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Polyelectrolyte Multilayers Fabricated from Antifungal β -Peptides: Design of Surfaces that Exhibit Antifungal Activity Against *Candida albicans*

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Abstract

The fungal pathogen *Candida albicans* can form biofilms on the surfaces of medical devices that are resistant to drug treatment and provide a reservoir for recurrent infections. The use of fungicidal or fungistatic materials to fabricate or coat the surfaces of medical devices has the potential to reduce or eliminate the incidence of biofilm-associated infections. Here, we report on (i) the fabrication of multilayered polyelectrolyte thin films (PEMs) that promote the surface-mediated release of an antifungal β -peptide and (ii) the ability of these films to inhibit the growth of *C. albicans* on film-coated surfaces. We incorporated a fluorescently labeled antifungal β -peptide into the structures of PEMs fabricated from poly-L-glutamic acid (PGA) and poly-L-lysine (PLL) using a layer-by-layer fabrication procedure. These films remained stable when incubated in culture media at 37 °C and released β -peptide gradually into solution for up to 400 hours. Surfaces coated with β -peptide-containing films inhibited the growth of *C. albicans*, resulting in a 20% reduction of cell viability after two hours and a 74% decrease in metabolic activity after seven hours when compared to cells incubated on PGA/PLL coated surfaces. In addition, β -peptide-containing films inhibited hyphal elongation by 55%. These results, when combined, demonstrate that it is possible to fabricate β -peptide-containing thin films that inhibit the growth and proliferation of *C. albicans* and provide the basis of an approach that could be used to inhibit the formation of *C. albicans* biofilms on film-coated surfaces. The layer-by-layer approach reported here could ultimately be used to coat the surfaces of catheters, surgical instruments, and other devices to inhibit drug-resistant *C. albicans* biofilm formation in clinical settings.

Introduction

Candida albicans is the most common human fungal pathogen, and its ability to form biofilms on the surfaces of implanted medical devices creates numerous therapeutic challenges. In particular, *C. albicans* biofilms exhibit increased resistance to antifungal therapies and often necessitate the removal of colonized devices to eliminate infections.¹ The formation of a *C. albicans* biofilm begins when yeast cells adhere to a surface. As cells grow on the surface, germ tubes emerge and the cells produce extracellular matrix, eventually resulting in an organized network of yeast and hyphal cells that is completely

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surrounded by extracellular matrix.² One approach to reducing biofilm formation and ensuing infections is to design surfaces that inhibit the adhesion of cells, halting biofilm formation at an early stage. Although designing surfaces that reduce microbial adhesion has had some success,^{3–7} substantial inhibition of fungal biofilm formation on biomedical materials via this approach has been difficult to achieve.

A second approach to disrupting the early stages of biofilm formation is to design surfaces that inhibit the growth and proliferation of fungal cells on surfaces. For example, antimicrobial agents have been incorporated into a range of different materials to kill cells that either adhere to or reside near the surfaces of biomedical devices.^{4–7} This approach does not generally seek to directly inhibit the adhesion of microbes; however, the presence and/or release of the antimicrobial agent in the material can reduce the number of live cells on or near a surface, thereby reducing the likelihood of fungal colonization and biofilm formation. In this study, we developed a materials-based approach to inhibit fungal growth by designing thin polymer films that promote the localized and surface-mediated release of β -peptide-based antimicrobial compounds.

Layer-by-layer methods for the fabrication of multilayered polyelectrolyte thin films (or “polyelectrolyte multilayers”, PEMs) provide control over both the compositions and properties of ultrathin films and coatings and have been investigated broadly in numerous biomedical contexts.^{8–11} These methods are entirely aqueous and permit the fabrication of thin films using a broad range of synthetic or natural polymers. Fabrication typically involves the deposition of alternating layers of positively and negatively charged polymers (i.e., polyelectrolytes) onto a surface.^{12,13} With the adsorption of each subsequent polyelectrolyte layer on a surface, the exposed charge on the surface is reversed, allowing a polymer of opposite charge to be adsorbed on top of the previous layer. This layer-by-layer process permits the incorporation of other charged species into the structures of these assemblies. Of particular relevance to the work reported here, this approach can be used to assemble thin films and conformal coatings on the surfaces of objects having complex geometries and surface structures,^{8,14} including a variety of materials used to fabricate optical lenses, surgical instruments, implantable devices,^{15–22} and catheters on which *C. albicans* infections and biofilm formation occur in clinical settings. Numerous past studies have demonstrated that PEMs can serve as thin-film platforms for the localized and surface-mediated release of biologically active molecules for therapeutic applications such as gene delivery, drug delivery, or the design of antimicrobial surfaces.^{8–11,23–26} The versatility of PEMs makes them an appealing platform for the incorporation of antifungal molecules to create surfaces that prevent *C. albicans* biofilm formation.

Past studies have demonstrated that cationic amphiphilic oligomers of β -substituted amino acids (β -peptides) designed to mimic the structures of naturally-occurring helical antimicrobial α -peptides can be used to inhibit the growth of bacteria^{27–30} and planktonic *C. albicans* cells.^{31,32} These antimicrobial β -peptides can incorporate a cyclic residue such as aminocyclohexanecarboxylic acid (ACHC) to form stable amphiphilic, helical structures in hydrophilic environments,^{30,31} while helical antimicrobial α -peptides are typically linear molecules in aqueous environments and adopt an amphiphilic secondary structure when associated with microbial cell membranes.³³ Studies with β -peptides have demonstrated that it is possible to design β -peptides that are toxic toward bacteria and *C. albicans* at concentrations that do not significantly disrupt the membranes of mammalian cells (e.g., in red blood cell lysis assays).^{27–31} β -Peptides exhibit toxicity towards *C. albicans* biofilms formed on surfaces, and, more importantly, the presence of antifungal β -peptides at sufficient concentrations in solution can completely inhibit surface colonization and biofilm formation by *C. albicans*.³² These results suggest that approaches based on the incorporation and release of β -peptides from surfaces could reduce *C. albicans* colonization and

potentially inhibit or prevent biofilm formation. Past studies have reported that the incorporation of cationic, antimicrobial α -peptides into PEMs can be used to design films with antimicrobial properties.^{34–36} We hypothesized that β -peptides, although structurally different from the α -peptides previously embedded in PEMs, could be incorporated into PEMs to design antimicrobial films. The work reported here sought to (i) investigate the fabrication of PEMs that contain an amphiphilic β -peptide with potent antifungal activity and (ii) characterize the ability of these thin films to disrupt or inhibit the growth of *C. albicans* on coated surfaces.

Materials and Methods

Materials

Test grade n-type silicon wafers were obtained from Silicon Inc. (Boise, ID). Glass microscope slides were purchased from Fisher Scientific (Pittsburg, PA). Poly-L-lysine hydrobromide (PLL, MW = 15000 – 30000), poly-L-glutamic acid sodium salt (PGA, MW = 50000 – 100000), poly(sodium 4-styrenesulfonate) (SPS, MW = 70000), and branched poly(ethylene imine) (BPEI, MW = 25000) were obtained from Aldrich (Milwaukee, WI). Poly(allylamine hydrochloride) (PAH, MW = 60000) was purchased from Alfa Aesar (Ward Hill, MA). All polyelectrolytes were used as received without further purification. Lab-Tek II coverglass-bottomed 4-well chamber slides were obtained from Nalgene Nunc International (Naperville, IL). 2,3-bis(2-Methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carboxanilide (XTT) was purchased from MP Biomedicals (Solon, OH). Menadione was obtained from Sigma (St. Louis, MO). 7-Methoxycoumarin-3-carboxylic acid (coumarin), concanavalin A-Alexa Fluor 488 (conA), and propidium iodide (PI) fluorescent stains were purchased from Invitrogen (Carlsbad, CA). Fmoc-protected *trans*-2-aminocyclohexanecarboxylic acid (ACHC) was synthesized as described by Schinnerl *et al.*³⁷ Fmoc-protected β^3 -amino acids were purchased from PepTech (Burlington, MA). NovaSyn TGR resin and O-benzo-triazol-1-yl-N,N',N'-tetramethyluronium hexafluorophosphate (HBTU) were purchased from EMD Biosciences (La Jolla, CA). N,N-Dimethylformamide (DMF), N,N-diisopropylethylamine (DIEA), 1-hydroxybenzotriazole (HOBT), and piperidine were purchased from Aldrich (St. Louis, MO). {2-[2-(Fmoc-amino)-ethoxy]-ethoxy}-acetic acid (linker) was obtained from Bachem (Torrance, CA). RPMI 1640 powdered medium (with phenol red and L-glutamate, without bicarbonate) was purchased from Invitrogen, and 3-(N-morpholino) propanesulfonic acid (MOPS) was purchased from Fisher Scientific.

General Considerations

Quartz (1.8 cm $\times$ 0.7 cm) and silicon (3.5 cm $\times$ 0.7 cm) substrates were cleaned with acetone, ethanol, methanol, and deionized water, dried under a stream of filtered air, and cleaned further by etching in an oxygen plasma (Plasma Etch, Carson City, NV) for 5 minutes prior to film deposition.^{38,39} The optical thicknesses of three different films deposited on silicon substrates were determined in at least five predetermined locations near the top, middle and bottom portions of the films using a Gaertner LSE Stokes ellipsometer (632.8 nm, incident angle = 70°). Data were processed using the Gaertner Ellipsometer Measurement Program software package. Relative thicknesses were calculated by assuming an average refractive index of 1.58 for the multilayered films. Measurements of the fluorescence of solutions used to characterize the release of a fluorescently labeled β -peptide from multilayered films were made using a Fluoromax-3 fluorometer (Jobin Yvon, Edison, NJ). Fluorescence microscopy was performed using an Olympus IX70 microscope and images were obtained using the Metavue version 4.6 software package (Universal Imaging Corporation). Images were processed using NIH Image J software and Adobe Photoshop CS3. *C. albicans* cells in phase contrast and fluorescence microscopy images were counted

and measured using plugins associated with the NIH Image J software. Laser scanning confocal microscopy (LSCM) was performed using a Bio-Rad Radiance 2100 MP Rainbow laser scanning confocal microscope equipped with a multiphoton laser. Images were processed using the Bio-Rad LaserSharp 2000 processing kit and Adobe Photoshop CS3. Absorbance measurements in the XTT assay were obtained at 490 nm using a Tecan Infinite M1000 microplate reader (Tecan Group Ltd., Durham, NC).

β -Peptide Synthesis

The coumarin-labeled β -peptide having the structure coumarin-linker-(ACHC- β^3 Val- β^3 Lys)₃, was synthesized in a microwave reactor using Fmoc solid-phase synthesis, as previously described.^{32,40} Briefly, the β -peptide was synthesized on Novasyn TGR resin, and β -amino acids were coupled in a microwave reactor (2 minute ramp to 80 °C, 2 minute hold at 80 °C) using a solution of HBTU (0.5 M), HOBT (0.5 M), and DIEA (1.0 M) in DMF. Fmoc protecting groups were removed in the microwave reactor (2 minute ramp to 90 °C, 2 minute hold at 90 °C) with piperidine (20% v/v) in DMF. The ethylene glycol linker and 7-methoxycoumarin fluorescent label were appended at room temperature using the same coupling and deprotection reagents and at least 2 hour coupling and 20 minute deprotection reaction times. β^3 -Lys side chains were deprotected and the β -peptide was cleaved from the resin using a trifluoroacetic acid solution containing 2.5% (v/v) triisopropylsilane and 2.5% (v/v) water. The β -peptide was purified by reversed-phase high-pressure liquid chromatography; matrix-assisted laser desorption/ionization time-of-flight mass spectrometry confirmed that the isolated material had the expected molecular weight.³²

Fabrication of Multilayered Films

Solutions of BPEI, SPS, PAH, PLL, PGA, and coumarin-linker-(ACHC- β^3 Val- β^3 Lys)₃ used for the fabrication of the multilayered films were prepared at a concentration of 1 mg/mL using a 0.15 M NaCl solution in deionized water. All PEMs were deposited on glass or silicon substrates pre-coated with multilayered films having the general structure BPEI(SPS/PAH)₂ [i.e., fabricated by the deposition of one layer of BPEI followed by the deposition of two pairs of layers (or “bilayers”) of SPS and PAH]. These precursor films were fabricated using the following general protocol: (1) substrates were submerged in a solution of BPEI for 10 minutes, (2) substrates were removed and immersed in a rinse bath of 0.15 M NaCl solution in water for 1 minute followed by a second rinse bath for 1 minute, (3) substrates were submerged in a solution of SPS for 10 minutes, (4) substrates were removed and rinsed in the manner described in step (2), (5) substrates were submerged in a solution of PAH for 10 minutes, (6) substrates were removed and rinsed in the manner described in step (2), and (7) steps (3 – 6) were repeated.

Films fabricated using the β -peptide were then deposited on the precursor layers described above using the following general method: (1) substrates were submerged in a solution of PGA for 10 minutes, (2) substrates were removed and immersed in a rinse bath of 0.15 M NaCl solution in water for 1 minute followed by a second rinse bath for 1 minute, (3) substrates were submerged in the β -peptide solution for 10 minutes, (4) substrates were removed and rinsed in the manner described in step (2), (5) substrates were submerged in a solution of PLL for 10 minutes, and (6) substrates were removed and rinsed in the manner described in step (2). This cycle was repeated until the desired number of PGA/ β -peptide/PLL “trilayers” (typically 20) had been deposited. Films fabricated in this manner are denoted (PGA/ β -peptide/PLL)_x, where x is the number of trilayers deposited. Control films not containing the β -peptide were fabricated following the procedure outlined above, with the exception that a 0.15 M NaCl solution in water was substituted for the β -peptide solution used in step (3). Control films fabricated in this manner are denoted as (PGA/PLL)_x, where x is the number of bilayers deposited. For experiments designed to characterize film growth,

the optical thicknesses of films fabricated on silicon substrates were characterized by ellipsometry at intermittent points during layer-by-layer fabrication (see text).

Characterization of Film Stability and the Release of Incorporated β -Peptide

Experiments designed to investigate film stability and the release of incorporated β -peptide were performed in the following manner. Film-coated silicon substrates were placed in a plastic UV-transparent cuvette, and 1.0 mL of RPMI 1640 medium buffered to pH 7.0 with MOPS was added to cover the film-coated portion of the substrate. The samples were incubated at 37 °C and removed at predetermined intervals for characterization of (i) film thickness by ellipsometry and (ii) solution fluorescence by fluorometry. Films were rinsed under deionized water and dried under a stream of filtered, compressed air prior to measurement. The optical thicknesses of the substrates were then measured in at least five predetermined locations. The amount of β -peptide released from the films was characterized by measuring the fluorescence of incubation buffer solutions using a fluorometer at an excitation of 336 nm and an emission of 399 nm, corresponding to the excitation and emission maxima of the coumarin-labeled β -peptide. Fluorescence measurements resulting from these experiments were converted to β -peptide mass using a calibration curve generated using known concentrations of β -peptide. After each measurement, the film-coated substrates were placed in a new cuvette containing fresh RPMI 1640 medium and returned to the 37 °C incubator.

Biofilm Inhibition Assays

C. albicans SC5314 cells were grown overnight at 30 °C in yeast extract-peptone-dextrose (YPD) medium. Cells were washed with phosphate-buffered saline (PBS) and resuspended in RPMI 1640 medium buffered with MOPS (pH 7.0). The cell density was adjusted to OD₆₀₀ = 0.02 with RPMI 1640, and PI was added to the cells at a concentration of 1 μ g/mL. Glass substrates coated with either control films or β -peptide-containing films were placed in the wells of Lab-Tek II coverglass-bottomed 4-well chamber slides. *C. albicans* cell suspensions were added to the wells (500 μ L/well), and the samples were incubated at 37 °C. Chamber slides were removed from the incubator at 2 hours, 7 hours, and 24 hours post-seeding for characterization by fluorescence microscopy and quantification of growth inhibition.

Microscopy

After 2 hours and 7 hours of incubation with *C. albicans*, film-coated substrates were imaged on an Olympus IX70 fluorescent microscope using a Texas red filter set for PI fluorescence and a DAPI filter set for coumarin fluorescence. At least five images were acquired from random locations over the length of the substrate. At 24 hours post-seeding, biofilms formed on the substrates were washed with PBS, and an RPMI 1640 solution containing 1 μ g/mL PI and 50 μ g/mL conA was added to the biofilms. Biofilms were incubated with these stains for 45 minutes and then imaged using LSCM. PI was excited at 543 nm, and emission was collected above 610 nm; conA was excited at 488 nm, and emission was collected above 500 nm; the coumarin label was excited at 720 nm with a multiphoton laser, and emission was collected at wavelengths below 490 nm.

Quantification of Antifungal Activity

To quantify the antifungal activity of β -peptide-containing films at the 2 hour time point, fluorescence microscopy images were analyzed using NIH Image J. Live cells (impermeable to PI) and dead cells (permeable to PI) on control and β -peptide-containing films were counted to calculate the viability of cells on the films. At least 500 cells were counted on each substrate. NIH Image J was used to measure the length of at least 250 hyphae per

positively charged surface suitable for the subsequent deposition of PGA. The fabrication of β -peptide-containing PEMs was performed using an alternate dipping procedure. The iterative dipping of substrates into PGA, coumarin-linker-(ACHC- β^3 Val- β^3 Lys)₃ (β -peptide), and PLL solutions resulted in the growth of β -peptide-containing films (referred to hereafter as having the general structure (PGA/ β -peptide/PLL)_x, where x denotes the number of “trilayers” deposited). Although the mechanism for β -peptide incorporation into the films is unknown, the cationic nature of the β -peptide presumably led to its interaction with the negatively charged PGA. However, the β -peptide did not reverse or completely neutralize the negative surface charge of the PGA layer, allowing the deposition of the positively charged PLL.

Figure 1 shows the optical thickness of a PGA/ β -peptide/PLL film and a PGA/PLL film as a function of the number of layers of material deposited on a silicon substrate (closed circles and open circles, respectively). These data demonstrate that film growth occurred in a non-linear manner, consistent with past reports on the fabrication of PGA/PLL-based films^{42,43} (see Materials and Methods for additional details). A comparison of the growth profiles for these two films reveals that they are essentially identical. This result suggests that either no β -peptide was incorporated during film fabrication or that incorporated β -peptide did not contribute significantly to increases in film thickness. The presence of the β -peptide in these films was verified by fluorescence microscopy. As shown in the inset of Figure 1, β -peptide-containing films exhibited blue fluorescence (B), whereas the control (no β -peptide) films appeared dark (A). Additional evidence that the β -peptide can be incorporated into the structures of these PEMs during layer-by-layer assembly is discussed below.

Characterization of Film Stability and the Release of β -Peptide

Past studies investigating PGA/PLL-based multilayered films have demonstrated that these films remain stable and intact when incubated under physiologically-relevant conditions (e.g., pH 7.4, 37 °C).^{19,34,35,43,44} The stability of these materials renders them particularly attractive as substrates for the attachment of cells^{43–47} and as thin-film reservoirs for the diffusion-controlled release of small molecules.⁴⁸ In addition, other studies have demonstrated that PGA/PLL multilayers can be degraded by proteolysis in the presence of enzymes or cells and that this approach can be used to deliver or release macromolecular species (e.g., proteins or DNA) embedded in the films.^{49–51} To characterize film stability, we incubated silicon substrates coated with β -peptide films or control (no β -peptide) films in RPMI 1640 medium at 37 °C. Substrates were removed at predetermined intervals to characterize film thickness by ellipsometry and to monitor β -peptide release into solution using fluorometry.

Figure 2A shows a plot of the optical film thickness of a β -peptide-containing film and a control film incubated in RPMI 1640 as a function of time. The thicknesses of both films did not change significantly for up to 400 hours (~17 days) of incubation. However, the β -peptide-containing film gradually released approximately 150 μ g of β -peptide (83 μ g/cm²) over this same time period (Figure 2B). At 400 hours fluorescence could not be detected in the film, suggesting complete or near-complete release of the β -peptide. We draw several important conclusions from the results of these experiments: (i) these β -peptide-containing PEM films were stable for prolonged periods in a culture medium commonly used to study *C. albicans* biofilm growth, (ii) the β -peptide used here was released from these materials without the need for additional processes (e.g., proteolytic degradation) that would promote physical film erosion, and (iii) the release of β -peptide from these films occurred gradually over a period of 17 days as opposed to a burst release during the first few hours due to differences in pH and salt concentration between the fabrication and erosion conditions. This combination of features provides an attractive platform for the development of antifungal surface coatings. Optimization of β -peptide loading into the films and film structure to

regulate β -peptide release will be required to evaluate effectiveness for specific antifungal applications.

Characterization of the Antifungal Activity of β -Peptide-Containing Films

To evaluate the antifungal properties of our β -peptide-containing films, a suspension of *C. albicans* cells was inoculated onto film-coated glass substrates, and cell growth and viability were characterized by phase contrast and fluorescence microscopy over a 24 hour period. The viability of cells on the surfaces of these substrates was characterized by staining the cells with PI, a nucleic acid-binding dye that is excluded from live cells and can be used to identify dead cells. Figure 3 shows representative phase contrast and fluorescence microscopy images of PI-stained substrates after 2 hours, 7 hours, and 24 hours of incubation with cells. Inspection of these images reveals a large number of PI-stained cells on β -peptide-containing films relative to the number of PI-stained cells on control films. These results demonstrate that the β -peptide in these films remained cytotoxic after being incorporated into the multilayered films. These results also demonstrate that the antifungal activity of the β -peptide-containing films was a result of the incorporation of the β -peptide rather than other physicochemical properties of the PGA/PLL films.

Although past studies of the interactions of cells with PEMs have demonstrated that cells can access bioactive agents embedded deep within these materials by penetrating or extending into the film,⁴⁴ the results of our experiments suggest that the antifungal activity of the β -peptide-containing films likely arises from the release of incorporated β -peptide into solution over the first 24 hours of incubation. This conclusion is supported by the observation of a higher number of dead cells on the uncoated bottoms of culture wells in areas adjacent to the coated substrates containing β -peptide in comparison to areas adjacent to coated substrates fabricated without β -peptide (data not shown). As discussed above, the release of β -peptide occurred readily in RPMI culture medium in the absence of cells and did not require processes that lead to film degradation. However, in these cell-based experiments, we note that it is possible that *C. albicans* could alter the release profile of β -peptide through degradation of the PLL and PGA via cell surface or secreted proteases, as previously demonstrated by Jessel *et al.*^{49,51}

We used confocal microscopy to characterize the localization of the fluorescently-labeled β -peptide during cell growth on the PEM substrates and to confirm that the cell toxicity observed in Figure 3 was the result of β -peptide released from the PEM films and incorporated into *C. albicans* cells. In these experiments, we used a fluorescent conjugate of concanavalin-A, which binds cell surface glucose and mannose residues, to identify all cells (both live and dead) on the surfaces of the substrates after 24 hours of incubation. β -Peptide and PI generally co-localized in both yeast and hyphal cells on β -peptide-containing films (Figure 4). The presence of β -peptide in dead cells is consistent with the results of our earlier study³² and suggests that β -peptide toxicity led to the death of cells growing on the surfaces of these films. Figure 4 also shows a small number of cells that contained β -peptide but were not stained with PI. These results may indicate that the β -peptide had not caused sufficient permeabilization of cell membranes to allow PI to access nucleic acids inside the cells.

We used several complementary methods to quantify the influence of β -peptide release on cell viability and biofilm formation. To provide a measure of these effects at early time points, we determined the percentage of cells stained with PI after 2 hours of incubation on film-coated substrates. As shown in Figure 5, there were 20% fewer viable cells on control films compared to β -peptide-containing films. The abundance of interwoven hyphae on control films after 7 hours necessitated non-visual methods of quantification. For these later time points, we used an XTT metabolic activity assay to assess growth inhibition. As shown in Figure 5, *C. albicans* cells growing on β -peptide-containing films were 74% less

metabolically active than cells growing on control films. Our results showed a similar level of toxicity towards *C. albicans* at 7 hours as those of a previous report on the characterization of PGA/PLL PEMs fabricated using the antifungal α -peptide chromofungin.³⁵ We note, however, that differences in the experimental approach used in this current study, including measuring cell growth on the surface rather than in the culture medium and challenging the antifungal films with a 20-fold higher cell density, prevent a direct comparison to the results of this past study. The ability of our PEM films to exhibit toxicity at relatively early times following cell seeding could be important, because this feature could prevent attached *C. albicans* cells from forming biofilms that may subsequently lead to device-associated, drug-resistant infections.

Influence of β -Peptide-Containing Films on Cell Morphology and Biofilm Growth

In addition to observing decreased viability of *C. albicans* cells on β -peptide-containing films, we discovered that the β -peptide-containing films had a pronounced effect on cell morphology. Under the assay conditions used here, *C. albicans* cells were primarily in the hyphal form (Figures 3 and 4). However, *C. albicans* hyphae growing on β -peptide-containing films were 55% shorter on average than the hyphae of cells growing on control films (Figure 6). Although the specific reasons for this stunted hyphae growth are not understood, we note that a similar effect was observed in antifungal activity assays using β -peptides administered in solution (data not shown).

The inhibition of normal hyphal development is an important observation, because hyphae play a significant role in *C. albicans* pathogenesis. The ability to produce normal hyphae is a virulence factor of *C. albicans*, and strains with defects in hyphal production and elongation, such as strains lacking *HWPI*, *FLO8*, and *NOT4*, exhibit reduced pathogenicity.^{52–54} Elongating hyphae can penetrate or invade host tissues and biomedical materials.^{55–58} As a result, approaches that inhibit hyphal formation and elongation could limit the progression of infections and reduce the deterioration of materials. A decrease in the length of hyphae or in the abundance of hyphae could also influence the architectures and stabilities of biofilms. Although hyphae are not absolutely necessary for biofilm formation, they are normally present in *C. albicans* biofilms, and mutations in hyphal-specific genes are commonly associated with biofilm defects. Richard *et al.* suggested that the biofilm formation defects of hyphal-deficient *C. albicans* strains were a result of the loss of adherence provided by the hyphae.⁵⁹ Hyphal-defective mutants lacking *HWPI* or *ALS3* genes, which encode cell-surface adhesins, exhibited decreased adherence to a substrate and decreased biofilm mass.⁶⁰ Mutants lacking the cell-surface adhesin *EAP1*, which is present in both yeast and hyphal cells, also had a reduced capacity for adherence to surfaces and to other cells and displayed significant defects in biofilm formation *in vitro* and *in vivo*.⁶¹

As shown in the images in Figure 3, our β -peptide-containing films were not able to completely prevent *C. albicans* biofilm formation at 24 hours post-seeding. However, significant toxicity of the β -peptide towards the *C. albicans* cells in the biofilm was evident at 24 hours, as indicated by the high level of PI staining. This result, in combination with the substantial levels of growth inhibition observed at earlier time points and the significant reductions in hyphal growth, suggests the potential for these films as surface coatings for inhibiting *C. albicans* biofilm growth. The levels of *C. albicans* growth inhibition observed in this present study could be limited by the amount of β -peptide that was incorporated into the PEMs or, more likely, by the relatively small fraction (~40 %, 63 μ g) of the β -peptide that was released from the films over the 24 hour course of these studies (Figure 2B). In this context, the modular nature of these layer-by-layer assemblies provides approaches that can potentially be used to manipulate the rates and extents of β -peptide release. For example, increasing the number of layers deposited onto the surface of the substrate should lead to an increased loading of β -peptide in the films. In addition, the incorporation of other agents or

polyelectrolytes, such as hydrolytically degradable polycations, which have been recently used to tune the release of α -peptides from surfaces, could help modulate the β -peptide release from these films.^{25,62–64} Future studies that evaluate methods for increasing the loading or release rate of β -peptide could enhance the inhibition of *C. albicans* biofilm formation by increasing the local concentration of β -peptide near the substrate surface. Additionally, improvements in antifungal efficacy of the multilayer coatings could be achieved by enhancing β -peptide potency.

Conclusions

We have reported on the incorporation of an antifungal β -peptide into multilayered polyelectrolyte films fabricated on the surfaces of silicon and glass substrates. These films were assembled using a layer-by-layer process to fabricate films composed of poly-L-glutamic acid, a fluorescently labeled antifungal β -peptide, and poly-L-lysine. The resulting films promoted the sustained release of the β -peptide into culture medium for up to 400 hours (~17 days). Surfaces coated with β -peptide-containing films inhibited fungal cell growth and resulted in a higher percentage of dead cells compared to surfaces coated with poly-L-glutamic acid and poly-L-lysine only. In addition, hyphal elongation and metabolic activity were substantially decreased for *C. albicans* grown on substrates coated with β -peptide-containing films. This work demonstrates the ability to incorporate antifungal β -peptides into multilayered polyelectrolyte films and the potential of the resulting β -peptide-containing films as antifungal surface coatings. Our strategy promotes the localized delivery of β -peptides to fungal cells near the coated surface, which could eliminate the need for the systemic delivery of high concentrations of β -peptides that may lead to host toxicity. It could also prove possible to design multifunctional PEMs with the added ability to kill fungal cells on contact by combining our current approach with approaches reported in past studies for the incorporation of microbicidal cationic polymers into PEMs.^{6,65} The versatility of the layer-by-layer assembly method presents a promising platform for functionalizing a wide range of surfaces (i.e., catheters, tubes, surgical instruments, and other devices) with β -peptides to inhibit the fungal growth and morphological transitions that contribute to drug-resistant biofilms.

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Figure 1.

Plot of the ellipsometric film thicknesses of (PGA/PLL)₂₀ films (○) and (PGA/β-peptide/PLL)₂₀ films (●) versus the number of dipping cycles deposited onto silicon substrates (see text). The data represent the average of three films fabricated on the same day. The inset shows representative fluorescence micrographs of a (PGA/PLL)₂₀ film (A) and a (PGA/β-peptide/PLL)₂₀ film (B). Scale bars = 1 mm.

Figure 2. (A) Plot of film thickness and (B) release of β-peptide over time for representative (PGA/PLL)₂₀ films (○) and (PGA/β-peptide/PLL)₂₀ films (●) fabricated on silicon substrates. Samples were incubated in RPMI 1640 at 37 °C. Error bars represent deviations in film thickness at five positions along the length of a single substrate.

Figure 3. Phase contrast and fluorescence micrographs of *C. albicans* cells incubated on (PGA/PLL)₂₀ films and (PGA/β-peptide/PLL)₂₀ films at 2, 7, and 24 hours after the initial seeding of *C. albicans* on film surfaces. PI staining (red) was used to identify dead cells. Scale bar = 50 μm.

Figure 4. Confocal microscopy images of *C. albicans* cells grown on (A) (PGA/PLL)₂₀ films and (B) (PGA/β-peptide/PLL)₂₀ films 24 hours after initial cell seeding. Green fluorescence corresponds to cell surfaces stained with conA-Alexa Fluor 488, red fluorescence corresponds to dead cells stained with PI, and blue fluorescence corresponds to the location of the coumarin-labeled β-peptide. Scale bars = 50 μm. Inset scale bar = 10 μm.

Figure 5.

Percent of viable *C. albicans* and the relative metabolic activity of *C. albicans* cells grown on (PGA/PLL)₂₀ films (black bars) and (PGA/β-peptide/PLL)₂₀ films (gray bars). Percent viability was determined by PI exclusion 2 hours after cell seeding, and metabolic activity was determined by XTT reduction 7 hours after cell seeding. The viability data represent an average of three experiments, each consisting of three control and three β-peptide-functionalized films. The metabolic activity data are from a single representative experiment consisting of three of each type of coated substrate. * $p < 0.001$

Figure 6.

Representative phase contrast images of cells growing on (A) a (PGA/PLL)₂₀ film and (B) a (PGA/β-peptide/PLL)₂₀ film. Scale bar = 50 μm. (C) Average hyphal length of *C. albicans* cells growing on (PGA/PLL)₂₀ films (black bar) and (PGA/β-peptide/PLL)₂₀ films (gray bar) 2 hours after cell-seeding on the substrates. The data represent the average of three experiments each consisting of three control and three β-peptide-functionalized films. * $p < 0.001$