Original Research

Local delivery polymer provides sustained antifungal activity of amphotericin B with reduced cytotoxicity

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Impact statement

Amphotericin B (AmB) is an effective and commonly used antifungal agent. However, nephrotoxicity and poor solubility limits its usage. The proposed polymerized cyclodextrin (pCD) system therefore is an attractive method for AmB delivery, as it retains the antifungal activity of AmB while decreasing toxicity, and confining drug release to the local environment. This system could potentially be used for both prevention and treatment of established fungal infections, as AmB is toxic to fungus whether associated or released from pCD.

Abstract

A diverse range of clinical infections are on the increase, resulting in part from disruption of the natural microbiome, or even mycobiome, as a result of many different medical interventions. Amphotericin B (AmB) is a leading drug for the treatment of clinical fungal infections. However, AmB is extremely cytotoxic to mammalian cells, making use of the drug problematic. In this work, a drug delivery system made of polymerized cyclodextrin (pCD) allows for the localized administration of AmB, reducing the toxicity to host cells while retaining antifungal activity. A slow, sustained delivery rate of AmB was achieved through exploiting molecular interactions between the CD pockets and the drug. Surface plasmon resonance (SPR) and Fourier transform infrared spectroscopy (FTIR) were used to characterize the interaction between AmB and cyclodextrins (CDs). Through these methods, it was found that amphotericin binds strongly ($K_D = 5.4 \times 10^{-7}$ M^{-1}) to β-cyclodextrin. Release studies showed slow, sustained release of AmB from pCD disks. Antifungal activity was tested against *Saccharomyces cerevisiae* in assays for zone of inhibition, contact killing, and solution killing. In all assays, AmB-loaded pCD disks were found to exhibit significant antifungal activity. These results indicate that AmB-loaded pCD disks are capable of both the prevention of fungal growth and the elimination of established colonies. Additionally, results suggest that AmB exhibits antifungal activity whether associated with or released from pCD. The usage of pCD as a delivery vehicle also significantly reduces the toxic side effects of AmB, as seen in mammalian cell culture studies. These results show that in addition to reducing side-effects from systemic dosing, local delivery of AmB from pCD disks has the potential to improve its usage both in antifungal efficacy and reduced mammalian cell toxicity.

Keywords: Drug delivery, amphotericin B, antifungal, cyclodextrin, infection, cytotoxicity

Introduction

Invasive fungal infections pose a significant burden upon health care systems due to limitations in both their diagnosis and treatment. One fungal pathogen, *Candida* spp, causes serious infections in approximately 46,000 patients each year in the United States.[1] The proliferation of aggressive antibiotic and chemotherapy regimens, hematopoietic stem cell transplants, organ transplantation, and placement of indwelling devices has led to a disruption of host microbiome (including mycobiome), leaving this population vulnerable to fungal infections.[1,2] Critically ill patients in

intensive care units are at elevated risk for fungal infections due to a high use of central lines, altered pharmacokinetics, and extreme illness.[2,3] The formation of fungal biofilms on in-dwelling devices such as catheters, stents, and shunts can cause bloodstream infections and poses a significant treatment challenge.[4,5] Invasive mold infections are also a source of high morbidity and mortality, with stem cell transplant and solid organ transplant patients being particularly vulnerable.[2] Additionally, evidence suggests that prophylactic treatment with antifungals and antimicrobials has further increased the incidence of fungal infection.[6]

Amphotericin B (AmB) is a polyene antifungal compound that has been used to combat fungal infections for over 50 years.[7] Despite the use of AmB for decades, resistance remains rare, as the adaptations weaken fungal species such as *Candida albicans* into non-virulent pathogens.[7] AmB is available in two main formulations: free AmB and liposomal AmB.[8] Free AmB is poorly soluble and comes with serious nephrotoxicity concerns.[9] Liposomal forms of AmB have slightly decreased toxicity and better pharmacokinetic properties,[10,11] but some systemic toxicity remains, and their high costs limit utility in developing countries. Lipid-based formulations have also been found to be more effective at shortening length of stay for patients.[12] In these formulations, AmB is typically retained within the liposome with the help of a lipid like deoxycholate (e.g. AmBisome), which transfers to the fungal membrane where it ultimately forms a pore leading to cell death.[7] This transfer from liposome to fungal cell membrane has been found to be energy dependent.[13]

Several groups have investigated various methods of delivering AmB from polymers to provide a sustained release[14–16] that would be ideally suited for residence in bone marrow where repeated intravenous applications are required. Cyclodextrin (CD) is a ring of 6, 7, or 8 glucose molecules with a hydrophilic exterior and slightly hydrophobic interior, sometimes used pharmacologically to solubilize hydrophobic drugs including AmB.[14,17,18] Importantly, liposomes containing AmB complexed with chemically modified β-cyclodextrin (βCD) achieved better area under the curve and maximum serum concentrations than liposomal AmB without βCD complexation.[17] In their work, Chakraborty and Naik hypothesized that the βCD–AmB complex slowed transfer of AmB from the liposome to the plasma and host tissues, causing the improved properties that were seen.

Our group takes a different approach, using an insoluble form of pCD as a local drug delivery platform to harness the inclusion complex between CD and drug. The rate of association/dissociation between drug and CD is the rate limiting step for drug release where only drug dissociated from CD is free to diffuse out of the device, compared to conventional delivery polymers where diffusion is the rate limiting step.[19] By tailoring the type of CD, we hypothesize that we can control delivery rates while still achieving high drug loading from organic and aqueous solvents. Previous research using pCD has focused on antimicrobial therapies to prevent bacterial device infection.[20,21] This work builds upon our extensive antibacterial expertise and extends protection to fungal pathogens.

The main hypothesis of this work is that pCD will load high amounts of AmB and retain that AmB to minimize host toxicity while providing a long-term drug reservoir capable of killing fungal cells through localized delivery. Based on our results, the loading of AmB into pCD disks improves its usage as an antifungal agent, making it potentially useful for prevention and treatment of established fungal infections through drug release and bioactivity on contact. Additionally, the association between AmB and pCD decreases the drug's cytotoxicity, while local delivery reduces concerns about system side-effects such as nephrotoxicity.

Materials and methods

Chemicals and prepolymers: Lightly cross-linked βCD (2–15 kDa, average 10 CDs per chain) and γ-cyclodextrin as a prepolymer were purchased from CycloLab Ltd (Hungary). Dextran (Dex, 15–20 kDa) was purchased from Polyscience. 1,6-diisocyanatohexane (HMDI, Sigma Aldrich), AmB (GenDEPOT), N,N-dimethylformamide (DMF, extra dry, Applied Biosystems), dimethyl sulfoxide (DMSO, Acros), Tween 80 (Acros Organic), yeast extract (Fisher Scientific), tryptone (Fisher Scientific), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (Sigma Aldrich), and N-hydroxysuccinimide (Sigma Aldrich) were used as received.

Cell cultures: [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfopheny)-2H-tetrazolium] (MTS) was purchased from Promega (Madison, WI). 3T3 mouse fibroblasts and *Saccharomyces cerevisiae* (ATCC® 9763™) were purchased from ATCC (Manassas, VA). Dulbecco's Modification of Eagles Medium (DMEM), fetal bovine serum, and penicillin–streptomycin were purchased from Fisher Scientific and used as received.

Polymerization of an insoluble CD drug delivery system

The lightly cross-linked CD prepolymers were further polymerized as previously published.[22–24] Briefly, 1.5 g of CD prepolymer was dissolved in 4.5 mL DMF (33.3% weight/volume) and polymerized with HMDI in a ratio of 1:0.32 (glucose residue:HMDI) for four days at room temperature. Disks (8 mm diameter) were washed with excess DMF, DMF/water, and water to remove unreacted CD and HMDI.

AmB loading into pCD

AmB was dissolved in DMSO (5 wt%) and loaded into pCD disks for three days at room temperature (25°C). Loaded disks were removed from solution, blotted on Kimwipes, and briefly washed in excess water to remove surface DMSO and free AmB prior to drying.

Surface plasmon resonance (SPR)

βCD prepolymer solubilized in HBS-N buffer was immobilized to a CM5 sensor chip via NHS/EDC chemistry (10 μL/min flow; 7 min contact time) on a Biacore X100 system. Coupling was terminated with ethanolamine. Binding was carried out at 25°C. The chip was primed

with three cycles of 10 μL 10% DMSO in PBS prior to 180 s binding with AmB (5, 10, 20, and 50 μM). Dissociation proceeded for 540 s prior to two regeneration cycles (30 s each) with 50 mM NaOH, 25% DMSO PBS, or 50% DMSO in PBS. The affinity of AmB to CD was determined from the dissociation constant (K_D) calculated by the evaluation software from Biacore X100.

Fourier transform infrared spectroscopy (FTIR)

FTIR spectra were obtained using an Excalibur FTS 3000.[23] AmB powder, βCD prepolymer, unloaded pCD-β, and an AmB-loaded pCD-β were pressed into a pellet with KBr (1:100, sample: KBr) and scanned from 5000 to 400 cm^{-1}.

In vitro AmB release

AmB-loaded pCD disks were incubated with 1 mL of phosphate buffered saline (PBS, pH 7.4). Every 24 ± 5 h release media was removed and replaced with fresh. The concentration of each time point sample was determined spectrophotometrically at 382 nm using a standard curve of AmB in PBS.

Zone of inhibition assay

A modified zone of inhibition assay was used to determine the antifungal efficacy of released AmB *in vitro.* AmB-loaded pCD-β disks were evaluated against *S. cerevisiae*. *S. cerevisiae* was cultured overnight and 70 μL of this culture was then spread on a YM Agar plate. AmB-loaded pCD-β disks were placed on this fresh *S. cerevisiae* lawn and incubated at 30°C for 72 h. The zone of inhibition around each disk was measured with calipers at three points and recorded. Images were captured for inhibition area analysis using Image J. Each disk was then transferred onto a new *S. cerevisiae* plate and the process was repeated.

Contact killing assay

S. cerevisiae culture (10,000 or 2000 CFU) was pipetted onto AmB-loaded pCD-β disks (n = 3). Empty pCD disks without any drug were used as a polymer control. The yeast-covered disk was incubated at room temperature (25°C) for 3 h and then was placed into a test tube containing 5 mL of YM broth and incubated at 30°C. After 72 h the medium containing empty pCD disks was diluted 1:10,000. Seventy microliters of the diluted solution (neat for AmB-loaded pCD-β disks) was spread onto YM agar plates. The agar plates were incubated for 72 h and colonies counted to determine CFU and growth.

Solution killing assay

S. cerevisiae was introduced to 5 mL YM broth and allowed to incubate at 30°C for 96 h. During this incubation process, AmB-loaded pCD-β disks were added to specific samples at t = 0, 24, 48, and 72 h. Every 24 h, a sample was taken from each YM broth tubes, diluted 1:10,000, and 70 μL of this diluted solution was spread onto YM agar plates. The agar plates were incubated for 72 h and colonies counted to determine growth. The viable cell count in each sample was used to create a growth curve for *S. cerevisiae* over time and with varying exposure times to AmB-loaded pCD-β disks.

Cytotoxicity of pCD-released AmB

Cell viability tests were performed using 3T3 mouse fibroblast cells (25,000 cells/well) with DMEM high glucose (HG) in 10% serum in a 24 well plate. 3T3 cells were allowed to adhere for 4 h prior to placing sterile, AmB-loaded pCD disks in Transwell inserts (Costar, 5.0 μm pore size) in the well. Disks were removed after 24 h, media replaced with fresh media containing 1% MTS and incubated for 4 h before reading absorbance at 490 nm (BioTek H1).

To compare free AmB toxicity to that of pCD-released AmB, free AmB was used at a concentration comparable to that released at t = 24 h in the AmB-loaded pCD *in vitro* release data (10 μg/mL). This was done to ensure that both the pCD-AmB and free AmB conditions resulted in an equal concentration of free/delivered AmB in the well, so that toxicity results were due to formulation differences and not concentration differences. Free AmB is solubilized in 1% DMSO, and a 1% DMSO group was used as a control.

AmB-loaded pCD disks were placed in fresh DMEM-HG with 10% serum and media changed every day to model infinite sink release. On day 6 AmB-loaded pCD disks were placed on 25,000 fresh 3T3s in a 24 well plate as above. After 24 h exposure, disks were removed and metabolic activity assayed with 1% MTS in media as before. For both time points, a standard curve of 3T3s in media only (no drug) and pCD disks with only DMSO treatment (solvent loading control) served as controls.

Statistical analysis

If not otherwise stated, experiments were carried out in triplicate for statistical analysis (n = 3). Data displayed is the mean of each condition, and error bars represent the standard deviation of the triplicate set. One-way ANOVA was done using Prism to determine reported statistical significance. Significance is defined to be a p-value < 0.05.

Results

Determining binding affinity using SPR

SPR was used to characterize the molecular interactions between AmB and pCD. Based on this information regarding binding affinity, we can better understand the binding and release kinetics associated with localized AmB delivery using this system. Increasing the amount of solvent in the regeneration buffer increased the value of K_D (Figure 1). Regeneration with 50 mM NaOH gave a K_D value of 5.4×10^{-7} M^{-1} compared to 50% DMSO (5.48 M^{-1}) for a range of 10^8. In physiological conditions, AmB binds tightly to βCD due to hydrophobic interactions. However, a decrease in these hydrophobic interactions (addition of solvent) increases dissociation.

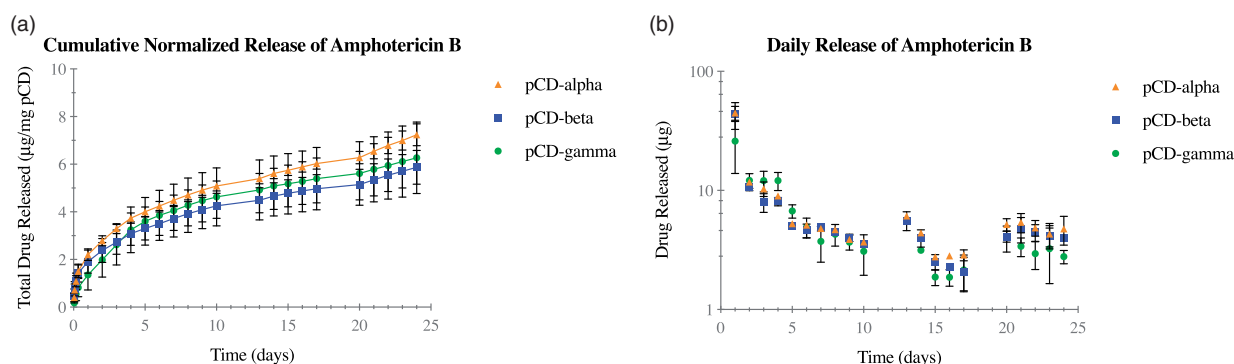## Characterizing AmB–pCD complex using FTIR

By using FTIR, we can characterize the physical association between AmB and pCD and compare it to our initial computer-generated model (Figure 1). Between 2000 and 950 cm^{-1} free AmB powder showed peaks at 1670, 1480, and 1242 cm^{-1}, which represent C=O, –OH, and C–O
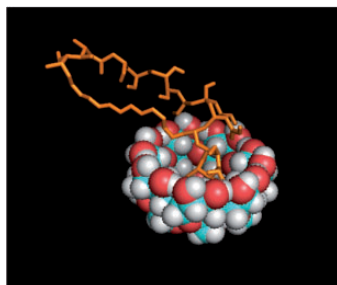
Figure 1. Image shown is a 3D conformer of modeled affinity binding between pCD-beta and AmB. (A color version of this figure is available in the online journal.)

FTIR: Amphotericin-Cyclodextrin Association

— AmB Powder
— AmB-loaded pCD-β
— pCD-β disk
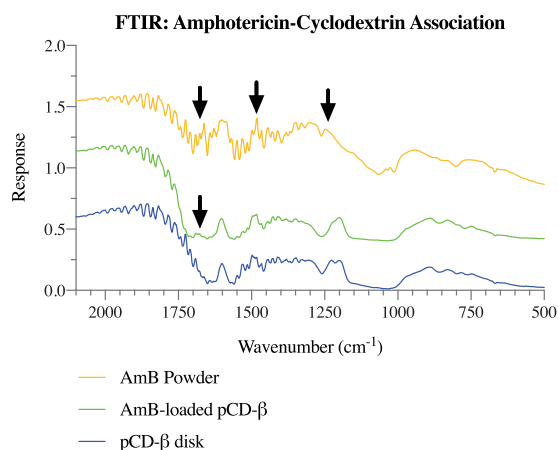
Figure 2. FTIR data for pCD-*β* disks, AmB-loaded pCD-*β* disks, and free AmB powder without polymer. Free AmB showed peaks at 1670, 1480, and 1242 cm^{-1}, which represent C=O, –OH, and C–O stretching of the carboxylic groups, respectively. AmB-loaded pCD showed a peak at 1670 cm^{-1} which corresponds to the C=O in free AmB. AmB: amphotericin B; FTIR: Fourier transform infrared spectroscopy; pCD: polymerized cyclodextrin. (A color version of this figure is available in the online journal.)

stretching of the carboxylic groups, respectively (Figure 2). The peaks at 1670 and 1242 cm^{-1} indicate the existence of an ester group. The AmB-loaded pCD spectrum showed a peak at 1670 cm^{-1} which corresponds to the C=O indicated in the AmB powder spectrum. This indicates that AmB is associated with the CD pocket and that the ester group on the AmB is outside of the hydrophobic pockets of the pCD.
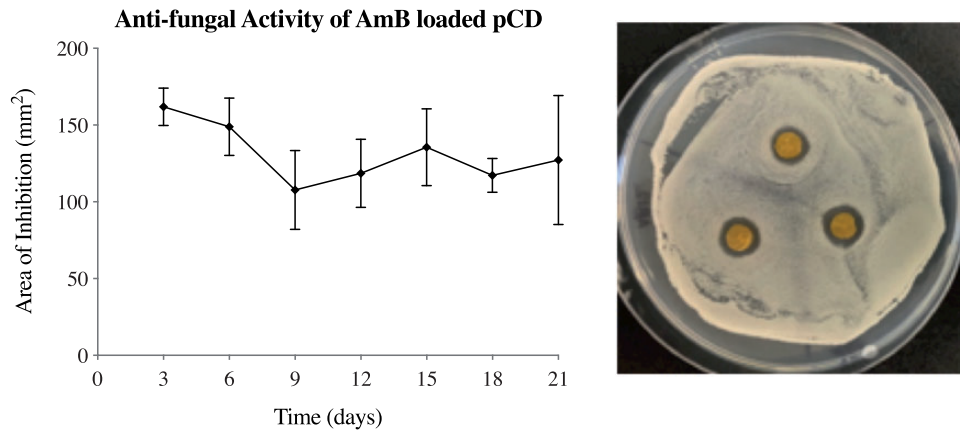
In vitro AmB release profile

SPR results show high affinity between AmB and pCD, indicating a slow and steady release of AmB over time. To confirm this release profile, the results of an *in vitro* drug release study are reported in Figure 3. Normalized cumulative release can be seen in Figure 3(a) and daily cumulative release is shown in Figure 3(b). All three CD types are shown and have similar release profiles. Release is seen out to at least 24 days. An initial burst release can be seen within the first 24 h (day 1), where an average of 38 μg is released.

## Determining antifungal activity using a zone of inhibition assay

A periodic zone of inhibition assay was used to show sustained, local antifungal effect of drug-loaded polymer implants. Areas cleared on plates replaced every three days are shown in Figure 4. Implants were capable of clearing new zones consistently throughout the three weeks of study, indicating release and antifungal activity capable of preventing fungal growth throughout this period. While the release study (Figure 3) shows an initial burst release of AmB, the zone of inhibition assay does not show significantly higher killing at the first time point. This is likely due to partitioning, as the hydrophobic nature of AmB makes it difficult for the drug to diffuse through the agar. To address this, we also tested the activity of AmB loaded into pCD disks to kill *S. cerevisiae* in solution (Figure 6).

Determining complexed activity using a contact killing assay

Based on the SPR data, which indicates AmB is more likely to be associated with pCD than released at early time points, we also tested the antifungal activity of AmB-

(a)
Cumulative Normalized Release of Amphotericin B

▲ pCD-alpha
■ pCD-beta
● pCD-gamma

(b)
Daily Release of Amphotericin B

▲ pCD-alpha
■ pCD-beta
● pCD-gamma

Figure 3. AmB released from all three types of pCD (*α*, *β*, and *γ* at similar rates for more than 24 days (a). Daily release shows a small initial burst followed by steady delivery rates out past day 5 (b). pCD: polymerized cyclodextrin. (A color version of this figure is available in the online journal.)

Figure 4. AmB-loaded pCD disks show consistent antifungal activity against *S. cerevisiae* for 21 days. AmB-loaded pCD disks were placed on fresh *S. cerevisiae* lawns at t = 0 and grown for 72 h at 30°C. Zone of clearance was measured and AmB-loaded disks placed on a fresh lawn every three days. ImageJ analysis was used to calculate the area of clearance; AmB released from pCD showed little variability in the area of inhibition over the course of the assay demonstrating long-term drug stability and efficacy. Image shown is t = day 15. AmB: amphotericin B; pCD: polymerized cyclodextrin. (A color version of this figure is available in the online journal.)
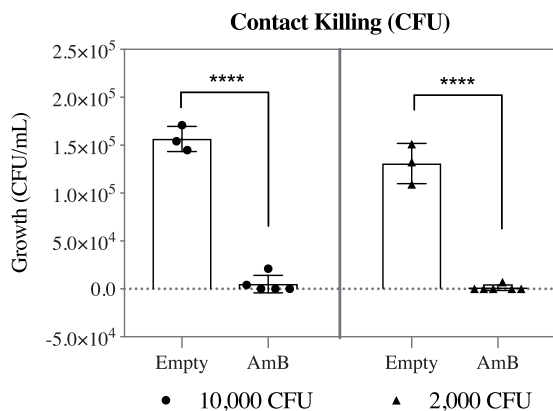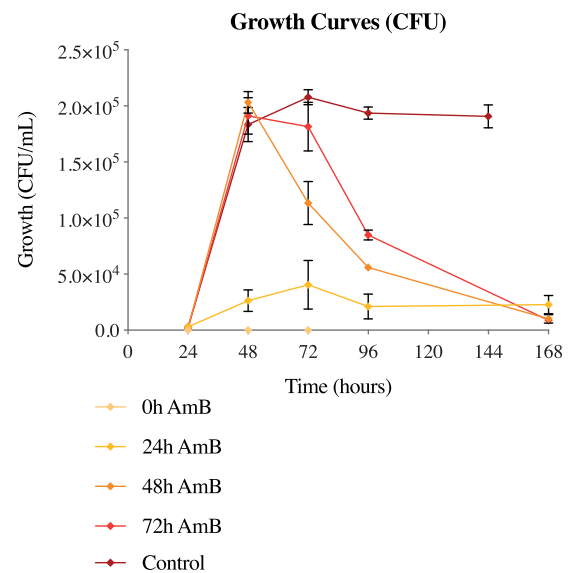

Figure 5. AmB does not need to dissociate from the CD pocket for antifungal activity. *S. cerevisiae* was cultured on AmB-loaded pCD disks or pCD without drug at two different yeast cell burdens (10,000 or 2000 CFU) for 3 h prior to culturing in sterile YM broth for 72 h, dilution and plating. AmB loaded into pCD significantly reduced viable *S. cerevisiae*. Statistics: ****represents $p < 0.0001$. AmB: amphotericin B; CFU: Colony forming units.

Figure 6. AmB-loaded pCD prevents progression of *S. cerevisiae* from lag to exponential growth phase and is capable of killing *S. cerevisiae* once established in late exponential or stationary phase. Growth curves of *S. cerevisiae* are shown with addition of AmB-loaded pCD-β disks at time = 0, 24, 48, and 72 h post-seeding. Control represents *S. cerevisiae* growth with no antifungal intervention. Addition of AmB-loaded pCD-β disks shows significant killing at all time points. AmB: amphotericin B; CFU: Colony forming units. (A color version of this figure is available in the online journal.)

loaded pCD without solution release. AmB-loaded pCD disks showed significant contact killing when challenged with *S. cerevisiae* on the pCD disk surface, in comparison to disks not loaded with AmB (Figure 5). These data show that AmB dissociating from a CD pocket and diffusing away (i.e. 'delivery') is not necessary for antifungal activity, and that AmB retains antifungal abilities when still associated with the CD pockets.

Determining activity against established colonies using a solution killing assay

The zone of inhibition assay may give indication of persistence of drug effect, but it does not represent all wound environments, particularly ones with high fluid content. Additionally, while the two previously mentioned assays might give indication as to how this device can prevent new infections, it does not show whether the device is capable of treating existing infections. To address these conditions, antifungal activity of AmB-loaded pCD was also tested in solution with varying time points of device introduction. When AmB-loaded pCD-β disks are added to a *S. cerevisiae* culture at t = 0 (time of seeding, inhibition), all yeast cells appear eradicated and no future growth is seen (Figure 6). When AmB-loaded pCD-β disks are added to the yeast cultures at t = 24 h, growth is contained at low levels. Treating yeast cultures with AmB-loaded pCD disks at t = 48 h and t = 72 h lines (when growth is established) resulted in a slow, steady killing of yeast cells. After 168 h, all AmB-loaded pCD-β disk curves show significant reduction of microbial load.
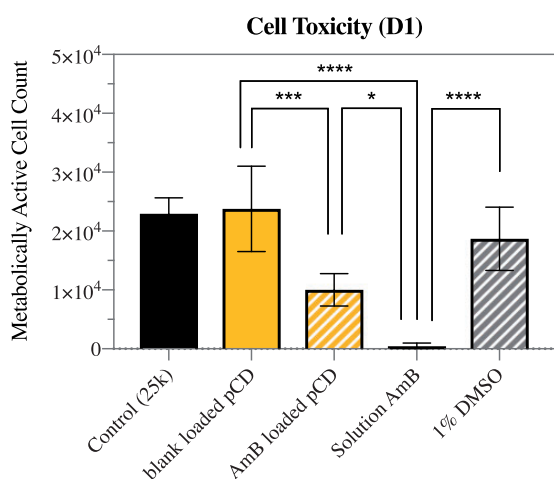
Cytotoxicity of pCD-released AmB

AmB-loaded pCD disks show reduced cell toxicity when compared to an equivalent dose of free AmB. Toxicity of free AmB is significant and is due to drug toxicity, not DMSO solvent concentration, which is shown by the relative high cell counts in the 1% DMSO and blank-loaded pCD experimental groups (p < 0.0001 and 0.001, respectively; Figure 7). While free AmB resulted in an average cell survival of 1%, pCD-delivered AmB showed an average survival of 40% after 24 h (day 1), when the small burst release occurs. However, after six days of release, AmB-loaded pCD disks are no longer significantly cytotoxic (Figure 8). This result is consistent with the release observed for AmB from pCD *in vitro,* where there is a small initial burst followed by a long, steady release after day 5. In addition, the type of pCD shows no impact on cytotoxicity, which is consistent with the release observed for AmB (Figure 3).

Discussion

Determining binding affinity using SPR

SPR data indicated a very strong affinity between AmB and βCD (5.44×10^{-7} M^{-1}; Table 1) using aqueous 50 mM NaOH as the regeneration buffer. SPR first allows AmB to flow past immobilized βCD and associate followed by buffer-only dissociation. The regeneration step is designed to interfere with the interaction and cause the drug to dissociate. The addition of DMSO solvent significantly lowered the K_D value by a magnitude of 10^8 indicating hydrophobic interactions are critical to AmB associating with βCD. Under normal physiological conditions, AmB is favored in the AmB-CD associated state. Adding DMSO to lower the solvent polarity shifts the equilibrium significantly toward dissociation. Understanding the binding strength and mechanism of AmB with βCD is critical to optimizing loading conditions and predicting release kinetics of drugs from pCD.

Characterizing AmB–pCD complex using FTIR

We used FTIR to confirm that AmB is associated with the CD pocket and begin determining how the drug positioned itself inside the CD pocket. The FTIR spectra of the AmB-loaded pCD showed the 1670 cm^{-1} C=O peak (Figure 2), which was not present in the unloaded βCD spectra indicating the presence of AmB. The peak at 1670 cm^{-1} corresponds to the ester bond on AmB and also shows that the carboxylic acid group of AmB is outside the hydrophobic CD pocket.

In vitro AmB release profile

The results from the drug release study (Figure 3) confirm the SPR findings that show a very high affinity for AmB to pCD. The small burst-type release seen in the first few days is likely the result of non-specifically bound AmB and the outer shell of CD pockets releasing AmB. After this initial burst, the release shifts into a slow, steady release rate approaching zero-order kinetics (Figure 3(b)). There was no significant difference in the release from pCD-α, -β, and -γ disks, indicating that the difference in the affinity profile of AmB for the three different types of CD is small

Time-Dependent Toxicity
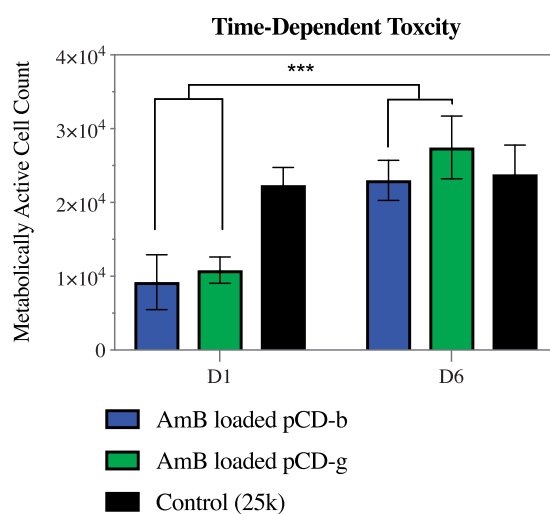
Figure 8. Cell toxicity from AmB-loaded pCD hydrogels is significantly reduced at later time points when a steady amount of AmB is released. AmB-loaded pCD was placed in serum containing media with daily media changes. After one or six days, AmB-loaded pCD disks were placed on 25,000 3T3 cells and after 24 h exposed to MTT for 4 h before reading absorbance. Statistics: *** represents p < 0.001. AmB: amphotericin B; pCD: polymerized cyclodextrin. (A color version of this figure is available in the online journal.)

Cell Toxicity (D1)

Figure 7. AmB released from pCD shows significantly reduced toxicity compared to free AmB. The cytotoxicity of free drug was confirmed by solvent control and blank-loaded pCD exposed to DMSO solvent but no drug, which both showed minimal toxicity compared to cell only control. All conditions were tested with 3T3 mouse fibroblast cells and 24 h exposure. Statistics: **** represents p < 0.0001, *** represents p < 0.001, and * represents p < 0.03. AmB: amphotericin B; DMSO: dimethyl sulfoxide; pCD: polymerized cyclodextrin. (A color version of this figure is available in the online journal.)

Table 1. K_D values of various regenerations determined via SPR.

Regeneration	K_D (M^{-1})
50 mM NaOH	5.44×10^{-7}
25% DMSO in PBS	5.45×10^{-5}
50% DMSO in PBS	5.48

DMSO: dimethyl sulfoxide; PBS: phosphate buffered saline.

enough to be overwhelmed by the equilibrium kinetics of dissociation into free solution.

Antifungal activity of pCD complexed and delivered AmB

The zone of inhibition data (Figure 4) shows that the AmB-loaded pCD disks were able to kill yeast out to at least 21 days with no significant reduction in inhibitory area. These results indicate that the AmB-loaded pCD is bioactive and able to kill a large number of yeast on a chronic timescale.

The SPR and release data show that pCD acts as a large sink for AmB meaning that at shorter timescales more drug stays in the pCD polymer than is released. We therefore tested whether AmB associated with pCD is capable of killing yeast through contact rather than direct release. AmB-loaded pCD significantly reduced the number of yeast cells compared to pCD without drug (p < 0.0001; Figure 5). Thus, AmB is cytotoxic to fungus whether associated or released from our pCD polymer.

AmB-loaded pCD was able to prevent *S. cerevisiae* from entering exponential growth phase (Figure 6) and was able to kill sufficient yeast cells in exponential growth to reduce the burden near initial seeding levels. This solution-based killing assay demonstrates that AmB-loaded pCD can kill even growing populations of fungal cells through a combination of drug release and contact killing. These data indicate further investigation of our AmB–pCD system for both prevention and treatment of fungal infections, up to full (72 h) growth is warranted. As fungal infections are often difficult to diagnose, a system that can be used for both prevention and treatment is a very clinically relevant goal.

Cytotoxicity of pCD-released AmB

The toxicity of free AmB is well known, and liposomal AmB is often used instead in an attempt to minimize the toxicity. Figure 6 shows that AmB-loaded pCD is significantly less toxic than free AmB, where both conditions resulted in a 10 µg/mL concentration of free/delivered AmB in the well (24 h time point). In fact, the AmB-loaded pCD condition could be considered to have more AmB overall, since the 10 µg/mL concentration occurs due to drug that has been released, and there is >100 µg of drug still held within the polymer. The decrease in toxicity seen in the AmB-loaded pCD condition is due to the occurrence of drug release over time, in comparison to the bolus dose used in traditional systemic administration (free AmB condition). While associated with CD, AmB does not appear to exhibit cytotoxic effects, meaning the consistent release of drug into media is what allows for the cytotoxicity that is seen. In this way, cells are likely exposed to fewer active AmB molecules at the beginning of treatment. Additionally, while there is some toxicity associated with the AmB-loaded pCD initially, this toxicity is eliminated once the burst release is over (Figure 8) and slow, steady release of AmB is achieved (Figure 3). This aligns with current usage, as the current burst release of 10 µg/mL is higher than clinically used AmB concentrations (plasma concentrations of 0.5 to 2 µg/mL), while later time points are more consistently within this range. The combination of solution-based

killing (Figure 6) with lack of long-term cytotoxicity indicates that AmB-loaded pCD is a potentially attractive option for treating fungal infections in medically fragile populations.

Overall, this work showed that AmB exhibits high affinity for CD and binds tightly to pCD. AmB releases for more than 24 days at near zero-order kinetics following an initial burst. AmB-loaded pCD was capable of clearing *S. cerevisiae* lawns for > 21 days and cells in contact with the drug-loaded polymer. In addition, AmB-loaded pCD prevented growth of *S. cerevisiae* in culture from lag into exponential growth. The AmB-loaded pCD also was capable of recovering established colonies of yeast. The toxicity of AmB-loaded pCD is significantly less than free AmB and quickly resolves to non-toxic levels after the initial burst release. Taken together these data indicate that AmB-loaded pCD can both prevent and treat fungal infections, but *in vivo* testing is required for further evidence.

In conclusion, we provided data demonstrating that AmB-loaded pCD is capable of preventing fungal growth and eliminating established fungal colonies with minimal toxicity to the host cells. This AmB delivery system represents a promising strategy for the treatment of localized invasive fungal infections, where systemic bloodstream administration is not necessary. With the current formulation, we could treat fungal growth at the site of implanted materials such as catheters and shunts, or in the abdominal region, as is the case in intra-abdominal candidiasis (*Candida* peritonitis). Our lab has also demonstrated the production of our pCD delivery system in a microparticle form.[25] This microparticle delivery system could potentially be used to adapt the AmB delivery for use in pulmonary fungal infections. Addressing fungal infections of all types continues to be a growing health care concern as we learn more about the mycobiome and its disruption through various medical interventions.

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