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Intraluminal Release of an Antifungal β-Peptide Enhances the Antifungal and Anti-Biofilm Activities of Multilayer-Coated Catheters in a Rat Model of Venous Catheter Infection

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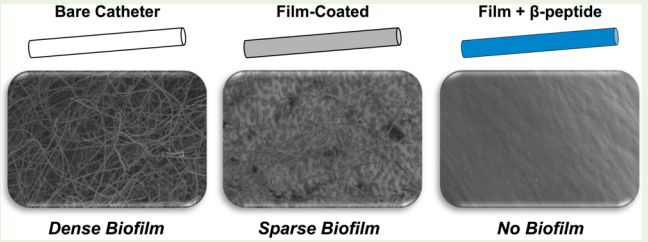
Supporting Information

ABSTRACT: *Candida albicans* is the most prevalent cause of hospital-acquired fungal infections and forms biofilms on indwelling medical devices that are notoriously difficult to treat or remove. We recently demonstrated that the colonization of *C. albicans* on the surfaces of catheter tube segments can be reduced in vitro by coating them with polyelectrolyte multilayers (PEMs) that release a potent antifungal β-peptide. Here, we report on the impact of polymer structure and film composition on both the inherent and β-peptide-mediated ability of PEM-coated catheters to prevent or reduce the formation of *C. albicans* biofilms in vitro and in vivo using a rat model of central venous catheter infection. Coatings fabricated using polysaccharide-based components [hyaluronic acid (HA) and chitosan (CH)] and coatings fabricated using polypeptide-based components [poly-L-lysine (PLL) and poly-L-glutamic acid (PGA)] both served as reservoirs for the loading and sustained release of β-peptide, but differed substantially in loading and release profiles and in their inherent antifungal properties (e.g., the ability to prevent colonization and biofilm growth in the absence of β-peptide). In particular, CH/HA films exhibited inherent antifungal and antibiofilm behaviors in vitro and in vivo, a result we attribute to the incorporation of CH, a weak polycation demonstrated to exhibit antimicrobial properties in other contexts. The antifungal properties of both types of films were improved substantially when β-peptide was incorporated. Catheter segments coated with β-peptide-loaded CH/HA and PLL/PGA films were both strongly antifungal against planktonic *C. albicans* and the formation of surface-associated biofilms in vitro and in vivo. Our results demonstrate that PEM coatings provide a useful platform for the design of new antifungal materials, and suggest opportunities to design multifunctional or dual-action platforms to prevent or reduce the severity of fungal infections in applied biomedical contexts or other areas in which fungal biofilms are endemic.



KEYWORDS: *antifungal, biofilms, catheters, polyelectrolyte multilayers, antimicrobial surfaces, β-peptides*

INTRODUCTION

Candida albicans is the most common cause of hospital-acquired fungal infections.[1] This pathogen causes invasive and life-threatening infections in humans, particularly in immune compromised individuals, with systemic infections having mortality rates as high as 30−60%.[2−4] Patients with implanted medical devices (e.g., central venous catheters) are at higher risk for systemic *C. albicans* infections because the surfaces of these devices serve as platforms for the introduction of the pathogen and as substrates for the subsequent growth of fungal biofilms that are both difficult to remove and more resistant to treatment with conventional antifungal drugs than planktonic (or non-biofilm-associated) *C. albicans*.[5−12]

The current standard of care for patients with device-associated *C. albicans* infections involves the removal and replacement of the infected device and simultaneous treatment with systemic antifungal drugs—a regimen that incurs high economic costs and is a substantial burden in human terms.[7,13−16] Surfaces and interfaces that can (i) kill *C. albicans* on contact, (ii) prevent *C. albicans* adhesion, or (iii) prevent or reduce the formation of mature biofilms on colonized surfaces, and thereby facilitate treatment using other antifungal agents, would improve patient care significantly and reduce costs associated with device-associated infections.[17−22] Here, we report a step toward these goals through the design of polymer coatings that have inherent antifungal properties and act as platforms for the local release of β-peptide-based structural mimics of antimicrobial peptides.[23−28] Our approach is based on the layer-by-layer fabrication[29−33] of polyelectrolyte multilayer

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(PEM) coatings as platforms for the immobilization and surface-mediated release of peptide-based antimicrobial agents.[34−39]

We recently demonstrated that PEM coatings fabricated on the inner surfaces of polymer-based catheter tube segments can serve as reservoirs for the intraluminal release of a potent and selective antifungal β-peptide and that this approach can reduce the formation of *C. albicans* biofilms in vitro.[38] That study sought to demonstrate proof of concept using a model PEM film system fabricated using two charged polypeptides [poly-L-lysine (PLL) and poly-L-glutamic acid (PGA)]. This current study sought to characterize the impacts of both polymer structure and film composition on the ability of PEM-coated catheters to prevent or reduce the formation of *C. albicans* biofilms in vitro and in vivo using a rat model of central venous catheter infection. With a view to developing new device/coating or device/coating/therapeutic combinations that exhibit inherent or enhanced antifungal properties, we focused in particular on characterization of the antifungal and antibiofilm activities of catheters coated using hyaluronic acid (HA) and chitosan (CH), a naturally occurring cationic polysaccharide that is reported to exhibit inherent antifungal properties in several other contexts.[40−47] Our results demonstrate that CH/HA films exhibit inherent antifungal and antibiofilm behaviors relative to PLL/PGA films, and that the antifungal properties of both classes of films can be improved significantly by the incorporation of β-peptide. Catheter segments coated with β-peptide-loaded CH/HA and PLL/PGA films were both strongly antifungal against planktonic *C. albicans* and the formation of surface-associated biofilms in vitro and in vivo. Overall, our results reveal PEM coatings to be a useful platform for the design of new antifungal materials and suggest opportunities to design multifunctional or "dual-action" platforms that prevent or reduce the severity of fungal infections in both fundamental and applied biomedical contexts.

MATERIALS AND METHODS

Materials. Catheter tubes (polyethylene, PE-100, inner diameter =0.034 in.) were obtained from Intramedic, Becton Dickinson & Co. Poly-L-lysine hydrobromide (PLL, MW = 15,000−30,000), poly-L-glutamic acid Na salt (PGA, MW = 50 000−100 000), chitosan (CH, medium molecular weight) and branched polyethylenimine (BPEI, MW = 25 000) were purchased from Sigma-Aldrich. Sodium hyaluronate (HA, MW = 1 500 000−2 200 000) was purchased from Acros Organic. 2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) and RPMI 1640 powder containing L-glutamine and phenol red (without HEPES or Na bicarbonate) were obtained from Invitrogen. Phosphate-buffered saline (PBS) concentrate was obtained from OmniPur, and formaldehyde, glutaraldehyde, and menadione were obtained from Sigma. Tween-20 was obtained from Acros, and osmium tetroxide (4%) was obtained from Electron Microscopy Sciences. NaCl, Tris-base, and 3-(N-morpholino)propanesulfonic acid (MOPS) were obtained from Fisher Scientific. All materials were used as received.

General Considerations. β-Peptide (coumarin-linker-(ACHC-β^3hVal-β^3hLys)$_3$) was synthesized using previously reported methods.[27] Fluorescence microscopy images were obtained using an Olympus IX71 microscope and MetaMorph Advanced version 7.8.1.0 software. Images were processed with NIH ImageJ and MS Powerpoint 2010. Fluorescence measurements to characterize release of coumarin-labeled β-peptide were made using a NanoDrop3300 (Thermo Scientific). When necessary (see text), the ends of catheter tubes were sealed reversibly by plugging open ends with custom-made metal stoppers consisting of a wide bore wire that fit snugly into the catheter tubes. Critical point drying and scanning electron microscopy (SEM) were performed using a Critical Point Dryer (Tousimis Samdri-815) and a LEO SEM microscope. All absorbance measurements were made at 490 nm with a plate reader (EL800 Universal Microplate Reader, Bio-Tek Instruments, Inc.).

Fabrication of Multilayered Films. Solutions of PLL, PGA, and BPEI (1 mg/mL) were prepared using a NaCl solution (0.15 M) in DI water. Solutions of HA and CH were prepared at 2 mg/mL in NaCl solution (0.15 M) and 0.1 M acetic acid containing 0.15 M NaCl, respectively. Multilayers were fabricated on the luminal (inner) surfaces of catheter tube segments using a fill-and-purge assembly protocol.[38] Briefly, (i) solutions of HA or PGA (negatively charged polymers) were infused and allowed to stand for 5 or 3 min, respectively, (ii) a rinse solution (0.15 M NaCl in water) was infused and allowed to stand for 1 min, followed by another rinse for 1 min, (iii) solutions of CH or PLL (positively charged polymers) were infused and allowed to stand for 5 or 3 min, respectively, and finally (iv) the tubes were rinsed again as described above. The cycle was repeated iteratively until a desired number of layers of each polymer was deposited.

Loading of Film-Coated Catheters. Film-coated catheters were infused with a solution of β-peptide (10 mg/mL in 0.15 M NaCl) and tube ends were reversibly sealed. The filled tubes were allowed to stand for 24 h at ambient temperature. The solution was removed after 24 h and tubes were washed using the following protocol: (i) PBS was infused and allowed to stand for 5 min, (ii) Tris-buffered saline Tween (TBST; 100 mM NaCl, 10 mM Tris-HCl, 0.1% Tween-20) was infused and allowed to stand for 1 h, and (iii) rinsing with PBS was again performed as described in above. Uncoated (bare) control tubes were treated with β-peptide as described above. Film-coated tubes (used as no-peptide controls) were prepared as described above but not loaded with β-peptide. Additional controls used in SEM studies were prepared as described above using buffer that did not contain β-peptide. For the catheters used in the in vivo studies, the catheter hub was removed and the lumen of the catheter tube was coated with PEM film over its entire length and loaded with peptide; PEM and peptide solutions were prepared using autoclaved water and PEM film fabrication and peptide loading were performed under sterile conditions in a biosafety cabinet.

Release of β-Peptide from Film-Coated Catheters. Segments (2 cm) of tubes treated with β-peptide were completely filled with PBS and the open ends of the tubes were sealed reversibly. The tubes were then incubated at 37 °C. Tubes were removed periodically to characterize the fluorescence of the buffer solution (excitation = 336 nm; emission = 402 nm; these wavelengths are the excitation/emission maxima for the coumarin label conjugated to the peptide). Measurements were converted to concentrations of β-peptide with calibration curves prepared with solutions of known β-peptide concentration. Tubes were then filled with fresh PBS and incubated further. Release plots were constructed by cumulative addition of the concentrations of peptide released at each individual time point.

Characterization and Analysis of Antifungal Activity. To evaluate the antifungal activities of film-coated catheter tubes, *C. albicans* inoculum was incubated inside tubes for 6 h, followed by removal of the inoculum solution to assess viability using colony counts. Yeast cells (*C. albicans* SC5314 cells) were allowed to grow overnight in liquid yeast extract-peptone-dextrose (YPD) medium at 30 °C. The cells were then washed using PBS and suspended again in RPMI 1640 medium (buffered with MOPS; pH 7.0). Cell densities were adjusted to 10^6 cfu/mL using RPMI 1640. All tubes were cut into small segments (3 cm). In short-term experiments, tube segments were filled completely with suspensions of cells and the open ends of the tubes were sealed reversibly. The tubes were then incubated at 37 °C for 6 h. The RPMI liquid containing the *C. albicans* inoculum was then removed from the tubes, and a 100-fold dilution was plated on YPD plates. The plates were incubated for ∼24 h at 37 °C, and manual counting was then used to count the number of colonies formed. These experiments were conducted using triplicate samples on at least 3 separate days. For longer-term experiments (see text), tube segments were incubated with PBS for 0, 3, 7, 14, 21, 28, 35, 42, 49, 56, 63, or 70 days at 37 °C prior to evaluating antifungal activity using the methods described above. After incubation of the *C. albicans* inoculum for 6 h, the solutions were transferred into the wells of a 96-well microtiter plate. After 24 h, an XTT assay was used to characterize the metabolic activity of the cells using methods described previously.[38] For multiple challenge experi-

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ments, catheter tubes were incubated with yeast as described earlier for the short-term antifungal evaluation experiments. At the end of the 6 h period, tube segments were incubated with PBS for ∼18 h. The next challenge was performed by removing the PBS solution and incubating tubes with fresh *C. albicans* inoculum for the next 6 h period. In all, six such challenges were performed.

Assays to Characterize Biofilm Inhibition in Vitro. For *in vitro* biofilm assays, *C. albicans* cells were allowed to grow overnight in liquid YPD medium at 30 °C. The cells were then rinsed using PBS and suspended again in RPMI 1640 buffered with MOPS (pH 7.0) and further supplemented using 5% fetal bovine serum (RPMI + 5% FBS) to grow a robust biofilm under controlled conditions. Cell densities were adjusted to 10^6 cfu/mL using RPMI + 5% FBS. A suspension of *C. albicans* (15 μL) was then infused into short segments of catheter tubes (3 cm), and the tubes were gently placed in individual wells of six-well plates containing RPMI + 5% FBS (2 mL) and incubated for 48 h at 37 °C. Growth of biofilms was characterized after 48 h using XTT assays and by SEM. For XTT assays, tubes were placed in individual microcentrifuge tubes and then placed in a sonication bath (Branson 2510R-MT; for 15 min) to dislodge any biofilm that was strongly adhered. The inoculum was removed from the tubes and transferred into the wells of 96-well microtiter plates. Tubes were rinsed two times using PBS (40 μL) and these rinse solutions were transferred to the respective wells for each sample. Microcentrifuge tubes were then centrifuged, and recovered fluid was also transferred to the respective wells on each microtiter plate. A solution of XTT [40 μL, 0.5 g/L in PBS, pH 7.4, and containing menadione (3 μM) in acetone] was then added to wells containing biofilm samples as well as wells containing RPMI + 5% FBS as negative controls. Samples were incubated for 2 h at 37 °C. The supernatant (75 μL) was then transferred to another 96-well microtiter plate and absorbance was measured at 490 nm to characterize relative metabolic activities. For characterization by SEM, segments of tubes were transferred into fixative solutions (1%, v/v, glutaraldehyde; 4%, v/v, formaldehyde) at 4 °C and allowed to sit overnight. Samples were then washed for 10 min using 0.1 M PBS and then treated for 30 min with osmium tetroxide (1%) followed again by 10 min in 0.1 M PBS. Samples were then dehydrated by exposure to a series of ethanol washes for 10 min each (30, 50, 70, 85, 95, and 100%), followed by final desiccation using a critical-point dryer. Samples were then mounted on Al stubs, sliced to expose the inner surfaces of the tubes, and then sputter-coated with Au−Pd and characterized by SEM at 5 kV in high-vacuum mode.

Characterization of Biofilm Inhibition in Catheters in Vivo. Specific-pathogen-free female Sprague−Dawley rats weighing 400 g were used for all animal studies. Animal maintenance and catheter placement surgery and maintenance were performed as described elsewhere.[48] Briefly, a vertical incision was made in the skin of the anterior neck just right of midline. The internal jugular vein was identified and a longitudinal incision a few millimeters long was then made in the vein wall. A catheter was then placed in the opening and advanced approximately 2 cm to a site above the right atrium, after which the catheter was secured to the vein with 3-O silk ties. The proximal end of the catheter was then tunneled subcutaneously to the midscapular space and externalized through the skin via a button, which was secured with sutures. Catheters were placed in animals 24 h prior to infection to allow a conditioning period for deposition of host protein on the catheter surface. After the conditioning time period, 100 μL of blood was obtained from the catheter and cultured to ensure sterility. An inoculum of *C. albicans* K1 was instilled in the catheter in a 500 μL volume (to fill the entire volume of the catheter). The inoculum was allowed to dwell for 6 h, after which it was withdrawn and the catheter was flushed with heparinized 0.85% NaCl and locked. Animals were sacrificed 48 h after catheter placement and the catheters were removed aseptically. The distal 2 cm of catheter was further cut perpendicular to the catheter length with an 11-blade scalpel into 2- to 3 mm-long segments, placed in fixative, and processed for imaging by SEM as described in the previous section. For all animal studies, animals were maintained in accordance with the American Association for Accreditation of Laboratory Care criteria. Animal studies were approved by the Animal Research Committee of the William S. Middleton Memorial Veterans Administration Hospital.

RESULTS

Fabrication of Film-Coated Catheters and Characterization of Inherent Antifungal Activity. We selected the HA/CH PEM film system for use in this study because (i) CH is a naturally occurring carbohydrate-based cationic polymer reported to exhibit inherent antimicrobial activity,[40−45] and (ii) PEMs fabricated from CH and HA are both biocompatible and have been well-studied as platforms for the loading and release of active agents in a range of fundamental and applied contexts.[37,39,46,47,49−53] This PEM system thus provided a useful starting point from which to develop PEM-based catheter coatings that could potentially also exhibit inherent antifungal activity. We performed a series of initial experiments to characterize and compare the physicochemical properties and antifungal activities of HA/CH and PGA/PLL films fabricated on the inner (luminal) surfaces of PE catheter tubes. For these and all other studies described below, we fabricated coatings 19.5 bilayers thick using an iterative fill-and-purge method reported previously for the fabrication of PGA/PLL coatings in PE tubing (Figure 1A).[38] Characterization of film-coated tubes by SEM revealed the inner surfaces of the tubes to be coated uniformly by both HA/CH and PGA/PLL films, with no substantial cracking or peeling (Figure S1). Characterization of film cross sections by SEM revealed HA/CH films to be substantially thicker (1340 ± 240 nm) than PGA/PLL films fabricated from the same number of polyanion/polycation pairs (710 ± 50 nm).[38]

Initial comparisons of the in vitro antifungal properties of HA/CH and PGA/PLL films against planktonic *C. albicans* were performed by incubating *C. albicans* inocula inside film-coated tubes for 6 h. As shown in Figure 2A, we observed an ∼25-fold reduction in the number of viable colonies in inocula recovered from catheter tubes coated with HA/CH films relative to inocula recovered from control (uncoated) PE tubes. In contrast, we observed no significant reduction in the number of viable colonies in inocula recovered from tubes coated with PGA/PLL films relative to the uncoated control (Figure 2B). These results demonstrate that HA/CH-based coatings exhibit inherent antifungal activity; the impacts of film thickness and structure on this activity are described below.

Loading and Release of β-peptide from Film-Coated Catheters. PEM-coated catheter tubes were loaded with antifungal β-peptide by infusion of aqueous solutions (10 mg/mL) of coumarin-labeled β-peptide for 24 h to allow β-peptide to diffuse into the films (followed by rigorous washing; see schematic in Figure 1A and Materials and Methods for additional details). Panels B and C of Figure 1 show representative fluorescence micrographs of β-peptide-loaded tubes and reveal the presence of β-peptide on the inner surfaces of tubes coated with HA/CH and PGA/PLL films, respectively (panels D and E show film-coated control catheters not loaded with β-peptide; panel F shows a bare (uncoated) tube treated with β-peptide for 24 h). Inspection of these images reveals fluorescence in tubes coated with HA/CH films to be substantially brighter than that observed in tubes coated with PGA/PLL films. Loading of β-peptide resulted in a significant increase in the thicknesses of HA/CH films, as determined by SEM (e.g., from 1340 ± 240 nm to 1730 ± 280 nm), similar to the increase in thickness observed in PGA/PLL films (from 710 ± 50 nm to 990 ± 50 nm) in past studies[38] and consistent with a rearrangement of the polyelectrolyte components of these materials that is likely to

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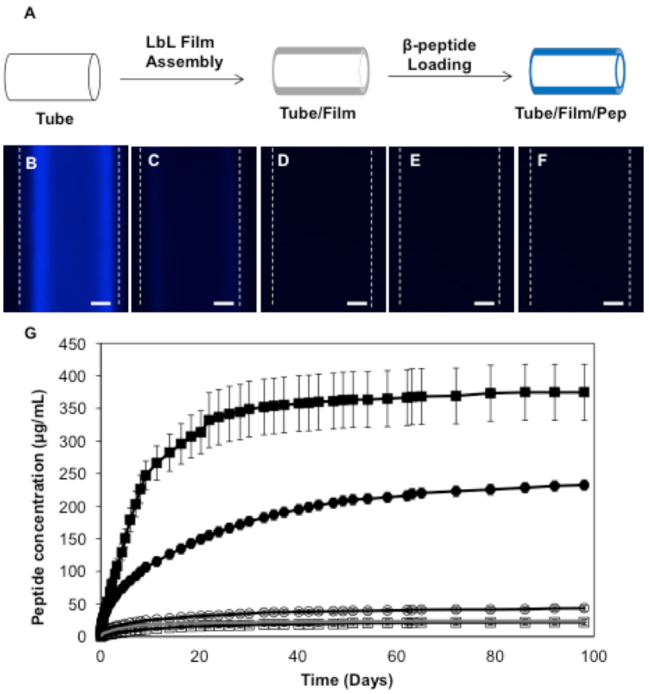

Figure 1. (A) Schematic illustration depicting layer-by-layer fabrication of films on the luminal surfaces of catheter tubes and the post-fabrication loading of the coatings with β-peptide. (B–F) Representative fluorescence micrographs of (B) a catheter coated with a HA/CH film 19.5 bilayers thick and (C) a catheter coated with a PGA/PLL film 19.5 bilayers thick after incubation in a solution of a coumarin-labeled β-peptide for 24 h, (D) a catheter-coated with a HA/CH film 19.5 bilayers thick (no peptide), (E) a catheter-coated with a PGA/PLL film 19.5 bilayers thick (no peptide), and (F) an uncoated catheter; scale bars = 250 μm. (G) Plot showing the release of β-peptide into the lumen of a HA/CH film-coated, β-peptide-loaded catheter (solid squares), a PGA/PLL film-coated β-peptide-loaded catheter (solid circles), and a no-film control catheter incubated with β-peptide (gray line); all samples were filled with PBS, sealed, and incubated at 37 °C. Background fluorescence measured using control HA/CH film-coated catheters (open squares) and PGA/PLL film-coated catheters (open circles) are also shown. Data points are the average of three replicates and error bars represent standard deviation.

occur upon the absorption and infiltration of the cationic β-peptide.

We next evaluated the release of antifungal β-peptide into the luminal spaces of film-coated, β-peptide-loaded catheters by infusing PBS and incubating for predetermined amounts of time. The plot in Figure 1G shows the cumulative release of β-peptide into fluid contained in tubes coated with HA/CH films (solid squares), PGA/PLL films (solid circles), and uncoated (bare) catheters (gray line). Measurements made with no-peptide control tubes coated with HA/CH (open squares) and PGA/PLL (open circles) are also shown for comparison. These results demonstrate that β-peptide was released gradually into solution from both film systems. However, whereas the PGA/PLL films released β-peptide over a period of ~100 days, release from tubes coated with HA/CH films plateaued after ~50 days. Tubes coated with HA/CH films also released ~1.6-fold more β-peptide than films coated with PGA/PLL films (~4.3 μg versus ~2.7 μg), consistent with the greater thicknesses of the HA/CH films (as discussed above) and the higher levels of fluorescence in these coatings observed after β-peptide loading (Figure 1B, C). No significant or sustained increase in solution fluorescence was

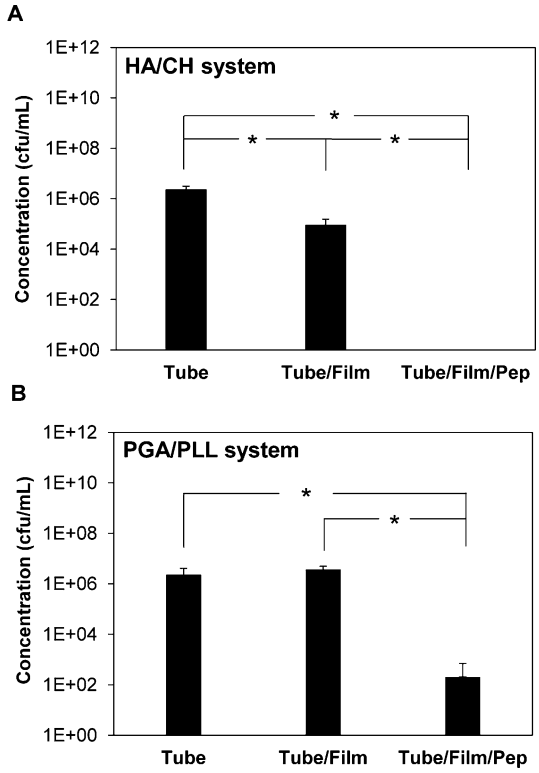

Figure 2. Antifungal activities of bare, untreated catheters (tube), PEM film-coated catheters (tube/film), and PEM film-coated catheters loaded with β-peptide (tube/film/pep) for experiments investigating (A) the HA/CH film system and (B) the PGA/PLL film system. Data points are averages of measurements from three independent experiments of three replicates each, and error bars denote standard deviation; (*indicates $p < 0.05$ by a two-tailed t test).

observed from uncoated β-peptide-treated tubes or tubes coated with untreated HA/CH or PGA/PLL films.

Quantification of the Activities of β-Peptide-Loaded Catheters Against Planktonic *C. albicans*. The antifungal activities of catheters coated with films containing β-peptide were characterized by infusing suspensions of *C. albicans* into the tubes for 6 h and then counting the number of viable colonies plated on agar plates. As shown in Figure 2A, we observed an essentially complete reduction in *C. albicans* viability in tubes coated with β-peptide-loaded HA/CH films. We also observed a significant (~10 000 fold) reduction in the number of viable cells in tubes coated with β-peptide-loaded PGA/PLL films (as compared to bare tubes; Figure 2B). As noted in the previous section, we observed a significant reduction in viable colonies in control tubes coated with HA/CH films that did not contain β-peptide as compared to uncoated control tubes (Figure 2A; middle column), a result we attribute to the inherent antifungal activity of these CH-containing films (compare to Figure 2B; middle column). These β-peptide-loaded CH/HA films provide the basis of a dual-functional approach to antifungal activity (that is, the films themselves exhibit distinct fungistatic or fungicidal activities that can be enhanced further by incorporating β-peptides into the films). However, the substantially more effective killing of planktonic *C. albicans* in the tubes coated with β-peptide-loaded CH/HA films demonstrated here (relative to experiments performed using analogous PGA/PLL films that are not inherently antifungal) is likely a result of the higher intraluminal concentrations of β-peptide released by these thicker films, as described above and shown in Figure 1G.

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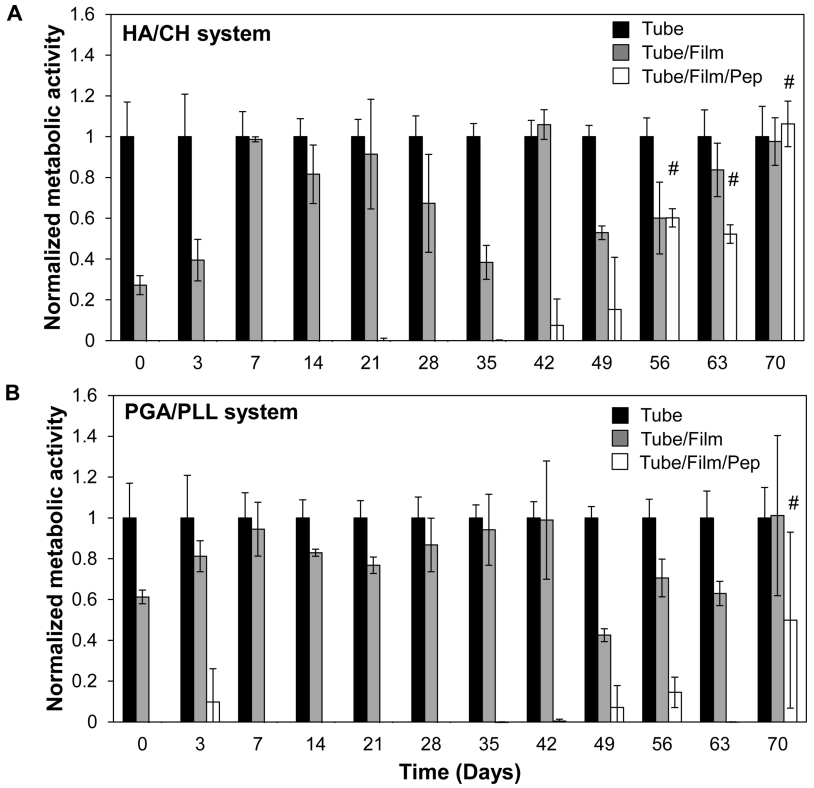

Figure 3. Antifungal activities of β-peptide-loaded film-coated catheters after pre-incubation in PBS for extended times. Bare, untreated catheters (tube; black bars), film-coated catheters (tube/film; gray bars), and film-coated catheters loaded with β-peptide (tube/film/pep; white bars) for experiments with (A) the HA/CH film system and (B) the PGA/PLL PEM system were pre-incubated with PBS for the indicated time periods (see text). That PBS solution was flushed prior to inoculation and incubation with *C. albicans* for 6 h at 37 °C. XTT was then used to measure differences in cell metabolic activities. Data points are averages of measurements from three replicates, normalized to the metabolic activities of the bare, uncoated tube for each data point; error bars denote standard deviation. # indicates lack of significance ($p > 0.05$ by two-tailed t test) between β-peptide-loaded films (tube/film/pep) and untreated controls (tube) under the same conditions. For all other pre-incubation conditions, reductions in metabolic activity for β-peptide-loaded films (tube/film/pep) were statistically different ($p < 0.05$ by two-tailed t test) from untreated controls (tube) under the same conditions.

To investigate the impact of film thickness and structure on the antifungal activities of PEM coatings (both with and without β-peptide), we performed an additional series of experiments in which (i) film thickness was varied from 4.5 to 19.5 bilayers thick (Figure S3) and (ii) the identity of the last polymer layer deposited (e.g., either CH or HA) was changed. For the HA/CH system, the onset of inherent antifungal activity in the absence of loaded β-peptide occurred at 9.5 bilayers. Consistent with results shown above, no inherent antifungal effects were observed for PGA/PLL-based coatings at any film thickness. As shown in Figure S3A, catheter tubes coated with HA/CH films loaded with β-peptide also exhibited antifungal activity at all film thicknesses whereas β-peptide-loaded PGA/PLL coatings only exhibited detectable antifungal activity for films greater than or equal to 14.5 bilayers thick (Figure S3B). Finally, results shown in Figure S4 reveal that HA/CH films remained inherently antifungal regardless of the identity of the last layer of polyelectrolyte deposited during fabrication. In contrast, the antifungal activities of PGA/PLL coatings were moderately improved when cationic PLL was deposited as the last layer (Figure S5). The identity of the final polyelectrolyte layer deposited during fabrication did not have a strong influence on the ability of either film system to host or release β-peptide; all β-peptide-loaded coatings exhibited strong antifungal activity regardless of the last layer deposited (Figures S4 and S5).

To evaluate the longer-term ability of β-peptide-loaded catheters to resist fungal challenges, we performed experiments in which PBS was incubated inside β-peptide-loaded catheter segments for up to 70 days prior to inoculation with *C. albicans*. Six hours after yeast were introduced into these "pre-incubated" tubes, the solutions were removed and an XTT assay was used to quantify differences in metabolic activity. Under these conditions, tubes coated with β-peptide-loaded HA/CH and PGA/PLL films exhibited consistent and significant antifungal activities after 49 and 63 days of PBS pre-incubation, respectively (Figures 3A, B, white bars; relative to levels of metabolic activity measured in uncoated control tubes, black bars). In many cases, the HA/CH coatings themselves (no loaded peptide) also exhibited inherent antifungal activity over 56 days, although these results were not statistically significant for all samples (Figure 3A; gray bars).

Finally, we characterized the ability of film-coated catheters to remain antifungal after multiple and repeated short-term challenges with planktonic *C. albicans* (e.g., as might be experienced during the deployment and regular use of a catheter in clinical contexts). Figures 4A, B show the antifungal activities of catheter tubes coated with HA/CH or PGA/PLL films after each of six different challenges with *C. albicans* inoculum. We observed reductions in yeast viability in inocula removed from tubes coated with HA/CH coatings alone (no loaded peptide) and in tubes coated with HA/CH films loaded with β-peptide after all six challenges (Figure 4A; gray bars and white bars, respectively). Tubes coated with PGA/PLL films also exhibited substantial and significant antifungal activity after all six microbial

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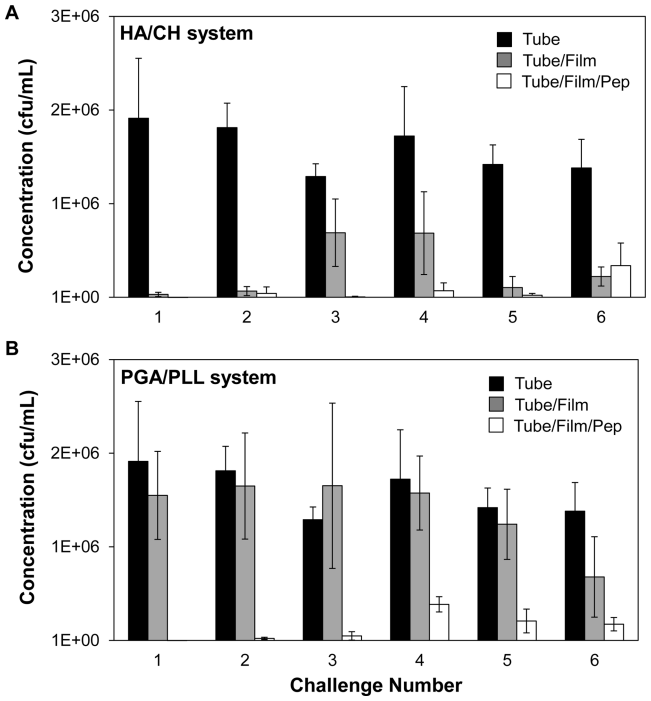

Figure 4. Antifungal activities of β-peptide-loaded, film-coated catheter tubes after multiple challenges with *C. albicans* inocula. Bare, untreated catheters (tube; black bars), film-coated catheters (tube/film; gray bars), and film-coated catheters loaded with β-peptide (tube/film/pep; white bars) for experiments using (A) the HA/CH film system and (B) the PGA/PLL PEM system were incubated with *C. albicans* inocula for six 6 h periods with 18 h PBS incubation periods in between. Colony counts were used to calculate viable cell concentrations in the catheters after every challenge; see text for additional details. Data points are averages of measurements from three replicates and error bars denote standard deviation. For all six challenges, viable cell concentrations of β-peptide-loaded catheters (tube/film/pep) were statistically different ($p < 0.05$ by two-tailed t test) from untreated controls (tube) under the same conditions.

challenges, but only when loaded with β-peptide (Figure 4B; white bars), consistent with the results of other experiments described above and in past studies.

β-Peptide-Loaded Catheters Inhibit Biofilm Formation in Vitro. *C. albicans* cells residing in biofilms differ phenotypically from planktonic cells, and biofilms exhibit complex structures (consisting of a dense network of yeast and hyphal cells encased in an extracellular matrix) that contribute to increased resistance to treatment with conventional antifungal drugs.[6,54] We characterized the inherent in vitro antibiofilm activities of catheter tubes coated with HA/CH and PGA/PLL films (no loaded peptide) by inoculating 1×10^6 cfu/mL of *C. albicans* in RPMI in the presence of 5% FBS at 37 °C and characterizing extents of biofilm growth qualitatively using SEM (Figure 5, S6) and quantitatively using XTT (Figure 6) after 48 h. Panels A and F of Figure 5 show representative SEM images of a bare (uncoated) tube, and reveal the presence of a robust biofilm, consisting of a dense network of hyphal cells with few yeast cells observed, on the inner surface. High levels of metabolic activity were also observed in these bare tubes by XTT assays (Figure 6). We also observed dense biofilm growth on tubes coated with PGA/PLL films (panels C and H of Figure 5, and Figure 6B) consistent with the results of past in vitro studies on this film system.[38]

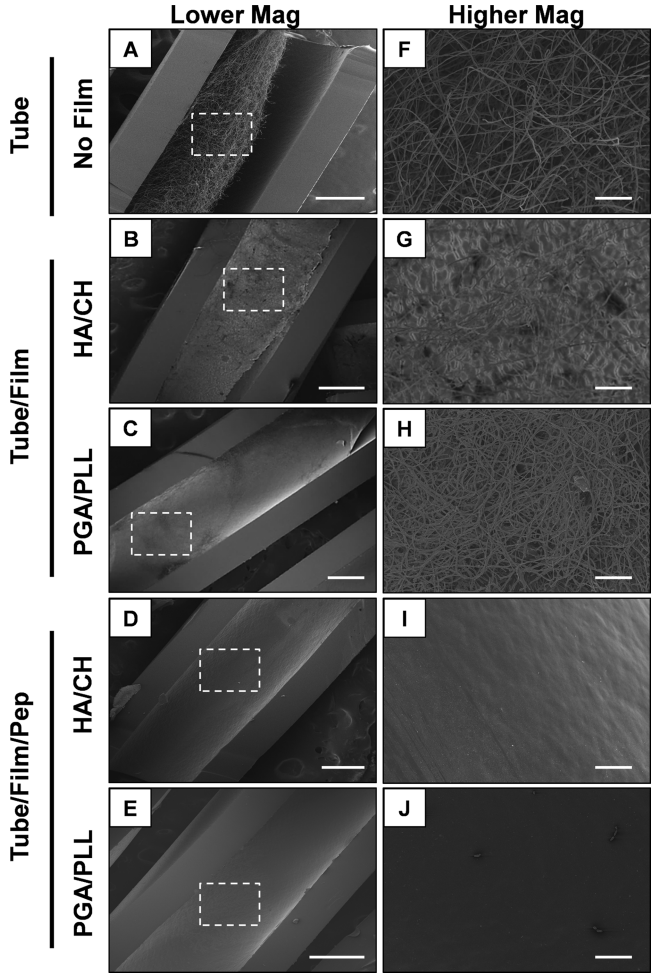

Figure 5. (A–E) Low- and (F–J) high-magnification scanning electron microscopy images showing the inner surfaces of catheter tubes after incubation with *C. albicans* in in vitro biofilm assays (catheter tubes were longitudinally sliced prior to imaging). Images show the surfaces of: (A, F) an untreated control tube (tube; no coating, no β-peptide); (B, G) a tube coated with a HA/CH film 19.5 bilayers thick (tube/film; no peptide), (C, H) a tube coated with a PGA/PLL film 19.5 bilayers thick (tube/film; no peptide), (D, I) a tube coated with a HA/CH film and loaded with β-peptide (tube/film/pep), and (E, J) a tube coated with PGA/PLL film and loaded with β-peptide (tube/film/pep) after incubation with *C. albicans* for 48 h. The white dotted boxes in panels A–E indicate the approximate region from which the corresponding higher magnification images in panels F–J were obtained. Scale bars = (A–E) 400 μm and (F–J) 40 μm.

Biofilm growth was also observed on the surfaces of catheter tubes coated with HA/CH films. In contrast to tubes coated with PGA/PLL films, however, biofilms on HA/CH coated surfaces were less dense (see panels B and G in Figure 5) and showed significant and substantial reductions in metabolic activity (Figure 6A), suggesting that the presence of CH in these films also confers a measure of inherent antibiofilm activity. For both film systems, loading of β-peptide resulted in substantial reductions in biofilm growth. In general, we observed no yeast cells or hyphal cells over the majority of the surfaces of β-peptide-loaded catheters (Figure 5D, E, I, J), with occasional yeast cells observed in some locations (e.g., Figure 5J). The addition of β-peptide to tubes coated with HA/CH films resulted in a small but significant decrease in metabolic activity compared to tubes coated with HA/CH films alone (Figure 6A).

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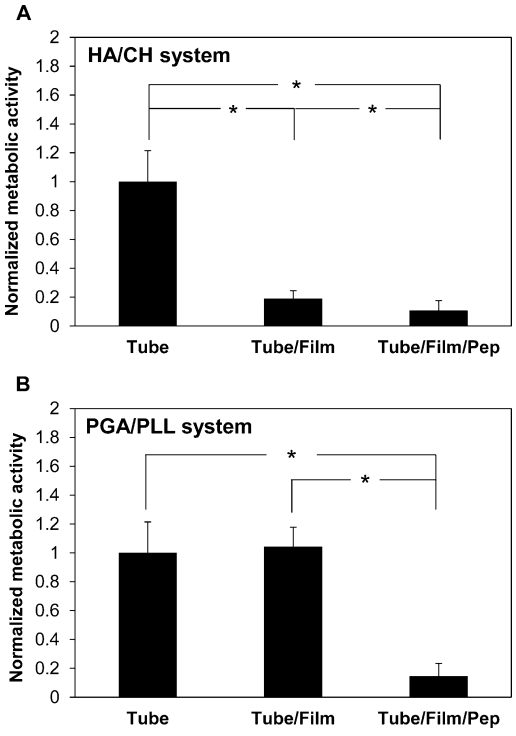

Figure 6. Inhibition of *C. albicans* biofilms by film-coated, β-peptide-loaded catheters. Bare, untreated catheters (tube), film-coated catheters (tube/film), and film-coated catheters loaded with β-peptide (tube/film/pep) for the HA/CH (A) or PGA/PLL (B) film system were incubated with *C. albicans* inoculum (1×10^6 cfu/mL) in RPMI containing 5% FBS at 37 °C. Data points are averages of measurements from three independent experiments of three replicates each, and error bars denote standard deviation; (*indicates $p < 0.005$ by two-tailed t test).

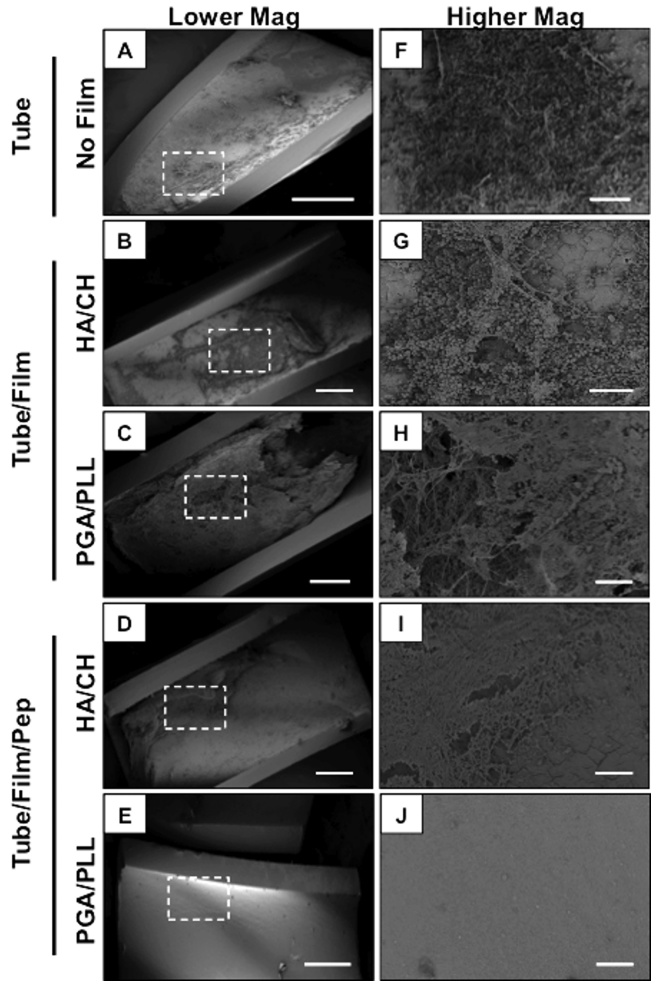

Figure 7. (A–E) Low- and (F–J) high-magnification scanning electron microscopy images showing biofilm formed in vivo on the inner surfaces of catheters using a rat central venous catheter biofilm model (catheter tubes were longitudinally sliced prior to imaging; see text for additional details). Images show the surfaces of: (A, F) an untreated control tube (tube; no film, no peptide); (B, G) a tube coated with a HA/CH film 19.5 bilayers thick (tube/film; no peptide); (C, H) a tube coated with a PGA/PLL film 19.5 bilayers thick (tube/film; no peptide); (D, I) a tube coated with a HA/CH film and loaded with β-peptide (tube/film/pep); and (E, J) a tube coated with a PGA/PLL film and loaded with β-peptide (tube/film/pep) after insertion into the jugular vein and incubation with a *C. albicans* inoculum. The white dotted boxes in A–E indicate the approximate region from which the corresponding higher magnification images in F-J were obtained. Scale bars = (A–E) 400 μm and (F–J) 40 μm.

β-Peptide-Loaded Catheters Inhibit Biofilm Formation in Vivo. We also characterized the ability of catheters coated with β-peptide-loaded HA/CH or PGA/PLL films to reduce or prevent *C. albicans* biofilm formation in vivo using a rat central venous catheter model.[48] For these experiments, uncoated catheters, film-coated catheters, or film-coated catheters loaded with β-peptide were placed in the jugular vein for 24 h prior to inoculation and incubation with *C. albicans* to condition the catheter and allow for the deposition of host protein on the surface. A *C. albicans* inoculum (1×10^6 cfu/mL) was then placed in the catheters for 6 h. After this period the inoculum was withdrawn and the catheter was flushed with heparinized NaCl solution. Catheters were removed after 24 h and characterized by SEM. The presence of the PEM coatings (with or without loaded peptide) did not promote thrombosis or any other adverse reactions under the conditions used in these experiments.

Panels A–E of Figure 7 show representative lower-magnification SEM images of the inner surfaces of catheter tubes used in these in vivo experiments. Panels F–J show corresponding higher-magnification images corresponding to the region enclosed in the dotted boxes shown in panels A–F. The surfaces of bare, uncoated catheters were covered with a dense network of yeast and hyphal cells (Figure 7A, F). Tubes coated with PGA/PLL films exhibited robust biofilms with dense networks of yeast and hyphal cells encased in an extracellular matrix (Figure 7C, H). Biofilms were also observed on the surfaces of HA/CH-coated catheters (Figure 7B, G) but were, in general, less robust than those observed on bare catheters and exhibited larger numbers of yeast cells as well as blood cells trapped in a network of host proteins.

Catheters coated with HA/CH or PGA/PLL and loaded with β-peptide led to a marked decrease in biofilm formation in vivo compared to bare, untreated catheters (Figure 7D, I and E, J), similar to behavior observed using β-peptide-loaded films in vitro (as discussed above; see Figures 5 and 6). Tubes coated with β-peptide-loaded HA/CH films exhibited either no or very few yeast cells over the majority of their surfaces, but were covered with a network of host proteins (Figure 7D, I). In contrast, the surfaces of tubes coated with β-peptide-loaded PGA/PLL films were almost entirely free of yeast cells, hyphal cells, or networks of host proteins observed on HA/CH films (Figure 7E, J). Low- and high-magnification views of other regions on the catheters

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used in these in vivo experiments are included in Figures S7 and S8 and additional higher-magnification images arising from areas surveyed in Figure 7 are shown in Figure S9. These additional images are consistent with the general observations reported above.

DISCUSSION

The design of new materials and surface coatings that can prevent the formation of fungal biofilms on the surfaces of indwelling medical devices would help reduce the occurrence of device-associated fungal infections.[20−22] The results of this study demonstrate (i) that polymer-based PEM coatings fabricated from HA and CH, two polysaccharide-based weak polyelectrolytes, have inherent antifungal properties, and (ii) that venous catheters coated with HA/CH films can substantially reduce the formation of *C. albicans* biofilms in challenging in vitro and in vivo environments. Our results also demonstrate that the inherent antifungal properties of these coatings can be improved upon further by loading them with potent antifungal β-peptides that are released slowly into surrounding media. These observations, when combined, provide a basis for the design of "dual-action" antifungal coatings for catheters or other devices that serve as entry points for fungal infections and recalcitrant device-associated biofilms. In addition to the ability to fabricate coatings with enhanced antifungal activities, the use of a materials platform with inherent antifungal properties as a matrix for the sustained and localized release of antifungal β-peptides also creates opportunities to (i) reduce the amount of β-peptide needed for prophylaxis (thereby reducing both cost and potential side effects) and has the potential to (ii) sustain prophylaxis when concentrations of the β-peptide in the surrounding environment fall, either temporarily or permanently, below those required for maximal antifungal activity.

Our observation that HA/CH films exhibit inherent antifungal activity is consistent with prior reports that CH possesses inherent antimicrobial properties in other contexts.[40−45,55] PEMs fabricated by the layer-by-layer assembly of HA and CH have been demonstrated in past studies to exhibit antibacterial properties,[46] but the most extensive studies on the antifungal properties of CH itself have been conducted, in large measure, in the context of applications in the food and packaging industries.[41−43,55] A study by Martinez et al. reported the ability of CH-coated catheters to reduce *C. albicans* colonization in vitro and in vivo using a central venous catheter model similar to that investigated here,[45] however the surfaces investigated in that study were modified simply by incubating catheters in solutions of CH, a method that provides limited control over surface coverage, film thickness, or the amount of chitosan adsorbed. The layer-by-layer method used here for the fabrication of HA/CH multilayers provides tunable control over these and other important parameters, and thus insight into factors (such as film thickness, or the number of HA/CH layers adsorbed; e.g., Figure S3) that influence the inherent antifungal behaviors of these films.

We note that two recent studies have investigated PEMs fabricated using CH in the context of developing new antifungal coatings. Cado et al. reported on the behaviors of HA/CH films functionalized with the host-defense antimicrobial peptide cateslytin against both bacteria (*S. aureus*) and yeast (*C. albicans*).[37] Jiang et al. also recently described the *in vitro* antifungal behavior of PEMs fabricated from alginate and N-trimethylchitosan, a synthetic derivative of CH, on planar surfaces.[39] To the best of our knowledge, our present study is the first to characterize the inherent antifungal activity of multilayer coatings containing CH. This study is also the first to investigate the activities of PEM coatings, with or without the addition of auxiliary antifungal agents, against fungal biofilm formation in vivo in the clinically relevant and challenging context of a short-term venous catheter infection model.

Our results demonstrate that catheters coated with HA/CH films are inherently antifungal against planktonic *C. albicans* for short times (e.g., over 6 h; Figure 2A) and upon multiple short-term challenges with *C. albicans* inocula (e.g., for up to six consecutive 6 h inoculations; Figure 4A). These coatings also maintain some antifungal activity, albeit at more modest and variable levels relative to uncoated controls, when inoculated after prolonged exposure to physiologically relevant fluids (e.g., for up to 49 days, see Figure 3A). The inherent antifungal activities of these HA/CH coatings stands in stark contrast to that exhibited by the polypeptide-based PGA/PLL coating system, which does not exhibit inherent antifungal activity (Figure 2B and Figures 3B and 4B, gray bars) and requires the infusion of β-peptide to achieve significant and substantial reductions in fungal cell viability under these conditions (Figure 2B and Figures 3B and 4B, white bars).

HA/CH films also exhibited an inherent ability to reduce the development of *C. albicans* biofilms on film coated catheters, both in vitro and in vivo, relative to uncoated control catheters and catheters coated with PGA/PLL films (Figures 5−7). While the inherent antifungal activity of the HA/CH films was not sufficient to completely inhibit biofilm growth, biofilms observed on the surfaces of HA/CH-film-coated catheters were less dense and robust than those observed in bare tubes or on catheters coated with PGA/PLL films (Figures 5 and 7). For both film systems, the controlled intraluminal release of β-peptide resulted in substantial reductions in biofilm growth in vitro and in vivo. Catheters coated with β-peptide-loaded HA/CH films exhibited few surface-associated yeast cells but were covered with a dense network of host proteins (Figure 7). The surfaces of catheters coated with β-peptide-loaded PGA/PLL films were almost completely free of any yeast cells, biofilm, or host protein networks. Additional studies will be necessary to understand the physicochemical differences between HA/CH films and PGA/PLL films that lead to these large differences in host response.

Finally, our results also reveal several other significant and potentially useful differences between the HA/CH and PGA/PLL films systems that influence the loading and release of β-peptide. For example, HA/CH films were substantially thicker than PGA/PLL films (at the same number of deposited bilayers; see Figure S2), and catheters with HA/CH coatings both loaded (Figure 1B, C) and released (Figure 1G) significantly higher amounts of β-peptide than those coated with PGA/PLL films. HA/CH films also released incorporated β-peptide more rapidly than PGA/PLL films (over ∼50 days versus ∼100 days, respectively; Figure 1G). However, our current results demonstrate that the materials investigated here release β-peptide at rates sufficient to maintain intraluminal concentrations above the minimum inhibitory concentration (8 μg/mL)[38] over times scales sufficient for many clinically relevant applications. For example, the results shown in Figure 1G demonstrate that catheter tubes coated with PGA/PLL and CH/HA films release β-peptide in amounts sufficient to reach concentrations of 15.2 and 31.5 μg/mL, or concentrations that are ∼1.9 and ∼3.9 times MIC, over the 24 h period of incubation used in our in vivo experiments (we note, however, that the results shown in Figure 1G were obtained by incubation in buffer,

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and that rates of release could vary in vivo or in the presence of media and yeast used in our in vitro studies[38]). It is likely that film fabrication parameters and β-peptide loading protocols could be optimized to increase loading levels or tune and extend release profiles further. In a broader context, we note that the modular nature of the approach to layer-by-layer assembly reported here also provides opportunities to incorporate other film components or other active agents that could act orthogonally or in synergy with those described here to design multifunctional coatings that are active against a wider range of fungal or bacterial cells.

CONCLUSIONS

We have demonstrated a layer-by-layer approach to the design of antifungal coatings that substantially reduce the formation of *C. albicans* biofilms both in vitro and in vivo using a rat central venous catheter model. Coatings fabricated on the luminal surfaces of catheter segments using either (i) two polysaccharide-based components (HA and CH) or (ii) two polypeptide-based components (PGA and PLL) served as reservoirs for the loading and subsequent release of a potent β-peptide-based antifungal and antibiofilm agent. These β-peptide-loaded coatings were strongly antifungal against both planktonic *C. albicans* and the formation of surface-associated biofilms in vitro and in vivo. Multilayer coatings fabricated using CH, a weak polycation that is known to exhibit inherent antifungal properties in other contexts, also exhibited antifungal and antibiofilm properties in the absence of β-peptide both in vitro and in vivo. Our results demonstrate that these PEM coatings provide a useful platform for the design of new antifungal materials, and suggest opportunities for the design of new multifunctional coatings of interest in the context of preventing device-oriented microbial infections or a range of other potential biomedical applications where fungal infections are endemic.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsbiomaterials.5b00427.

Additional characterization of film thickness and morphology, the influence of film thickness and structure on loading, and additional SEM images showing surfaces resulting from in vitro and in vivo biofilm assays (PDF)

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Notes

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