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Nylon-3 Polymers Active against Drug-Resistant *Candida albicans* Biofilms

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Supporting Information

ABSTRACT: *Candida albicans* is the most common fungal pathogen in humans, and most diseases produced by *C. albicans* are associated with biofilms. We previously developed nylon-3 polymers with potent activity against planktonic *C. albicans* and excellent *C. albicans* versus mammalian cell selectivity. Here we show that these nylon-3 polymers have strong and selective activity against drug-resistant *C. albicans* in biofilms, as manifested by inhibition of biofilm formation and by killing of *C. albicans* in mature biofilms. The best nylon-3 polymer (poly-**βNM**) is superior to the antifungal drug fluconazole for all three strains examined. This polymer is slightly less effective than amphotericin B (AmpB) for two strains, but the polymer is superior against an AmpB-resistant strain.

Fungal infections represent a major problem in human health care.[1,2] *Candida albicans* is the most common fungal pathogen, causing invasive infections that are associated with high mortality.[1] Treatment of *C. albicans* infections is imperfect because current drugs have significant side effects, and resistance is developing for these drugs, including fluconazole and amphotericin B (AmpB).[3,4] The majority of *C. albicans* infections are associated with biofilms.[5] The development of strategies to attack biofilms formed by drug-resistant *C. albicans* while limiting toxicity to the human host is challenging because both organisms are eukaryotes.

Host-defense peptides have been widely explored for antimicrobial properties,[6−8] as have synthetic peptide analogues and unnatural, sequence-specific oligomers intended to mimic host-defense peptides,[9−18] but relatively few of these compounds have been reported for activity toward fungal biofilms.[19] Histatin 5, a natural peptide from saliva, is active against planktonic *C. albicans*,[20] i.e., free-floating cells, and this peptide can inhibit biofilm formation,[21] but the effect of histatin 5 on mature biofilms has not be reported. ApoEdpL-W, an 18-mer derived from human apolipoprotein E, inhibits *Candida* biofilm formation but has relatively little effect on mature biofilms.[22] β-Amino acid oligomers developed by our group inhibit the growth of planktonic *C. albicans*, and they inhibit biofilm formation by *C. albicans*; however, these β-peptides show little activity toward mature biofilms, and they are relatively toxic toward mammalian cells, as manifested by their hemolytic activity.[12]

The high cost of chemical synthesis for peptides or unnatural, sequence-specific oligomers such as β-peptides has inspired many groups to evaluate the antimicrobial actions of synthetic polymers. Antibacterial activity has been reported for a number of polymers,[23−38] but antifungal activity has been documented in only a few cases. Peptidopolysaccharides that are active against planktonic forms of *C. albicans* have been described.[32] Polyester−polycarbonate block copolymers that form hydrogels at high concentrations (20 mg/mL) are moderately active against fungal biofilms.[39] We recently described nylon-3 polymers that display potent and selective activity against planktonic forms of multiple fungal species.[40,41] The best antifungal nylon-3 polymers contain the cationic subunit **βNM** and the hydrophobic subunit **CH** in varying proportions (Figure 1) and display minimum inhibitory concentration

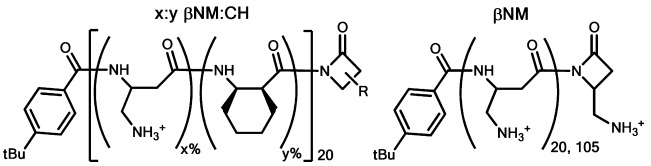

Figure 1. Structures of nylon-3 **βNM**:**CH** copolymers (left) and **βNM** homopolymers (right). R can be the side chain of either subunit for **βNM**:**CH** copolymers. All of the polymers used in this work have an average ∼20-mer length, except for a long version of the **βNM** homopolymer (∼105-mer). All of the polymers are heterochiral.

(MIC) = minimum fungicidal concentration (MFC) = 3 μg/mL for the planktonic form of the pathogenic K1 strain of *C. albicans*.[40] Here we show that such polymers can inhibit *C. albicans* biofilm formation and kill *C. albicans* in mature biofilms.

Three strains of *C. albicans* were examined in this study: clinical isolate K1, which is resistant to fluconazole;[42] clinical isolate Gu5, which is resistant to fluconazole;[43] and lab strain E4, which is resistant to both fluconazole and AmpB.[44] The activities of nylon-3 polymers were evaluated in terms of the minimum concentration necessary to inhibit 80% biofilm formation (the "sessile minimum inhibitory concentration", $SMIC_{80}$).[45] These values were determined by measuring cell viability within biofilms via an XTT assay.[46] The antifungal drugs fluconazole and AmpB were used as controls.

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Mammalian cell toxicity was assessed in terms of human red blood cell lysis ("hemolysis"), specifically the minimum concentration necessary to cause 10% hemolysis (HC_{10}).

Nylon-3 polymers were initially evaluated for inhibition of *C. albicans* biofilm formation (Table 1). In this study, *C. albicans* cells were combined with varying concentrations of polymer and then incubated for 48 h to allow biofilm formation. Each of the nylon-3 polymers could inhibit biofilm formation by all three strains of *C. albicans*, although the efficacies varied, with $SMIC_{80}$ values ranging from 9.4 to 75 μg/mL (Table 1). Polymers with a higher content of the cationic subunit (**βNM**) displayed stronger inhibitory activity (lower $SMIC_{80}$ values). The longest homopolymer, $(\beta NM)_{105}$, proved to be the most active, with $SMIC_{80}$ values of 9.4, 9.4, and 12.5 μg/mL toward the K1, Gu5, and E4 strains, respectively. For most of the polymers, no lysis of human red blood cells could be detected at the maximum concentration evaluated (2000 μg/mL); the most hydrophobic polymer, 70:30 **βNM:CH**, displayed limited hemolysis (~9%) at this concentration (Figure 2). Therefore, all of the nylon-3 polymers examined here demonstrate excellent selectivity in terms of their activity against the three *C. albicans* strains examined. The behavior of AmpB was inferior by some measures. This drug is very effective at inhibiting biofilm formation by the K1 and Gu5 strains ($SMIC_{80}$ = 2.4 and 0.8 μg/mL, respectively) but shows much lower activity for the E4 strain ($SMIC_{80}$ > 100 μg/mL). In addition, AmpB is highly hemolytic, in sharp contrast to the nylon-3 polymers (Figure 2). All three strains of *C. albicans* are highly resistant to fluconazole, and the nylon-3 polymers were superior to this drug in each case.

Table 1. Inhibitory Effect of Nylon-3 Polymers and Antifungal Drugs against *C. albicans* Biofilm Formation[a]

	$SMIC_{80}$ (μg/mL)		
polymer	K1	Gu5	E4
70:30 **βNM:CH**	37.5	37.5	75
80:20 **βNM:CH**	18.8	18.8	37.5
90:10 **βNM:CH**	12.5	9.4	18.8
βNM	18.8	18.8	18.8
$(\beta NM)_{105}$	9.4	9.4	12.5
AmpB	2.4	0.8	>100
fluconazole	>500	>500	375

[a]$SMIC_{80}$ is the concentration to inhibit 80% biofilm formation, as measured by assessing biofilm viability with an XTT assay. All of the nylon-3 polymers are of ~20-mer length except for $(\beta NM)_{105}$, which is of ~105-mer length.

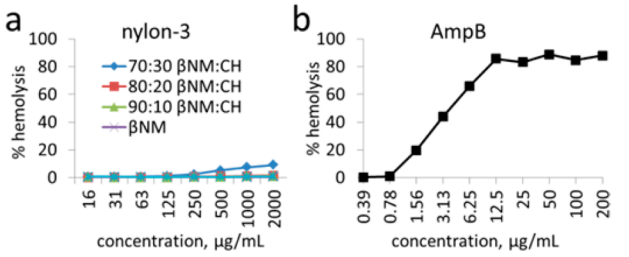

Figure 2. Dose-dependent hemolysis upon treatment with (a) nylon-3 polymers or (b) AmpB. The difference between the horizontal scales in (a) and (b) should be noted.

In the next set of experiments, we asked whether the nylon-3 polymers could kill *C. albicans* cells in pre-established biofilms (Table 2). This type of activity is known to be more difficult to achieve than inhibiting growth of planktonic cells or inhibiting biofilm formation because an established biofilm presents physical barriers to permeation by antifungal agents, and the sessile cells within a biofilm are physiologically distinct from planktonic cells.[5] Biofilms were allowed to form for 24 or 48 h and then treated with polymers or antifungal drugs for another 48 h, at which point the viability of *C. albicans* within the biofilm was assessed. In contrast to the biofilm formation assay results, where significant differences were observed among nylon-3 polymers, these polymers were similar to one another in terms of activity against 48 h biofilms. For the most hydrophobic polymer, 70:30 **βNM:CH**, the activity in this assay was comparable to that observed for inhibition of biofilm formation; however, for the polymers with greater cationic charge density, the activity against 48 h biofilms was somewhat lower (higher $SMIC_{80}$ values) than the inhibitory activity toward biofilm formation. Biofilms often contain polyanionic constituents, such as DNA,[47] which may hinder entry by the polycationic nylon-3 chains. A polymer that is more hydrophobic than 70:30 **βNM:CH** (40% **CH**) displayed similar or lower activity toward mature *C. albicans* biofilms and significantly lower activity in terms of inhibiting biofilm formation (Table S2 in the Supporting Information), which is consistent with the trend in nylon-3 activities toward planktonic *C. albicans*.[40]

Table 2. Inhibitory Effect of Nylon-3 Polymers and Antifungal Drugs against 48 h Mature *C. albicans* Biofilms[a]

	$SMIC_{80}$		
polymer	K1	Gu5	E4
70:30 **βNM:CH**	37.5	50	75
80:20 **βNM:CH**	37.5	75	50
90:10 **βNM:CH**	37.5	75	37.5
βNM	37.5	75	37.5
$(\beta NM)_{105}$	50	75	37.5
AmpB	6.3	6.3	>200
fluconazole	>1000	>1000	>1000

[a]$SMIC_{80}$ is the concentration to inhibit 80% biofilm growth, as measured by biofilm viability using an XTT assay. All of the nylon-3 polymers are of ~20-mer length except $(\beta NM)_{105}$, which is of ~105-mer length. See Table S1 for results with 24 h fungal biofilms.

AmpB is more active than any polymer against 48 h biofilms formed by susceptible K1 or Gu5 strains; however, the polymers have a clear advantage against biofilms formed by the AmpB-resistant E4 strain. Biofilms from all three strains are unaffected by fluconazole. Comparison of the $SMIC_{80}$ values for the polymers and AmpB against susceptible strains measured for 24 h (see Table S1) versus those for 48 h biofilms reveals a modest but interesting trend: the polymers seem to be slightly more active at the later time point. In contrast, AmpB seems to be slightly *less* active at the later time. The origin of this behavior is unclear.

We conducted live–dead staining followed by fluorescence microscopy to assess the impact of selected agents on K1 *C. albicans* cells within preformed biofilms (Figure 3). Mature *C. albicans* biofilms (48 h) were treated with $(\beta NM)_{105}$, AmpB, or fluconazole; for the polymer and AmpB, the concentrations used for these studies correspond to the $SMIC_{80}$ (50 and 6.3

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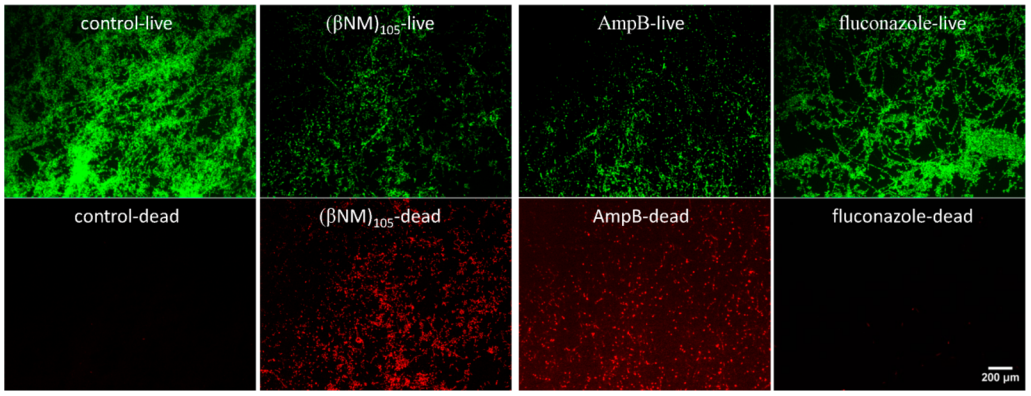

Figure 3. Fluorescence micrographs of mature K1 *C. albicans* biofilms (48 h) treated with antifungal agents and then subjected to live−dead staining. Biofilms were treated with antifungal agents at concentrations corresponding to the $SMIC_{80}$ for $(\boldsymbol{\beta}\mathbf{NM})_{105}$ and AmpB (50 and 6.3 μg/mL respectively) and at 1000 μg/mL for fluconazole. Untreated biofilms were used as controls. Scale bar is 200 μm.

μg/mL respectively), while for fluconazole the concentration was 1000 μg/mL. After treatment of the biofilm with each agent for 48 h, the biofilms were incubated with SYTO 9 and propidium iodide to stain live cells (green fluorescence) and dead cells (red fluorescence), respectively. Biofilms treated with $(\boldsymbol{\beta}\mathbf{NM})_{105}$ or AmpB showed a high density of dead cells, but the fluconazole-treated biofilm was similar to the control (no antifungal agent), with many cells evident, very few of which were dead. Biofilms treated with other nylon-3 polymers described here gave results qualitatively similar to those for $(\boldsymbol{\beta}\mathbf{NM})_{105}$ (data not shown). These imaging results are consistent with biofilm viability assays (Table 2) in indicating that fungicidal nylon-3 polymers can enter mature *C. albicans* biofilms and kill cells residing within.

We have shown that nylon-3 polymers containing the $\boldsymbol{\beta}$**NM** subunit can block biofilm formation by *C. albicans* and target cells within a mature biofilm. This activity is manifested against strains that are resistant to the antifungal drugs fluconazole and/or AmpB. The activity observed against *C. albicans* resident in existing biofilms is noteworthy because such cells typically display features that dampen drug efficacy, such as upregulation of efflux pumps in the outer membrane.[48] These observations suggest that the nylon-3 polymers discussed here may be useful for blocking fungal biofilm formation on biomedical device surfaces or for disinfecting those surfaces after infection has become established. The large-scale preparative accessibility of the nylon-3 polymers makes them attractive for surface-modification applications.

■ ASSOCIATED CONTENT

Supporting Information

Bioassay protocols and results. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare the following competing financial interest(s): S.H.G. and B.W. are co-inventors on a patent covering nylon-3 copolymers.

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