Lipase-responsive antifungal nanoplatform for synergistic photodynamic/photothermal/pharmacotherapy of azole-resistant *Candida albicans* infection

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Experimental section

Materials

Thieno[2,3-b]indole-diketopyrrolopyrrole (DPP) was synthesized as our previously described.[1] PEG5k-PCL2k (PGL) was purchased from Xi'an ruixi Biological Technology Co., Ltd (Xi'an, China). Fluconazole (FLU), menadione, glucose and 2,3-bis(2-methoxy- 4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carboxanilide (XTT) were obtained from Macklin (Shanghai, China). Propidium iodide (PI), dichlorodihydrofluorescein diacetate (DCFH-DA), peptone and yeast extract were provided by Shanghai Sangon Biotech Co., Ltd. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), human umbilical vein endothelial cells (HUVEC), phosphate-buffered saline (PBS), crystal violet staining solution and dulbecco's modified eagle's medium (DMEM) were procured from the Keygen Biotech Co., Ltd (Nanjing, China).

Characterization

The morphology of nanoparticles was characterized by scanning electron microscopy (SEM, Hitachi, S-4800, Japan). The average size and size distribution of nanoparticles were detected by dynamic light scattering (DLS, Brookhaven Instruments, USA). UV-vis absorption spectra were performed using UV-3600 Shimadzu UV-vis spectrometer (Shimadzu, Japan). Fluorescence spectra were determined by RF-5301PC spectrofluorophotometer (Shimadzu, Japan). The temperature of samples was monitored by infrared camera (FLIR, Arlington, VA). The cell fluorescence images were taken using Olympus IX70 inverted microscope (Olympus, Japan). The loading efficiency of FLU and DPP were measured using UV-vis spectrometer as Ren *et al.*'s reported.[2]

Preparation of PGL, PGL-FLU, PGL-DPP, and PGL-DPP-FLU nanoparticles

PGL and drug-loaded PGL nanoparticles were prepared as previously described.[3] Briefly, 1 mL tetrahydrofuran solution containing PGL (10 mg), PGL (10 mg) + FLU (2 mg), PGL (10 mg) + DPP (2 mg) or PGL (10 mg) + FLU (2 mg) + DPP (2 mg) was added to 10 mL of Mili-Q water under unremitting ultrasonication. The tetrahydrofuran in aqueous solution was volatilized by nitrogen stripping. Then the resulting solution was dialyzed and filtered using a 0.45 μm syringe filter before store in 4 ℃ refrigerator for further biomedical application.

Singlet oxygen detection

Singlet oxygen ($^1O_2$) generation was determined using singlet oxygen sensor green (SOSG) as $^1O_2$ fluorescent probe. 2 mL of PGL, PGL-FLU, PGL-DPP or PGL-DPP-FLU nanoparticles (all concentration at 20 μg mL^{-1}) were mixed with 20 μL of SOSG stock solution (5 mM) and added to 10 mm quartz cuvette, respectively. Then fluorescence spectra were immediately measured after the quartz cuvette was irradiated with a 660 nm laser (1 W cm^{-2}).

For intracellular reactive oxygen species detection, *C. albicans* (1×10^6 cells) was incubated with PGL, PGL-FLU, PGL-DPP or PGL-DPP-FLU nanoparticles (200 μg mL^{-1}) for 3 h, respectively. The solution was discarded through centrifugation. *C. albicans* cells were then stained with 200 μL of DCFH-DA (10 μM) for 30 min. After the sample washed with PBS, the *C. albicans* cells were exposed to a 660 nm laser (1 W cm^{-2}) for 20 min. Finally, *C. albicans* cells were collected through centrifugation and the cell suspension was dropped to a glass slide for fluorescence imaging.

***C. albicans* culture**

Azole-resistant *C. albicans* ~~stain~~ Nos. 108 was kindly provided by Professor Wanqing Liao

from Changhai hospital (Shanghai, China)[4] and culture overnight in YPD medium (1% yeast extract, 2% tryptone, 2% glucose) at 35 °C. The fungal cells were spinned down and washed thrice with PBS. Finally, *C. albicans* cells were redispersed in PBS solution and the cell density was determined using cell-count boards.

***In vitro* antifungal assay**

The antifungal activity of PGL, PGL-FLU, PGL-DPP, and PGL-DPP-FLU nanoparticles was evaluated by turbidimetry and plate counting method. For turbidimetric assay, 200 μL of YPD medium containing a series of concentration of nanoparticles/FLU solution (0, 10, 40, 80, 120 and 160 μg mL^{-1}) and 10^4 *C. albicans* cells was added to 96-well plate and culture at 35 ℃ in dark environment for testing the biocompability of nanoparticles. After 12 h of incubation, the cell density of each well was measured by a microplate spectrophotometer at 600 nm. To test the photofungicidal activity of nanoparticles, a similar procedure was carried out and each well was irradiated with a 660 nm laser (1 W cm^{-2}) for 20 min after the medium was added to 96-well plate. For plate counting assay, *C. albicans* cells (1×10^6 cells) were added to PGL, PGL-FLU, PGL-DPP, and PGL-DPP-FLU nanoparticles solution (200 μg mL^{-1}). The samples were exposed to 660 nm laser (1 W cm^{-2}) for 20 min after 3 h incubation. Then the samples were diluted and spread on YPD plates for the quantification of fungal cell density. To further examine the fungicidal activity of nanoparticles, *C. albicans* cells were also stained with PI for 30 min after the samples were treated with nanoparticles and 660 nm laser simultaneously. The dead cells emitted a red fluorescence can be observed under a fluorescence microscope.

Anti-biofilm assay

Biofilm was developed as our previously described.[5] 200 μL of RPMI 1640 medium containing

1×10^6 *C. albicans* yeast cells were seeded to 96-well plate and culture at 37 ℃ for 2 h for fungi cells adherence to the surface of each well. The unattached cells were removed and the attached cells were replenished with 200 μL of RPMI 1640 medium. Then the plate was placed in an incubator for 24 h.[5] After the biofilm formation, the supernatant was carefully discarded and 200 μL of PGL, PGL-FLU, PGL-DPP and PGL-DPP-FLU nanoparticles and FLU solution (200 μg mL^{-1}) were gently transferred into each well, respectively. Finally, each well was irradiated by 660 nm (1 W cm^{-2}) laser for 20 min and incubation for another 6 h.

For crystal violet staining assay, the solution was aspirated and 100 μL of 2.5% glutaraldehyde was added to each well to fix the *C. albicans* biofilms. After 20 min incubation, the supernatant was aspirated again and 100 μL of crystal violet staining solution was added to each well. Then the biofilm was incubated at room temperature for 30 min and washed with sterile distilled water for further microscope observation.

For biofilm quantification assay, the supernatant was thoroughly aspirated and the biofilm was washed with PBS. 100 μL of XTT/menadione mix solution containing 5.2 μg mL^{-1} menadione and 0.2 mg ml^{-1} XTT was added to each well. After incubation in dark environment for 2 h at 37 ℃, the supernatant was transferred to a new plate and the optical density at 490 nm of each well was determined using a microplate spectrophotometer.[6]

In vivo antifungal therapy

Female ICR mice (6 weeks) were ordered from QingLong mountain animal breeding center (Nanjing, China). All the animal experimental procedures were approved by the School of Pharmaceutical Science, Nanjing Tech University. To evaluate the antifungal efficacy of nanoparticles, 20 of ICR mice were equally divided into four groups (five mice per group).

After female ICR mice were anesthetized with 10% chloral hydrate, the hair on the back was removed and a circular wound on the back were created in each mouse. Then a commercial band aid added with 200 μL of *C. albicans* (10^8 cells mL^{-1}) was used to cover the wounds for 12 h. On day after infection, each group's wounds were treated with PBS, PGL-FLU, PGL-DPP, and PGL-DPP-FLU NPs, respectively. ~~The~~ wound was ~~irradiated with 660 nm laser (1 W cm^{-2}, 20 min),~~ observed and photographed every day to monitor healing process. Seven days later, the infected wound tissues were excised and homogenized in sterile PBS. To evaluate the wound fungal burden, the tissue homogenate samples were diluted and plated on YPD agar plate for measuring the quantity of fungi.

Histological examinations

The mice were anesthetized after 3 days of therapy. The wound tissues were harvested, fixed in 4% paraformaldehyde and embedded in paraffin for histologic sectioning. Then masson and periodic acid schiff (PAS) staining were performed to examine the formation of collagen fiber and the morphology of *C. albicans* cell, respectively. After that, the slides were observed and photographed with an Olympus IX 70 inverted microscope.

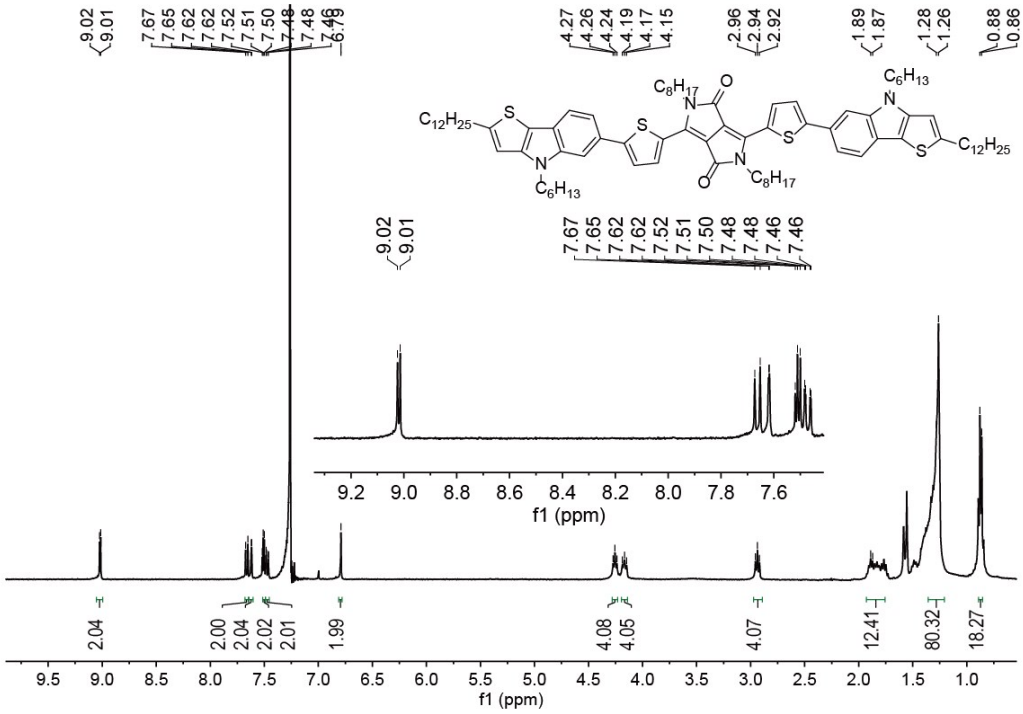

Fig. S1 H NMR of thieno[2,3-b]indole-diketopyrrolopyrrole ($CDCl_3$, 400 MHz)

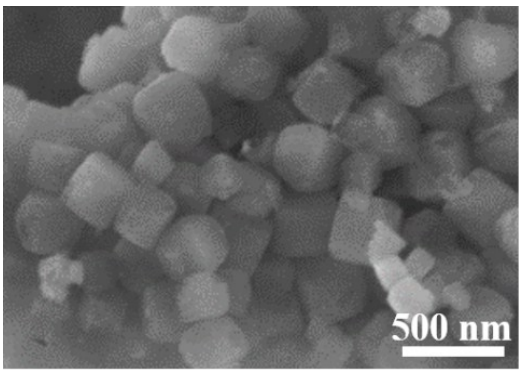

Fig. S2 SEM image of PGL NPs

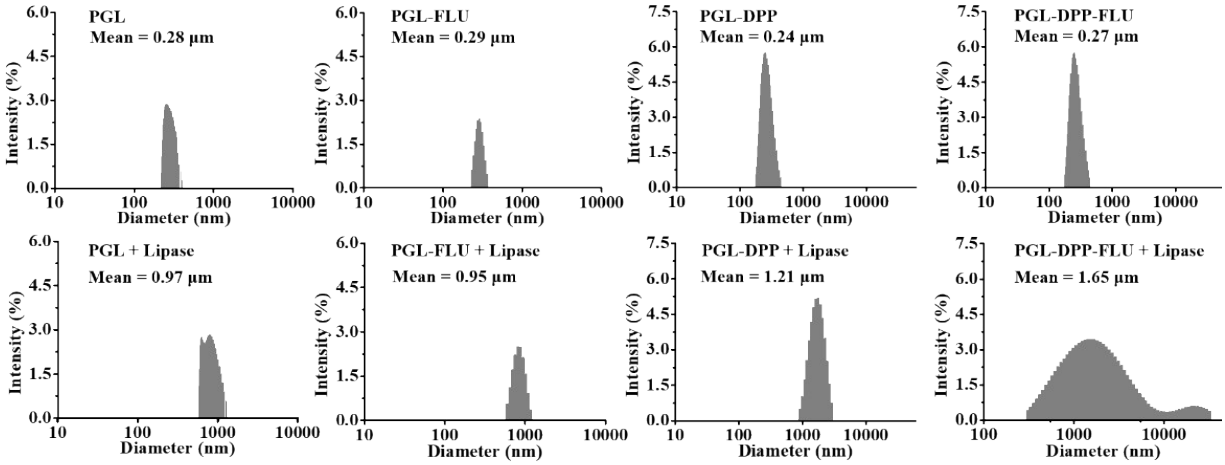

Fig. S3 Hydrodynamic size of PGL NPs, PGL-FLU NPs, PGL-DPP NPs, PGL-FLU-DPP NPs with and without lipase.

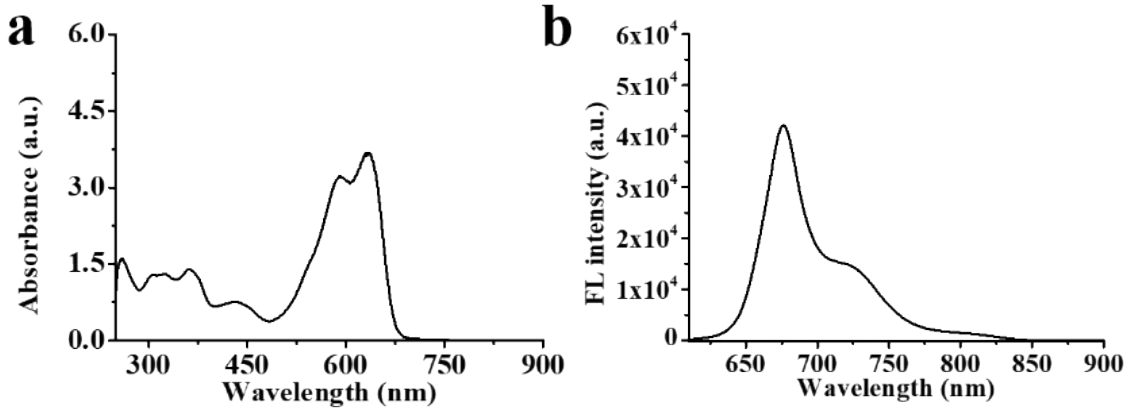

Fig. S4 The absorption and fluorescence spectra of DPP in THF solution.

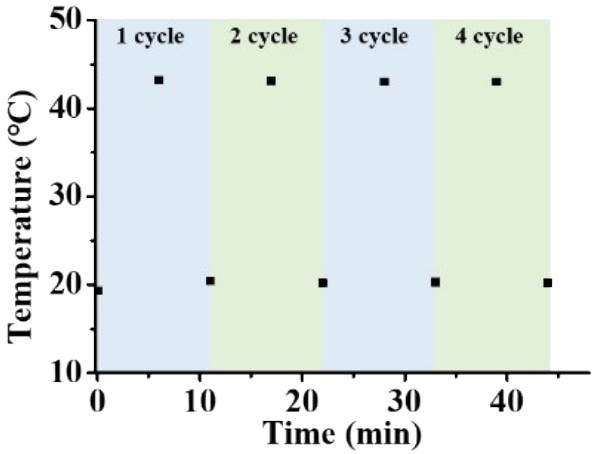

Fig. S5 The initial and eventual temperatures of the PGL-DPP-FLU NPs solution over 4 on/o cycles of 660 nm laser irradiation.

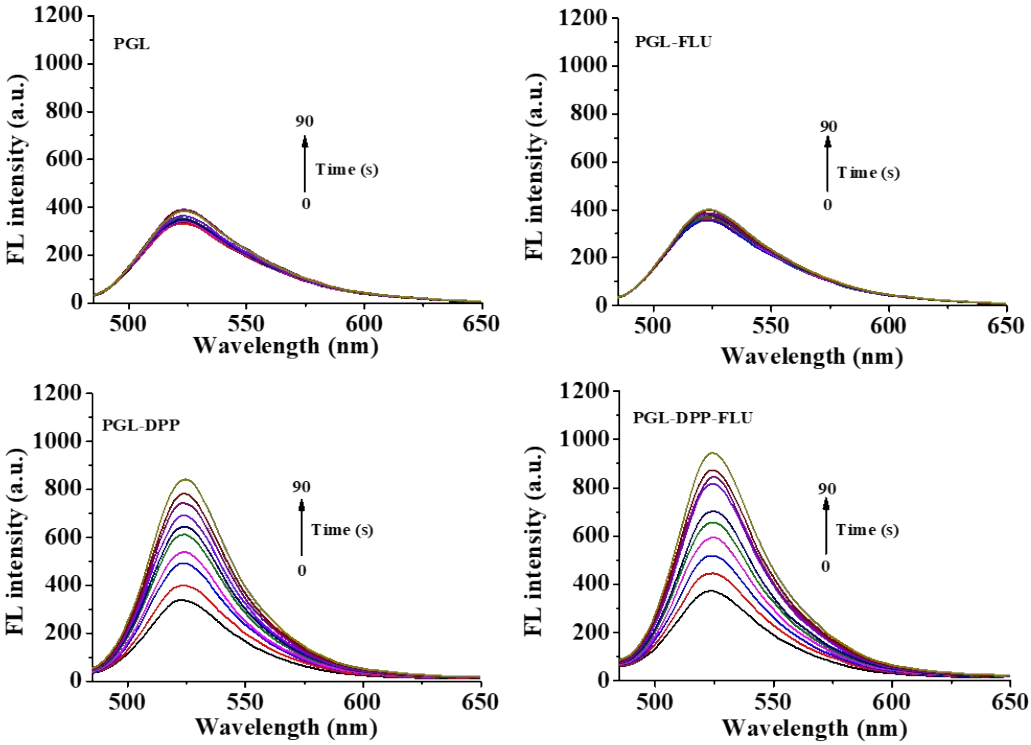

Fig. S6 The $^1O_2$ generation capacity of the PGL, PGL-FLU, PGL-DPP and PGL-DPP-FLU NPs under 660 nm laser irradiation was measured using SOSG probe.

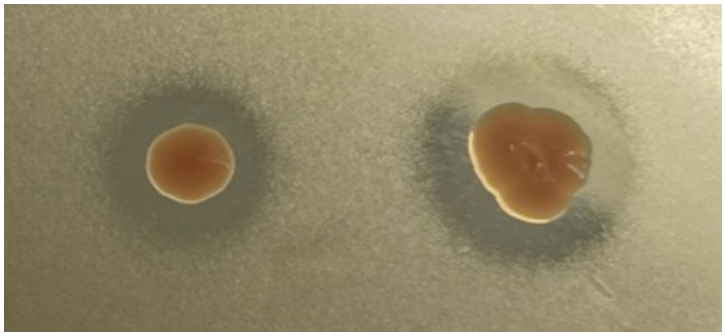

Fig. S7 Fungal lipase assay. Azole-resistant *C. albicans* ~~stain~~ Nos. 108 cells were dripped and cultured on the YPD-tributyrin agar. A clear halo surrounds the colony can be observed when *C. albicans* produces lipase and breaks down the tributyrin.[7]

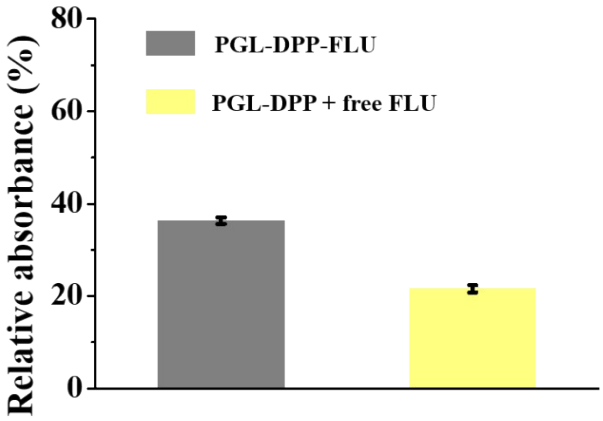

Fig. S8 Relative absorbance value of azole-resistant *C. albicans* cultured with 160 μg mL^{-1} of PGL-DPP-FLU NPs and PGL-DPP NPs + FLU with laser irradiation.

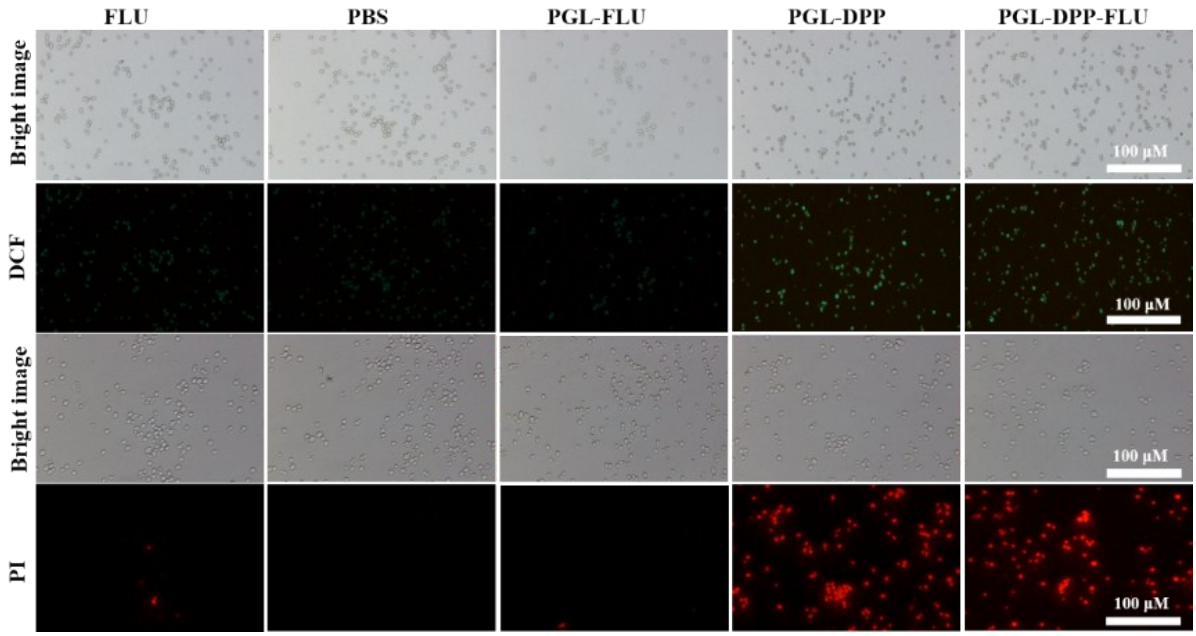

Fig. S9 Intracellular ROS (green fluorescence) and dead cell (red fluorescence) detection of azole-resistant *C. albicans* cells after receiving different treatments. All samples were irradiated with a 660 nm laser.

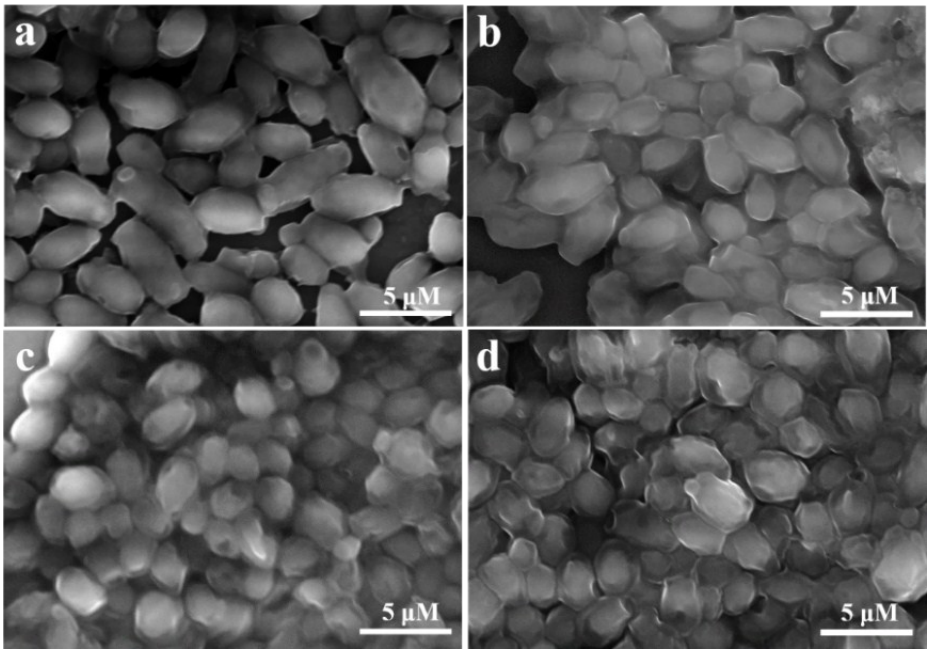

Fig. S10 SEM images of azole-resistant *C. albicans* cells after receiving different treatments: (a) PBS + laser, (b) PGL-FLU NPs + laser, (c) PGL-DPP NPs + laser, (d) PGL-DPP-FLU NPs + laser.

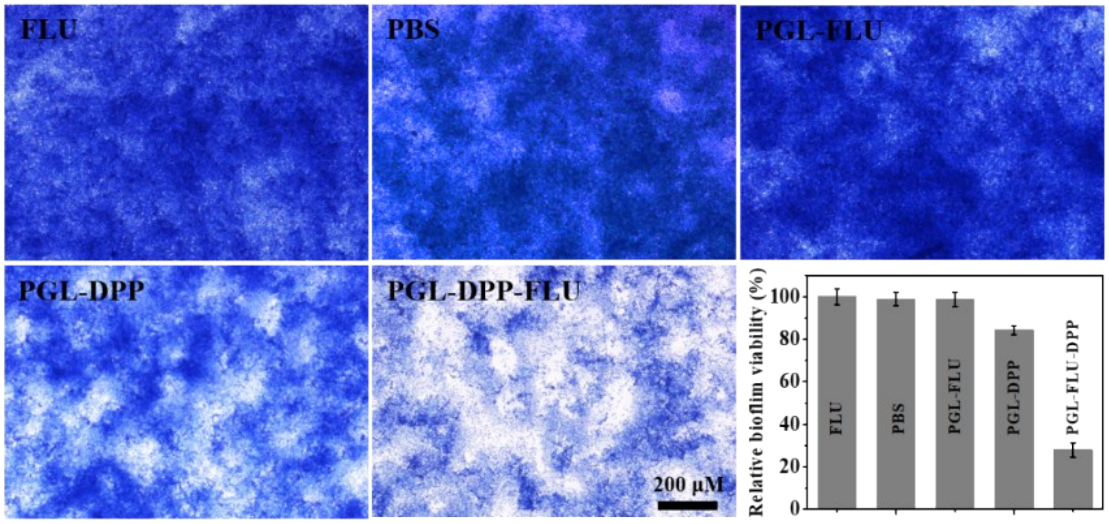

Fig. S11 Representative crystal violet stained images of *C. albicans* biofilms and corresponding biofilm mass after treated with FLU, PGL-FLU NPs, PGL-DPP NPs, and PGL-DPP-FLU NPs (200 μg mL^{-1}), respectively. Then all samples were irradiated with 660 nm laser.

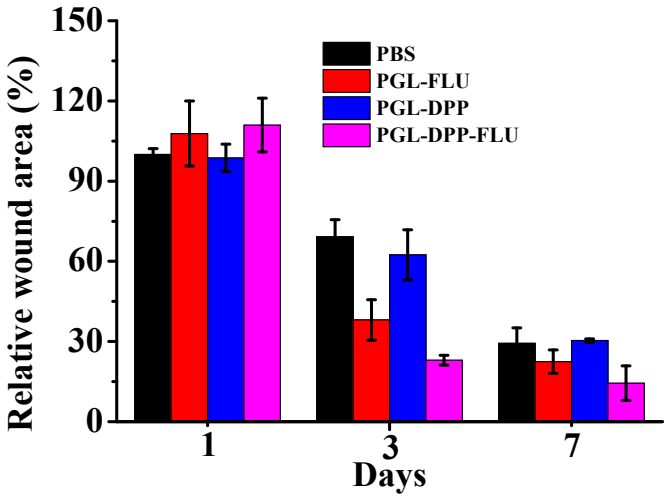

Fig. S12 The relative wound area change.

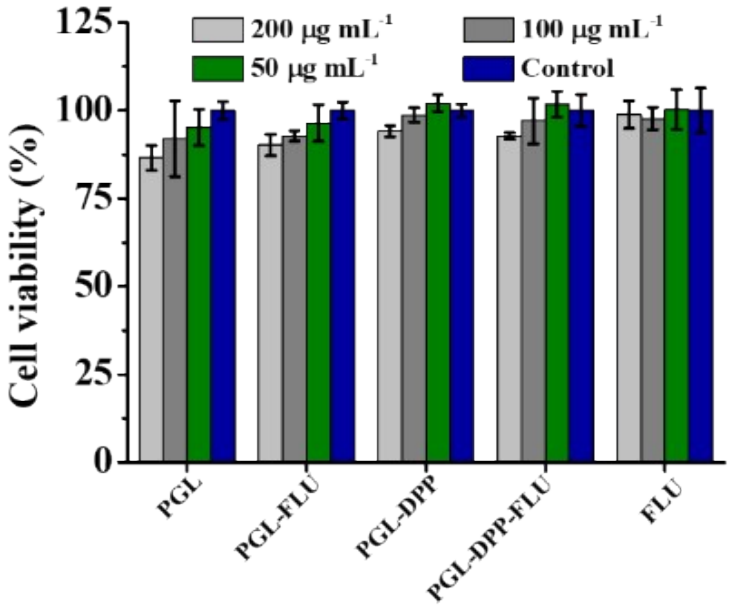

Fig. S13 *In vitro* viability of HUVEC cells after incubated with different concentrations (0, 50, 100 and 200 μg mL^{-1}) of PGL-FLU NPs, PGL-DPP NPs, PGL-DPP-FLU NPs and FLU for 24 h.

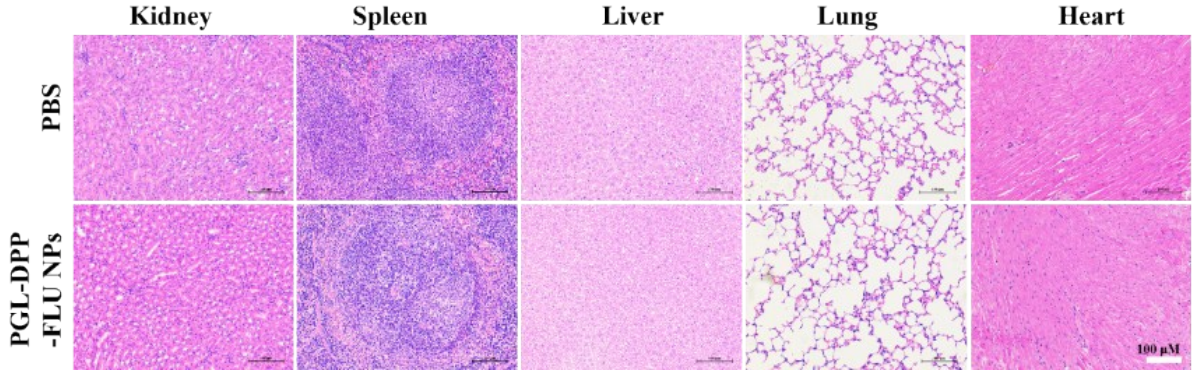

Fig. S14 Hematoxylin and eosin stained tissue sections from mice injected with 25 μL of PBS or PGL-DPP-FLU NPs (2 mg mL^{-1}) for 1 d.

Reference

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