied by 4-min intervals and the balance of the 60 min of exposure was in the dark.

Figure 2A illustrates the biocidal activity of the two concentrations of EO-OPE-DABCO, 1 and 10 μg/ml. In the absence of light, a 1-μg/ml concentration of OPE killed 34% of *C. albicans* yeast cells; however, killing drastically increased with just minimal light exposure, as 2-log cell death was observed after just 8 min. Increasing the concentration to 10 μg/ml greatly improved the killing capacity of the OPE in the dark, resulting in 97% cell death. With minimal light exposure, 10 μg/ml EO-OPE-DABCO exhibited a profound biocidal effect, exceeding a 3-log reduction after just 4 min in UVA light. Both OPE concentrations exceeded 3-log kill (over 99.9% cell death) after 20 min of light exposure, and a 4-log reduction (99.99% cell death) was nearly achieved after 60 min of light exposure. Interestingly enough, lowering the concentration of OPE to just 1 μg/ml had little effect on light-activated biocidal activity but a far greater effect on killing in the dark. Some level of PE photodegradation was notable by 60 min (data not shown), which is why testing durations were limited to 1 h, as photodegradation limits $^1O_2$ generation.

Figure 2B illustrates the viability of *C. albicans* following exposure to PPE-DABCO. It is quite evident that unlike EO-OPE-DABCO, its PPE counterpart is nontoxic in the absence of light; even at a relatively high concentration of 10 μg/ml, little to no cell death was observed even after 8 min of exposure to 420-nm light. After 52 min of continuous light exposure, 10 μg/ml PPE was able to kill 99% of all *C. albicans* yeast cells. In summary, we found the killing by the OPE in the dark to be concentration dependent, while the activity in the light was not. Conversely, the PPE's killing in the dark was not dependent on concentration, since it failed to elicit membrane damage in that case. The biocidal activity of PPE-DABCO is predicated on light exposure.

The inability of PPE-DABCO to kill *C. albicans* yeast cells in the dark led us to question whether the polymer may bind extensively to cell wall components, which might limit its ability to access the cell membrane. We evaluated the interactions between the two PEs with soluble β-(1,3)-glucan extracted from *Saccharomyces cerevisiae* yeast cell walls (32). The structures of *S. cerevisiae* β-(1,3)-glucan and *C. albicans* β-(1,3)-glucan are quite similar, and the polysaccharide is an important part of *Candida* drug resistance and pathogenicity, amounting to 40% of the cell wall (33). Size-fractionated β-glucan samples (low MW, 11 kDa; medium MW, 150 kDa; high MW, 450 kDa) were tested (Fig. 3A to D; see Fig. S1 in the supplemental material). Excitation and emission spectra of EO-OPE-DABCO and PPE-DABCO were evaluated in the absence and presence of the soluble β-glucan.

We observed enhanced emission of both PEs upon the introduction of the high-molecular-weight β-glucan, which is indicative of complexation (34). Emission enhancement is more profound in the case of PPE-DABCO (Fig. 3A to D), suggesting that complexation with soluble β-glucan promotes disaggregation of the PE polymer. In addition, a small degree of red-shifting was observed, implying that rotation of the conjugated regions of the

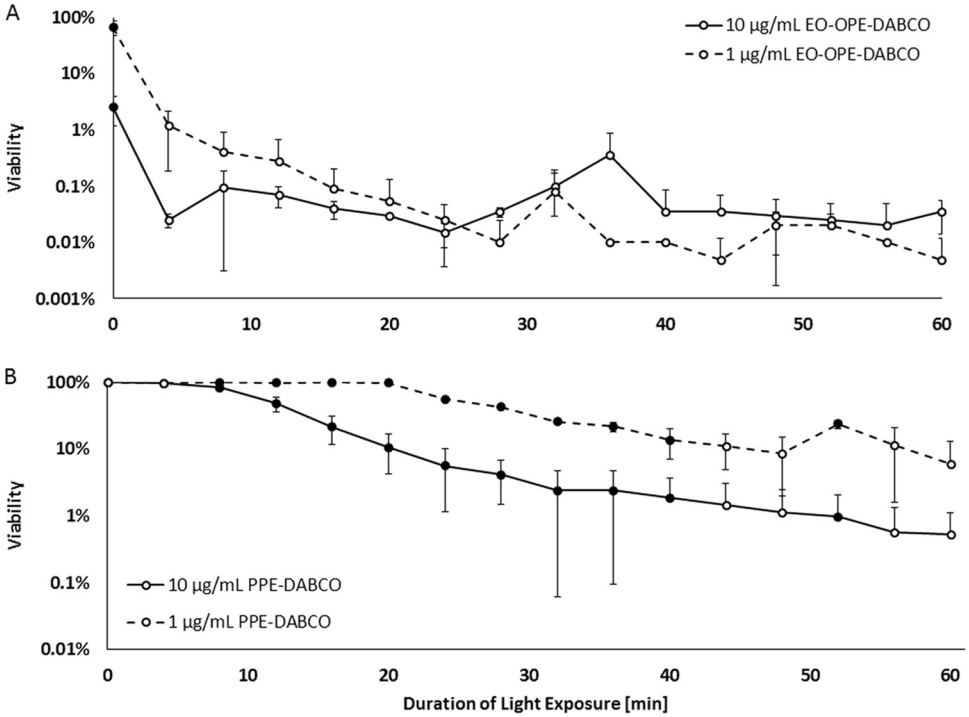

FIG 2 *C. albicans* yeast viability as a function of light exposure duration and antimicrobial concentration. Viability is shown on a logarithmic scale for EO-OPE-DABCO (A) and PPE-DABCO (B) at 1 μg/ml (dashed lines) and 10 μg/ml (solid lines). Significant differences in viability are indicated by filled data markers (P value ≤ 0.01).

PEs is restricted due to complexation with soluble β-glucan (35). The complexation between PPE-DABCO and β-glucan suggests that it may be largely sequestered in the cell wall; such positioning promotes interaction with components of the outer cell wall but may limit its ability to directly disrupt the plasma membrane.

These results shed light on the mechanisms by which EO-OPE-DABCO kills *C. albicans* yeast cells (Fig. 2A; see Discussion). With this in mind, the OPE became a focal point of this study from a biocidal perspective. With the determination that EO-OPE-DABCO was highly effective at killing standard laboratory strain *C. albicans* (SC5314), the question remained whether or not its biocidal efficacy would carry over to *C. albicans* clinical isolates.

Using a modified biocidal assay, six *C. albicans* clinical isolates were surveyed for their susceptibility to 10 μg/ml EO-OPE-DABCO in the dark. In this instance, the cells were stained with SYTO 9 and TO-PRO-3 before the introduction of EO-OPE-DABCO. Taking a flow cytometry dual-fluorescence measurement of 10,000 events every minute allowed for real-time reporting of OPE-induced membrane perturbation. The susceptibility of clinical isolates was gauged relative to that of *C. albicans* SC5314, as shown in Fig. 4. Three of the six isolates, TRL 001 (P value = 0.006), TRL 051 (P value = 0.0013), and TRL 057 (P value = 0.0003), showed significantly increased levels of OPE resistance within 15 min in the form of slower kinetics of killing and higher residual viability after 15 min of treatment. Conversely, no OPE resistance was observed in TRL 037, TRL 040, and TRL 052.

The variability in susceptibility to EO-OPE-DABCO among clinical isolates of one species (*C. albicans*) suggested that pathogenic non-*albicans Candida* species might also exhibit variable sensitivity to this biocide. To test this, we revised the above-mentioned 15-min flow cytometry assay to determine if EO-OPE-DABCO was more or less effective against *C. parapsilosis* and *C. glabrata* than it was to *C. albicans* SC5314. Figure 5 shows similar levels of biocidal activity against *C. albicans* and *C. parapsilosis* but less activity against *C. glabrata*, with about 50% surviving after 15 min of exposure. This result is consistent with the fact that *C. albicans* and *C. parapsilosis* share a closer phylogenetic relationship than is found between *C. albicans* and *C. glabrata* (36).

β-Glucan is highly immunogenic upon recognition by the innate immunoreceptors Dectin-1 and Mac-1 (37). Several prominent genera of fungal pathogens, including *Candida*, are known to employ an innate immune evasion strategy of masking β-glucan to restrict its exposure on the cell wall surface (38–41). We hypothesize that PE antimicrobials bound to cell wall constituents and exposed to light will generate singlet oxygen, leading to local cell wall damage, unmasking β-glucan and increasing immunogenicity. Using an anti-β-(1,3)-glucan primary antibody in tandem with a secondary fluorescently labeled antibody allowed for the comparison of β-glucan exposures following treatment conditions: PE in the dark, PE in the light, and a 60-min light negative control. *C. albicans* yeast treated with EO-OPE-DABCO in the dark, using conditions associated with high biocidal activity (Fig. 2), exhibited no increase in β-glucan exposure. Likewise, we observed no β-glucan unmasking with light-activated EO-OPE-DABCO. We then treated *C. albicans* with PPE-DABCO and observed β-glucan exposure. PPE-DABCO clearly binds to the fungal cell wall, as evidenced by strong PPE-DABCO emission upon 405-nm excitation using confocal imaging (see Fig. S2 in the supplemental material). In the absence of stimulation by light, PPE-DABCO treatment results in no significant increase in β-glu-

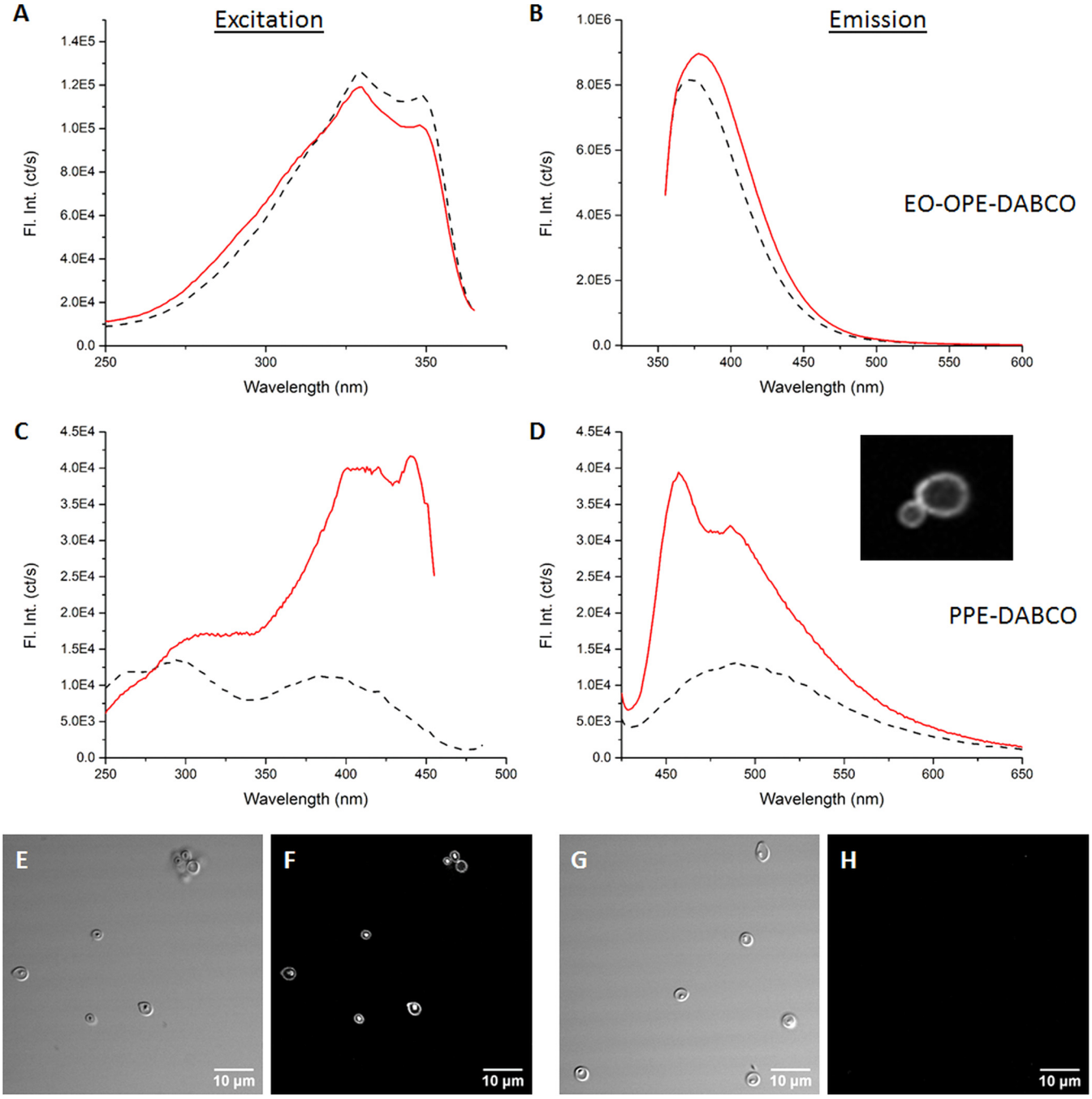

FIG 3 Excitation (A, C) and emission (B, D) spectra illustrating the interactions between high-molecular-weight soluble β-glucan and EO-OPE-DABCO (A, B) or PPE-DABCO (bottom, D). Dashed lines represent the spectra of PE compounds alone, and solid lines represent the spectra observed in PE–β-glucan mixtures. The inset in panel D illustrates fluorescent PPE-DABCO concentrated in the cell wall of a *C. albicans* yeast cell, as shown in Fig. S2 in the supplemental material. Fl. Int. (ct/s), fluorescence intensity (counts/second). Additionally, confocal microscopy images illustrate PPE-DABCO bound to glucan microparticles. A transmitted light image (E) and reflected light image (F) are shown, with 405-nm excitation being used to generate fluorescence of bound PPE-DABCO. No fluorescent signal is observed in the absence of PPE-DABCO (G, H).

can exposure. After illumination, PPE-DABCO-treated cell walls do show evidence of significant β-glucan unmasking (Fig. 6). These results suggest that β-glucan masking in *Candida* cell walls is light dependent and, presumably, mediated by $^1O_2$ and other ROS.

Given the evidence that PPE-DABCO can increase β-glucan exposure on *C. albicans* yeast cells, we tested whether the unmasking achieved by this treatment resulted in greater recognition of yeasts through the β-glucan receptor Dectin-1. HEK-293 cells were transfected with mApple-tagged human Dectin-1a, whereby expression is sufficient to drive phagocytosis of *C. albicans* yeast cells (42). Our transfection conditions resulted in Dectin-1-posi-

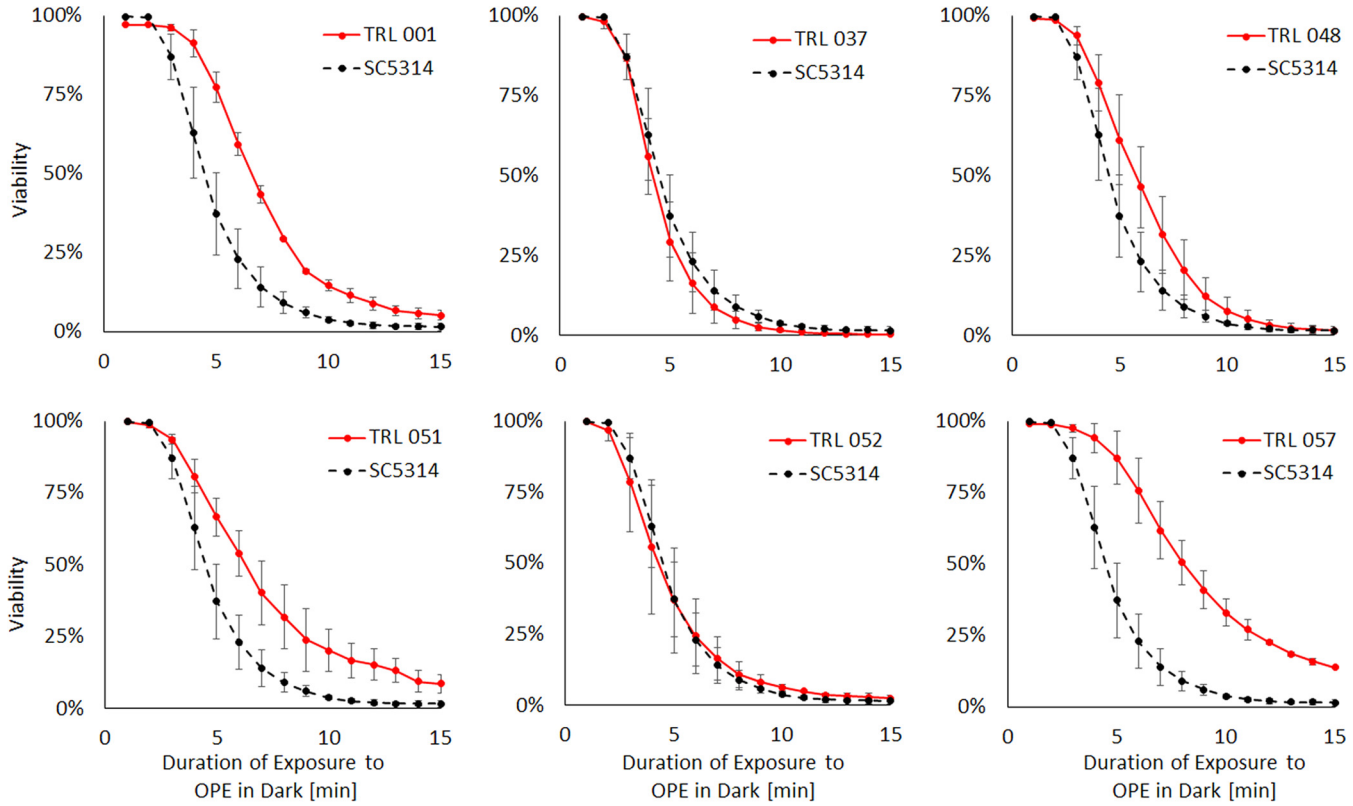

FIG 4 Susceptibility of various *C. albicans* clinical isolates to 10 μg/ml EO-OPE-DABCO in the dark. Laboratory strain SC5314 is shown for reference in all cases. Strains with the prefix "TRL" are recent (circa 2015) clinical isolates obtained as described in Materials and Methods.

tive HEK-293 cells, discriminated by a positive mApple signal, and nontransfected cells, which were negative for mApple and served as an internal control to assess Dectin-1 dependence of binding and phagocytosis. We employed a flow cytometric assay of yeast cell binding to and internalization by HEK-293 transfectants. Yeast cells were labeled with the pH-sensitive dye CypHer5E, which increases dramatically in emission intensity after internalization within acidic phagosomal compartments (43, 44). The CypHer5E signal was used to measure binding and internalization of yeast cells. Flow cytometry data were gated on HEK-293 cell-containing events for analysis, as defined by a high side scatter signal, which was significantly greater than that of free yeast cells. Yeast cells that were bound to HEK-293 cells registered a low CypHer5E signal. If yeast cells were internalized, the CypHer5E signal was much higher. The percentage of HEK-293 cells with yeast bound (for mApple-Dectin-1-positive and -negative cells) was determined by the percentage of SSC-gated events with a low or high CypHer5E signal. The extent of phagocytosis was assessed by the median CypHer5E fluorescence intensity within these populations (see Fig. S3 in the supplemental material).

As can be seen in Fig. 7, we observed minimal binding between mApple-Dectin-1-negative HEK-293 cells and untreated *C. albicans* yeast cells. Even if the HEK-293 cell has been transfected and is expressing Dectin-1 (mApple positive), β-glucan masking permits very little β-glucan to be accessible at the cell wall surface for Dectin-1 binding (as seen in Fig. 6). Conversely, PPE-treated *C.*

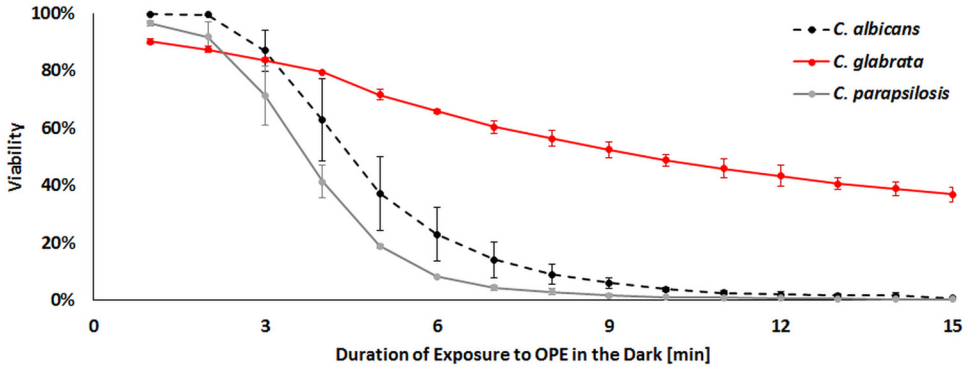

FIG 5 Viability of *C. albicans*, *C. glabrata*, and *C. parapsilosis* in the presence of 10 μg/ml EO-OPE-DABCO in the dark.

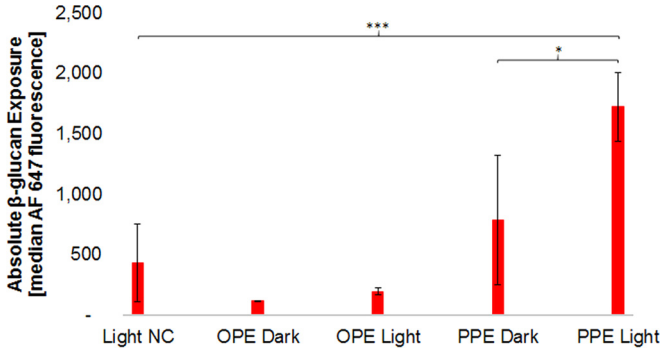

FIG 6 Absolute β-glucan exposure of *C. albicans* following various treatments. β-Glucan exposure was estimated from the median fluorescence signal of Alexa Fluor 647 (AF 647). The exposure duration for all samples was 60 min, with the exception of EO-OPE-DABCO exposure in the light, for which the exposure duration was limited to 10 min. NC, negative control; *, $0.01 \leq P$ value < 0.05; ***, P value < 0.001.

albicans yeast cells bind avidly to HEK-293 cells, and this binding is independent of excitation of PPE-DABCO or Dectin-1 expression by the HEK-293 cells (Fig. 7A). These data suggest that the binding of PPE-DABCO to *Candida* cell walls alters their surface properties in ways that promote Dectin-1-independent adhesion to human cells, perhaps through electrostatic and/or hydrophobic mechanisms. The extent of the interaction between the yeast cell and the HEK-293 cell is not dependent on the degree of incurred cell membrane damage, as *C. albicans* killed with light-activated PPE were no more likely to bind HEK-293 cells. Despite their ability to bind HEK-293, internalization of PPE-DABCO-treated *C. albicans* yeast cells required Dectin-1 expression and excitation of PPE-DABCO prior to binding (Fig. 7B). These data indicate that the β-glucan unmasking caused by light activation of PPE-DABCO on *C. albicans* cell walls results in the biological outcome of increased Dectin-1-dependent phagocytosis.

DISCUSSION

Cationic phenylene ethynylenes exhibit biocidal effects against *Candida* yeast. Despite its intrinsic resistance to cationic and oxidative stresses (45, 46), *C. albicans* was highly susceptible to EO-OPE-DABCO and, to a lesser extent, to PPE-DABCO. The biocidal activity of these compounds against *C. albicans* utilizes a dual mechanism combining light-independent cationic stress and light-dependent oxidative stress. These results resemble those of previous studies, which demonstrate the effectiveness of a dual-mechanism mode of biocidal action (47). Unlike other broad-spectrum antimicrobials, PEs exhibit low levels of *in vitro* toxicity

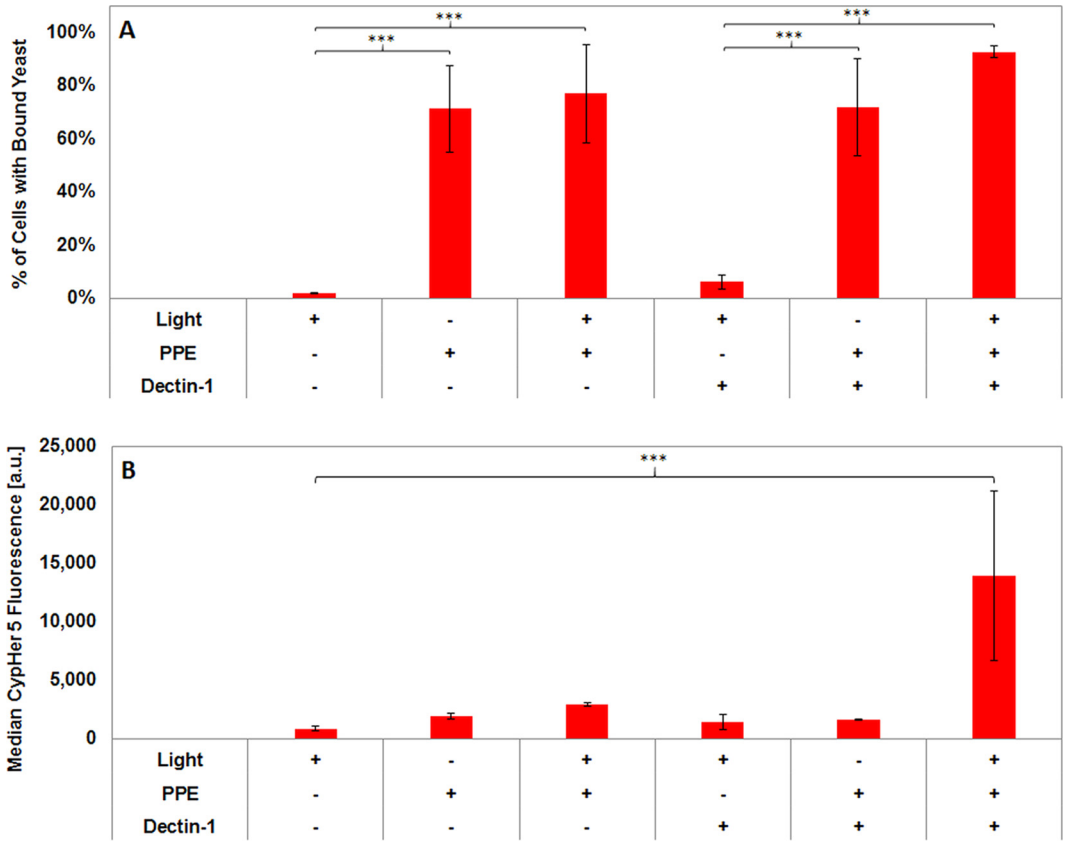

FIG 7 PPE-DABCO induces *C. albicans* yeast phagocytosis by HEK-293 cells in a manner that requires illumination of PPE-DABCO and expression of the β-glucan receptor Dectin-1. Prior to the addition of HEK cells, samples were first treated with 10 μg/ml PPE-DABCO for 1 h and then subsequently stained with CypHer5E and SYTO 9. (A) PPE-DABCO treatment, with or without illumination, induces increased binding of *C. albicans* to HEK-293 cells in a Dectin-1-independent fashion. (B) Phagocytosis of *C. albicans* yeast bound to HEK-293 cells requires Dectin-1 expression and is induced by light treatment of PPE-DABCO. a.u., arbitrary units; ***, P value < 0.001.

against mammalian cells (48), making them attractive candidates in numerous clinical applications.

Therefore, it is relevant to note that all clinical isolate strains exhibited significant levels of killing during a 15-min exposure to EO-OPE-DABCO. The partial resistance of some clinical isolate strains may derive from adaptations of the pathogen to growth in the host, which may cause changes in cell wall structure and upregulation of mechanisms that permit growth under adverse conditions, such as leukocyte-derived ROS in the phagosomal environment (49, 50). Previous research has also suggested a correlation between *Candida* resistance to the antifungal drug amphotericin B and cell wall structure and composition (51, 52).

While *C. parapsilosis* was found to be as susceptible to EO-OPE-DABCO in the dark as *C. albicans*, *C. glabrata* displayed an inherent resistance. *Candida* spp. experience cationic stress as they interact with innate immune defenses. For example, cationic antimicrobial peptides, such as Histatin-5, are deployed in host defense against *Candida* spp. and are thought to work by disrupting fungal plasma membrane integrity (53). *C. glabrata* is noted for its resistance to killing by cationic antimicrobial peptides in comparison to *C. albicans* and other pathogenic *Candida* species (54–56). Furthermore, the decreased ability of EO-OPE-DABCO to kill *C. glabrata* resembles the results of a previous study, in which a 10-μg/ml concentration of the compound failed to kill 99% of *S. cerevisiae* yeast cells, even after an hour in the light (23). Although *S. cerevisiae* is benign, it is closely related to *C. glabrata* (32, 36). Finally, *C. glabrata* is also known to have robust antioxidative defenses that allow it to survive in the phagosome (57, 58) and that may impact its ability to resist oxidative killing by cationic phenylene ethynylenes.

Interactions with β-glucan. An interesting result of this study is that PPE-DABCO strongly associates with soluble β-(1,3)-glucan (Fig. 3C and D), which is important for structural support of the cell wall of *C. albicans* (32), as well as glucan microparticles (Fig. 3E and F). We speculate that the PPE-DABCO–β-glucan interaction may directly cause more global disruption to the cell wall, and it is likely that the targeting of polymeric phenylene ethynylenes to cell wall polysaccharides places them in an ideal position to induce reactive oxygen-mediated damage to cell wall components after photoactivation. Using transmission electron microscopy and other techniques, Wang et al. demonstrated this behavior with PPE-DABCO and Gram-negative *Escherichia coli* (31). However, we also note the alternative possibility that glucan exposure upon treatment with PPE-DABCO and light might require cell wall remodeling downstream of cell wall stress responses due to ROS generated by excited PPE-DABCO.

Conversely, the OPE appears far less prone to complexation with the soluble β-(1,3)-glucan. Although this may limit the ability of OPE to unmask mannoproteins and reveal more β-(1,3)-glucan (Fig. 6), the lack of interaction with the glucan likely allows the molecule to quickly penetrate the cell wall and to access and damage the cell membrane. Abundant lateral noncovalent interactions between β-glucan and PPE-DABCO may promote PE–β-glucan complexation, analogous to the role of interpolymeric hydrogen bonding in stabilizing lateral interactions of individual β-glucan polymers in aqueous solution (59, 60). PPE-DABCO is far larger than its oligomeric counterpart and has numerous sites where weak interactions with β-glucan polymers may form; furthermore, extensive valency of laterally aggregated β-glucan would make this interaction very strong. Emission enhancement of the PE polymer (Fig. 3) resembles that of a previous study, in which PPE-DABCO was shown to exhibit similar photophysical effects in methanol, as opposed to water (29). Therefore, we suggest that abundant complexation with soluble β-glucan disaggregates PPE-DABCO in aqueous buffer.

These results help to rationalize the inability of PPE to kill *C. albicans* in the dark. It is likely that PPE-DABCO, which exhibits a strong propensity to interact and associate with β-glucan, is limited in its ability to fully penetrate the cell wall and that much of the compound is sequestered on β-glucan in the cell wall. Given the limited radius of destruction of singlet oxygen and the density of organic material in the cell wall capable of quenching singlet oxygen, this association may limit the depth of cell wall permeation by PPE-DABCO and its capacity to directly perturb the yeast's plasma membrane, in comparison to EO-OPE-DABCO. However, an advantage of PPE-DABCO's ability to bind β-glucan may be an increased specificity for and targeting to the fungal pathogen's cell wall, as opposed to host tissues, which are devoid of β-glucan. The oligomer, on the other hand, appears far less likely to interact with β-glucan, which may allow it to permeate the fungal cell wall more readily and to better access the yeast's plasma membrane.

PPE-DABCO unmasks β-glucan. Furthermore, PPE-DABCO displays immunostimulatory attributes, particularly in the light. This polymer was found to unmask the outer cell wall of *C. albicans* yeast cells in such a way that β-(1,3)-glucan could more easily be recognized and bound by pattern recognition receptor Dectin-1. PPE-DABCO binds to yeast cell walls (see Fig. S2 in the supplemental material). The chemical basis of this binding may relate to direct interactions between PPE-DABCO and β-(1,3)-glucan, as discussed above. Additionally, PPE-DABCO may interact electrostatically with anionic moieties in the outer cell wall. Ultrastructural studies have described the presence of evenly dispersed anionic sites on the *C. albicans* yeast surface (61). Also, *C. albicans* N-linked mannans contain abundant oligomannose side chains attached via anionic phosphodiester linkages that could provide sites of electrostatic binding for polycations like PPE-DABCO (62, 63). In either configuration, PPE-DABCO would be ideally positioned in the outer cell wall to damage mannoproteins that are thought to provide β-glucan masking. Our results suggest that merely the binding of PPE-DABCO to *C. albicans* increases the adherence of the yeast to HEK-293 cells in a receptor-independent fashion, suggesting that PPE-DABCO alters cell wall surface characteristics in ways that impact the interaction with host cells nonspecifically (Fig. 7A). However, increases in both β-glucan exposure and Dectin-1-dependent phagocytosis require excitation of PPE-DABCO, which probably results in direct oxidative damage to the cell wall, leading to β-glucan unmasking. This is the first instance in which PEs have been demonstrated to elicit immunostimulatory attributes. Our work demonstrates that the biocidal and immunostimulatory properties of phenylene ethynylene antimicrobials make them promising candidates for novel antimicrobial applications to improve the health outcomes of patients with life-threatening fungal infectious diseases.

Potential antimicrobial applications. Some antifungal medical applications envisioned for phenylene ethynylenes concern the treatment of medical devices or topical applications in wound care. For instance, prevention of catheter infections by the use of improved antimicrobial materials or preventive device treatment regimens could save lives, improve quality of life, and reduce costs

associated with treating catheter-associated infections. However, providing light to activate phenylene ethynylenes in the catheter context is an important challenge. First, we showed that EO-OPE-DABCO has strong biocidal activity against *C. albicans*, even without light activation, so it is possible that this compound could be used in antifungal lock solutions even without illumination. Second, central venous catheters are susceptible to contamination via their hubs and injection ports. These device parts are external and accessible for illumination by external sources or integrated light-emitting diode (LED) sources. The port and hub lumen could be regularly filled with a phenylene ethynylene antimicrobial and subjected to light-induced disinfection as part of a routine infection prevention program. Third, phenylene ethynylene compounds could be grafted to external medical device materials (e.g., central venous catheter hubs, ports, and tubes) to incorporate surfaces that could be regularly disinfected by the application of light. In addition, phenylene ethynylenes could find medical use in the area of wound care as topically applied antimicrobials and by incorporation into wound dressings. In this setting, light activation could be applied manually from external sources during dressing changes or in a more automated fashion from an integrated dressing LED light source, to prevent contamination of the wound. Additional costs associated with the use of antimicrobial catheter treatments and associated illumination systems would be offset by prevention of the substantial costs associated with the treatment of catheter-associated infections, as well as by improvements in patient morbidity and mortality.

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