Synergistic Antifungal Effect of Amphotericin B-Loaded Poly(Lactic-Co-Glycolic Acid) Nanoparticles and Ultrasound against *Candida albicans* Biofilms

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ABSTRACT *Candida albicans* is a human opportunistic pathogen that causes superficial and life-threatening infections. An important reason for the failure of current antifungal drugs is related to biofilm formation, mostly associated with implanted medical devices. The present study investigated the synergistic antifungal efficacy of low-frequency and low-intensity ultrasound combined with amphotericin B (AmB)-loaded poly(lactic-co-glycolic acid) (PLGA) nanoparticles (AmB-NPs) against *C. albicans* biofilms. AmB-NPs were prepared by a double-emulsion method and demonstrated lower toxicity than free AmB. We then established biofilms and treated them with ultrasound and AmB-NPs separately or jointly *in vitro* and *in vivo*. The results demonstrated that the activity, biomass, and proteinase and phospholipase activities of biofilms were decreased significantly after the combination treatment of AmB-NPs with 42 kHz of ultrasound irradiation at an intensity of 0.30 W/cm² for 15 min compared with the controls, with AmB alone, or with ultrasound treatment alone ($P < 0.01$). The morphology of the biofilms was altered remarkably after joint treatment based on confocal laser scanning microscopy (CLSM), especially in regard to reduced thickness and loosened structure. Furthermore, the same synergistic effects were found in a subcutaneous catheter biofilm rat model. The number of CFU from the catheter exhibited a significant reduction after joint treatment with AmB-NP and ultrasound for seven continuous days, and CLSM and scanning electron microscopy (SEM) images revealed that the biofilm on the catheter surface was substantially eliminated. This method may provide a new noninvasive, safe, and effective therapy for *C. albicans* biofilm infection.

KEYWORDS amphotericin B, *C. albicans* biofilm, PLGA nanoparticles, synergistic antifungal, ultrasound

Candida albicans is a normal commensal organism found on human mucosal surfaces that acts as an opportunistic pathogen in immunocompromised patients. In the past decades, the infection and mortality rates of *C. albicans* infections have been increasing annually in response to the widespread use of antibiotics and hormone treatments. Approximately 90% of *C. albicans* infections are related to biofilm formation associated with medical devices, such as indwelling catheters, venous catheters for parenteral nutrition, prosthetic valves, and joint implants (1, 2). Biofilms are communities of microorganisms that attach to the surface of tissues or biological materials and encase themselves in a self-secreted extracellular matrix (ECM) (major sugars and proteins), which forms a dense natural barrier to evade the immune function of the host and prevent permeation of antibiotics (3). Biofilm formation is an important reason for the failure of current antifungal drugs because it makes microbes less susceptible to

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antifungal agents, imparting 10- to 2,000-times-higher resistance to the effects of antimicrobial agents (4, 5). Currently, there are only a few antimycotics, including azoles (miconazole), polyenes (amphotericin B [AmB]), and echinocandins, that are partially effective against biofilm-associated infections. However, these drugs sometimes cause significant adverse effects when applied via parenteral administration, such as nephrotoxicity, hepatotoxicity, hematotoxicity, and hypersensitivity reactions, which have limited their widespread use (6, 7). Therefore, newer antifungal drugs with greater hypotoxicity and greater efficacy, as well as newer treatment methods that can reduce the amounts of drugs and achieve effective antifungal effects, have become a focus of studies investigating antibiofilm infections.

Naturally degradable and synthetic biodegradable polymer nanomaterials for alternative antibacterial therapy have been extensively reported as innovative tools for combating high rates of antimicrobial resistance, including in multidrug-resistant (MDR) bacterial and biofilm-associated infections (8). Among them, poly(lactic-co-glycolic acid) (PLGA) biodegradable polymeric nanoparticle (NP)-based drug delivery systems have been proposed as a potential alternative to various biomedical applications for vaccination, cancer, inflammation, and other diseases (9, 10). Moreover, oral or parenteral administration of AmB polymeric nanoparticle formulations has been reported to have antifungal efficacy and low toxicity with increased bioavailability compared to intravenous (i.v.) AmBisome or Fungizone (11, 12). Thus, we investigated the ability of synthesized AmB-loaded PLGA nanoparticles (AmB-NPs) to reduce the adverse effects of free AmB and explored the antifungal efficacy of AmB-NPs against *C. albicans* biofilms in this study.

During biofilm formation, surface-adherent extracellular matrices of the biofilm act as a barrier to antibiotic diffusion, which limits the exposure of intracellular bacteria in the biofilms to superficial antibiotics. Ultrasound (US) has been widely acknowledged as a promising approach to overcome these obstacles by enabling increased permeability for gene/drug delivery (13, 14). Ultrasound exposure induces sonoporation, which may transiently disrupt the cell membrane and increase the permeability of the membrane to promote transportation of membrane-impermeable agents, such as nanoparticles or antibodies, into cells or tissues in preclinical studies. Ultrasound radiation also creates many holes in the extracellular matrix of bacterial biofilms, which can benefit the rapid uptake of bulk antibiotics, oxygen, and nutrition by cells (15, 16). More importantly, the presence of nanoparticles can enhance acoustic cavitation by increasing cavitation nuclei to reduce the initial energy of the cavitation threshold (17, 18). Our previous study also revealed that low-frequency and low-intensity ultrasound combined with AmB-NPs has higher antifungal efficacy than free AmB alone against planktonic *C. albicans* *in vitro* (19).

Therefore, we investigated the effects of low-frequency and low-intensity ultrasound on biofilms and the antifungal efficiency of ultrasound combined with AmB-NPs against *C. albicans* biofilms *in vitro*. We also investigated the synergistic antifungal efficacy *in vivo* using a rat model of *C. albicans* biofilm-associated catheter infection. The results presented here provide a new noninvasive, safe, and effective treatment for *C. albicans* biofilm infection therapy.

RESULTS

Nanoparticle characteristics and drug release. Transmission electron microscopy (TEM) (Hitachi High-Technologies, Tokyo, Japan) photomicrographs demonstrated that a majority of nanoparticles were spherical, in the nanometric range, with smooth surfaces and favorable dispersibility. Compared with blank NPs, AmB-NPs clearly revealed that the drug was loaded in nanoparticles (Fig. 1A). The characteristics average diameter (D), zeta potential (ZP), polydispersity index (PDI), content (in percent) of drug loaded onto a nanoparticle (LC%), and percent encapsulation efficiency (EE%) of NPs and AmB-NPs are summarized in Table 1; they show that the drug loading content reached 5.7% and that the encapsulation efficiency reached 85%.

FIG 1 (A) TEM photomicrographs of plain PLGA nanoparticles (NPs) and amphotericin B-loaded nanoparticles (AmB-NPs) at a $\times 20,000$ or a $\times 50,000$ magnification. (B) Percentage of AmB cumulative release from nanoparticles with and without sonication during 0 to 72 h of incubation. The double asterisks denote a significant difference ($P < 0.01$) between release with and without sonication. US, ultrasound.

The kinetic release of AmB from nanoparticles *in vitro* with ultrasonication or natural release is shown in Fig. 1B. The results revealed an initial burst release of about 15% of AmB in the first 2 h, followed by a continuous slow release of the drug over 72 h. Moreover, a significantly higher cumulative concentration of AmB was observed after ultrasonic irradiation than with natural release over the same time frame. After 72 h, drug release following ultrasonic irradiation was twice as likely as natural release.

***In vitro* hemolytic activity and macrophage viability of AmB-NPs.** The hematological toxic reactions on red blood cells, hemolytic activity, and macrophage viability of AmB-NPs relative to free AmB are depicted in Fig. 2. In the hemolytic assay with red blood cells, free AmB induced higher hemolytic activity (65.81%) than AmB-NPs (30.24%) at the same concentration of 32 $\mu\text{g}/\text{ml}$ of AmB, and AmB was more likely to be hemolytic, even at very low concentrations. Moreover, NPs showed negligible hemolysis, even with increased concentrations of NPs (Fig. 2A). The viability of macrophages was also significantly decreased after 24 h of cocubation with free AmB compared to AmB-NPs at the same concentration of 4.0 $\mu\text{g}/\text{ml}$ of AmB ($P < 0.05$) (Fig. 2B). Furthermore, the AmB-loaded nanoparticles that underwent ultrasound preirradiated increased the hemolysis rate and decreased macrophage activity compared to AmB-loaded nanoparticles alone, but the toxicity was still lower than that of free AmB, which confirms that the ultrasound-mediated AmB release of drug-loaded nanoparticles had no significant effect on its biosafety.

Biofilm activity following a combination of ultrasound and AmB-NP treatment. To investigate the synergistically antimicrobial effect of ultrasound combined with AmB-NPs on *C. albicans* biofilms, the activity of biofilms was quantified by 2,3-bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carboxanilide (XTT) reduction assays, and biofilm biomass was quantified by crystal violet (CV) staining after different treatments. The results are shown in Fig. 3. The color change presented by XTT reduction assays is correlated with biofilm activity, which was quantified by the optical density at 490 nm (OD_{490}). All the AmB-related treatments significantly reduced the

TABLE 1 Physical characteristics of nanoparticle formulations^a

Formulation	Mean particle size (nm) $\pm$ SD	Mean ZP (mV) $\pm$ SD	PDI	Mean LC% $\pm$ SD	Mean EE% $\pm$ SD
NPs	227.1 $\pm$ 5.06	-1.84 $\pm$ 0.25	0.223		
AmB-NPs	287.8 $\pm$ 8.64	-10.9 $\pm$ 1.9	0.205	5.7 $\pm$ 0.12	85 $\pm$ 2.4

^aNPs, plain PLGA nanoparticles; AmB-NPs, amphotericin B-loaded PLGA nanoparticles; ZP, zeta potential; PDI, polydispersity index; LC, loading content; EE, entrapment efficiency.

FIG 2 (A) Hemolysis of red blood cells following incubation with free AmB, AmB-NPs, and AmB-NPs with ultrasound preirradiated at an intensity of 0.30 W/cm² for 15 min at final AmB concentrations of 0 to 32 μg/ml. (B) Toxicity analysis of macrophage viability following incubation with free AmB, AmB-NPs, and AmB-NPs with ultrasound preirradiated at an intensity of 0.30 W/cm² for 15 min at the same AmB concentration of 4.0 μg/ml. **, $P < 0.01$ compared with the controls; *, $P < 0.05$ compared with AmB-NPs or AmB-NPs preirradiated by ultrasound treatment.

activity of biofilms compared to the control group. The inhibition rates for the AmB group, the US-plus-AmB group, the AmB-NP group, and the US-plus-AmB-NP group were 46.65%, 56.75%, 41.98%, and 74.64%, respectively. The activity of biofilms in the joint treatment group also significantly declined compared to those with the other AmB-related treatments ($P < 0.01$) (Fig. 3A). Additionally, the total biofilm biomass of the group jointly treated with ultrasound and AmB-NPs was also significantly decreased by 31.26% compared with the control group ($P < 0.01$) (Fig. 3B).

Biofilm architecture observation and analysis *in vitro*. The changes in the architecture of biofilms and living and dead fungi in the biofilm after different treatments were observed by confocal laser scanning microscopy (CLSM) (A1+R; Nikon, Tokyo, Japan). Figure 4 shows viable cells (green fluorescence) and dead cells (red fluorescence) in the bottom biofilm layer of three-dimensional (3-D) reconstructed images. The control group was dominated by green fluorescence, which showed dense growth of live fungal cells. However, after ultrasound irradiation with AmB-NP treatment, the biofilm mainly consisted of red fluorescence (dead cells) and was characterized by sparse structures of voids, channels, pores, reduced thickness, and basically no remaining active biofilm. To further quantify the different parameters of biofilm architecture changes, COMSTAT software was applied to analyze the mean thickness (MT),

FIG 3 Activity of biofilm (A) and biofilm biomass (B) are decreased significantly following treatment with a combination of ultrasound and AmB-NP compared with the control group. Ultrasonic irradiation parameters with a sound intensity of 0.30 W/cm² for 15 min were chosen, and the final concentration of drug employed in the AmB group and AmB-NP group was 4.0 μg/ml. The double asterisks denote a significant difference ($P < 0.01$) compared with the control group.

FIG 4 Living and dead fungal cells of the bottom biofilm layer of 3-D-reconstructed CLSM images of different treatment groups at a $\times 400$ magnification (green, live cells; red [PI], dead cells).

textural entropy (TE), areal porosity (AP), and average diffusion distance (ADD) (Table 2). Following ultrasound irradiation with AmB-NP treatment, the MT of the biofilm was reduced by more than half, TE and ADD were significantly decreased, and AP was markedly increased compared with the control group. Taken together, these findings confirmed that ultrasonic treatment combined with AmB-NPs caused obvious damage to the biofilm structure of *C. albicans* *in vitro*.

Proteolytic and phospholipase enzymatic activities. The proteolytic and phospholipase enzymatic activities of *C. albicans* biofilms, both of which are important virulence factors contributing to host tissue damage, were analyzed, as shown in Fig. 5. Compared with the control group, the activities of protease and phospholipase in the combined ultrasound and AmB-NP group were reduced by 68.41% and 68.57%, respectively, ($P < 0.01$), while the enzyme activity was lower than that of the AmB group ($P < 0.05$). These findings indicated that the combination of ultrasound and AmB-NPs could increase the ability to inhibit protease phospholipase activity while weakening the expression of virulence factors, thereby reducing the pathogenicity of biofilms.

Quantization and morphological structure changes of biofilms *in vivo*. Fungus loading onto the implantation catheter was quantified by CFU after the corresponding treatments for 3 or 7 days. Colonies incubated on the plates are shown in Fig. 6. The colony counts were obviously reduced on the plates following treatment with ultrasound combined with AmB-NPs (Fig. 6A). Moreover, both free AmB and AmB-NPs could reduce the amount of fungus on the catheter. However, the fungal burden of rats treated with ultrasound combined with AmB-NPs was significantly lower than that of free-AmB-treated rats or those that received other treatments ($P < 0.01$), while ultrasound treatment alone did not significantly decrease the fungal counts compared with the control group ($P > 0.05$). The fungal burden on the catheters showed a similar trend after the 7-day treatment experiment; moreover, it significantly decreased after the

TABLE 2 COMSTAT analysis of structural parameters of *C. albicans* biofilm in different treatments^a

Group	Mean thickness (μm) $\pm$ SD	Avg diffusion distance (μm) $\pm$ SD	Mean textural entropy $\pm$ SD	Mean areal porosity $\pm$ SD
Control	30.56 $\pm$ 0.62	10.94 $\pm$ 0.18	4.40 $\pm$ 0.19	0.553 $\pm$ 0.037
US	29.38 $\pm$ 0.56	8.27 $\pm$ 0.15**	3.77 $\pm$ 0.12	0.697 $\pm$ 0.021
AmB	25.69 $\pm$ 0.13**	4.21 $\pm$ 0.03**	3.15 $\pm$ 0.07**	0.757 $\pm$ 0.023**
US + AmB	20.51 $\pm$ 0.65**	2.82 $\pm$ 0.14**	2.22 $\pm$ 0.03**	0.897 $\pm$ 0.032**
AmB-NPs	26.72 $\pm$ 0.24**	4.53 $\pm$ 0.11**	3.17 $\pm$ 0.06**	0.797 $\pm$ 0.015**
US + AmB-NPs	11.56 $\pm$ 1.22** Δ	1.55 $\pm$ 0.11** Δ	1.60 $\pm$ 0.09** Δ	1.173 $\pm$ 0.067** Δ

^a**, $P < 0.01$ compared to the control group; Δ , $P < 0.01$ compared to the AmB group. US, ultrasound; AmB, amphotericin B; AmB-NPs, amphotericin B-loaded PLGA nanoparticles.

FIG 5 Proteolytic (A) and phospholipase (B) enzymatic activities of *C. albicans* biofilms of different treatment groups. Significant reductions in proteinase and phospholipase enzyme activities were observed with the combination treatment with 4.0 $\mu\text{g/ml}$ AmB. The double asterisks denote a statistically significant difference ($P < 0.01$).

7-day treatment experiment following combined treatment with ultrasound and AmB-NPs compared to the 3-day treatment ($P < 0.01$) (Fig. 6B). These results suggest a remarkably synergistic antifungal effect of ultrasound combined with AmB-NPs on *C. albicans* biofilms relative to AmB alone *in vivo*, which was in accordance with the results observed *in vitro*.

To further observe the morphological structural changes of the biofilms in catheters after different treatments, explanted catheters were observed by CLSM and scanning electron microscopy (SEM). Figure 7 shows a mature biofilm architecture with abundant fungal cells embedded within the extracellular matrix on the catheter surface in the controls. Following treatment with AmB with or without ultrasound treatment, the biofilm structure was destroyed, the extracellular matrix was reduced, mycelia were shortened, and only a small amount of biofilm remained on the surface of the catheter. However, after the joint effects of ultrasound and AmB-NPs, only a single fungal colony remained on the catheter surface, and the biofilm was substantially eliminated. At the same time, Fig. 8 shows SEM observations of biofilms attached to the catheter surface, and a complex structure with hyphae, yeast, and extracellular matrix can be visualized in the control sample. The dense structure of the biofilm became dispersed after

FIG 6 Catheter fungal loading of *C. albicans* with different treatments after 24 h of incubation on plates (A) and fungal colony counts ($\log_{10}$ CFU) after 3- or 7-day treatments (B). The double asterisk denotes a significant difference ($P < 0.01$). Rectangles at the bottom of the panels, 1 cm.

FIG 7 Morphological changes in catheter biofilm after seven consecutive days of treatment. *C. albicans* cells were stained with ConA and visualized by CLSM at a $\times 400$ magnification. The control group of mature biofilms shows a dense structure on the catheter surface. In the group treated with ultrasound and AmB-NPs, only a single fungal colony remained on the catheter surface, and the biofilm was substantially eliminated. Bars, 50 μm .

ultrasonic irradiation, and the biofilm was deformed after AmB-related treatments such that the extracellular matrix was reduced or disappeared, and the structure was loosened. But ultrasound combined with AmB-NPs led to the complete inhibition of the biofilm, and cells remained in the yeast form and were swollen and inactivated.

DISCUSSION

Fungal colonization of medical devices is a widespread problem and is responsible for nosocomial infections. *C. albicans* biofilm formation, which is commonly associated with implanted medical devices, is less susceptible to antifungal agents than other fungi and is therefore difficult to cure (20). AmB has been used for decades to treat common invasive fungal infections. Although AmB has high nephrotoxicity, nanoparticle formulations of AmB have been shown to have reduced toxicity compared to its monomer state (21, 22). Similarly, the results of the present study revealed lower toxicity of AmB-NPs than free AmB, including the hemolytic activity of red blood cells and the activity of macrophages (Fig. 2), which was associated with drug encapsulation

FIG 8 Scanning electron microscopy images of *C. albicans* biofilms on the surface of a rat subcutaneous catheter after 7-day treatments. The biofilm is attached to the catheter surface and has a complex structure with hyphae, yeast, and extracellular matrix in the control samples. The AmB-related treatments show deformed morphology of the biofilm, and treatment with ultrasound combined with AmB-NPs led to the complete inhibition of the biofilm, with cells remaining in the yeast form and being swollen and inactivated.

and its subsequent slow release from nanoparticles. However, the nephrotoxicity (blood urea and creatinine levels) of AmB and AmB-NPs was not obvious in *in vivo* experiments (data not shown), which may be related to the dose used and the method of injection. The dose of AmB (total of 7.0 mg/kg of body weight/animal) in this experiment may not be enough to cause renal impairment. The results of research by Souza et al. also showed no changes of urea or creatinine levels between AmB and AmB-NP treatments in which the dose of AmB in their experiments was a total of 18.0 mg/kg/animal (23), and the local injection method in this experiment will significantly reduce the amount of drug metabolized through the kidneys. It is arguable that after the same dose of AmB is injected, AmB will be detected in the kidneys following intravenous (i.v.) delivery but not local intraperitoneal (i.p.) dosing, as in the report by Chang et al. (24).

In vitro US-triggered drug release experiments showed that the release of AmB-NPs was delayed when allowed to proceed naturally but significantly increased after sonication. These findings indicate that ultrasound promotes the diffusion of nanoparticles within the shells, resulting in increased drug release rates, which may allow for the targeted release of drug-loaded nanoparticles at specific positions in which the drug vehicle receives ultrasound energy (25, 26).

The use of low-frequency ultrasound (20 to 100 kHz) as a noninvasive treatment has been shown to improve cell membrane permeability, increase drug penetration, and achieve synergistic antibacterial effects by previous research (27–29). In the *in vitro* experiments, we demonstrated a highly effective synergistic antifungal effect of low-frequency ultrasound combined with drug-loaded nanoparticles against *C. albicans* biofilms through XTT reduction assays and CLSM observation. Joint treatment with ultrasound irradiation and AmB-NPs not only significantly reduced the activity of biofilm fungal cells (Fig. 3A) but also had a destructive effect on the structure of the biofilm itself; specifically, the distribution of the biofilm structure was uneven, with high porosity, as shown in Fig. 4 and quantified in Table 2. The synergistic antifungal mechanism may be related to the cavitation effect with the accompanying extreme physical phenomena (30), such as high pressure and high shear force, that could loosen the densified structure of the biofilm extracellular matrix, thereby enhancing drug permeability. Moreover, drug-loaded nanoparticles, acting as exogenous systemically administered microbubbles, can greatly increase the number of cavitation nuclei, reduce the threshold of localized cavitation, and enhance the cavitation effect (31–33). Therefore, ultrasound-irradiated nanoparticles break and release drugs to exert a highly effective antifungal effect.

In the *in vivo* experiments, we established an animal model of a subcutaneous catheter *C. albicans* biofilm. The CFU of the catheter fungus were significantly reduced in the treatment with ultrasound combined with AmB-NPs relative to AmB alone, or ultrasound combined with AmB, after 7-day treatments (Fig. 6). Specifically, CLSM and SEM revealed that the attached biofilm structure on the catheter was destroyed, the mycelium had disappeared, and only a few colonies remained (Fig. 7 and 8). These findings indicated that combined treatments with ultrasound and drug-loaded nanoparticles exert synergistic antifungal activity and achieve comparable or even better therapeutic effects at lower doses, which reduces the incidence of adverse effects. Although some studies have provided evidence that combining amphotericin B and echinocandin or azoles is effective *in vitro* and in animal models of invasive fungal infections, the combination of drugs often increases side effects to various degrees, and the clinical treatment effect is not satisfactory (34, 35). Thus, ultrasound is a potential way to realize synergistic antifungal effects compared to current traditional combination therapies.

In summary, we prepared AmB-NPs that slowly released the drug, resulting in lower toxicity than free AmB, and more importantly, the synergistic antifungal efficacy of low-frequency and low-intensity ultrasound combined with AmB-loaded PLGA nanoparticles against *C. albicans* biofilms was successfully supported by *in vitro* and *in vivo* assays. The combination of ultrasound with drug-loaded nanoparticles is a promising

strategy to improve efficacy, reduce dosages, and shorten the duration of antifungal therapy. Future perspectives include the potential to use ultrasound with drug-loaded nanoparticles to treat common clinical *C. albicans* biofilm-related infections, such as those associated with central venous catheter insertion, catheters, and artificial joint replacement.

MATERIALS AND METHODS

Materials. AmB (99.8% purity), polyvinyl alcohol (PVA), 2,3-bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carboxanilide (XTT), menaquinone, 3-(4-5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), crystal violet (CV), concanavalin A (ConA), azocasein and phosphatidylcholine substrates, and dialysis bags (molecular weight cutoff of 12 kDa) were purchased from Sigma-Aldrich (St. Louis, MO, USA). PLGA polymer material with a molecular weight of 21 kDa (molar ratio of lactide to glycolic acid of 50:50) was purchased from RuiJia Biological (Xian, China). RPMI 1640 medium, fetal bovine serum (FBS), and phosphate-buffered saline (PBS) were purchased from Gibco BRL (Carlsbad, CA, USA). Sabouraud dextrose (SD) agar (SDA) and SD broth were obtained from Huankai Microbial Co., Guangdong, China. A Live/Dead BacLight bacterial viability kit was acquired from Invitrogen (catalog no. L7012).

***C. albicans* strains and biofilm formation.** *C. albicans* ATCC 10231 was obtained from the China General Microbiological Culture Collection Center. Individual colonies were selected from the SDA plate and inoculated into 100 ml of SD broth at 37°C for 24 h with agitation (150 rpm) to prepare *C. albicans* suspensions, which were preserved at 4°C for future experiments.

For biofilm formation, we developed a model of fungal biofilm growth of *C. albicans* in RPMI 1640 medium supplemented with 10% FBS and grown in 35-mm-diameter plastic-bottom petri dishes at 37°C for 48 h of continuous incubation *in vitro*. Biofilm formation was confirmed and quantified by CV staining and XTT reduction assays (36) (see <http://www.isminim.org/index.php?c=article&id=108>).

Animal species. Sixty healthy female Sprague-Dawley (SD) rats weighing about 200 g were obtained from the Laboratory Animal Administration Center of Chongqing Medicine University. All animals were housed in monomer independent air cages and given a standard diet *ad libitum*. The animal experiments conducted in this study were performed with the approval of the Chongqing Medical University Institutional Animal Care and Use Committee.

Preparation and characterization of AmB-loaded nanoparticles. AmB-loaded PLGA nanoparticles (AmB-NPs) and plain PLGA nanoparticles (NPs) were formulated by a double-emulsification method as previously described (19). Briefly, a preweighed amount of AmB powder was dissolved in dimethyl sulfoxide (DMSO) that was miscible with water (20:80, vol/vol). PLGA polymer material was dissolved completely in dichloromethane and mixed with the drug or water for the first ultrasonic oscillation (XL2020; Qsonica) at 100 W for 2 min. Next, an aqueous solution of 1% PVA was added to the polymeric mixture for the second ultrasonic oscillation at 100 W for 5 min. NPs were prepared according to a similar method, except that the drug was exchanged for an equal amount of deionized water. The nanoparticles were resuspended, washed, and collected after the organic solvent was evaporated at room temperature for 6 h. The physical characteristics of nanoparticles, including the average diameter (D), zeta potential (ZP), and polydispersity index (PDI), were measured using a Malvern laser particle size analyzer (3000HS zeta sizer), and morphological characterization of nanoparticles was conducted by TEM. The drug loading content (LC%) and the encapsulation efficiency (EE%) of AmB-NPs were then calculated using the following equations: $LC\% (wt/wt) = (\text{mass of drug in NPs} / \text{mass of loaded NPs recovered}) \times 100\%$ and $EE\% (wt/wt) = (\text{mass of drug in NPs} / \text{amount of drug used for encapsulation}) \times 100\%$.

***In vitro* investigations of ultrasound-triggered drug release.** The kinetic release of AmB from nanoparticles *in vitro* with ultrasonication was assessed. A sample of AmB-NP lyophilized powder was diluted in PBS with or without sonication (fixed frequency of 42 kHz) at an intensity of 0.30 W/cm² for 15 min. After sonication, the samples were then individually transferred into dialysis bags, which were subsequently immersed in a container filled with 50 ml PBS and shaken at 100 rpm. Dialysate (1-ml) samples were collected, and the percentage of AmB released from nanoparticles was evaluated by UV-visible (UV-vis) spectrophotometry (UV-2600; Shimadzu, Japan) at each predetermined time point. Samples of AmB-NPs that underwent the same procedure, but without sonication, were used as controls.

Hemolytic activity of AmB-NPs *in vitro*. AmB sometimes causes significant adverse effects, such as hematological toxic reactions, which induce hemolytic anemia by allowing normal red blood cells to undergo hemolysis (7). The hemolytic activity of AmB-NPs was assessed using a previously reported method (37). Briefly, 1.0 ml of a healthy rabbit red cell suspension at 1% hematocrit was mixed with 1.0 ml of a solution containing free AmB, AmB-NPs, or NPs, and final AmB concentrations of 0 to 32 µg/ml were applied for the free-AmB and AmB-NP groups, respectively. The corresponding concentrations of NPs were 0 to 800 µg/ml (based on drug loading content). The same amount of AmB-NPs underwent ultrasound preirradiated at an intensity of 0.30 W/cm² for 15 min to investigate the biosafety of ultrasound-mediated AmB release from the nanoparticles. After 24 h of incubation at 37°C, the supernatants were collected, and the absorbance (Abs) at 540 nm was measured using a spectrophotometer. Additionally, red cells incubated with PBS alone served as a negative-control group (to estimate natural hemolysis), and those incubated with distilled water served as a positive control (serving as 100% hemolysis). The percentage of hemolysis induced by free AmB or AmB-NPs was then calculated using the equation $\% \text{ hemolysis} = (Abs_s - Abs_0) / (Abs_{100} - Abs_0) \times 100\%$, where Abs_s is the average absorbance of the sample, Abs_0 is the average absorbance of the negative control, and Abs_{100} is the average absorbance of the positive control.

FIG 9 Schematic illustration of low-frequency ultrasound irradiation *in vitro* (A) and *in vivo* (B).

Effects of AmB-NPs on macrophage activity. Macrophages are major anti-inflammatory cells that play an important role in eliminating extraneous pathogens and participate in immune responses (38); therefore, it is necessary to investigate the effects of AmB-NPs on macrophage activity. Free AmB, AmB-NPs, and NPs were cocultured with RAW264.7 macrophages (1×10^6 cells per well) in the logarithmic phase for 24 h at 37°C, and the final concentration of the drug used in this experiment was 4.0 $\mu\text{g}/\text{ml}$ (based on a test of the susceptibility of *C. albicans* biofilms to AmB). NPs at corresponding concentrations (70 $\mu\text{g}/\text{ml}$, calculated by drug loading content) were used in this experiment. Similarly, the same amounts of AmB-NPs underwent ultrasound preirradiation at an intensity of 0.30 W/cm^2 for 15 min and were then cocultured with macrophages to investigate the biosafety of ultrasound-mediated release of AmB. Finally, the activity of macrophages in each group was detected by an MTT assay based on the absorbance at 490 nm (23).

Ultrasonic irradiation method *in vitro* and *in vivo*. The ultrasound device provided by Haina Science and Technology Co., Suzhou, China, was used in this study for *in vitro* and *in vivo* experiments. The instrument parameters were a fixed frequency of 42 kHz, a transducer diameter of 5.0 cm, and an adjustable sound intensity output of 0 to 0.6 W/cm^2 , which was monitored using a UPM-DT-1AV acoustic power detector (Ohmic Instruments, USA). The formula used to calculate the spatially and temporally averaged intensity (I_{ATA}) (in watts per square centimeter) was equal to the ultrasonic power (in watts) divided by the transducer surface area (in square centimeters) (39).

For sonication *in vitro*, a biofilm-growing plastic-bottom petri dish was fixed in a degassed distilled water bath using an aluminum wire. The ultrasonic transducer was submerged directly on the bottom of a biofilm plate, and degassed distilled water was used as the ultrasonic medium. The irradiation setup was submerged completely in a degassed distilled water bath and maintained at room temperature (22°C). For sonication *in vivo*, the top of the transducer was uniformly coated with the medical ultrasonic coupling agent, after which the transducer was affixed tightly to the skin of the rat for irradiation after injection of the drug into the local infection site. A schematic diagram of the ultrasonic irradiation method is shown in Fig. 9.

Synergistic effects of ultrasound combined with AmB-NPs on *C. albicans* biofilms *in vitro*. *C. albicans* biofilm in the mature period after 48 h of incubation was observed microscopically and evaluated using a growth dynamics curve (see Fig. S1 at <http://www.isminim.org/index.php?c=article&id=108>). To further investigate whether there is a synergistic effect of ultrasound combined with AmB-NPs on *C. albicans* biofilm *in vitro*, the formation of biofilm was performed by ultrasound and AmB-NPs separately or jointly in the following treatments: untreated (control), ultrasound alone (US), free AmB alone (AmB), ultrasound combined with free AmB (US plus AmB), AmB-loaded nanoparticles (AmB-NPs), and ultrasound combined with AmB-loaded nanoparticles (US plus AmB-NPs). The susceptibility of AmB and AmB-NPs to *C. albicans* biofilms was determined, and the sessile MIC_{50} (SMIC_{50}) of AmB was 4.0 $\mu\text{g}/\text{ml}$ (see Fig. S2 in the URL mentioned above). Therefore, the final concentration of drug employed in the AmB group, AmB-NP group, US-plus-AmB group, or US-plus-AmB-NP group was 4.0 $\mu\text{g}/\text{ml}$, and the corresponding concentration of AmB-NPs was 70 $\mu\text{g}/\text{ml}$, calculated by the drug loading content. Furthermore, the effects of ultrasound on *C. albicans* biofilm according to different acoustic parameters showed that ultrasonic irradiation at 0.30 W/cm^2 for 15 min may provide favorable conditions for synergistic effects of ultrasound combined with AmB-NPs (see Fig. S3 in the URL mentioned above); therefore, ultrasound irradiation in this experiment was performed at an intensity of 0.30 W/cm^2 for 15 min.

For quantitative analysis of *C. albicans* biofilm changes after experimental treatment, the activity and total biomass of biofilms were measured by XTT and CV assays as described above and below,

respectively. For XTT reduction assays, after 24 h of biofilm incubation following experimental treatment, the biofilm was rinsed twice in the petri dish and suspended in 1 ml PBS, and the cell suspension (100 μ l) was then transferred to 96-well microtiter plates, mixed with 100 μ l of an XTT-menaquinone solution prepared fresh (see <http://www.isminim.org/index.php?c=article&id=108>), and incubated at 37°C for 2 h in the dark. After incubation, the OD₄₉₀ was measured by a spectrophotometer (UV-2600; Shimadzu, Japan) to quantify biofilm activity. In addition, the total biomass of the biofilm was quantified by using a previously reported CV assay (40, 41). After treatment, the supernatant of the biofilm was removed, the biofilm was washed with PBS four times, fixed with 95% methanol, and stained with 500 μ l of 0.1% CV at 37°C for 10 min in the dark. After incubation, it was washed with PBS and transferred to another plate to measure the OD₅₇₀ with a spectrophotometer.

For analysis of the biofilm structure changes after treatment, a Live/Dead BacLight bacterial viability kit (catalog no. L7012; Invitrogen, CA, USA) including SYTO9 and propidium iodide (PI) dye was used to stain *C. albicans* biofilms according to the manufacturer's instructions; this assay is specifically designed for rapid and accurate detection of fungal cell activity. Following treatment of *C. albicans* biofilms with ultrasound and AmB-NPs separately or jointly, 50 μ l SYTO9-PI fluorescent dye was added to the biofilm petri dish, and the dish was incubated at 37°C for 30 min in the dark. The morphology and distribution of live and dead fungal cells of the biofilm were then visualized by CLSM. Next, the biofilm in the field from three randomly selected positions was scanned from the bottom layer to the top layer at a layer spacing of 0.5 μ m, and a three-dimensional (3-D) structural image of the biofilm was reconstructed. The structure of *C. albicans* biofilms was then assessed using COMSTAT software (Haluk Beyenal, Montana State University, USA) to determine the mean thickness (micrometers), textural entropy (TE) (reflects biofilm heterogeneity), areal porosity (AP) (reflects biofilm gap channels), and average diffusion distance (ADD) (reflects biofilm nutrient supply distance) (42).

Proteinase and phospholipase enzyme secretion assay. Secretory acidic proteases and extracellular phospholipase are two important virulence factors of *C. albicans* that are closely related to its invasiveness and pathogenicity. To investigate whether ultrasound affected the enzyme secretion activity, proteinase and phospholipase enzyme secretion assays were conducted as previously described (43, 44). Briefly, after the biofilm was treated by ultrasound combined with AmB-NPs, it was rinsed twice in a petri dish and then suspended in PBS. For determining proteinase activity, the following steps were carried out: the supernatant of the biofilm was mixed with 1% azocasein at a 1:9 (vol/vol) dilution and incubated for 1 h at 37°C in 5% CO₂; 500 μ l of 10% trichloroacetic acid was added to stop the reaction; the mixture was then incubated for another 10 min at room temperature, after which it was centrifuged at 10,000 rpm for 5 min; and 500 μ l of the supernatant was mixed with 500 μ l of 0.5 M sodium hydroxide and incubated for 15 min at 37°C in 5% CO₂, after which the OD₄₄₀ was measured. The phospholipase enzyme activity was determined by mixing the supernatant of the biofilm solution with an equal volume of the phosphatidylcholine substrate for 1 h at 37°C in 5% CO₂, after which the absorbance at 630 nm was read in a spectrophotometer. Finally, the enzyme activity was reported as a specific activity unit that increases the absorbance by 0.001 per min and normalized by the dry weight of biofilms (units per gram [dry weight]).

Subcutaneous catheter biofilm formation in a rat model and antifungal therapy. The method for establishing biofilm formation on a subcutaneous catheter in an *in vivo* rat model was conducted as described previously by Říčicová et al. (45) and as described in detail at <http://www.isminim.org/index.php?c=article&id=108>. The intravenous catheters (diameter of 2.4 mm) were cut into segments about 1.0 cm long, and fungal colonies were then allowed to adhere via coincubation with a *C. albicans* suspension (10⁷ CFU/ml) for 3 h at 37°C with shaking at 120 rpm. The catheters were subsequently implanted under the skin of SD rats. Forty-eight hours after implantation, adhesion of fungal colonies to the catheter reached a stable period, and a biofilm was formed on the surface of the subcutaneous catheter, which was confirmed by calculation of catheter fungus loading and microscopic observation (see Fig. S4 in the URL mentioned above).

To further investigate the synergistic antifungal efficacy of ultrasound and AmB-NPs against *C. albicans* biofilms *in vivo*, the infected rats were randomly divided into the following six groups (with 10 rats in each group): untreated (control), US, AmB, US plus AmB, AmB-NPs, and US plus AmB-NPs. The infected rats in each group were further divided into two subgroups (5 rats in each subgroup) for 3-day treatments and 7-day treatments. The dose of free AmB was selected based on recommendations for the clinical treatment of fungal infections converted to the body weight of rats, which was 1 mg/kg/day for 3 days (total of 3.0 mg/kg/animal) or 7 days (total of 7.0 mg/kg/animal). The dose of AmB-NPs was 10 mg/kg/day for 3 days or 7 days. The animals in some groups were subjected to once-daily ultrasonic irradiation for 15 min at 0.30 W/cm² with a continuous wave. This level of ultrasonic treatment was previously shown not to cause skin lesions during biosafety assessments of percutaneous irradiation in our previous experiments (19).

The explanted catheters were removed 24 h after the last treatment, and biofilms were subsequently stripped from the catheter surface and dispersed in PBS by sonication for 5 min at 40 kHz in a water bath sonicator (catalog no. KQ5200DE; SuZhou, China). Our preliminary results indicated that this process did not affect cell culturability. Samples were then seeded onto SDA plates at a 1:10 dilution for 24 h at 37°C, after which the numbers of CFU on the catheters were counted and the levels of blood urea and creatinine after 7-day treatments were determined. All animals were euthanized after the experiment.

For observation of morphological changes in the catheter biofilm, explanted catheters were stained with 50 μ g/ml ConA (which binds to polysaccharides to outline the cell walls of the yeast and produce intense green fluorescence) for 1 h in the dark. Fluorescence images were observed by CLSM at

excitation/emission wavelengths of 488/530 nm. In addition, the explanted catheters were dried and processed as described previously by Nett et al. (46) for SEM observation.

Statistical analysis. Qualitative data in this study are described as the means $\pm$ standard deviations (SD) from three experiments and were analyzed using GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla, CA, USA). Results were considered to be significant when *P* values were <0.05 .

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We declare that, to the best of our knowledge, there is no conflict of interest regarding the publication of this paper.

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