Support Information

Synergistic antifungal effect of amphotericin B-loaded PLGA nanoparticle with ultrasound against *C. albicans* biofilms

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1. Preparation of XTT-menaquinone solution

The activity of cells within the biofilms was quantified through color change in a XTT reduction assay with XTT-menaquinone solution. XTT-menaquinone solution was prepared freshly. Briefly, 0.5 mg/mL stock solution of XTT tetrazolium salt was dissolved in PBS, sterilized through a filter with 0.22 μm pore size, and stored as aliquots in the dark at -70°C. Menaquinone was aliquoted into 10 mmol/L acetone solutions and stored in dark at -70°C. Prior to each assay, XTT-menaquinone solution was prepared at a final menaquinone concentration of 1 μM. In the process of the assay, 100 μL XTT-menaquinone solution was added to each well and mixed with the biofilm, and the plates were incubated at 37°C for 2 h in the dark. The absorbance was measured by a microplate plate reader (ELX800, BIO-TEK instruments, INC.) at 490 nm to measure the metabolic activity of cells within the biofilms.

2. *C. albicans* biofilm formation observation and growth dynamics measurement

C. albicans cells were resuspended in RPMI 1640 medium supplemented with 10% FBS and grown in 35-mm diameter plastic bottom petri dishes. After an initial incubation for 3 h at 37°C, the culture medium was discarded and the non-adherent colonies were gently rinsed off with PBS, and adherent cells were further cultured for 72 h for biofilm formation by adding fresh RPMI 1640 medium containing 10% FBS. During the three developmental phases of *C. albicans* biofilm formation, early phase (0-11 h), intermediate phase (12-30 h), and maturation (31-72 h)[1], the biofilms were stained with crystal violet to observe morphological changes under an optical microscope, and the growth dynamics of biofilms were tested by XTT reduction assays at 2 h, 6 h, 12 h, 24 h, 48 h, and 72 h of incubation.

The morphological changes and growth activity dynamics curve of *C. albicans* biofilms during the formation phases are shown in Fig. S1. After 48 h of inoculation, a typical biofilm characteristic-like structure of a certain thickness with predominant extracellular matrix wrapping

were observed by microscopy (A). XTT reduction assays revealed that the growth activity of the biofilm was saturated after 48 h of incubation, which indicated that it was in a mature and stable state (B). We thus used mature biofilms after a 48 h inoculation for subsequent experiments.

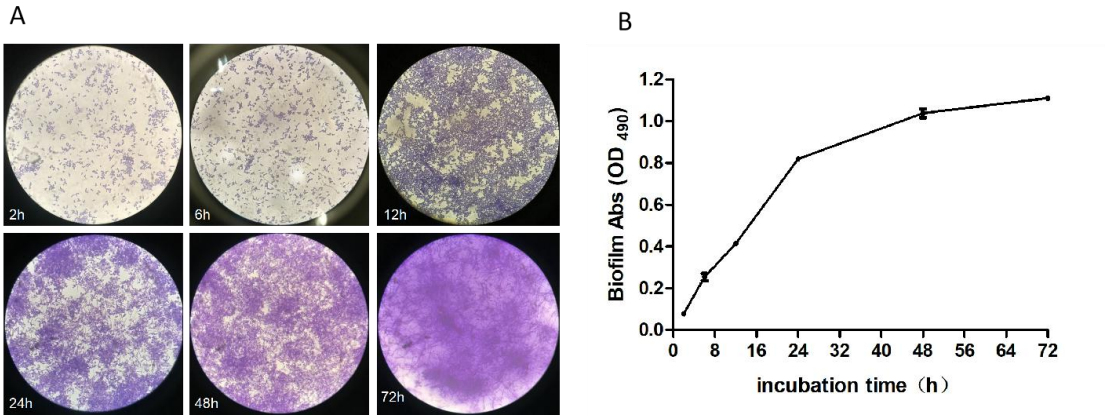

Fig. S1. Morphological changes (A) and growth dynamics curve (B) of *C. albicans* biofilms during the formation phases *in vitro*.

3. Susceptibility of AmB and AmB-NPs against *C. albicans* biofilms

The susceptibility of AmB and AmB-NPs against C. albicans biofilms was tested in vitro with XTT reduction assays in accordance with the NCCLS (National Committee for Clinical Laboratory Standards) guidelines[2]. AmB and AmB-NPs were added to the biofilms on polyethylene 96-well microtiter plates in serially double-diluted concentrations of 0.5 μg/mL to 32 μg/mL, and RPMI 1640 medium without AmB was used as the control. The biofilms were incubated for a further 48 h at 37 °C. Sessile minimum inhibitory concentration (SMIC) was determined at 50% ($SMIC_{50}$) of biofilm activity inhibition, which was calculated by optical density values at 490 nm (OD_{490}) with co-cultures of different concentrations of AmB compared to biofilms in the control group.

The antifungal susceptibility of AmB-NPs against biofilms after 24 h incubation is shown

in Fig. S2. The activity of the biofilms gradually decreased with the drug concentration increasing, and $SMIC_{50}$ of free AmB and AmB-NPs was found to be 4.0 μg/mL and 8.0 μg/mL, respectively; the higher $SMIC_{50}$ of AmB-NPs could be related to the slow release of AmB from AmB-NPs.

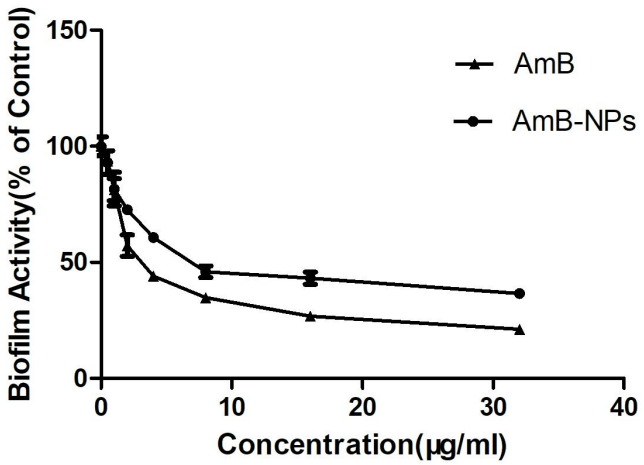

Fig. S2. Activity of *C. albicans* biofilms after co-incubation with different concentrations of AmB and AmB-NPs for 24 h. The biofilm activity (%) was calculated by OD_{490} of biofilms with co-cultures of different concentrations of AmB compared to the OD_{490} in the control group.

4. Ultrasound effect on *C. albicans* biofilms with different acoustic parameters

After mature *C. albicans* biofilms formed after 48 h incubations, we investigated the effect of ultrasound with different acoustic parameters *in vitro*. The biofilms were treated at different ultrasonic intensities (0.15, 0.30, and 0.60 W/cm^2) for 15 min and at different irradiation periods (5, 10, 15, 20 min) with an intensity of 0.30 W/cm^2. Biofilms that did not undergo ultrasound irradiation were used as the control group. The biofilm activity was measured with XTT reduction assays as mentioned above.

The results revealed that biofilm activity did not significantly decrease by ultrasonic irradiation for 5 min or 10 min or at 0.15 W/cm^2 intensity compared with the control group ($P >$

0.05), but biofilm activity was significantly decreased when the ultrasonic intensity reached 0.3 W/cm^2 or the irradiation time reached 15 min ($P < 0.05$). Thus, ultrasonic irradiation at 0.30 W/cm^2 for 15 min provided favorable conditions for follow-up experiments combining ultrasound with AmB-NPs.

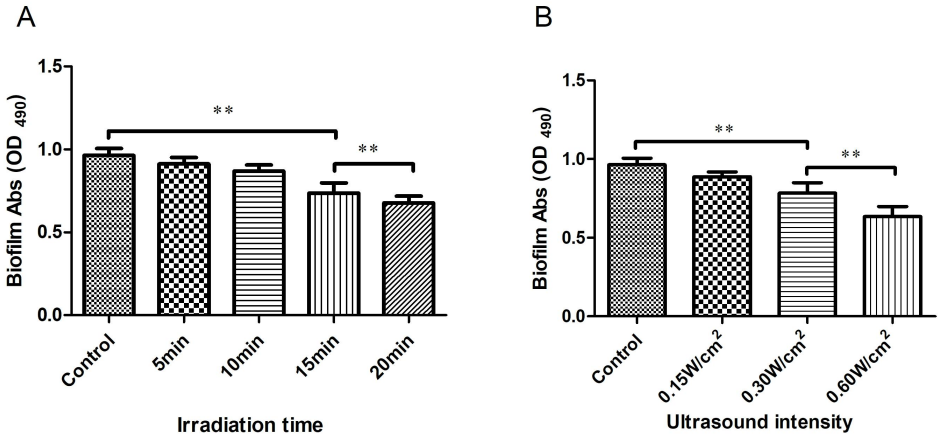

Fig. S3. Activity of *C. albicans* biofilms after ultrasonic treatment at different irradiation periods (A) and for different ultrasonic intensities (B). The control group did not undergo ultrasound irradiation. Significant statistical difference ($P < 0.01$) is denoted by (**).

5. *C. albicans* biofilm growth in a rat subcutaneous catheter model

Before the experiments, all catheters were cut into small pieces about 1.0 cm in length and dipped in 75% medical alcohol for disinfection, then incubated overnight in bovine serum at 37°C. Serum-coated catheters were incubated in *C. albicans* suspensions (10^7 CFU/mL) for 3 h at 37°C with 120 rpm shaking. After initial attachment, catheters were gently washed twice with PBS to remove non-adhering colonies. Before catheters were implanted under the skin, all rats were anesthetized with 3% chloral hydrate (1 mL/kg body weight). Then the lower back of the rat was shaved with an electric clipper and disinfected with 75% alcohol followed by betadine solution, an

incision of approximately 1 cm was made longitudinally in the dorsal skin with a sterile surgical blade, and the catheters with pre-adhering colonies were carefully inserted subcutaneously through the incision. The wound was then closed and disinfected with 75% alcohol followed by betadine solution. To observe the growth dynamics of biofilms in the rat subcutaneous model, the colony forming units (CFUs) of the catheter fungus were calculated over a period of 72 h. All explanted catheters were removed and sonicated for 5 min at 40 kHz in a water bath sonicator (KQ5200DE, SuZhou, China) at 6 h, 12 h, 24 h, 48 h, and 72 h after implantation. Samples were seeded on SDA plates at 1:10 dilution for 24 h at 37°C to determine CFUs. In addition, the morphology of the biofilms was observed by crystal violet staining for optical microscopy and by scanning electron microscopy.

The CFUs of the catheter fungus were calculated over a period of 72 h as shown in Fig. S4. The number of cells significantly increased in the 48-h following implantation of the catheters ($P < 0.05$), and at 72 h, the CFUs were not significantly increased compared to 48 h (Fig. S4A). The morphology of the biofilms was further observed by optical microscopy and scanning electron microscopy at 48 h post-implantation, which showed the biofilms in a dense network of hyphal cells on the surface of the subcutaneous catheter that had reached a stable period, similar to biofilms *in vitro* (Fig. 4B and C).

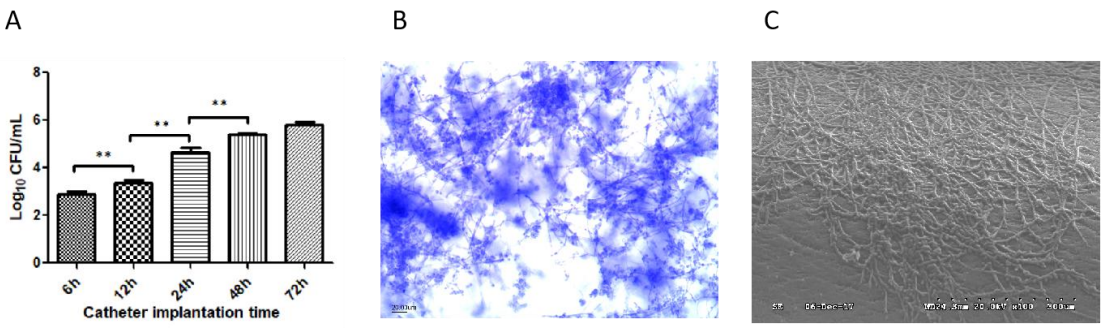

Fig. S4. Fungal colony counts (Log_{10} CFU) on implanted catheters at five time points by 72 h (A). The morphology of biofilms was observed by crystal violet staining with optical microscopy (B) and by scanning electron microscopy (C) at 48 h post-implantation.

Reference

[1] Chandra J, Kuhn D M, Mukherjee P K, et al. Biofilm formation by the fungal pathogen Candida albicans: development, architecture, and drug resistance. J Bacteriol. 183(18), 5385-5394(2001).

[2] Ramage, G., Vande Walle, K., Wickes, B. L. & Lopez-Ribot, J. L. Standardized method for in vitro antifungal susceptibility testing of Candida albicans biofilms. Antimicrob Agents Chemother. 45, 2475-2479 (2001).