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

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Abstract

Candida albicans is the most common fungal pathogen in humans, and most diseases produced by *C. albicans* are associated with biofilms. Previously, we developed nylon-3 polymers with potent activity against planktonic *C. albicans* and excellent *C. albicans* vs. 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- β NM) 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} *C. 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 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 evaluated for activity toward fungal biofilms.¹⁹ Histatin 5, a natural peptide from saliva, is active against

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Notes: SHG and BW are co-inventors on a patent covering nylon-3 copolymers.

Associated Content: Supporting Information: Bioassay protocols and results. This information is available free of charge via the Internet at <http://pubs.acs.org/>.

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 been reported. ApoEdpLW, 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 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 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 Recently, we have 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 β NM and the hydrophobic subunit CH in varying proportions (Figure 1), and display minimum inhibitory concentration (MIC) = minimum fungicidal concentration (MFC) = 3 μ g/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;⁴² clinical isolate Gu5, which is resistant to fluconazole;⁴³ and lab strain E4, which is resistant to both fluconazole and AmpB.⁴⁴ The activity of nylon-3 polymers was evaluated in terms of the minimum concentration necessary to inhibit 80% biofilm formation (“sessile minimum inhibitory concentration,” SMIC₈₀).⁴⁵ These values were determined based by measuring cell viability within biofilms via an XTT assay.⁴⁶ The antifungal drugs fluconazole and AmpB were used as controls. Mammalian cell toxicity was assessed in terms of human red blood cell lysis (“hemolysis”), specifically, the minimum concentration necessary to cause 10% hemolysis (HC₁₀).

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 48 hours to allow biofilm formation. Each of the nylon-3 polymers could inhibit biofilm formation by all three strains of *C. albicans*, although efficacies varied, and SMIC₈₀ values ranged from 9.4 to 75 μ g/mL (Table 1). Polymers with a higher content of the cationic subunit (β NM) displayed stronger inhibitory activity (lower SMIC₈₀ values). The longest homopolymer, (β NM)₁₀₅, proved to be the most active, with SMIC values of 9.4, 9.4 and 12.5 μ g/mL toward K1, Gu5 and E4 strains, respectively. For most of the polymers, no hemolysis could be detected at the maximum concentration evaluated (2000 μ g/mL); the most hydrophobic polymer, 70:30 β NM: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 (SMIC₈₀ = 2.4

and 0.8 $\mu\text{g}/\text{mL}$ respectively), but shows much lower activity for the E4 strain ($\text{SMIC}_{80} > 100$ $\mu\text{g}/\text{mL}$). 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.

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 hours and then treated with polymers or antifungal drugs for another 48 hours, 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-hour biofilms. For the most hydrophobic polymer, 70:30 $\beta\text{NM}:\text{CH}$, activity in this assay was comparable to the activity observed for inhibition of biofilm formation; however, for the polymers with greater cationic charge density, activity against 48-hour biofilm was somewhat lower (higher SMIC_{80} values) than 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 $\beta\text{NM}:\text{CH}$ (40% CH) displays similar or lower activity toward mature *C. albicans* biofilms and significantly lower activity in terms of inhibiting biofilm formation (Table S2), which is consistent with the trend in nylon-3 activities toward planktonic *C. albicans*.⁴⁰

AmpB is more active than any polymer against 48-hour 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 SMIC_{80} values for polymers and AmpB against susceptible strains measured for 24-hour (see Figure S1) vs. 48-hour 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 pre-formed biofilms (Figure 3). Mature *C. albicans* biofilms (48-hour) were treated with (βNM)₁₀₅, AmpB or fluconazole; for the polymer and AmpB, the concentrations used for these studies correspond to the SMIC_{80} (50 and 6.3 $\mu\text{g}/\text{ml}$ respectively), while for fluconazole the concentration was 1000 $\mu\text{g}/\text{ml}$. After treatment of the biofilm with each agent for 48 hours, 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 (βNM)₁₀₅ or AmpB showed a high density of dead cells, but 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 (βNM)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 β NM subunit can block biofilm formation by *C. albicans* and target cells within a mature biofilm. This activity is manifested even 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 make them attractive for surface-modification applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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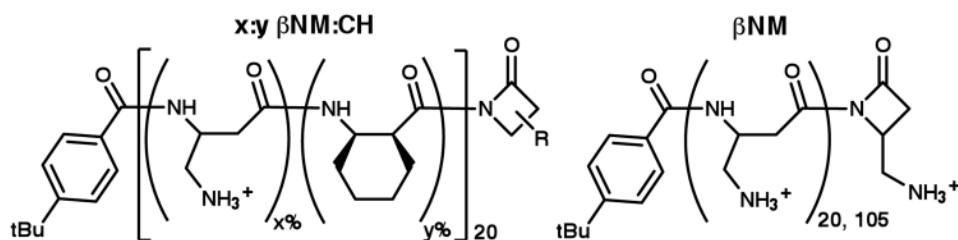


Figure 1. Structures of nylon-3 $\beta\text{NM}:\text{CH}$ copolymers (left) and βNM homopolymers (right). R can be the sidechain of either subunit for $\beta\text{NM}:\text{CH}$ copolymers. All of the polymers used in this work have an average ~ 20 -mer length, except for a long version of the βNM homopolymer (~ 105 -mer). All polymers are heterochiral.

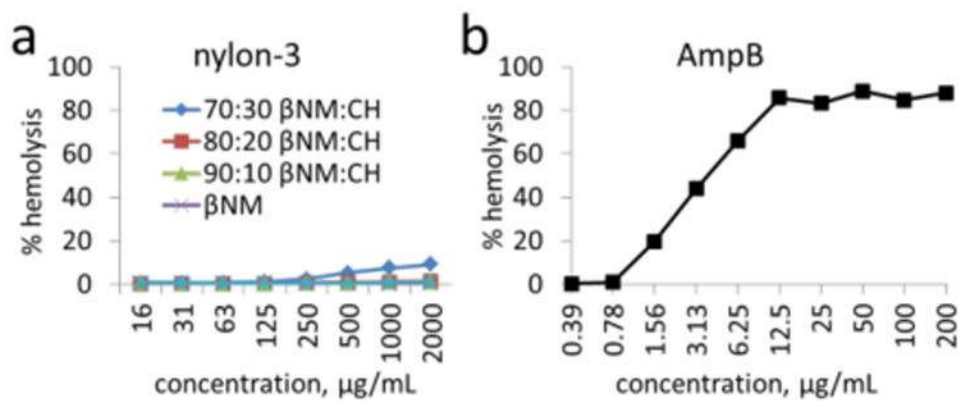


Figure 2. Dose-dependent hemolysis upon treatment with (a) nylon-3 polymers or (b) AmpB. Note the difference in horizontal scale between (a) and (b).

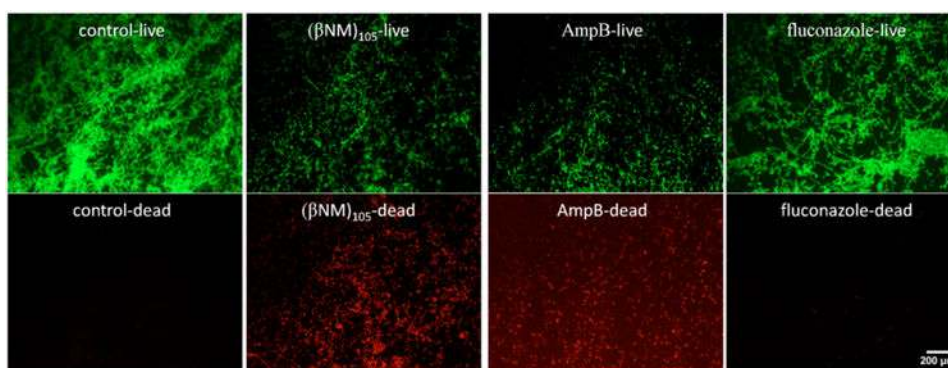


Figure 3. Fluorescence micrographs mature K1 *C. albicans* biofilms (48 hr) treated with antifungal agents and then subjected to Live-Dead staining. Biofilms were treated with antifungal agents at a concentration corresponding to the SMIC₈₀ for (βNM)₁₀₅ and AmpB (50 and 6.3 μg/ml respectively), and at 1000 μg/ml for fluconazole. Untreated biofilms were used as a control. Scale bar is 200 μm.

Table 1

The inhibitory effect of nylon-3 polymers and antifungal drugs against *C. albicans* biofilm formation^a

polymer	SMIC ₈₀ , µg/ml		
	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
(βNM) ₁₀₅	9.4	9.4	12.5
AmpB	2.4	0.8	>100
fluconazole	>500	>500	375

^aSMIC₈₀ is the concentration to inhibit 80% biofilm formation, as measured by assessing biofilm viability with an XTT assay. All nylon-3 polymers are of ~20 mer length except (βNM)₁₀₅, which is of ~105 mer length.

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Table 2

The inhibitory effect of nylon-3 polymers and antifungal drugs against 48 hr mature *C. albicans* biofilms^a

polymer	SMIC ₈₀		
	K1	Gu5	E4
70:30 β NM:CH	37.5	50	75
80:20 β NM:CH	37.5	75	50
90:10 β NM:CH	37.5	75	37.5
β NM	37.5	75	37.5
(β NM) ₁₀₅	50	75	37.5
AmpB	6.3	6.3	>200
fluconazole	>1000	>1000	>1000

^aSMIC₈₀ is the concentration to inhibit 80% biofilm growth, as measured by biofilm viability using an XTT assay. All nylon-3 polymers are of ~20 mer length except (β NM)₁₀₅ which is of ~105 mer length. See Table S1 for results with 24 hr fungal biofilms.