Surface-Active Antifungal Polyquaternary Amine

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There is a distinct need for antimicrobial compounds that can act at surfaces without leaching into the environment. Such materials should be easy to synthesize, be easy to apply to surfaces, and display reasonable levels of antimicrobial and antifungal activity. Here we describe such a surface-active compound and demonstrate its ability to inhibit the growth of the filamentous fungus *Aspergillus niger*. We have synthesized a series of polyquaternary ammonium compounds by atom transfer radical polymerization. Two members of this series were tested for their ability to inhibit the growth of *A. niger*. The compounds were dried onto surfaces, and the treated surfaces were then used as growth chambers for *A. niger*. A water soluble polyquaternary amine compound was shown to effectively kill *A. niger* in solution in a dose-dependent manner. Conversely, a water insoluble polyquaternary amine compound was shown to kill only the fungi in direct contact with the material on the surface. These results have important implications for the development of effective, environmentally benign, surface-active anti-fungal compounds.

Introduction

In a world where the use of soluble antimicrobial compounds leads to rapid emergence of resistant strains, there is a need for materials that can kill bacteria and fungi but remain bound to surfaces and are thus less likely to induce resistance. Cationic antimicrobials are especially well positioned to play a role in the development of self-disinfecting surfaces[1]. The ability of these compounds to kill or inhibit a wide range of microorganisms allows them to be used for a number of applications from hospital surfaces and medical devices to building materials and filtration devices.

Among the most commonly used of the cationic antimicrobials are the quaternary ammonium salts,[1] and within this group, the polymeric quaternary amines show great promise in the realm of surface active compounds. Our current interest is to investigate the properties of polyquaternary amines with regard to their use as surface active antifungals. Since much of the recent investigation into the antimicrobial activity of polyquaternary amines has been directed toward activity against bacteria, it is useful to review briefly the growth of filamentous fungi as a contrast to the strictly single-celled lifestyle of the bacteria and yeasts.

Vegetative growth of filamentous fungi begins with the germination of spores. Spore germination leads to the formation of tubular hyphae which grow by apical extension and subapical branching.[2−3] Macroscopically, the mycelium forms as a radially symmetric colony that expands at a constant rate from the site of spore germination[4−6] The hyphal mat, or mycelium, grows on the surface and, whenever possible, the subsurface of the substrate. For the species of fungi commonly referred to as molds, reproduction is accomplished through the formation of spores which are disseminated primarily as aerosols.

The process of sporulation occurs in specialized structures which form on aerial hyphae which project through the water film that covers the substrate mycelium. The aerial structures must push through this water film to produce spores and release them into the atmosphere for distribution to new sites.[7] It is the requirement for the water film over the substrate mycelium that makes moist conditions ideal for fungal growth. During the sporulation process, the fungus must be able to overcome the surface tension of the water film and extend mycelia into the air.[7,8] Prevention of mold growth in moist environments is a challenge for researchers as well as commercial workers and consumers. In addition to the extracellular enzymes the fungi release to degrade their substrata, they are known to produce a large number of other types of molecules. Many of these molecules, such as the mycotoxins, are toxic to other organisms and can be quite dangerous. These toxins are thought to be responsible for many of the symptoms associated with sick building syndrome.[9,10]

There remains a need within the building industry for a stable, surface-active, antifungal that can be applied either pre- or post-construction. An attractive compound would be stable to transient immersion in water and would prevent fungi from growing on the treated surface. To address this need, we have synthesized a polymeric quaternary amine via the living polymerization, atom transfer radical polymerization (ATRP). Previous work has shown that the surface-bound and the soluble polymer have significant biocidal activity against the bacteria *Escherichia coli* and *Bacillus subtilis*.[11]

Activity against bacteria does not ensure activity against fungi, but there are indications that synthetic polymeric quaternary amines, which are polycations, will have activity against fungi. Supporting the possibility that poly-quaternary amines will be effective at reducing fungal growth are recent reports that

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chitosan, a polycationic polymer of glucosamine, shows antifungal activity.[12,13] The activity of this material is thought to be related to its polycationic nature. Several reports have suggested that surface bound polycations are capable of killing various microbes, including yeasts by disrupting the integrity of the cell membrane.[11,14−18] Several mechanistic hypotheses have been put forward to explain the wide range of cells that are susceptible to polyquaternary amines including recruitment of membrane lipids into membrane blebs causing disruption of function[1] and direct insertion of the polymer into the membrane.[14] A somewhat simpler and perhaps more universal mechanism posits that the charge density of the surface induces an ion exchange, causing essential structural divalent ions to move out of the membrane resulting in a loss of membrane integrity.[18] Recently, preparations of quaternized poly(2-dimethylaminoethyl methacrylate) (poly-DMAEMA) have been synthesized as cationic surfactants,[19] as polymer microspheres,[20] and on glass and paper surfaces,[10] and all demonstrate high levels of antibacterial activity, and it is possible that the mechanism by which they kill cells is dependent upon the physical form that they take. Soluble polymers are more likely to be able to penetrate cell walls and membranes, whereas surface bound ones are more likely limited to a charge-transfer mechanism.

One way to begin to answer mechanistic questions is to compare the behavior of very similar molecules with slightly different physical properties. To that end, we have synthesized two derivatives of poly-DMAEMA, These molecules, PQA-C6 and PQA-C12, differ in that the monomers were quaternary amines where one substituent on the nitrogen was 6 or 12 carbons, respectively (see below). The major physical difference between the two polymers is solubility, with PQA-C6 being highly water soluble and PQA-C12 being virtually water insoluble. We have tested these compounds for their ability to inhibit the growth of *Aspergillus niger*. Herein we show that both PQA-C6 and PQA-C12 inhibit the growth of *A. niger*. PQA-C6 is solution-active, and PQA-C12 is particularly effective as a surface-active agent.

Materials and Methods

Materials for Polymer Synthesis. *N*,*N*-Dimethylaminoethyl methacrylate (DMAEMA), fluorescein-*O*-methacrylate, ethyl 2-bromoisobutyrate, 1,1,4,7,10,10-hexamethyltriethylenetetramine (HMTETA), copper(I) bromide (CuBr), 1-bromohexane, 1-bromododecane, 2-bromo-2-methylproionic acid bromide, acetone, acetonitrile, chloroform, methanol, and *N*,*N*-dimethylformamide (DMF) were purchased from Sigma-Aldrich Chemical Co.

Measurement. ^{1}H NMR spectra were recorded on a Bruker Avance (300 MHz) spectrometer in DMSO-d_6 and $CDCl_3$. Routine Fourier transform infrared (FT-IR) spectra were obtained with an ATI Mattson Infinity series FT-IR spectrometer. Melting points (mp) were measured with a Laboratory Devices Mel-Temp. Number average molecular weights (M_n) and the distributions (M_w/M_n) were estimated by gel permeation chromatography (GPC) on a Waters 600E Series with a data processor, equipped with three polystyrene columns (Waters styragel HR1, HR2 and HR4), using DMF with LiBr (50 mM) as an eluent at a flow rate of 1.0 mL/min.

Monomer Synthesis. 1-Bromohexane (21.5 mL, 152.7 mmol) or 1-bromododecane (35 mL, 133.7 mmol) were added to a solution of DMAEMA (21.4 mL, 127.2 mmol) in acetonitrile (100 mL)/ chloroform (50 mL) and stirred at 40 °C overnight. The resulting residues were precipitated into diethyl ether and filtered. The filtrates were dried in vacuo and analyzed. **MAQAC**$_6$; yield 37.8 g (92%), mp 85∼88 °C, ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.87 (t, 3 H, J = 6.6 Hz, $N^+(CH_3)_2(CH_2)_5CH_3$), 1.29 (broad m, 6 H, $N^+(CH_3)_2CH_2CH_2$ ($CH_2)_3CH_3$), 1.69 (m, 2 H, $N^+(CH_3)_2CH_2CH_2$ $(CH_2)_3CH_3$), 1.91 (s, 3 H, CH_2═C(CH_3)), 3.15 (s, 6 H, $N^+(CH_3)_2(CH_2)_5CH_3$), 3.43 (m, 2 H, $N^+(CH_3)_2CH_2(CH_2)_4CH_3$), 3.78 (m, 2 H, $OCH_2CH_2N^+(CH_3)_2(CH_2)_5CH_3$), 4.64 (m, 2 H, $OCH_2CH_2N^+(CH_3)_2(CH_2)_5CH_3$), 5.76 and 6.08 (s, 2 H, CH_2═C(CH_3)) ppm. IR (KBr) 3421, 3003, 2955, 2925, 2859, 1721, 1636, 1464, 1318, 1296, 1158, 957, 929, 809, 650 cm^{-1}. **MAQAC**$_{12}$; yield 44.1 g (86%), mp 80∼83 °C, ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.86 (t, 3 H, J = 6.6 Hz, $N^+(CH_3)_2(CH_2)_{11}CH_3$), 1.30 (broad m, 18 H, $N^+(CH_3)_2CH_2CH_2$ $(CH_2)_9CH_3$), 1.74 (m, 2 H, $N^+(CH_3)_2CH_2CH_2$ $(CH_2)_9CH_3$), 1.93 (s, 3 H, CH_2═C(CH_3)), 3.50 (s, 6 H, $N^+(CH_3)_2(CH_2)_{11}CH_3$), 3.63 (m, 2 H, $N^+(CH_3)_2CH_2(CH_2)_{10}CH_3$), 4.15 (m, 2 H, $OCH_2CH_2N^+(CH_3)_2(CH_2)_{11}CH_3$), 4.64 (m, 2 H, $OCH_2CH_2N^+(CH_3)_2(CH_2)_{11}CH_3$), 5.65 and 6.12 (s, 2 H, CH_2═C(CH_3)) ppm. IR (KBr) 3421, 3003, 2955, 2925, 2859, 1721, 1636, 1464, 1318, 1296, 1158, 957, 929, 809, 650 cm^{-1}.

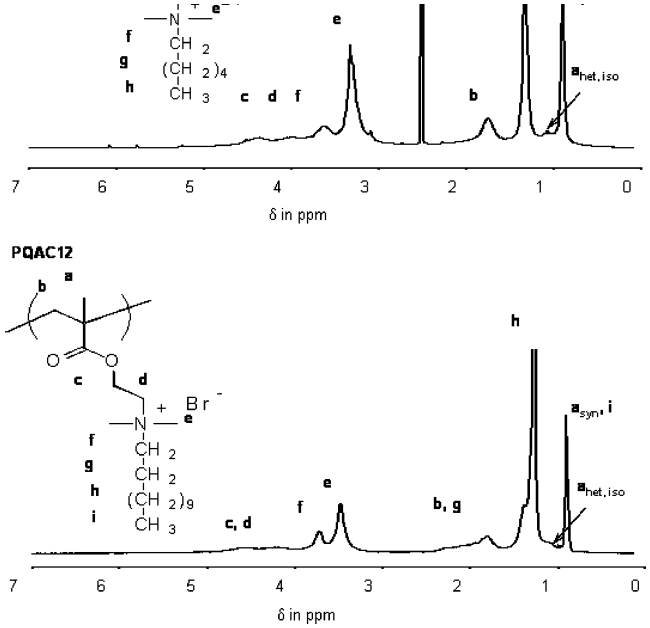

Figure 1. 1H NMR for PQA-C6 and PQA-C12.

Polymerizations. The monomer **MAQAC**$_6$ (3.2 g, 10.0 mmol) or **MAQAC**$_{12}$ (4.1 g, 10.0 mmol) was placed in a polymerization tube and covered with 2-bromoisobutyrate (14 μL, 0.1 mmol) as an initiator and HMTETA (46 μL, 0.2 mmmol) as the ligand in a solvent of acetone (35 mL)/ DMF (5 mL). The monomer solutions were degassed by five freeze−pump−thaw cycles. Freeze−pump−thaw was performed by placing the solution in a Schlenk tube, freezing in liquid N_2, exposure to a 30 mmHg vacuum for 5 min, and thawing at room temperature. Following the freeze−pump−thaw, CuBr was added (28.7 mg, 0.2 mmol) as a catalyst. The resulting mixture was heated to 40 °C for 20 h prior to dilution with acetone (10 mL). The polymerization solution was passed through a basic alumina column to remove the CuBr and then precipitated in hexane. The resulting polymer was dried in vacuo and analyzed. **PQAC**$_6$: yield 2.8 g (88%), M_n = 35 000 g/mol and the distributions (M_w/M_n) 1.25, ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.73 and 1.07 (broad s, total 6 H, $-CH_2C(CH_3)-$ and $N^+(CH_3)_2(CH_2)_5CH_3$), 1.31 (broad s, 8 H, $N^+(CH_3)_2CH_2(CH_2)_4CH_3$), 1.71 (broad s, 2 H, $-CH_2C(CH_3)-$), 3.29 (broad s, 6 H, $N^+(CH_3)_2(CH_2)_5CH_3$), 3.61 (broad s, 2 H, $N^+(CH_3)_2CH_2(CH_2)_4CH_3$), 4.00 and 4.41 (broad s, 2 and 2 H, $OCH_2CH_2N^+(CH_3)_2(CH_2)_5CH_3$) ppm. IR (KBr) 3343, 2956, 2927, 1725, 1632, 1485, 1266, 1235, 1151, 1057, 961, 752 cm^{-1}. **PQAC**$_{12}$: yield 3.1 g (76%), M_n = 93 000 g/mol and the distributions (M_w/M_n) 1.35, ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.84,1.23, and 1.75 (broad s, total 26 H, $-CH_2C(CH_3)-$ and $N^+(CH_3)_2CH_2(CH_2)_{10}CH_3$), 2.26 (broad s, 2 H, $N^+(CH_3)_2CH_2CH_2(CH_2)_9CH_3$), 3.30 (broad s, 6 H, $N^+(CH_3)_2(CH_2)_{11}CH_3$), 3.67 (broad s, 2 H, $N^+(CH_3)_2CH_2(CH_2)_{10}CH_3$), 4.02 and 4.55 (broad s, 2 and 2 H, $OCH_2CH_2N^+(CH_3)_2(CH_2)_{10}CH_3$) ppm. IR (KBr) 3432, 3005, 2955, 2926, 2860, 1725, 1639, 1475, 1266, 1235, 1151, 1050, 961, 752 cm^{-1}. Figure 1 shows the 1H NMR traces for the polymers.

Fluorescein containing polymer was synthesized by a similar procedure to that of PQAC12 synthesis except that DMAEMA containing 1 mol % of Fluorescein-O-methacrylate was used as the monomer solution. The resulting polymer was then quaternized with 1-bromohexane and 1-bromododecane to obtain the fluorescein-PQAs.

Synthesis of Covalently Attached Quaternary Ammonium Polymer Brush on Planar Glass Slides. Attempts to directly polymerize the quaternary amine polymers to the surface of a glass slide were unsuccessful, so slides were prepared similarly to the method previously reported[11] with changes in the initiator and procedure as described below.

Synthesis of 3-(2-Bromoisobutyroyl)-animopropyltrimethoxysilane. Allylamine (7.5 mL, 100 mmol), triethylamine (21 mL, 150 mmol), and dichloromethane (200 mL) were slowly added at 0 °C to a solution of 2-bromo-2-methylproionic acid bromide (13.6 mL, 110 mmol) in dichloromethane (50 mL). Next the mixture was stirred for 4 h at room temperature and was then washed with water followed sequentially by, a saturated aqueous solution of $NaHCO_3$, 0.5 M HCl, and a saturated aqueous solution of NaCl. The organic layer was dried over anhydrous $MgSO_4$, filtered, and concentrated by rotary evaporation to a final volume of 20 mL. A solution of the allylic compound (5 mL, 24.2 mmol) and toluene (20 mL) were mixed with trimethoxysilane (7.7 mL, 60.5 mmol) and Pt/C (10% Pt, 100 mg), and then the mixture was stirred at 60 °C overnight. Toluene and excess trimethoxysilane were removed by evaporation in vacuo. The crude residue was used for initiator immobilization after the Pt/C had been removed by filtration.

Initiator Immobilization of 3-(2-Bromoisobutyroyl)-animopropyltrimethoxysilane on Planar Glass Slides. Planar glass slides were placed into the initiator solution of 3-(2−bromoisobutyroyl)-animopropyltrimethoxysilane (100 μL), triethylamine (100 μL), and toluene (100 mL) at 80 °C for 1 h. The treated slides were rinsed sequentially with toluene, methanol, and acetone.

"Surface-Initiated" ATRP of DMAEMA on Slides. The initiator-modified slides were placed in a polymerization tube and covered with DMAEMA (8.4 mL), HMTETA (68 μL), and acetone (50 mL). The monomer solution was degassed by five freeze−pump−thaw cycles (as above), and Cu(I)Br (36 mg) was added. The mixture was heated to 40 °C and incubated for 20 h. The slides were then extracted in acetone for at least 6 h in a shaker to remove free polymer from the layer followed by a final rinse with methanol and then acetone.

Quaternization of Poly(DMAEMA) Brush on Glass Surfaces with 1-Bromododecane. Acetonitrile (50 mL), chloroform (10 mL), and 1-bromododecane (5 mL) were added to glass slides that had been modified with ester polymer brushes and incubated at 55 °C overnight. The glass slides were then extracted in chloroform, and rinsed with methanol and then with acetone.

Solubility Test of PQAs by Gel Permeation Chromatography. Stock solutions of PQA-C6 and PQA-C12 (50 mg/ml in 2-propanol) were applied to the wells of 24 well tissue culture dishes (Fisher, Pittsburgh, PA) at 1.5 mg/well. The tissue culture dishes with the PQAs were dried completely under a laminar flow hood for 1−2 h. Residual solvent was further removed by keeping the dishes overnight in a vacuum desiccator. For each well, 1 mL of sterile distilled water was added, and the plates were incubated at room temperature for 24 and 48 h. At the designated time, the entire volume was removed from the well, passed through a PTFE filter (0.2 μm) (Whatman, U.K.), and analyzed by gel permeation chromatography with a Waters model 2414 HPLC (Waters, Milford MA). Standard curves were generated using measured concentrations of the PQAs.

Fungal Growth. *Aspergillus niger* ATCC 9642 (American Type Culture Collection, Manassas VA) was cultured in Potato Dextrose Broth (PDB) or Potato Dextrose Agar (PDA) (Difco, Detroit, MI.). *A. niger* was cultured on PDA for 3−4 days to allow sporulation. Spores were collected by rubbing a sterile cotton swab gently on the surface of the plate followed by immersion in a 1 mL solution of sterile distilled water with 10% Tween 20 (Sigma Chemical Co., St. Louis MO). The number of spores was estimated with a hemocytometer and stocks of $(1-3) \times 10^6$ spores/ mL were prepared.

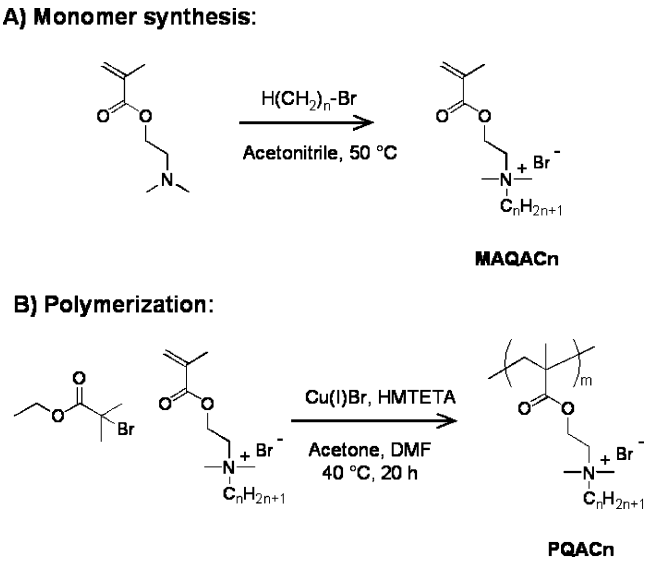

Figure 2. Synthesis of a poly-quaternary amines (PQA) by atom transfer radical polymerization (ATRP). For PQA-C6, $n = 6$, whereas for PQA-C12, $n = 12$; m is about 100 for PAQ-C6 and about 220 for PAQ-C12.

Fungal Growth Inhibition Studies. PQA C-6 or PQA C-12 (1.5, 1.0 or 0.5 mg) was coated on 24 well tissue culture plates as described above and dried under vacuum. Control wells were treated with IPA without added PQA. Depending on the experiment, each well was inoculated with between 10^3 and 10^4 *A. niger* spores and the plates were incubated at 30 °C for various times.

Antifungal activity was assessed by the MTT assay as described previously.[21,22] Briefly, growth was assessed by the reduction of the tetrazolium dye 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) (Sigma, St. Louis MO). After different time points (usually 24, 48, and 72 h), the medium was removed from each well and 0.1 mL of fresh PDA broth was added, followed by 20 μL of MTT solution (1 g/ L). After 4−12 h of incubation at 30 °C, reduced MTT was extracted with 1 mL of acidic isopropyl alcohol (1 mL of 12 N HCL in 100 mL of IPA) The solution containing the dissolved colored formazan was centrifuged, and the A_{570nm} of the supernatant was determined in a UV−vis spectrophotometer (Perkin-Elmer).

Fluorescent Probe Staining. FungaLight (Molecular Probes, Eugene OR), a fluorescent live/dead assay, was used as described by the manufacturer. Stained samples (100 μL) were applied to clean, L-lysine-coated (Sigma, St. Louis MO) glass slides, air-dried, and washed with sterile double distilled water. The slides were observed using a Leica inverted microscope (Leica, Wetzlar Germany) equipped with differential interference contrast (DIC) optics and a multiple fluorescent filter turret. In instances where the results were quantitated, images from 10 random fields were obtained using both DIC and epifluorescence. The number of viable and nonviable *A. niger* mycelia or spores were counted and tabulated as described in the text.

Results and Discussion

Synthesis and Characterization of the Quaternary Ammonium Polymers. Figure 2 outlines the synthesis of the polymeric quaternary amines used in this study. The polymers were assembled from monomers synthesized by quaternization of dimethylaminoethyl methacrylate (DMAEMA) with 1-bromohexane or 1-bromododecane.

The monomers, $MAQAC_6$ and $MAQAC_{12}$, were then polymerized by atom transfer radical polymerization (ATRP). One advantage of ATRP for preparing polymers is that the molecular weight distribution of the obtained polymer is relatively narrow. The molecular weight and the molecular weight distribution of

Table 1. Solubilization of PQAs in Water

sample	% recovery at 24 h	% recovery at 48 h
PQA-C6	83*	80
PQA-C12	0*	0

*The lower detection limit for both PQA-C6 and PQA-12 was 50 μg/ml.

the polymers, $PQAC_6$ and $PQAC_{12}$, were estimated by GPC measurement as 35 000 (degree of polymerization, ca. 100) and 93 000 (degree of polymerization, ca. 220) with relatively narrow poly-dispersity indices of 1.25 and 1.35, respectively.

Solubility of PQA-C6 and PQA-C12. Surface active biocides that could be applied to construction materials need to be hydrophobic, stable to environmental stresses, and insoluble in water. On the basis of previous studies demonstrating the capacity of polyquats to disrupt the membranes of bacteria,[10] we decided to synthesize water- and organic-soluble PQAs and compare their ability to kill fungi on surface and in solution.

When PQA-C6 is dried onto the bottom of a well in a 24 well tissue culture plate, it can be completely resolubilized in 1 mL of water. On the other hand, PQA-C12 deposited on the surface from a solution in 2-propanol (100 μl) is insoluble in water. Table 1 shows the results of one method used to determine the relative solubilities of PQA-C6 and PQA-C12 after they had been dried onto polystyrene surfaces. In this experiment, water was added to wells containing 1.5 mg of the PQAs, and the concentration of the PQA in the water solution was determined after 24 and 48 h. The results show that PQA-C6 is quickly solubilized, with most of the material in solution within 24 h. By contrast, the PQA-C12 was still undetectable after soaking in water for 48 h.

PQA-C12 Remains on the Surface after Immersion in Water. The surface activity and nonleachability of PQA-C12 can be investigated elegantly because when PQA-C12 is dried onto the surface of the culture dish, the polymer dries as a series of concentric rings or ripples. An example of these ripples is shown in Figure 3. A variant of PQA-C12 which contains a small amount of fluorescein covalently as part of the polymer chain was synthesized. Observation of ripples made with this fluorescent PQA-C12 shows that the PQA is concentrated in the ridges of the ripples (Figure 3B) with the troughs virtually free of the polymer. When wells prepared in this manner are soaked in water for 48 h (Figure 3C) the ripples are not dissolved. The same experiment performed with PQA-C6 shows

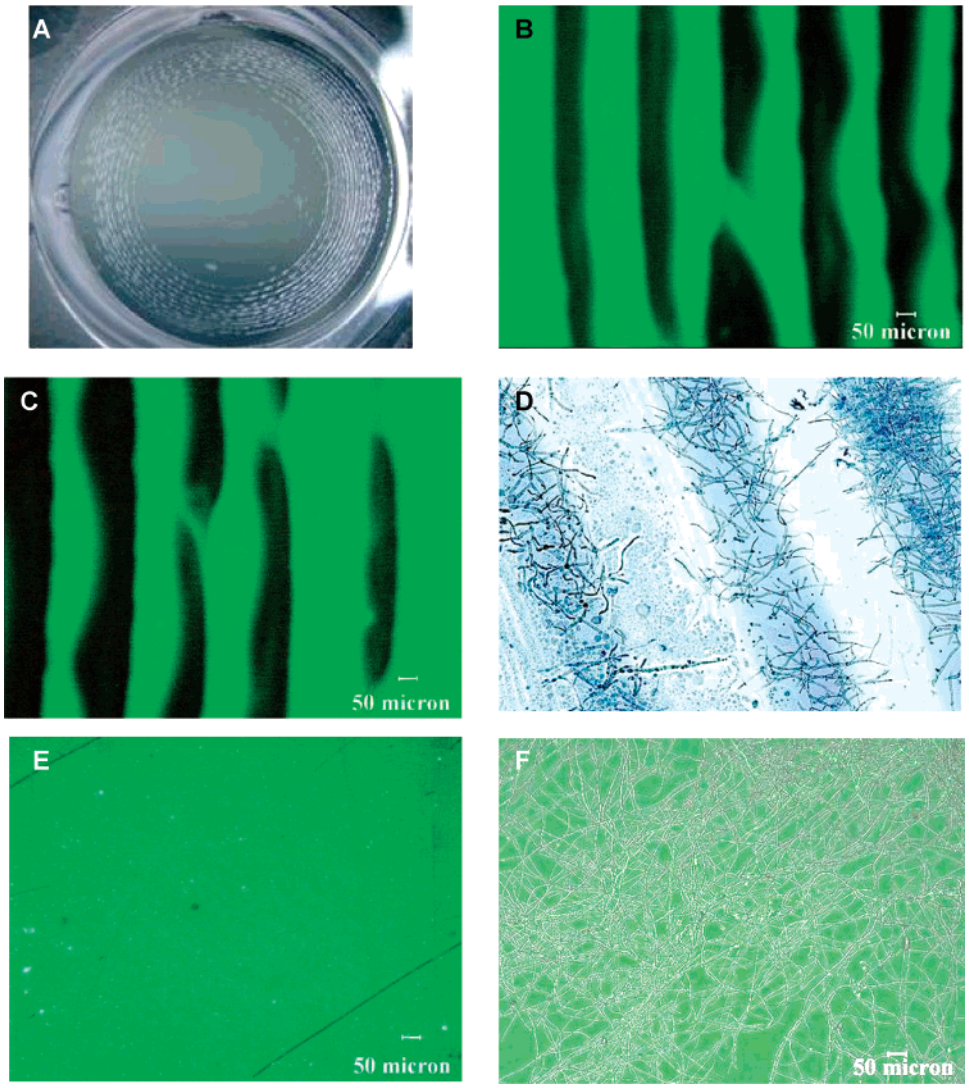

Figure 3. Impact of PQA-C12 on fungal growth. (A) Ripples formed in the well of a tissue culture plate. (B) Magnification of the ripples formed by the fluorescein containing PQA-C12. (C) Ripples in a well that was soaked with water for 48 h. (D) Growth of fungi in a well containing rippled PQA-C12. (E) Fluorescein staining of a PQA-C12 modified glass slide (fluorescein binds tightly to quaternary amines and fungi) after incubation with *A. niger* for 48 h showing a complete absence of fungal mycelia. (F) Fluorescein staining of an unmodified glass slide after incubation with *A. niger* for 48 h showing extensive fungal growth (note: the contrast and brightness of the image are enhanced relative to those in panel E).

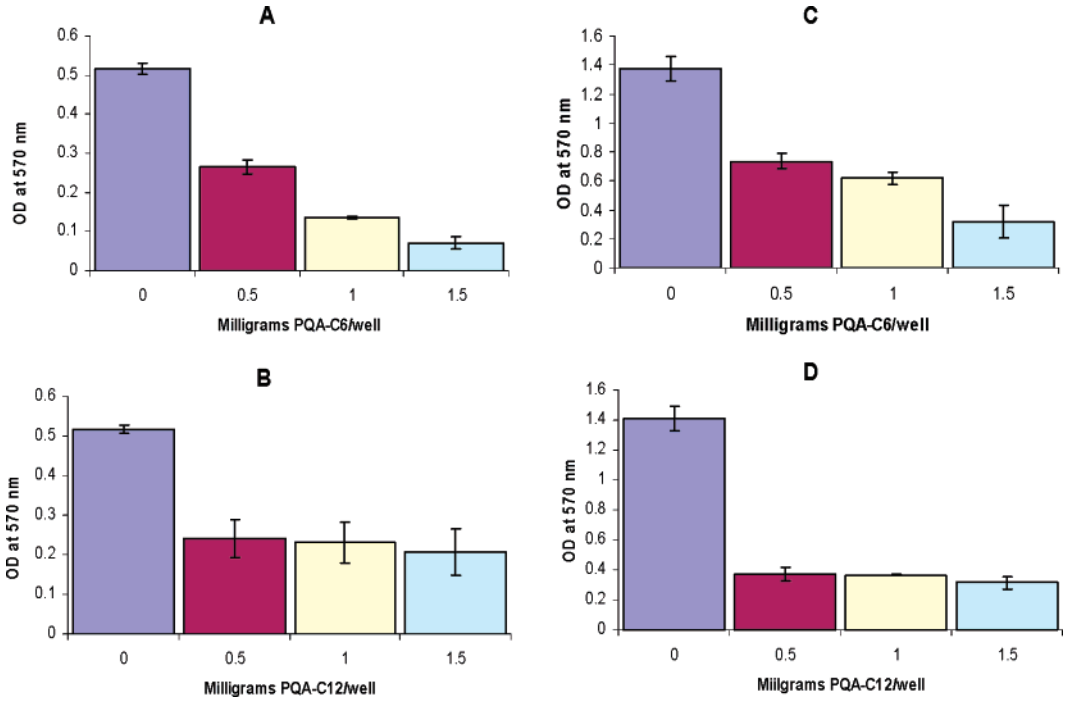

Figure 4. Growth inhibition of *A. niger* by PQAs. *A. niger* was grown in wells of 24 well culture dishes containing PQA at various concentrations. The extent of growth was measured at 24 (A and B) and 48 h (C and D) by MTT reduction. Results are reported as OD 570 of the extracted formazan. Results are the average of six determinations.

that the ripples disappear after as little as 30 min in water (data not shown). Interestingly, when rippled wells are inoculated with fungi, the mycelia grow in the troughs between the ripples (Figure 3D). This observation further confirms that PQA-C12 remains on the surface after extensive washing, that it is indeed a surface active anti-fungal, and that it does not leach from the surface.

We have also grown PQA-C12 from the surface of glass slides using ATRP. In this experiment, the covalent attachment of the polymer to the glass provides a uniform and nonleaching surface. Ellipsometry demonstrates that a 60−70 nm thick coating is routinely achieved by this method (data not shown). When fungi cultured on a PQA-C12 modified slide are compared to fungi on a plain glass surface, the results are striking (Figures 3 E and F). Fungi grow rapidly on the untreated glass surface, whereas the covalently coupled PQA-C12 prevents any fungal accumulation of the surface.

Dose Response Measurement of Inhibition of Fungal Growth by PQA's. PQA-C6 and PQA-C12 were dissolved in 2-propanol (50 mg/ml), applied to the wells of 6-well dishes at 0.5, 1.0, and 1.5 mg/well, and dried as described above. Each well was then inoculated with 5000 *A. niger* spores in 1 mL of media. After 24 or 48 h, the relative mass of actively metabolizing fungi was determined with the MTT assay. Figure 4 shows the results of these experiments.

The results of these inhibition studies are interesting in that the two very similar compounds display very different inhibition patterns. The PQA-C6 shows a classic dose response to the increasing amounts of polymer, whereas the PQA-C12 response is flat across all of the concentrations and both time points tested.

These results can be reconciled by again considering differences in the solubility of PQA-C6 and PQA-C12 in water. Since PQA-C6 is soluble in water, it will have complete access to the growing fungi throughout the media. Conversely, since PQA-C12 is insoluble, only the fungi that come in contact with the bottom of the well can be killed. This explains the flat dose−response of PQA-C12 because the effects would be more

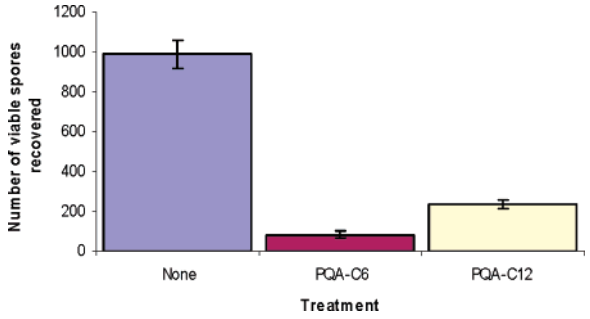

Figure 5. Reduction in the number of viable colonies following incubation with PQAs. *A. niger* spores were treated by PQAs (1.5 mg/well) for 8 h. Aliquots were taken, diluted, and plated on PDA agar to assess viability.

dependent on the surface area of the well than on the concentration of the polymer. In the PQA-C12 wells, the growing fungi are visible as a floating mat of hyphae on the surface of the media. Because PQA-C12 does not diffuse from the surface of the plate, the fungus on the surface of the media escapes the lethal activity of PQA-C12.

Effect of PQAs on the Number of Colony-Forming Units. To confirm that the viability reduction was due to reduced growth and not inhibition of oxidative metabolism, the number of viable fungal colonies was determined. In this experiment, 1000 spores were incubated in wells with 1 mL of media with or without PQAs at 1.5 mg/well. After 8 h incubation, aliquots were spread on potato dextrose agar plates to determine viable counts. As can be seen in Figure 5, the PQAs dramatically reduced the number of colonies that appeared. Interestingly, the relative reduction in colony forming units was similar to the reduction measured by the MTT assay for both compounds in the longer term experiments.

Since 8 h incubations approximate the time required for the added spores to undergo germination, the results in Figure 5 could be interpreted as representing either a reduction in the viability of hyphae or as interference at the level of the

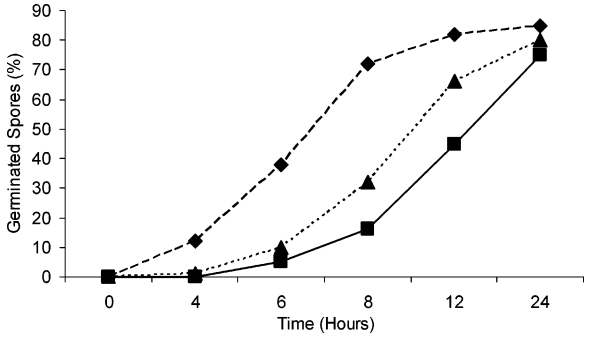

Figure 6. Spore germination in the presence and absence of PQAs. Spores in media were inoculated into wells and incubated at 30 °C. At various times, samples were removed and loaded into a hemocytometer. The proportion of spores with visible germ tubes was determined for each sample at each time point. Control (◆); PQA-C6 (■); PQA-C12 (▲).

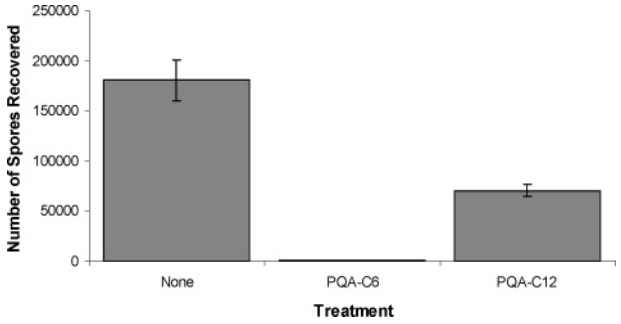

Figure 7. Number of spores recovered after one week incubation with and without PQAs.

germination of the fungal spores. Further experiments were conducted to differentiate between these possibilities.

PQAs Reduce the Rate of Germination but Do Not Reduce the Extent of Germination. To test whether PQAs were acting as sporicides, we inoculated plates with spores as above, removed aliquots at various time points, and visually counted the number of germinated spores. The surprising result of these observations is seen in Figure 6. For spores incubated without PQAs, nearly 75% were germinated by 8 h of incubation and >90% were germinated by 24 h. Spores in the presence of PQA-C6 and PQA-C12 were only 15 and 30% germinated at 8 h but were nearly 90% germinated by 24 h. While the 8 h results agree with the viable count assay (Figure 5), they do not account for the fact that germination was completely recovered by 24 h. Indeed, the results in Figure 5 show that all of the spores used in the test were recovered as colonies in the controls even though only 70% had germinated within 8 h. The spores from the PQA-treated wells would presumably complete germination on the agar plates after removal from the PQAs since they do so even in the presence of the PQAs.

Table 2. Germination of *A. niger* Spores in the Presence of PQAs

	number of spores per microscopic field	viable spores	dead spores
control	27.2 ± 1.8	24.0 ± 1.2	3.0 ± 1.2
PQA-C6 treated	26.0 ± 2.8	23.0 ± 2.6	3.0 ± 1.4
PQA-C12 treated	25.6 ± 2.8	22.6 ± 2.3	3.0 ± 1.2

A further possibility for the action of the PQAs is that they are killing the germinating spores before they can form a viable mycelium. Direct observation cannot differentiate between a viable germ tube and a dead one; however, specific dyes are available to differentiate living versus dead fungal cells and spores. Aliquots from the 12 h germination sample and the 24 h PQA treated germination samples were stained with FungaLite (Invitrogen) to differentiate live from dead spores with or without germ tubes. Ten full field images were captured for each condition, and the average number of live (green fluorescent) and dead (red fluorescent) spores was determined (Table 2). There was no difference between the control surfaces and the PQA surfaces with respect to the number of viable germinated spores.

It is apparent that the PQAs are not inhibiting the germination or the emergence of the germ tube so the growth inhibition must occur at a later stage of fungal development.

Sporulation of PQA-Treated *Aspergillus*. As noted above, mats of fungal growth could be seen floating above the PQA-

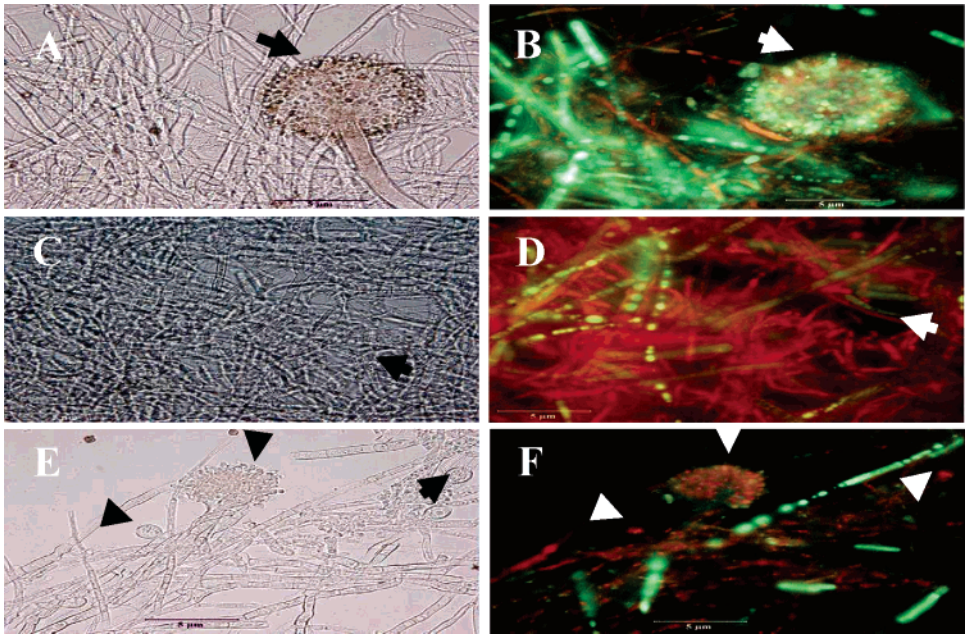

Figure 8. Observation of growth in the presence of PQAs. Cultures were grown for 48 h, harvested, stained with FungaLight, and observed at 640× with white light and with epi-fluorescence. A, C, and E are observed with Nomarski optics and B, D, and F under fluorescence. (A and B) Control culture without PQA. (C and D) Growth in the presence of PQA-C6. E and F: Growth in the presence of PQA-C12.

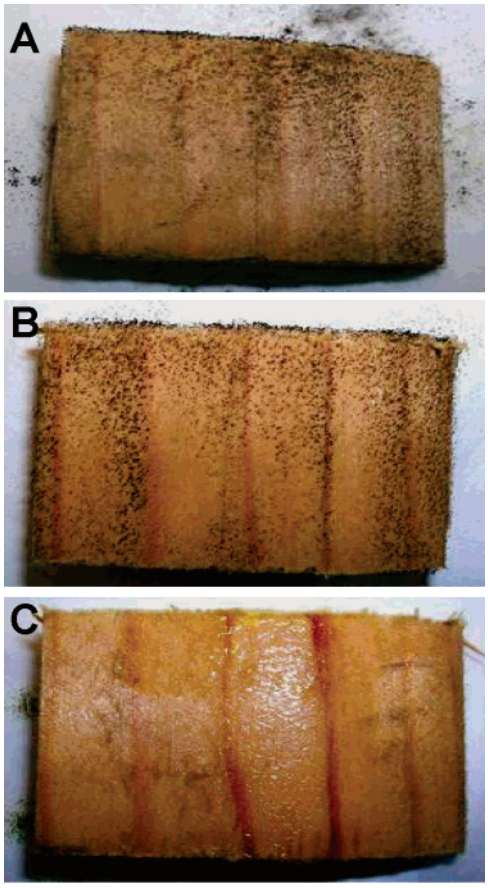

Figure 9. Wood coupons (2 × 2.5 cm) incubated with *A. niger* spores. (A) Untreated. (B) Wood coupon treated with isopropyl alcohol. (C) Wood coupon treated with PQA-C12.

C12 surfaces. The possibility that a portion of the mycelia might survive to produce spores was tested. *A. niger* was grown in plates containing 1.5 mg/well of the two PQAs. After one week of growth, the entire contents of the wells were harvested and the number of spores was determined. The results are shown in Figure 7. Again the relative number of spores produced approximates the results from the MTT assays (Figure 4). When all of the foregoing results are considered, the apparent difference in activities between the two PQAs can be attributed to the solubilities of the compounds and that PQA-C12 remains on the surface of the dish.

PQA Activity Against Mycelia. Because germination goes to completion with both PQAs, the FungaLight stain was used to test the activity of the compounds against mycelia. Observation of 48 h cultures growing in wells containing PQAs shows the presence of some mycelia. These were harvested and stained with the FungaLight stains (Figure 8). With the FungaLight, the live cells fluoresce green and the dead cells fluoresce red. In the control culture, there is abundant mycelial growth and the formation of conidiophores and very few dead cells. When grown in the presence of the water soluble PQA-C6, the very few mycelia that can be found show an overwhelming abundance of dead cells. The situation is different with surface-bound PQA-C12. In this case there is a mixture of live and dead mycelia and the presence of some conidiophores. In the example shown, the conidiophore and most of the attendant spores appear to be dead but other examples show some viable spores (not shown).

PQA-C12 Can Inhibit Fungal Growth on Wood. Reducing fungal growth in laboratory dishes helps us understand how PQAs work, but we are also interested in utilizing that knowledge. With this in mind, we painted the surface of wood coupons (2 × 2.5 cm) with PQA-C12 (1.5 mg/mL dissolved in 2-propanol) or with 2-propanol alone. The coupons were placed in the wells of a 6-well culture dish with 1 mL of potato dextrose broth and inoculated with 5000 *A. niger* spores. After 4 days in culture the coupons were photographed (Figure 9). Numerous small fungal colonies can be seen on the control and 2-propanol treated coupons, whereas the PQA-C12 coupon remains free from fungal growth.

Conclusions

PQA-C12 is a surface-active antifungal agent that effectively prevents the proliferation of fungal mycelia. PQA-C12 is sufficiently hydrophobic that, once it adsorbs to a surface, water will not remove it. In addition to a facile solution based synthesis, PQA-C12 can be grown directly from a surface, thereby imparting antifungal properties to that surface. The depth of mechanistic understanding that we have been able to gather has also led to a simple method to produce a nonleaching antifungal wood.

In summary, we have extensively studied the impact of PQAs on fungal growth and we find the following conclusions:

1. PQA-C6 and PQA- C12 significantly inhibit fungal growth.
2. PQA-C6 is soluble in water whereas PQA-C12 is not soluble in aqueous solution and acts as a nondiffusible antifungal coating.
3. The PQAs have the ability to kill fungal mycelia. The PQAs retard but do not inhibit germination of spores. Differences between PQA-C6 and PQA-C12 on the reduction in the rate of germination are likely to be a result of the solubility differences between these compounds. Both compounds thus apparently kill fungi at the more vulnerable mycelial stage.
4. The insolubility of PQA-C12 would argue more strongly for the ion exchange mechanism of cell kill. In order for the polymer to penetrate the fungal cell wall and penetrate the membrane, it would need to be configured in such a way that the polymer chains extended a considerable distance from the surface. The low solubility of PQA-C12 would suggest that this is unlikely in an aqueous environment. It is much more likely that the polymer is in a compact form near the surface on which it is coated and would not likely be able to see the cell membrane. The polycationic nature of the polymer would, however, persist in whatever architecture the polymer obtained, and this would be the active factor in the mycelial kill.

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