

Antifungal hydrogels

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Fungi are increasingly identified as major pathogens in bloodstream infections, often involving indwelling devices. Materials with antifungal properties may provide an important deterrent to these infections. Here we describe amphogel, a dextran-based hydrogel into which amphotericin B is adsorbed. Amphogel kills fungi within 2 h of contact and can be reused for at least 53 days without losing its effectiveness against *Candida albicans*. The antifungal material is biocompatible *in vivo* and does not cause hemolysis in human blood. Amphogel inoculated with *C. albicans* and implanted in mice prevents fungal infection. Amphogel also mitigates fungal biofilm formation. An antifungal matrix with these properties could be used to coat a variety of medical devices such as catheters as well as industrial surfaces.

antibiotic | *Candida albicans* | matrix

Intravascular catheter infections are a major cause of morbidity and mortality in hospitalized patients, accounting for the majority of the 200,000 nosocomial bloodstream infections occurring in the United States annually (1). *Candida* spp. are the fourth most common cause of bloodstream infection in hospitalized patients (2). Up to 40% of patients with *Candida* spp. isolated from i.v. lines have fungemia, and the mortality rate of patients with catheter-related candidemia approaches 40% (3). Fungal sepsis is a leading cause of death in patients with indwelling vascular catheters, particularly in immunocompromised individuals.

Candida spp. can form biofilm on intravascular catheters and other medical implants (4). These biofilms enhance antimicrobial resistance and can render infections refractory to antifungal therapy. Persistence of an infection can necessitate removal of the device (5–7), which can be undesirable or even life threatening. Therefore, the design of materials or surfaces that mitigate or prevent fungal colonization or infection with subsequent biofilm formation would be beneficial to patients, especially because no medical device surfaces with specific and efficient antifungal properties currently exist. Surfaces treated with chlorhexidine and benzalkonium chloride (8), modified with polyethylene glycols (PEGs) (9, 10), or that mimic cell membranes (11) reduce the adhesion of *Candida* spp. but do not eliminate it. Effective killing of airborne fungi onto textiles has been shown with a surface bearing cationic polymers (12–14); however, these antifungal agents are not currently approved by the Food and Drug Administration (FDA), and their antifungal spectrum and cytotoxicity profile in contact with human cells is unknown.

Here we report the design and development of a dextran-based hydrogel containing the polyene macrolide amphotericin B (AmB), an FDA-approved, potent antifungal agent widely used in clinical practice. Amphotericin's indications, use, chemistry, and toxicity are well characterized. It has a broad spectrum of antifungal activity, and few resistant strains have been reported after 40 years in clinical use (15–17). Dextran, a polymer widely used in medicine (18), itself is known to be protein

repellent (19) and therefore could mitigate biofilm formation (20); the biocompatibility of cross-linked dextran has been described (21). The dextran hydrogels, loaded with AmB (amphogels) described here, have marked antifungal efficacy for at least 53 days and prevent fungal infection when implanted in mice.

Results and Discussion

Dextran-based hydrogels containing AmB (“amphogels” below) were produced by placing cross-linked dextran disks in AmB-containing dimethylformamide (DMF) solutions overnight, followed by 6 days of washing (Fig. 1a). These gels had a constant swelling ratio for up to 33 days [see supporting information (SI) Fig. 5a], suggesting that there was minimal degradation during that period. The elastic behavior of amphogels was evaluated by the rheologic determination of storage modulus (G'), which is a measure of the energy stored and recovered per cycle of oscillatory deformation (22). The G' at equilibrium for these networks was 53.4 ± 10.0 kPa (SI Fig. 5b), which is comparable with values reported for similar polysaccharide-based hydrogels (23, 24), indicating a soft material.

The viability of yeast cells on the surfaces of amphogels or drug-free gels was assessed by incubating them in medium containing *C. albicans* for 2 h, after which the disks were crushed and vigorously stirred. The suspension was plated on yeast extract peptone dextrose (YEP)–agar plates for 24 h, and the number of yeast colonies was counted. Drug-free hydrogels did not inhibit fungal viability, whereas amphogels showed a marked reduction in fungal viability (Figs. 1b and 2a). Comparison of scanning electron micrographs (SEM) of the surface of hydrogels without AmB and amphogel (Fig. 1c) showed that the latter dramatically reduced the fungi on the surface.

To assess the drug-loading capacity of the hydrogels, they were incubated in solutions containing increasing concentrations of AmB, and their AmB content was determined by extraction of the drug in dimethyl sulfoxide (DMSO) with subsequent analysis via HPLC. The maximal loading capacity of the hydrogels was ≈ 1.1 mg of AmB per disk (SI Fig. 5c). The disks had average dry and wet weights of 13.8 ± 0.8 and 71.4 ± 2.7 mg ($n = 7$), respectively. Because there was no benefit in cell killing from

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The authors declare no conflict of interest.

Abbreviations: AmB, amphotericin B; DMF, dimethylformamide; YEP, yeast extract peptone dextrose; YNB, yeast nitrogen base.

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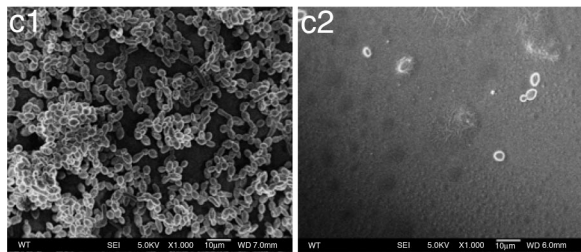
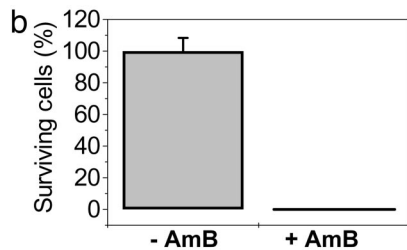
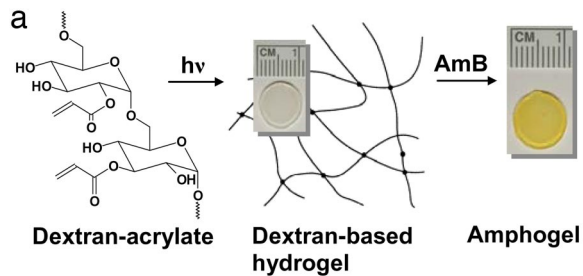


Fig. 1. Antifungal properties of dextran-based hydrogels with or without AmB. (a) Schematic representation of the preparation of amphogels. (b) *C. albicans* viability on the hydrogel surface as assessed by a colony growth assay after a 2-h exposure to dextran-based hydrogels with or without AmB. (c) SEM images of dextran-based gels without (c1) and with (c2) AmB incubated with *C. albicans* for 48 h.

using higher AmB loadings (SI Fig. 5d), we used amphogels with 0.17 ± 0.14 mg of AmB per gel for subsequent studies (made with a loading solution containing 1.3 mg/ml AmB).

Fungi were rapidly killed by amphogel. Amphogels ($n = 4$) were placed in 1 ml of yeast nitrogen base (YNB) medium containing 1×10^7 *Candida albicans*, and the number of fungal colonies arising from cells surviving in the medium and on the amphogel surfaces was determined after 24 h in YEP-agar plates (Fig. 2a). After 2 h, no viable fungi were found either in the medium (Fig. 3a) or on the surface of the amphogel disks (data not shown).

To assess the relative contributions from drug release and surface killing, amphogels ($n \geq 5$) were placed into YNB medium, and at predetermined time points the medium was collected (Fig. 2b). The viability of *C. albicans* in these medium samples that had been exposed to amphogel (i.e., into which drug had been released) decreased significantly at 2 and 24 h ($P < 0.001$) compared with cells grown in fresh YNB medium (Fig. 3b). After 24 h, the medium no longer killed *Candida*. These studies suggest that there is some early release of fungicidal activity from the gel, which subsequently ceases. In contrast, the disks themselves maintained the capability to kill *C. albicans* for an extended period. Amphogel disks ($n = 4$) were placed into 1 ml of YNB medium containing 1×10^7 *C. albicans* for 2 h. The gels were then removed from the culture medium, which was assayed for fungal cell survival (Fig. 2a). The amphogel disks were stored in the dark in PBS 7.2 at 37°C until use, when they were placed into a fresh aliquot of YNB medium containing fungi as above. This process was repeated

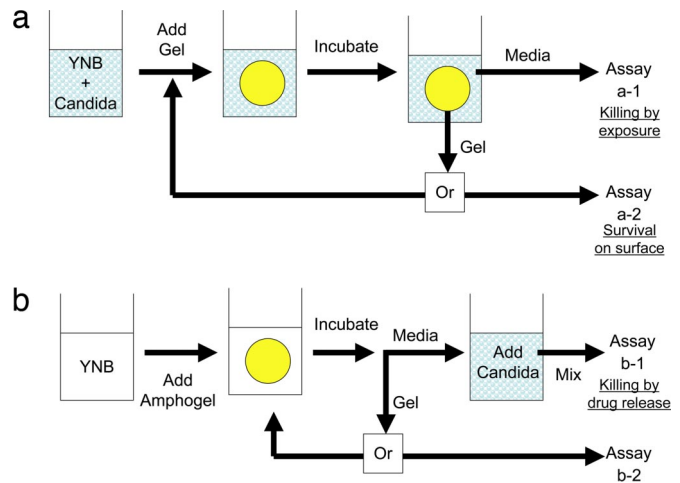


Fig. 2. Schematic of fungal survival experiments. (a) In one type of experiment that studied the effect of direct exposure to the disks, the disks were placed in medium containing fungi, and the extent of fungal survival in the medium (a-1) and on the disks' surfaces (a-2) was determined. (b) In a second type of experiment designed to determine the effect of AmB released from the disks, they were first incubated in medium without fungi. After that incubation, the medium was removed, fungi were added to that incubation medium, now without the disk, and survival was assessed (b-1). In both contexts, disks were used repeatedly in serial experiments describing the time course of fungicidal activity.

for 53 days. At all time points, there was minimal or no survival in the medium after a 2-h exposure to amphogel (Fig. 3c). There was no survival on disk surfaces during the same period (data not shown).

In the absence of drug release from the amphogels after 24 h, a possible explanation for their fungicidal activity is that *Candida* is killed on contact. This possibility is supported by an experiment where amphogels were incubated for 2 h without mechanical agitation, either in 1 ml of medium containing 1×10^7 *C. albicans* in 1.9-cm-diameter wells or in 2 ml of medium containing 2×10^7 *C. albicans* in 6-cm-diameter wells. Whereas the cells in the 1.9-cm wells were completely killed, the majority of the cells in the 6-cm wells survived ($\approx 65\%$; see SI Fig. 6). However, if the 6-cm wells were gently agitated during the 2 h of incubation, there was no fungal cell survival in the medium. We observed similar results in disks that were preincubated in YNB for 5 days and which therefore were no longer releasing fungicidal activity. These findings are consistent with the possibility that direct contact with the amphogel disk is necessary for fungal cell killing. In these experiments as well, no viable cells were recovered from the surfaces of the amphogels.

Amphogels were amenable to storage by freeze-drying. Disks lyophilized then rehydrated in PBS for 24 h showed the same effectiveness in killing *Candida* as observed for amphogels that were not freeze-dried (data not shown).

Different materials were investigated for their suitability for loading with AmB. Hydrogels made from cross-linked PEG diacrylates did not retain AmB to visual inspection: the yellow color was lost after the first 24 h wash in DMF. When the PEG gel was incubated in medium with *C. albicans*, 80–100% of the cells survived in the medium (Fig. 3d). Hydrogels made from the polysaccharide inulin retained AmB to visual inspection throughout the washes in DMF and showed the same high efficiency as dextran-based amphogels in killing fungi in the medium.

The hemocompatibility of amphogel was tested by exposing red blood cells to amphogel for 1 h and quantifying the release

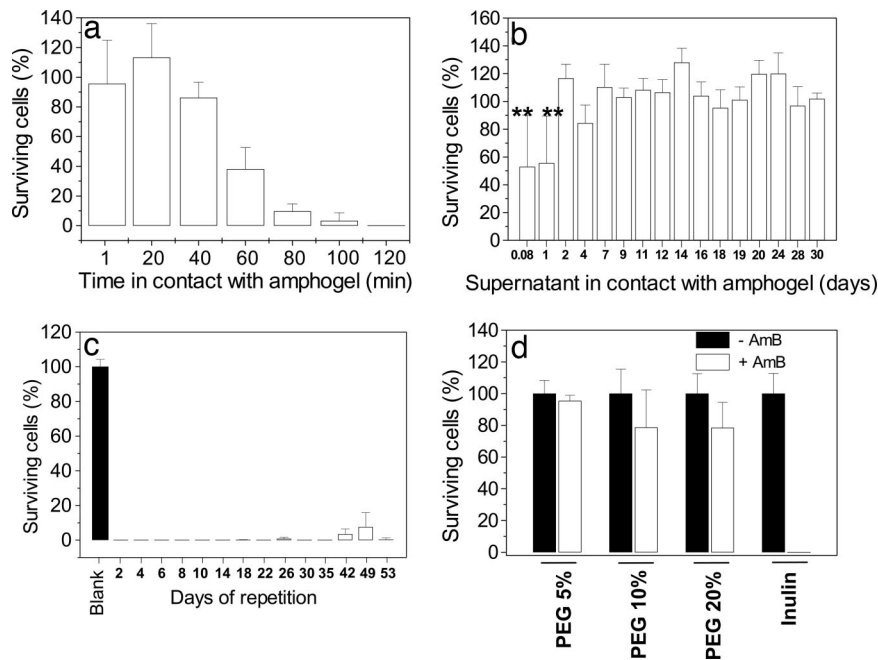


Fig. 3. Yeast killing by amphogels. Survival was assessed by a colony growth assay. Data are means with standard deviations. (a) Time course of yeast cell killing in medium upon contact with amphogel. More than 99% of *C. albicans* cells exposed to amphogel were killed after 120 min. (b) Survival of fungi added to medium that had been exposed to amphogels over the course of 30 days (release medium; see Fig. 2b). The individual medium samples had been exposed to the gels for a time period equal to the interval between the predetermined time points. ** denotes statistical significance ($P < 0.001$, $n \geq 5$) between the various samples and cells cultured without amphogels. (c) Effect of 2-h exposure to amphogels on survival of *C. albicans* in medium over the course of 53 days (see Fig. 2a). Dextran-based hydrogels without AmB ("Blank") were used as controls. (d) Fungal survival in the medium after exposure for 2 h to PEG and inulin gels loaded with AmB. Hydrogels without AmB were used as controls.

of free hemoglobin as a measure of cell lysis (SI Fig. 7). No release was detected. The biocompatibility of amphogels and dextran gels without AmB *in vivo* was evaluated by s.c. implantation in mice. In all samples from both groups, there was minimal to mild inflammation at 3 days after implantation (Fig. 4a) and only mild to moderate inflammation at 3 weeks (Fig. 4b), both to gross inspection and by light microscopy. Tissue reaction was similar in both groups at both time points, and disks maintained their structural integrity.

In vivo activity of amphogel in killing *C. albicans* was evaluated in a mouse model. Amphogels or hydrogels without AmB were inoculated with *C. albicans*, then implanted s.c. in mice for 3 days. Animals then were killed, and the disks were removed for enumeration of cells and microscopic examination. No fungal survival was observed with amphogels (log CFU per g of disk: 0 ± 0 , $n = 5$), whereas dextran hydrogels without AmB showed an average of 5.7 ± 0.4 ($n = 5$) log CFU per g of disk, i.e., almost 10^6 CFU per g of disk. SEM showed that amphogel surfaces did not have any *Candida* cells or biofilm attached (Fig. 4c). In some areas of the amphogels, a few host cells (mainly white blood cells) were observed (Fig. 4d). In contrast, dextran hydrogels without AmB were covered with *Candida* biofilm (Fig. 4e), *Candida blastospores*, and white blood cells (Fig. 4f).

Materials like the antifungal matrix described here could be suitable for coating a variety of medical and industrial surfaces or as stand-alone antifungal systems. Amphogel kills *C. albicans* within 2 h of contact and remains biologically active for at least 53 days. This is a timeframe that is useful for many medical devices, particularly central venous catheters. This hydrogel is biocompatible, does not cause hemolysis in human blood, and is effective in killing *C. albicans* when incubated with microorganisms and implanted in mice. Previously described hydrogels containing antifungal compounds did not

demonstrate these properties, or they used compounds with relatively limited antifungal potencies and/or spectra or that are not generally used internally (25, 26). The matrix itself is of interest because of the unanticipated adsorption of AmB to a specific hydrophilic matrix resulting in a very simple manufacture process, and the marked killing of *C. albicans* in the absence of detectable release of fungicidal activity. As fungi appear to gain entry into patients via percutaneous devices, the ability to reduce entry through these portals could prevent the introduction of the microorganism and thereby reduce the probability of ensuing disease.

Materials and Methods

Preparation of Dextran-Based Hydrogels With and Without AmB.

Dextran acrylate with a degree of substitution of 23.3% was synthesized as reported (27). Dextran-based hydrogels (10-mm diameter and 1-mm thickness, before swelling) were obtained by a photopolymerization reaction of aqueous solutions of dextran acrylate. Briefly, dextran acrylate (400 mg) was dissolved in 1.8 ml of 0.2 M sodium citrate buffer (pH 5.0) after which 2-hydroxy-1-[4-(hydroxyethoxy) phenyl]-2-methyl-1-propanone (Irgacure 2959; CIBA, 0.5% wt/vol in H_2O , 200 μ l) was added. The polymerization reaction was initiated by UV light (≈ 4 mW/cm²), between two glass-slides with a 1-mm spacer, for 10 min. The gel was subsequently removed from the slides and punched by using stainless steel cork-borers to yield disks with 10 mm diameter. For the preparation of dextran-based hydrogels loaded with AmB (denoted as amphogels), 6 disks were immersed in 16 ml of a solution of DMF: triethylamine (15:1) containing 20 mg of AmB (Sigma-Aldrich, St. Louis, MO) and 5 mg of 4-dimethylaminopyridine. The loading was performed for 12 h, and then the gels were washed with DMF (3 days) followed by PBS (pH 7.2; 3 days). Gels

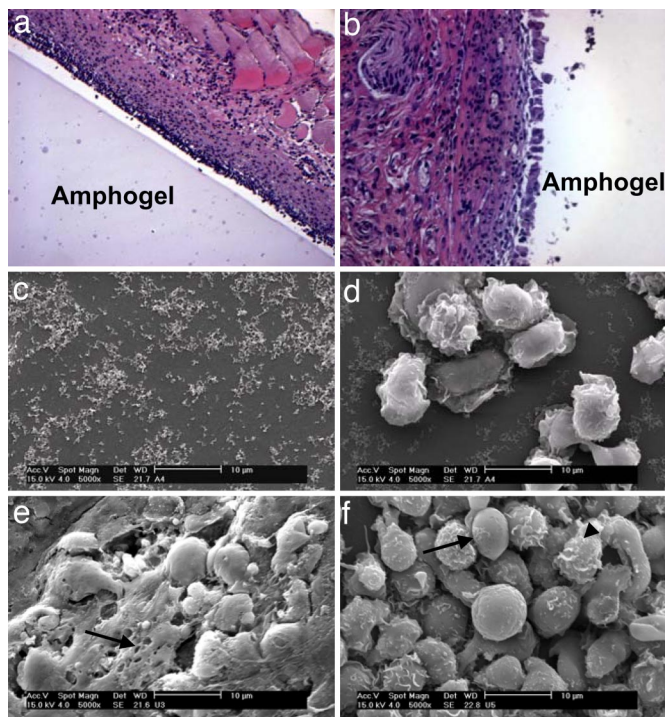


Fig. 4. *In vivo* biocompatibility studies and biological activity of amphogels. (a and b) Representative light micrographs of amphogel implanted s.c. and surrounding tissue after 3 days (a) or 3 weeks (b), stained with hematoxylin/eosin. Minimal to mild inflammation was observed at day 3 and only mild to moderate inflammation at 3 weeks. (Original magnification: $\times 50$, a; $\times 200$, b.) (c–f) SEM photographs from the surface of amphogel (c and d) or dextran gel without AmB (e and f) incubated with *C. albicans* that were implanted into mice and then removed after 5 days. Amphogels did not have any *Candida* biofilm (c) and had only a few host cells (mainly white blood cells) (d). Dextran gels without AmB were covered with fungal biofilm (e, arrow). Certain areas of the disks were covered with a large number of *Candida* cells (f, arrow) mixed with white blood cells (arrowhead).

containing no AmB were prepared by an identical process except that AmB was not included.

Preparation of Inulin and PEG Hydrogels. Inulin acrylate with a degree of substitution of 28.7% was synthesized as reported (28). Inulin hydrogels were obtained by the photopolymerization reaction of aqueous solutions of inulin acrylate (600 mg, in 0.2 M PBS) containing 0.056% (wt/vol) Irgacure 2959. PEG gels were prepared by the photopolymerization of aqueous solutions of PEG diacrylate (5, 10, or 20% vol/vol, M_w of 700 Da; Sigma–Aldrich) in PBS containing 0.056% (wt/vol) of Irgacure 2959. In both cases, the polymerization reaction and gel loading with AmB was performed as described for dextran hydrogels.

Growth of *C. albicans*. *C. albicans* strain SC5314 was regrown from frozen stocks from the Whitehead Institute or Case Western Reserve University. In all cases, *Candida* were maintained on YEP–agar plates. For experiments, YPD (50 ml) was inoculated with one colony of *Candida* overnight. After that time, the suspension was centrifuged (5 min at $930 \times g$), the supernatant was discarded, and the cells were washed twice with PBS 7.2 (50 ml). The cells were counted on a hemocytometer and diluted to 1×10^7 or 4×10^7 cells/ml in YNB medium with 50 mM glucose.

Assessment of Disk Effectiveness. There were two basic contexts in which the disks' fungicidal activity was assessed (Fig. 2). In all

these experiments, the actual assay of fungal survival was a colony growth assay. The details of these experiments follow.

Fungicidal effect of direct exposure to disks (Fig. 2a). Polymer disks (1 cm diameter) were placed in the wells of a 24-well tissue culture plate with 1 ml of the *Candida* suspension (1×10^7 cells in YNB). The disks were incubated for 2 h, unless specified otherwise, at 37°C while shaking at 100 rpm. Then the disks were removed, and the remaining medium was vigorously stirred then diluted to a concentration of 1:1,000. Next, 200 μl of the diluted medium was plated on YEP agar plates (Fig. 2a1). The disks were washed gently in 3×1 ml of fresh PBS to remove any nonadherent cells. The disks were crushed and vigorously stirred in 1 ml of PBS, and the suspension was diluted to a concentration of 1:1,000. Then, 200 μl of the diluted suspension was plated on YEP–agar plates (Fig. 2a2). The YEP plates were incubated at 37°C for 24 h, and yeast colonies were counted.

Fungicidal effect of drug release (Fig. 2b). To assess the fungicidal effect of AmB from amphogel, hydrogels were immersed in 1 ml of YNB medium with 50 mM glucose and incubated at 37°C at 100 rpm. At predetermined intervals, the incubation medium was removed completely, and the gel was immersed in fresh medium. To 225 μl of YNB medium collected at each time point, we added 75 μl of fresh YNB containing 4×10^7 cells/ml *C. albicans* SC5314. After 2 h the suspension was diluted to a concentration of 1:1,000, plated onto YEP agar plates, and incubated at 37°C for 24 h, after which viable colonies were counted. Control samples were prepared by adding 75 μl of YNB containing 4×10^7 cells/ml *C. albicans* SC5314 to 225 μl of YNB not exposed to amphogels.

In Vivo Biocompatibility Studies. Male SV129 mice weighing 25 g (Charles River Laboratories, Wilmington, MA) were cared for in accordance with protocols approved by the Animal Care and Use Committee at the Massachusetts Institute of Technology and the *Guide for the Care and Use of Laboratory Animals* of the U.S. National Research Council. They were housed in groups, in 6 a.m.–6 p.m. light–dark cycles. Dextran-based hydrogels with or without AmB were sterilized by several washes in ethanol followed by washes in sterile PBS before implantation. Mice were anesthetized with isoflurane in oxygen, shaved, and prepped in a sterile manner. An incision was produced in the dorsal midline, and s.c. pouches were extended down either flank by blunt dissection. One gel was deposited in each flank. At predetermined intervals, animals were euthanized with carbon dioxide, and the gels were removed along with adherent tissues and placed in 4% (vol/vol) neutral buffered formalin. After fixation for 24 h, the blocks were sectioned and stained (hematoxylin/eosin) by using standard techniques and analyzed by a blinded observer (D.S.K.).

Biological Activity of Amphogel in Vivo. All animals were cared for in accordance with protocols approved by the Animal Care and Use Committee at the Massachusetts Institute of Technology and Case Western Reserve University, and the *Guide for the Care and Use of Laboratory Animals* of the U.S. National Research Council. In each group, a total of five female BALB/c mice weighing 20–25 g were used. The mice were anesthetized with ketamine (100 mg/kg) and xylazine (5 mg/kg) i.p. Their backs were shaved, a midline incision was made in the skin above the midthoracic spine, and a pocket was made s.c. by blunt dissection, extending 2–3 cm anteriorly. Individual amphogels or dextran gels without AmB were inoculated with 1×10^7 cells of *C. albicans* and incubated at 37°C for 90 min, then placed in a s.c. pocket. The incision was closed with 3–0 vicryl sutures. Three days after inoculation, animals were killed with carbon dioxide, and the disks were removed aseptically, weighed, and used for enumeration of cells or microscopical examination. For the enumeration of cells the

disks were homogenized, and the suspension was diluted and cultured in Sabouraud Dextrose Agar plates at 37°C. After 48 h, the number of CFU were counted and expressed as CFU per gram of disk.

Statistical Analysis. The statistical significance of differences between groups was assessed by one-way ANOVA with Bonferroni correction as necessary for subsequent tests, by using Prism

4.0 (GraphPad, San Diego, CA). Results were considered significant at $P \leq 0.05$.

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