

Selective Antifungal Activity of Hyperbranched Polylysine Mediated by Charge-dependent Membrane Interactions

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Electronic Supplementary Information

Abstract Invasive fungal infections remain a significant global health threat, and the development of antifungal agents that selectively target fungi remains a critical challenge. This study investigates hyperbranched polylysine (HPL) with tunable molecular weight and charge density as a potential selective antifungal candidate. HPL1, with the lowest molecular weight, showed negligible antimicrobial activities, while HPL3, with the highest molecular weight, exhibited broad-spectrum antimicrobial effects against both bacteria and fungi. Notably, HPL2, the mediate molecular weight, demonstrated selective antifungal activities against clinically relevant *Candida* species, including *C. albicans*, *C. krusei*, *C. parapsilosis*, *C. tropicalis*, and *C. glabrata*. This selectivity is mainly ascribed to its optimal zeta potential and appropriate hydrodynamic size, enabling HPL2 to penetrate through the fungal cell wall while stuck in bacterial cell envelopes. Mechanistic studies revealed that HPL2 initially adheres to the fungal surfaces induced *via* electrostatic interactions, then passively penetrates the fungal cell wall, disrupts membrane integrity, induces intracellular damage, and ultimately leads to cell death. Furthermore, HPL2 exhibited excellent biocompatibility and *in vivo* therapy efficacy with minimal disruption to host gut microbiota. These results highlight HPL2 as a promising antifungal agent with potent efficacy and favorable safety, which can be easily synthesized at kilogram scale.

Keywords Selective antifungal activity; Hyperbranched polylysine; Electrostatic interaction; *Candida albicans*

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INTRODUCTION

Invasive fungal infections have emerged as an increasingly pressing global health threat, particularly since the World Health Organization (WHO) published a priority list of invasive fungal pathogens in 2022.^[1] Among these fungal pathogens, *Candida* species remain the main cause of systemic infections worldwide, with attributable mortality rates reaching as high as 65% according to epidemiological studies.^[2] The rapid emergence of multidrug-resistant fungal strains has intensified this problem, further limiting the utility of conventional antifungal drugs. Thus, the development of novel antifungal agents with lower propensity to induce resistance is of critical importance.

Cationic antimicrobial agents are characterized by a high density of positive charges that disrupt the cell membranes of microorganisms. Unlike traditional antibiotics that target specific molecular pathways, cationic antimicrobial agents destroy the integrity of the entire membrane, thereby reducing the risk of resistance development.^[3,4] However, the application of cationic antimicrobials is challenging. Antimicrobial

activity is often accompanied by cytotoxicity, including hemolysis, plasma coagulation, and damage to host cells.^[5]

To date, most cationic antimicrobial agents have exhibited either primary or broad-spectrum antimicrobial activities.^[6–8] This limited selectivity towards fungi may be associated with structural differences in microbial cell envelopes. Gram-negative bacteria possess a trilaminar, "sandwich-like" architecture composed of a negatively charged outer membrane containing lipopolysaccharides (LPS) as the major barrier for gram-negative pathogens, a periplasmic space containing a thin layer of peptidoglycan, and an inner phospholipid bilayer plasma membrane.^[9–11] Gram-positive bacteria lack an outer membrane, but have a peptidoglycan cell wall modified by teichoic acids that provide a negative charge.^[11–14] In contrast, fungi, particularly pathogenic yeasts such as *Candida* species, exhibit fundamentally different architectures composed of a negatively charged outer layer rich in β -glucans and mannoproteins that confer electrostatic shielding and an inner structural layer composed of chitin and long-chain polysaccharides, forming a cross-linked scaffold that provides structural rigidity.^[15–17] Notably, passive diffusion into the fungal cells is restricted. Studies have shown that only molecules with hydrodynamic diameters smaller than about 6 nm can penetrate the cell wall of *Candida*.^[18] These struc-

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tural distinctions underscore the importance of optimizing the structure-activity relationships in cationic polymers to achieve fungal selectivity while minimizing cytotoxicity.

Hyperbranched polylysine (HPL) is a promising cationic antimicrobial polymer. The hyperbranched architecture provides HPL with a high density of terminal amino groups, and the adjustable molecular weight provides variable charge distributions.^[19] Our previous studies have demonstrated that HPL possesses broad-spectrum antimicrobial activity. Its efficiency and cytotoxicity are highly dependent on its molecular weight and charge density. Guanidination modification further enhanced antifungal activity. With an increase in the molecular weight, HPL exhibited selective antibacterial effects without significant activity against fungal pathogens.^[20] To date, no systematic studies have explored the selective antifungal activity of HPL.

The aim of this study was to investigate the antimicrobial activity of HPL. An optimal molecular weight range was identified in which HPL selectively targeted fungi over bacteria, with low cytotoxicity and hemolysis. Mechanistic studies suggested a cell wall-bypassing, membrane-disrupting mode of action that may reduce the likelihood of resistance development. *In vivo*, the optimized HPL exhibited low acute oral toxicity, significant therapeutic efficacy in a gastrointestinal *C. albicans* infection model, and preservation of the gut microbial balance. These results highlight HPL as a promising candidate for a selective and safe antifungal agent, which is favorable for further development of cationic polymer antimicrobial agents.

EXPERIMENTAL

Materials

L-Lysine hydrochloride, 3-(*N*-morpholino) propane sulfonic acid (MOPS), acetic acid at HPLC grade, sodium acetate at GC grade, methylthiazolyldiphenyl-tetrazolium bromide (MTT), 3,3'-dipropylthiadicarbocyanine iodide (DiSC₃(5)) and cyclophosphamide were purchased from Aladdin Chemistry Co., Ltd. and used as received. Luria-Bertani agar (LB-Agar), Mueller-Hinton Broth (MHB), Sabouraud Dextrose Broth (SDB), and Sabouraud Dextrose Agar (SDA) were purchased from QingDao Hopebio-Technology Co., Ltd. The R/MINI 1640 medium was purchased from Gibco, (Grand Island NY, USA). All bacterial and fungal strains were obtained from Shanghai Luwei Technology Co., Ltd.: *Escherichia coli* (*E. coli*, ATCC 8739), *Pseudomonas aeruginosa* (*P. aeruginosa*, ATCC 9027), *Staphylococcus aureus* (*S. aureus*, ATCC 25923), *Methicillin resistant Staphylococcus aureus* (MRSA, ATCC 43300), *Candida albicans* (*C. albicans*, ATCC 10231, ATCC 90028, ATCC 14053, and CMCC 98001), *Candida tropicalis* (*C. tropicalis*, ATCC 750), *Candida krusei* (*C. krusei*, ATCC 6258), *Candida glabrata* (*C. glabrata*, ATCC 15126), *Candida parapsilosis* (*C. parapsilosis*, AS 2.590).

Propidium Iodide (PI), fluorescein 5-isothiocyanate (FITC), *N*-phenyl-1-naphthylamine (NPN), a mitochondrial membrane potential assay kit with JC-1 (M8650), and a Reactive Oxygen Species Assay Kit (CA1410) were purchased from Beijing Solarbio Science & Technology Co., Ltd. The succinate dehydrogenase (S0530S) and alkaline phosphatase (P0321S) assay kits were purchased from Beyotime Biotechnology Co., Ltd. Mouse IL-6 ELISA Kit (EK206) and Mouse TNF- α ELISA Kit

(EK282) were purchased from MultiSciences Biotech Co., Ltd.

All other materials were purchased from Aladdin Chemistry Co., Ltd., and used as received.

Synthesis and Characterization

HPL was synthesized *via* thermal polycondensation of L-lysine hydrochloride in an aqueous medium, according to a previously reported method.^[19] Briefly, an equimolar amount of KOH was added to an aqueous solution of L-lysine hydrochloride and the reaction was conducted at 180 °C under nitrogen protection. By systematically varying the reaction time, a series of HPLs with controlled molecular weights was obtained.

Structural characterization was performed using ¹H-NMR spectroscopy (Bruker AV-500 MHz) with D₂O as the solvent, which confirmed the expected hyperbranched architecture. Molecular weights were characterized using a size-exclusion chromatography system (SEC) equipped with a multi-angle light scattering detector (MALS), an online differential viscometer (VIS), and a refractive index detector (RI) (DAWN[®] HELEOS-II MALS, Viscostar[®] viscometer, Optilab[®] T-REX RI detector, Wyatt Technology, USA). The HPL concentrations were dissolved at 10 mg/mL in CH₃COOH/CH₃COONa buffer (pH=3.6) and eluted with the same buffer at 1 mL/min (35 °C). The system provided a precise determination of the refractive index increment (dn/dc), weight-average (M_w), number-average (M_n) molecular weights, polydispersity index ($\bar{D}$), and hydrodynamic radius (D_h). zeta potentials of HPL solutions (5 mg/mL in PBS buffer, pH=7.4) were measured at 25 °C using a Malvern Zetasizer.

In vitro Antibacterial Assay

The antibacterial activity of HPL was evaluated using the standardized microbroth dilution method, following CLSI guidelines M07-A9. Bacterial strains stored at -80 °C were initially streaked onto Luria-Bertani (LB) agar plates and incubated at 37 °C for 24 h to obtain isolated colonies. Subsequently, several well-isolated colonies were transferred to Mueller-Hinton (MH) broth using a sterile inoculation loop and cultured overnight at 37 °C with shaking (130 r/min) to achieve mid-logarithmic phase growth. The bacterial suspension was adjusted to approximately 1×10^6 CFU/mL using fresh MH broth.

For the determination of the minimum inhibitory concentration (MIC), 175 μ L of the standardized bacterial suspension was added to each cell of the sterile 96-well microtiter plate, followed by the addition of 25 μ L of serially diluted HPL solutions (final concentrations range from 1.5 mg/mL to 3 μ g/mL) to achieve the desired test concentrations. Appropriate controls included bacterial suspension in MH broth as a positive control and sterile MH broth as the negative control. After 24 h incubation at 37 °C, bacterial growth inhibition was evaluated by both optical density measurements at 600 nm (OD₆₀₀) using a microplate reader and visual examination of culture turbidity. The MIC value was defined as the lowest HPL concentration that inhibited visible bacterial growth relative to the positive control.

In vitro Antifungal Assay

The antifungal properties of HPL were evaluated according to CLSI guideline M27-A3 using a broth microdilution method similar to the antibacterial testing protocol, with modifications for fungal assays. Fungal stocks, stored at -80 °C, were initially re-

covered on Sabouraud dextrose agar (SDA) plates and incubated at 35 °C for 24–48 h. Isolated colonies were then inoculated into Sabouraud dextrose broth (SDB) and cultured to the mid-logarithmic phase (35 °C, 24 h with shaking at 130 r/min). The fungal suspension was standardized to 1×10^3 CFU/mL in R/MINI 1640-MOPS medium (0.165 mol/L, pH=7.0).

175 μ L of the fungal suspension was added to each cell of the sterile 96-well microtiter plate, followed by the addition of 25 μ L of serially diluted HPL solutions (final concentrations range from 1.5 mg/mL to 3 μ g/mL) to achieve the desired test concentrations. After 48 h of static incubation at 35 °C, inhibition was assessed using OD₆₀₀ measurements and visual turbidity scoring. The minimum inhibitory concentration (MIC) was defined as the lowest HPL concentration that resulted in $\geq 90\%$ growth inhibition compared to the fungal-containing positive controls.

Fungal Resistance Study

The potential for resistance development was assessed by serially passaging *C. albicans* at sub-inhibitory concentrations of HPL. Mid-logarithmic phase *C. albicans* cells (about 10^3 CFU/mL) were exposed to HPL at $1/8 \times$ MIC in 10 mL cultures, with continuous shaking at 35 °C. After 24 h incubation (designated as the first generation), the cells were harvested by centrifugation, washed with PBS, and resuspended in fresh medium to about 10^3 CFU/mL. Subinhibitory HPL solutions were re-induced and the cultures were incubated for another 24 h. This procedure was repeated for a total of 14 generations. The MIC was determined every two generations. Fluconazole (FLC) and PBS served as the clinical drug and blank controls, respectively. MIC₀ denotes the baseline MIC, whereas MIC_n represents the MIC of the *n*th generation of *C. albicans*.

Time-kill Kinetics

For the killing kinetics analysis, *C. albicans* suspensions (about 10^6 CFU/mL) were treated with HPL at $4 \times$ MIC, with fluconazole and PBS as controls. Aliquots (100 μ L) of the suspensions were collected at predetermined intervals (0, 0.5, 1, 2, 3, 4, 6, 8, and 12 h), serially diluted, and plated on SDA. After 24 h of incubation at 35 °C, viable colonies were enumerated to determine the time-dependent killing profiles. All experiments were conducted in triplicate under standardized conditions (35 °C, 130 r/min shaking).

Cytotoxicity Assay

The cytotoxicity of HPL was evaluated in mouse embryonic fibroblasts (NIH/3T3 or L929). Cells in the logarithmic growth phase were trypsinized and adjusted to a density of approximately 40000 cells/mL in complete DMEM. The cell suspensions were seeded into 96-well plates (200 μ L per well), allowed to adhere for 12 h under standard culture conditions (37 °C, 5% CO₂), and then treated with a series of gradient-diluted HPL solutions (25 μ L per well) for 24 h. Cell viability was assessed using the MTT assay. The half-maximal inhibitory concentration (IC₅₀) was determined as the concentration of HPL required to reduce cell activity by 50% compared with untreated control cells.

Hemolysis Assay

For the hemocompatibility assessment, fresh rabbit blood (1 mL) was centrifuged at $1000 \times g$ for 10 min to isolate erythrocytes. After three washes with sterile PBS, packed red blood cells were resuspended in PBS to prepare a 2% (V/V) working sus-

pension. For the assay, 175 μ L of the erythrocyte suspension was incubated with 25 μ L of test samples (serially diluted HPL), a positive control (0.5% Triton X-100), or a negative control (PBS) in sterile 1.5 mL microcentrifuge tubes. After 1 h incubation at 37 °C, the suspensions were centrifuged, and 150 μ L of the supernatant was transferred to a 96-well plate for absorbance measurement at 540 nm. The percentage of hemolysis was calculated relative to the positive and negative hemolysis controls. The hemolytic concentration (HC₁₀) was defined as the lowest HPL concentration that induced 10% hemolysis.

Prothrombin Time Assessment

The anticoagulant activity of HPL was evaluated through prothrombin time (PT) measurements according to the CLSI guideline H47-A2, with some modifications. Fresh rabbit blood (1.8 mL) was immediately collected into citrate anticoagulant tubes containing 0.2 mL of 109 mmol/L sodium citrate solution (maintaining a strict 9:1 blood-to-anticoagulant ratio) and gently inverted to ensure complete mixing while preserving platelet integrity. Anticoagulated blood was then centrifuged at $1000 \times g$ for 10 min at room temperature to obtain platelet-poor plasma.

For PT determination, 70 μ L of the prepared plasma in a polypropylene centrifuge tube was equilibrated at 37 °C for 3 min in a water bath to achieve optimal assay temperature. Subsequently, 140 μ L of pre-warmed HPL test solution at a concentration of 2 mg/mL (or thrombin standard solution as a positive control) was added to the plasma. The mixture was immediately mixed by gentle inversion, while maintaining constant 37 °C conditions. The coagulation endpoint was visually determined by the appearance of fibrin strands, with the elapsed time recorded as the PT. To ensure reliability, each test condition was performed in triplicate and the mean PT value was calculated for analysis.

Morphological Characterization

To evaluate the antimicrobial effects of HPL, mid-logarithmic phase *C. albicans* (about 10^6 CFU/mL in PBS) and bacterial suspensions (*E. coli* and *S. aureus*, about 10^7 CFU/mL in PBS) were treated with HPL at $4 \times$ MIC (or 1500 μ g/mL for HPL1) in 1.5 mL centrifuge tubes at 35 °C, with PBS-treated samples serving as negative controls. The cells were harvested by centrifugation at $3000 \times g$ for 5 min, washed three times with sterile PBS to remove residual HPL, and then followed by fixation in 4% glutaraldehyde at 4 °C overnight. The fixed cells were pelleted ($3000 \times g$, 5 min) and washed three times with PBS to eliminate excess glutaraldehyde. Three independent replicates were performed for the SEM analysis, and ten images were obtained from each independent experiment. For the TEM analysis, the experiment was conducted once with three resin sections, and ten images were gathered from each resin section. All SEM and TEM samples were prepared with the pathogen cells treated with HPLs or PBS for 4 h.

For SEM observation, a graded ethanol series (10%, 30%, 50%, 70%, 90%, and 100%, with 15-min incubations at each concentration) was employed for progressive dehydration. Fully dehydrated samples were resuspended in anhydrous ethanol, deposited onto silicon wafers, air-dried, and sputter-coated with gold for surface morphology observation using a field-emission scanning electron microscope (Merlin, Zeiss).

For TEM observation, the fixed samples were embedded in 1% agarose solution and post-fixed with 1% osmium tetrox-

ide in PBS at room temperature for 2 h, followed by three PBS washes. Dehydration was achieved through a graded ethanol series and two 15-min acetone treatments. Samples were then infiltrated with 812 embedding resins and heated at 60 °C for 48 h. Ultrathin sections (60–80 nm) were stained sequentially with 2% uranyl acetate in ethanol and 2.6% lead citrate solution for contrast enhancement before TEM observation.

Exudation of Contents

To quantitatively assess the membrane-disrupting activity of HPL against *C. albicans*, a UV absorption-based nucleic acid release assay was performed. Mid-logarithmic phase fungal cells were adjusted to about 10^6 CFU/mL in PBS, and 10 mL of *C. albicans* suspensions were treated in 15 mL conical tubes with either HPL at 4× MIC (HPL1 at 1500 µg/mL) or PBS as a control. Cultures were maintained at 35 °C. At predetermined intervals (2, 4, 6, 8, 10, 12, 24, and 48 h), 1 mL of the suspension was collected and centrifuged (3000× g, 5 min) to pellet the intact cells. Then, 200 µL of each supernatant was carefully aspirated and transferred to a UV-transparent 96-well plate. The absorbance at 260 nm (OD_{260}) was measured using a microplate reader. Three technical replicates were performed at each time point with background subtraction using the corresponding sterile solutions.

Isothermal Titration Calorimetry (ITC)

The interaction between pathogen cells and HPLs was measured using Nano ITC (TA INSTRUMENTS). Mid-logarithmic phase *E. coli*, *S. aureus*, *C. albicans* cells were suspended in PBS at a concentration of about 10^7 CFU/mL. For each titration, 300 µL of cell suspension was added to the sample cell at 25 °C. A solution of HPL was injected in 2 µL increments (20 injections total) with a 120 s interval between injections. The thermodynamic parameters were derived by fitting the data to an independent model.

Cell Wall Permeability Assay

Mid-logarithmic phase *C. albicans* cells were harvested and adjusted to approximately 10^6 CFU/mL in PBS. For the cell wall permeability assay, 1 mL of the cell suspension was mixed with 1-naphthylphenylamine (NPN) at a final concentration of 10 µmol/L and incubated at 35 °C in the dark for 30 min. Subsequently, 175 µL of cell suspension was transferred to a black 96-well microplate. After signal stabilization, the following test compounds were added: 25 µL of hyperbranched polylysine (HPL) solution (final concentration of 4× MIC) for the experimental group, 25 µL of 0.5% Triton X-100 aqueous solution as the positive control, and 25 µL of PBS as the negative control. Real-time fluorescence intensity data ($\lambda(E_x) = 388$ nm, $\lambda(E_m) = 425$ nm) were collected immediately after the compound addition at 35 °C for 2 h using a microplate reader.

Alkaline phosphatase (ALP) activity was quantified using the Beyotime ALP Activity Assay Kit (S0131S) according to the manufacturer's instructions.

Cytoplasmic Membrane Depolarization Assay

Mid-logarithmic phase *C. albicans* cells were suspended in PBS at a concentration of approximately 10^6 CFU/mL. One milliliter of suspension was added to 10 µL DiSC₃(5) solution (final concentration of 5 µmol/L) and incubated at 35 °C in the dark for 30 min. The stained cell suspension was centrifuged (3000×

g, 5 min) and washed three times with PBS to remove the unbound dye. The final cell pellet was resuspended in PBS and 175 µL of the suspension was transferred to a black-bottom 96-well plate. After establishing a stable baseline fluorescence ($\lambda(E_x) = 620$ nm, $\lambda(E_m) = 670$ nm) using a microplate reader maintained at 35 °C, 25 µL of HPL solution was added to each well (final concentration of 4× MIC). Changes in fluorescence intensity were monitored immediately and continuously for 40 min at 1 min intervals to assess membrane depolarization kinetics.

Real-time Live/Dead Fluorescence Monitoring Assay

For real-time imaging, mid-logarithmic phase *C. albicans* cells were adjusted to about 10^4 CFU/mL in saline and allowed to adhere to glass slides for 5 min. After establishing a baseline bright-field image, a time-lapse program at 1 min intervals with a total length of 2 h was initiated. Calcofluor white CFW (final concentration of 1 mmol/L), Cy3-labeled HPL (final concentration of 4× MIC), and SYTOX Deep Red (final concentration of 5 µmol/L) were added, and continuous capture began immediately to monitor HPL localization (orange fluorescence) and loss of membrane integrity (red fluorescence).

To verify the selective fungicidal activity of HPL, real-time PI uptake was monitored using a multifunctional microplate detector. Mid-logarithmic phase cultures of *C. albicans* (about 10^6 CFU/mL) and other bacterial strains (*E. coli* and *S. aureus*, about 10^7 CFU/mL) were suspended in saline containing 10 µmol/L PI. 175 µL of each suspension was distributed into black-bottom 96-well plates equilibrated at 35 °C in a multifunctional microplate reader. After baseline stabilization ($\lambda(E_x) = 535$ nm, $\lambda(E_m) = 617$ nm), 25 µL of the HPL solution (final concentration of 4× MIC) was added to each well. Fluorescence was monitored continuously for 2 h at 1 min intervals, capturing species-specific membrane damage kinetics, with PI signal increases directly correlating with the loss of membrane integrity in non-viable cells.

Energy Consumption Assay

The antifungal efficacy of HPL under energy-depleting conditions with sodium azide (NaN₃) treatment or under low-temperature (4 °C) incubation was tested to investigate whether HPL intake of HPL is an energy-dependent process. *C. albicans* cells in mid-log phase (about 10^6 CFU/mL) were suspended in R/MINI 1640-MOPS medium and divided into three groups: (1) NaN₃-treated (1%, 35 °C, incubated for 1 h, NaN₃ group), (2) PBS-treated (4 °C, 1 h, 4 °C group), and (3) PBS-treated (35 °C, 1 h, blank group). After the treatments, each group was divided into two groups: one added with 25 µL of HPL (final concentration of 2× MIC) and the other added with 25 µL PBS to distinguish between the effects of HPL and energy-depleting conditions on cell viability. Then the NaN₃ and blank groups were incubated at 35 °C for 24 h, whereas the 4 °C group was incubated at 4 °C for 24 h. Viability was quantified by plating serial dilutions on SDA plates at 35 °C for 24 h and counting the colonies. The assay was performed in triplicate.

ROS Accumulation Monitoring

Mid-logarithmic phase *C. albicans* cells were adjusted to approximately 10^6 CFU/mL in sterile PBS. For reactive oxygen species (ROS) detection, 1 mL of cell suspension was loaded with DCFH-DA at a final concentration of 25 µmol/L and incubated at 35 °C in the dark for 30 min. Cells were harvested by

centrifugation at 3000× g for 5 min and washed three times with PBS to remove excess probe. The washed cells were resuspended in PBS and 175 μ L of the cell suspension was transferred to a black-bottom 96-well plate. After baseline stabilization, 25 μ L of the HPL solution was added to achieve a final fixed concentration of 375 μ g/mL. fluorescence intensity ($\lambda(E_x) = 488$ nm, $\lambda(E_m) = 530$ nm) was monitored continuously for 30 min at 35 °C using a microplate reader, with readings taken every 30 s.

Mitochondrial Membrane Potential Assay

Mid-logarithmic phase *C. albicans* cells were adjusted to approximately 10^6 CFU/mL in sterile PBS. The cell suspension was treated with HPL at 4× MIC final concentration and incubated at 35 °C for 4 h. Cells were then harvested by centrifugation at 3000× g for 5 min and washed three times with PBS to remove residual HPL. For the mitochondrial membrane potential assessment, the washed cells were resuspended in PBS and stained with 10 μ mol/L JC-1 at 35 °C for 30 min in the dark. After removing the unincorporated dye by centrifugation (3000× g, 5 min) and washing with PBS, 100 μ L of the suspension was transferred to a glass-bottomed confocal dish and allowed to settle for 5 min. Fluorescence imaging was performed using a laser scanning confocal microscope (Zeiss LSM 700) with the following settings: $\lambda(E_x) = 488$ nm and $\lambda(E_m) = 530$ nm for JC-1 monomers (green fluorescence), and $\lambda(E_x) = 585$ nm and $\lambda(E_m) = 590$ nm for JC-1 aggregates (red fluorescence).

Comet Assay

Mid-logarithmic phase *C. albicans* cells were adjusted to about 10^7 CFU/mL in PBS and cultured with HPLs at a final concentration of 4× MIC at 35 °C for 4 h. Cells were then washed three times with PBS to remove the remaining HPLs. After the last centrifugation, the cells were lysed, and single-stranded DNA was extracted for the next step of gel electrophoresis and staining. DNA damage in *C. albicans* was assessed using a commercial Comet Assay Kit (G010-1-1; Nanjing Jiancheng, China) according to the manufacturer's instructions. The quantity of tail DNA was analyzed using the Comet Assay Software Project. Three biological replicates were used for each experiment.

Effect of ROS Scavengers on the Antimicrobial Properties of HPL

To investigate the role of reactive oxygen species (ROS) in the antifungal activity of HPL, ROS scavengers were added to the culture medium, following a previously reported method with a few modifications. Mid-logarithmic phase *C. albicans* cells were adjusted to 1×10^3 CFU/mL in R/MINI 1640 medium. ROS scavengers, *N*-acetylcysteine (NAC, 25 mmol/L), glutathione (GSH, 25 mmol/L), and ascorbic acid (AA, 25 mmol/L), were added to the suspensions. The scavenger-free R/MINI 1640 group was used as a control. Each treated suspension (175 μ L) was added to 96-well plates, followed by 25 μ L of serially diluted HPL. Plates were sealed with breathable membranes and incubated at 35 °C for 48 h. The OD₆₀₀ value was recorded to determine the MIC using a microplate reader.

For the minimum fungicidal concentration (MFC) assessment, 100 μ L of suspensions from wells corresponding to the MIC and the two preceding dilutions were plated onto SDA in triplicate. After incubation at 35 °C for an additional 48 h, the MFC was defined as the lowest concentration yielding ≤ 3 CFU per plate. Three biological replicates were used for each ex-

periment.

In vivo Biosafety Testing

Female KM mice (6–8 weeks old, weighing 22–25 g) were randomly assigned to four groups ($n=6$ per group), following a modified protocol based on the *Hygienic Standard for Disinfection* (2002 version, Ministry of Health of the PRC). After an overnight fasting period with free access to water, mice in the treatment groups received HPL solutions via a single oral gavage at a dose of 5000 mg/kg body weight (200 μ L suspension in sterile saline), while the control group received 200 μ L sterile saline. Body weights were recorded daily at 9:00 AM (± 1 h).

On day 14, the mice per group were anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneally) for terminal blood collection via cardiac puncture. After euthanasia, the liver, spleen, kidneys, ileum, and colon were harvested, fixed in 4% paraformaldehyde at 4 °C for 24 h, and processed for paraffin embedding. Tissue sections were stained with hematoxylin and eosin (H&E) and examined histopathologically under a microscope.

For serum cytokine analysis, whole blood samples were allowed to clot at room temperature for 2 h, followed by centrifugation at 1000× g for 10 min at 4 °C. The supernatant was transferred to fresh tubes and centrifuged again under the same conditions to obtain particle-free serum, which was stored at –80 °C until analysis. Serum levels of interleukin-6 (IL-6) and tumor necrosis factor- α (TNF- α) were quantified using commercial ELISA kits, according to the manufacturer's protocol. Absorbance was measured at 450 nm, with a reference wavelength of 570 nm, using a microplate reader.

In vivo Infection Model

Female KM mice (6–8 weeks old, 22–25 g) were immunocompromised by intraperitoneal injection of cyclophosphamide (100 mg/kg/day) for three consecutive days before infection. The mice were randomly assigned to five groups ($n=8$ per group): (1) HPL1-treated (10 mg/kg), (2) HPL2-treated (10 mg/kg), (3) HPL3-treated (10 mg/kg), (4) fluconazole-treated (10 mg/kg, positive control), and (5) saline-treated (infected, untreated control). After fasting overnight with free access to water, the mice were infected via oral gavage with 200 μ L of mid-logarithmic phase *C. albicans* suspension (1×10^8 CFU/mL in sterile saline). After 24 h of infection, the treatment groups received daily oral administrations of the respective compounds (200 μ L per dose) for seven consecutive days, while the control groups received an equivalent volume of sterile saline. Body weights were recorded daily at 9:00 AM (± 1 h).

On day 7 post-treatment, the mice were euthanized, and the liver, spleen, kidneys, colon, and ileum were harvested. For six mice per group, organs were homogenized in 10 mL of sterile PBS and serially diluted for quantitative culture on SDA plates at 35 °C for 24 h. The fungal burden was expressed as colony-forming units (CFU) per organ. For the remaining two mice per group, organs were fixed in 4% paraformaldehyde at 4 °C for 24 h and processed for paraffin embedding. Tissue sections were stained with hematoxylin and eosin (H&E) and examined histologically using a microscope. All animal experiments were conducted in accordance with relevant ethical regulations. The procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the Changchun Institute of Applied Chemistry, Chinese Academy

of Sciences (No. 20230040).

Microbial Diversity Testing

Fecal samples were collected aseptically from mice into sterile 2 mL cryotubes and immediately flash-frozen in liquid nitrogen before being stored at -80°C . Microbial genomic DNA was extracted using the E.Z.N.A.[®] Soil DNA Kit (Omega Bio-tek, D5625-01) following the manufacturer's protocol, with the addition of a bead-beating step (0.1 mm zirconia/silica beads, 6.0 m/s for 60 s) for enhanced cell lysis. DNA quality was verified by 1% agarose gel electrophoresis and quantified using a Qubit 4.0 Fluorometer (Thermo Fisher Scientific).

The hypervariable V3-V4 region of bacterial 16S rRNA genes was amplified using barcoded primers 338F (5'-ACTCC-TACGGGAGGCAGCAG-3') and 806R (5'-GGAC-TACHVGGGTWCTAAT-3') with the Phusion High-Fidelity PCR Master Mix (Thermo Scientific, F531L). Thermal cycling conditions consisted of initial denaturation at 98°C for 30 s, followed by 25 cycles of 98°C for 15 s, 55°C for 30 s, and 72°C for 30 s, with a final extension at 72°C for 5 min. Amplicons were purified using AMPure XP beads (Beckman Coulter) and quantified using the PicoGreen dsDNA assay.

Sequencing was performed on the Illumina NovaSeq 6000 platform (2×250 bp paired-end reads) at Shanghai Majorbio Bio-Pharm Technology Co., Ltd. Raw sequencing data were processed on the Majorbio Cloud Platform using QIIME2 (v2022.2). Sequences were quality filtered (quality score ≥ 20), denoised (DADA2), and clustered into amplicon sequence variants (ASVs) at 100% similarity. Taxonomic assignment was performed against the SILVA 138.1 database with a 70% confidence threshold.

Statistical Analysis

All experiments were performed with at least three replicates. Data are presented as mean $\pm$ standard deviation (SD). Statistical significance was determined using one-way analysis of variance (ANOVA) with multiple comparisons using Tukey's post-hoc test. Statistical significance was defined as a p -value of less than 0.05, denoted as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

RESULTS AND DISCUSSION

HPL Synthesis and Characterization

A series of hyperbranched polylysine samples (HPL1-HPL3) with progressively increasing molecular weights was synthesized by varying the polycondensation reaction time on a kilogram scale (Fig. 1a). The H-NMR spectrum confirmed the hyperbranched structure of the HPL (Fig. S1 in the electronic supplementary information, ESI). SEC-MALS-VIS-RI analysis confirmed a stepwise increase in the weight-average absolute molecular weight (M_w) from 1380 g/mol (HPL1) to 3130 g/mol (HPL3), with corresponding number-average molecular weights (M_n) ranged from 940 g/mol to 2420 g/mol and polydispersity indexes ($\mathcal{D}$) between 1.30 and 1.67 (Fig. 1b), indicating moderate control over molecular weight distribution. The hydrodynamic diameter (D_h) increased proportionally with the molecular weight, ranging of 1.68 nm for HPL1, 2.88 nm for HPL2 and 4.36 nm for HPL3 (Fig. 1b). Zeta potential measurements showed a corresponding rise in surface charge, with $+14.6 \pm 0.6$ mV, $+21.6 \pm 1.2$ mV, and $+32.2 \pm 0.7$ mV for HPL1-HPL3, respectively (Fig. 1c), which can

be attributed to the higher density of terminal amine groups in large polymers.

The gradual increase in D_h reflects larger spatial requirements for incorporating additional lysine units while maintaining the hyperbranched structure. The relatively consistent increase in zeta potential values suggests that the polymers retain a compact, surface-exposed amine-rich surface conformation even as the molecular weight increases.

In vitro Antimicrobial Activities

The antimicrobial activity of the HPLs was evaluated using the microbroth dilution method following the CLSI guidelines (M07-A11 for bacteria, M27-A4 for fungi). Representative Gram-negative (*E. coli* ATCC 8739 and *P. aeruginosa* ATCC 9027) and gram-positive strains (*S. aureus* ATCC 25923 and *MRSA* ATCC 43300) were tested. HPL1 and HPL2 displayed no detectable antibacterial activity (MIC >1500 $\mu\text{g/mL}$), whereas HPL3 exhibited MIC values of 188 and 48 $\mu\text{g/mL}$ against *E. coli* and *MRSA*, indicating an enhanced antibacterial potential at higher molecular weights. Antifungal activity was evaluated against five major *Candida* species: *C. albicans* (four strains along with multiple drug-resistant clinical isolates FCA001), *C. glabrata*, *C. tropicalis*, *C. parapsilosis*, and *C. krusei*. Different strains of *C. albicans* were selected to evaluate the robustness of HPLs. ATCC 10231 and ATCC 90028 are standard reference strains recommended by the US Pharmacopoeia, and CLSI M27-A, CMCC 98001 is the standard strain recommended by the Chinese Pharmacopoeia. ATCC 14053 was included because of its filamentation and biofilm-forming capabilities^[21]. HPL1 showed limited activity, inhibiting only *C. krusei* and *C. tropicalis* at 188 $\mu\text{g/mL}$, with MICs of >1500 $\mu\text{g/mL}$ for other species. In contrast, HPL3 showed broad-spectrum antimicrobial activity against both bacteria and fungi. Notably, HPL2 exhibited interesting selective fungicidal efficacy, with antifungal activities against all tested *Candida* species, including a multiple drug-resistant clinical isolate (Fig. 1d, Fig. S2 and Table S2, S3 in ESI). The MIC values were 48–96 $\mu\text{g/mL}$ for HPL2 and 24–48 $\mu\text{g/mL}$ for HPL3, respectively, against the four strains of *C. albicans*. Liu *et al.* reported broad-spectrum antimicrobial activity of HPLs.^[20] Briefly, HPLs maintain broad-spectrum antimicrobial activity with M_n of up to 4600 g/mol. However, when M_n reached 6600 g/mol, the antifungal efficiency against *Candida spp.* began to decline, and the MIC value against *C. albicans* increased from 96 $\mu\text{g/mL}$ to 188 $\mu\text{g/mL}$. Further increasing the M_n of HPL to 12000 g/mol led to the loss of activity against *C. albicans*, with an MIC value of 1500 $\mu\text{g/mL}$. In contrast, the antibacterial activity was slightly enhanced as the molecular weight increased. Therefore, this study extends the structure-activity relationship of HPL across a broader molecular weight spectrum in the lower M_n range.

Based on these differential activity profiles, HPL1 (inactive), HPL2 (selective), and HPL3 (broad-spectrum) were selected for further evaluation, *E. coli* ATCC 8739, *S. aureus* ATCC 25923, and *C. albicans* ATCC 10231 were chosen as the test strains.

The time-kill kinetic assay of HPL2, HPL3, FLC, and AmB was carried out with *C. albicans*. HPL3 induced >1 order of reduction at 8 h, the killing kinetics of HPL2 was lower than that of

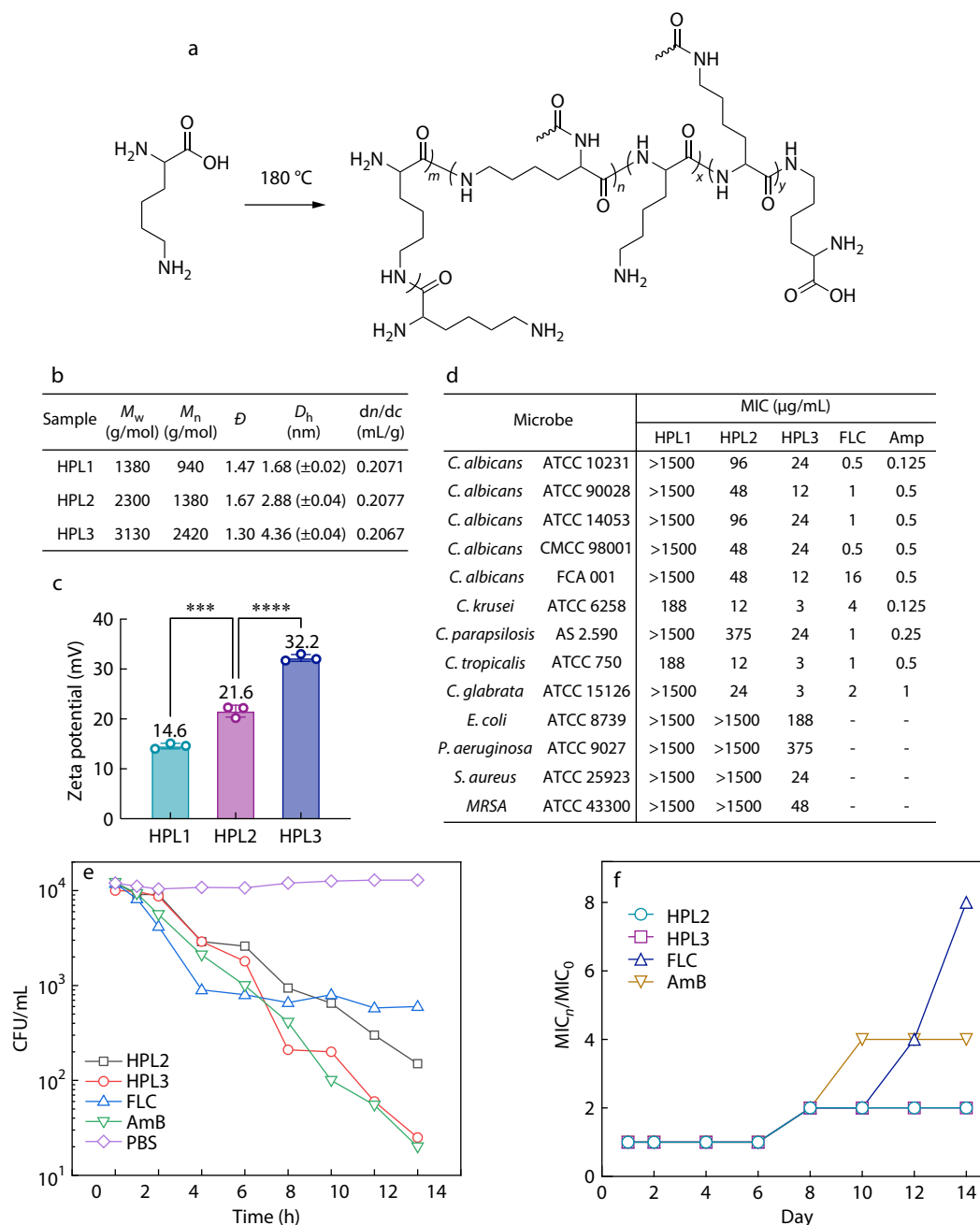


Fig. 1 Synthesis, characterization and antimicrobial activities of HPLs. (a) Synthesis scheme of HPL; (b) Summary of SEC-MALS-VIS-RI characterization. M_n , number average molecular weight; M_w , weight average molecular weight; D_h , hydrodynamic radius; $\bar{D}$, polydispersity index. (c) Zeta potential of HPLs; (d) The antimicrobial activities of HPLs or antifungal drugs. FLC, fluconazole. AmB, Amphotericin B. (e) The time-kill kinetic study of *C. albicans* treated with HPL2, HPL3, FLC, AmB and PBS. (f) Resistance development study of HPL2, HPL3, FLC and AmB.

HPL3, and >1 order of reduction was observed at 10 h. Although FLC can reduce the CFU by >1 order at 4 h, the CFU of *C. albicans* remained stable in the later test hours, indicating a fungistatic effect. AmB exhibited fungicidal activity (Fig. 1e). Resistance development assays revealed that FLC exposure led to an 8-fold increase in MIC by the 8th passage, and AmB led to a 4-fold increase in MIC at the 10th passage. In contrast, the HPL2- and HPL3-treated strains exhibited only a 2-fold increase (Fig. 1f). This slight shift may be attributed to the phenotypic adaptation of *C. albicans*, such as a transition

from yeast to hyphal forms over generations, rather than stable genetic resistance, given that hyphal cell walls contain approximately three times more chitin than yeast forms.^[22]

In vitro Biocompatibility

Cytotoxicity and hemocompatibility assays were conducted to evaluate the *in vitro* biocompatibility of the HPLs. Cytotoxicity of HPL1-HPL3 was assessed using MTT assays in L929 and NIH/3T3 murine fibroblast cell lines. For L929 cells, HPL1 and HPL2 maintained >90% cell viability at concentrations up to 1500 $\mu\text{g/mL}$, indicating minimal toxicity, whereas HPL3 had an IC_{50} value of

about 1388 $\mu\text{g/mL}$. In NIH/3T3 cells, all HPLs exhibited low toxicity, with an IC_{50} value $>1500 \mu\text{g/mL}$ (Table S1 in ESI). These results indicate that lower molecular weight HPLs possess superior biocompatibility across multiple mammalian cell types. Hemocompatibility assays further demonstrated that none of the HPL variants induced detectable hemolysis at concentrations up to 1500 $\mu\text{g/mL}$ (Table S1 in ESI), as evidenced by the negligible hemoglobin release from erythrocytes. In prothrombin time (PT) assays, HPLs showed no significant impact on coagulation kinetics ($\text{PT}>60 \text{ s}$), even at concentrations up to 2 mg/mL , in contrast to the thromboplastin-positive control ($\text{PT}=15 \text{ s}$). These results demonstrate the excellent *in vitro* biosafety of HPLs.

Antimicrobial Mechanisms of HPLs

Morphology Characterization

To investigate the antimicrobial mechanisms of HPLs at the cellular level, SEM and TEM were performed on representative bacterial (*E. coli* and *S. aureus*) and fungal (*C. albicans*) strains treated with HPL1-HPL3 (Fig. 2, Fig. S3 in ESI). The first row shows a large view of the cell situation, while the second row shows the representative zoom-in images of single cells with different treatments. Untreated and HPL1-treated microbial cells retained intact morphology, including smooth surfaces and well-preserved internal structures, which is consistent with the negligible antimicrobial activity of HPL1 (Fig. 2a, Fig. S3 in ESI). Exposure to HPL2 induces distinct species-dependent ultrastructural changes. Bacterial cells maintained smooth membranes and discernible cytoplasmic contents, consistent with the non-observable antibacterial activities of HPL2 (Fig. 2a, Fig. S3 in ESI). In contrast, significant structural damage has been observed in *C. albicans*. SEM analysis revealed localized surface depressions and TEM analysis showed marked intracellular disorganization, including membrane invaginations (yellow arrows), membrane ruptures (red arrows), and organelle degradation (Fig. 2). HPL3 caused severe damage to all the tested microorganisms. SEM images showed shallow depressions on the cell surface (Fig. 2a), and TEM revealed membrane rupture and profound loss of organelle integrity. Notably, the cell wall remained structurally intact in all HPL-treated *C. albicans* cells, suggesting that HPL may penetrate, while not destroying, the cell wall, exerting killing effects through membrane disruption (Fig. 2b, Fig. S3 in ESI). These morphological disruptions indicated a membrane disruption mechanism, consistent with the potent and broad-spectrum antimicrobial activity of HPL3 observed in susceptibility assays.

Together, these ultrastructural observations revealed a molecular weight-dependent mode of action for the HPLs. Lower molecular weight polymers (HPL1) exhibit non-observable membrane damage, mid-range polymers (HPL2) selectively penetrate fungal cells and disrupt internal organization, and higher molecular weight polymers (HPL3) cause severe membrane damage and cellular disruption in both bacterial and fungal cells.

Cell wall penetrating action

Isothermal titration calorimetry (ITC) was used to evaluate the electrostatic interactions between the HPL molecules and pathogen cells. In all groups, the enthalpy change (ΔH) increased (Fig. S4 in ESI), indicating that a primary binding inter-

action occurred despite the antimicrobial activity.^[23,24] These results indicate that electrostatic association is the initial step in HPL-cell surface interactions.

To evaluate whether HPLs disrupt the cellular barrier of *C. albicans*, the leakage of intracellular contents was assessed by monitoring the absorbance at 260 nm (OD_{260}), which reflects the release of nucleic acids and other UV-absorbing macromolecules.^[25] Since HPLs exhibited baseline absorbance at this wavelength in a concentration-dependent manner, appropriate blank controls (HPLs in PBS without cells) were included to subtract the background signals. As shown in Fig. 3(a), compared to the blank control, OD_{260} values of the *C. albicans* suspensions increased gradually over 48 h upon exposure to HPL2 or HPL3, indicating the exudation of intracellular contents and disruption of the cell envelope integrity.

The fungal cell wall integrity was further evaluated by measuring the extracellular activity of alkaline phosphatase (ALP), which is a periplasmic enzyme localized between the cell wall and plasma membrane that cannot be detected extracellularly unless the cell wall is disrupted.^[26] ALP activity in the HPL-treated group was not different from that in the PBS control group, with no detectable enzymatic release. This suggests that the structural integrity of the cell wall was preserved, and that HPLs may selectively permeabilize the plasma membrane without lysing the outer wall.

Since the results from TEM analysis revealed the cell wall integrity of all HPL-treated *C. albicans* (Fig. 2b), which was in contradiction with the increase in OD_{260} in the HPL2- and HPL3- groups, fungal cell wall permeability was further evaluated using *N*-phenyl-1-naphthylamine (NPN) uptake assays. NPN is a hydrophobic fluorescent probe that fluoresces only when it interacts with the exposed phospholipid bilayers in compromised cell walls.^[27,28] No significant change in fluorescence was detected over 2 h after the addition of HPLs to *C. albicans* suspensions preloaded with NPN, indicating that the cell wall remained impermeable to NPN. In contrast, the positive control (0.1% Triton X-100) induced a rapid and stable increase in fluorescence intensity (Fig. 3b). Collectively, these findings demonstrate that HPL2 exerts antifungal effects by destroying the cell envelope while preserving cell wall integrity, which is consistent with the intact cell wall structure revealed by TEM analysis (Fig. 2b).

Membrane-disrupting action

Cationic antimicrobial polymers typically act *via* electrostatic interactions with negatively charged microbial surfaces, leading to membrane destabilization and permeabilization.^[3,4] To assess whether HPLs follow this mechanism, effects on fungal and bacterial cell membranes were evaluated using a series of approaches.

The potentiometric dye, DiSC₃(5), was used to monitor membrane depolarization. Depolarization of the membrane causes dye release and fluorescence recovery, providing a quantitative measure of the membrane perturbation.^[29] As shown in Fig. 3(c), rapid and pronounced fluorescence increases, indicative of membrane depolarization, were observed in DiSC₃(5)-pretreated *C. albicans* upon treatment with HPL2 or HPL3. In contrast, the HPL1 group remained unchanged over the tested period, consistent with its lack of membrane activity and antifungal efficacy. A similar trend

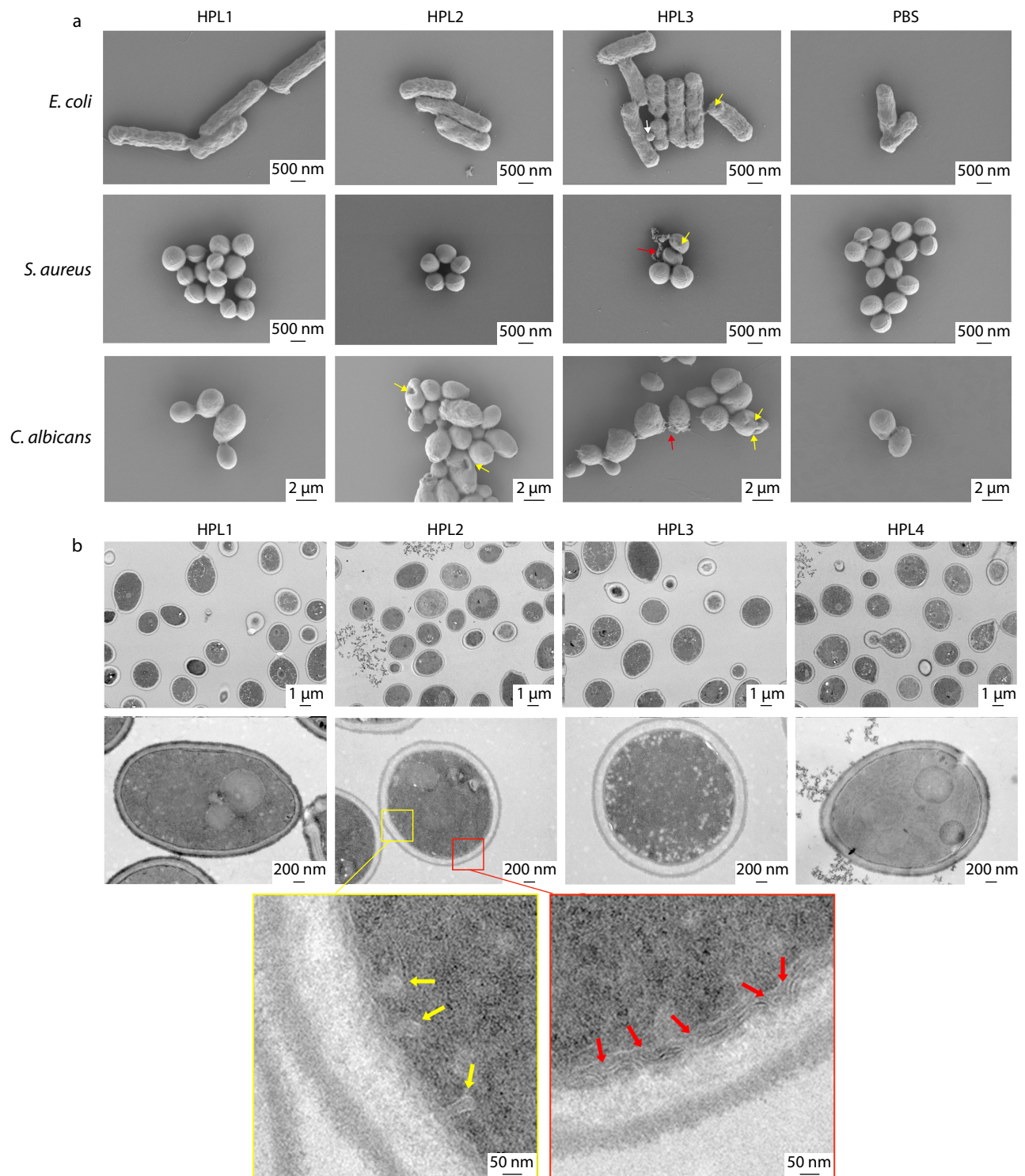


Fig. 2 Morphology studies of HPLs against bacteria and fungi. (a) Representative SEM images of *E. coli*, *S. aureus* and *C. albicans* treated with HPL1, HPL2, HPL3 and PBS control. The white arrow indicates a surface vesicle. The yellow arrows indicate surface collapse, the red arrows indicate content exudation. (b) Representative TEM images of *C. albicans* treated with HPL2 and PBS. Yellow arrows indicate membrane invaginations, red arrows show membrane rupture. Three independent repeats were done for SEM analysis, and ten images were gathered from each independent experiment. For TEM analysis, the experiment was conducted once with three resin sections, and over ten images were gathered from each resin section.

was observed for *S. aureus*; significant fluorescence increase was observed in the HPL3 groups, while HPL1 and HPL2 in-

duced unchanged trends comparable to the PBS group (Fig. S5 in ESI), which is consistent with their antibacterial proper-

ties.

To confirm the membrane compromise and fungicidal activity of HPLs, propidium iodide (PI), a membrane-impermeable DNA-binding dye, was used as a marker for cell mem-

brane damage.^[30] Exposure to HPL3 caused a rapid and significant increase in red fluorescence in bacterial suspensions, which plateaued after about 30 min, indicating widespread bacterial death. In contrast, HPL1 and HPL2 caused negligible

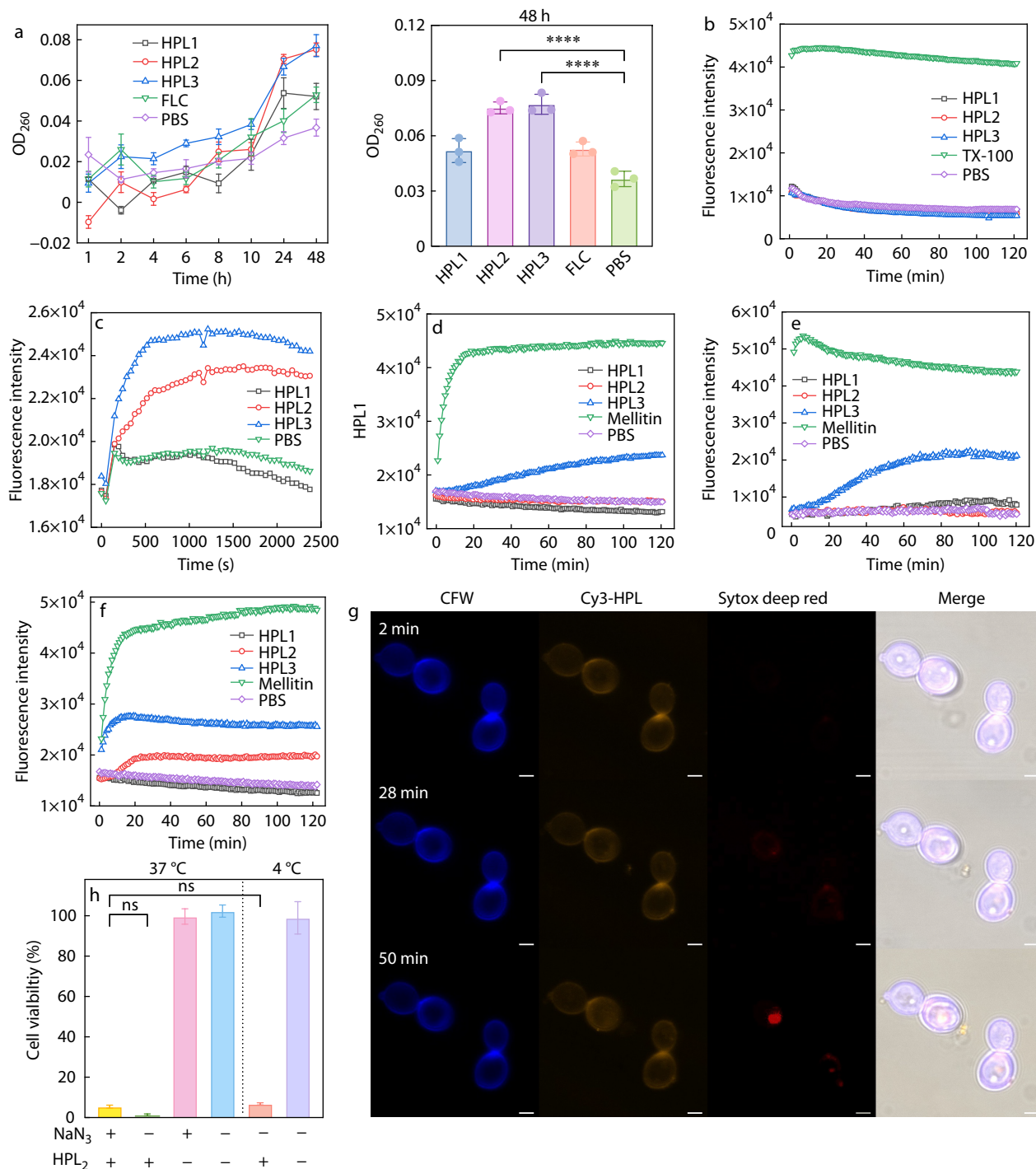


Fig. 3 Membrane-disrupting mechanisms study. (a) Time-lapse leakage of cytoplasmic contents from *C. albicans* treated with HPLs, FLC and PBS negative control; (b) Cell wall integrity study of *C. albicans* cells treated with HPLs and Triton X-100 positive control; (c) Cytoplasmic membrane depolarization of HPL-treated *C. albicans*; (d-f) Live/Dead staining of pathogen cells over time measured by PI dye, treated with HPLs, melittin positive control and PBS negative control; (g) Time-lapse fluorescence confocal images of the *C. albicans* cells treated with CFW (blue fluorescence), Cy3-HPL2 (orange fluorescence) and PI (red fluorescence). Scale bars: 2 μ m. (h) Killing efficiency assay of HPL2 against *C. albicans* under the energy depletion state of culture with 1% sodium azide or at 4 °C. Data are presented as mean $\pm$ SD.

fluorescence changes, consistent with their non-observable antibacterial activity. For *C. albicans*, both HPL2 and HPL3 triggered marked increases in PI fluorescence over time, whereas HPL1 and the PBS control group showed minimal signals, confirming that HPL2 and HPL3 compromised fungal membrane integrity. This time-dependent increase further supports a model of progressive membrane damage rather than instantaneous lysis (Figs. 3d–3f).^[31]

To further visualize the dynamic interaction between HPL2 and *C. albicans* cells in real time, time-lapse confocal laser scanning microscopy (CLSM) was performed using Cy3-labeled HPL2 (orange) co-incubated with *C. albicans*, calcofluor white CFW (blue), and SYTOX Deep Red (red). CFW was used to localize the cell wall of *C. albicans*. Orange fluorescence appeared almost immediately after the addition of Cy3-HPL2 to *C. albicans* cells, indicating rapid binding of HPL2 to the fungal surface. Approximately 30 min later, faint red fluorescence began to emerge, reflecting that HPL2 caused early membrane compromise in a subset of cells. The fluorescence intensity increased with time, and the red fluorescence expanded across other cells at approximately 60 min, reflecting extensive membrane permeabilization and cell death caused by HPL2 (Fig. 3g). These real-time imaging results not only corroborate the time-kill assays and membrane rupture in the TEM observations but also provide mechanistic insight into the action of HPL2. The process begins with surface adsorption, followed by membrane disruption, and ultimately cell death.^[32]

To elucidate the cellular entry mechanism of HPLs, an energy-depletion assay was conducted using sodium azide (NaN_3), an ATP synthesis inhibitor that blocks energy-dependent transport.^[33] *C. albicans* cells were pretreated with NaN_3 to deplete intracellular ATP, thereby blocking its active transport. The cell viability of the HPL2-treated group remained unchanged after NaN_3 treatment or at low temperatures, indicating that HPL uptake was energy-independent (Fig. 3h). These findings suggest that HPL may cross the fungal cell envelope *via* passive transport, potentially bypassing ATP-dependent resistance mechanisms, such as efflux pumps.^[34]

In summary, HPL2 killed *C. albicans* by selectively penetrating the cell wall without damaging the cell wall (Fig. 3b), binding to the plasma membrane, leading to depolarization (Fig. 3c), and damaging membrane integrity (Figs. 3d–3g). Notably, this process was energy-independent (Fig. 3h). HPL is distinguished from conventional antifungal drugs such as azoles or echinocandins, which often face resistance mediated by active efflux or stress response pathways^[35] indicating its potential as a promising antifungal agent with a novel mechanism of action.

ROS accumulation and mitochondrial dysfunction

Succinate dehydrogenase (SDH) is a key mitochondrial enzyme, the activity of which is related to mitochondrial function and metabolic status.^[36] SDH activity was significantly reduced in both HPL2- and HPL3-treated *C. albicans* cells, similar to the cells treated with FLC (Fig. 4a), while SDH activity did not change in HPL1-treated *C. albicans* cells, indicating that HPL2 and HPL3 destroyed the mitochondrial activity of *C. albicans* to a certain extent. (Fig. 4a). Mitochondrial membrane potential was monitored using the JC-1 dye, which shifts from red to green fluorescence upon depolarization.^[37] After 4 h of co-culture with HPL2,

green fluorescence was observed in HPL2-treated *C. albicans*, similar to the positive control CCCP group (Fig. 4b), suggesting that the mitochondrial membrane potential shifted in *C. albicans*, indicating that the mitochondrial activity of *C. albicans* was destroyed to a certain extent.

Since mitochondrial disruption is closely associated with the accumulation of reactive oxygen species (ROS), intracellular ROS levels were monitored with DCFH-DA, which emits fluorescence upon ROS release in the cell.^[38] The green fluorescence intensity increased immediately in all HPL-treated groups and continued to increase in the test time range of 1800s, with the rate positively correlated with molecular weight (Fig. 4c). This result indicates that HPL induces the production of ROS in *C. albicans* cells.^[39] Since HPL1 slightly increased ROS despite its limited antifungal activity, it is necessary to further investigate the causal relationship between ROS production and cellular death.

A comet assay was performed to verify the relationship between ROS production and DNA damage in *C. albicans*.^[40] The DNA leakage of both HPL2- and HPL3-treated cells increased significantly, with obvious trailing visible in the fluorescence images, indicating that the DNA had been damaged to a certain degree. In contrast, cellular DNA leakage in the HPL1 group was similar to that in the control group (Fig. 4d). These results suggested that although HPL1 could induce ROS production in *C. albicans* cells, it was insufficient to cause cell death.

Mitochondrial dysfunction can lead to ROS production, and the excessive accumulation of ROS can induce mitochondrial dysfunction and apoptosis.^[38] Various ROS scavengers (*N*-acetylcysteine [NAC], glutathione [GSH], and ascorbic acid [AA]) were induced in the culture medium. The addition of scavengers slightly affected antifungal activity, increasing the MIC by one concentration gradient in some cases, whereas the minimum fungicidal concentration (MFC) remained unchanged (Fig. 4e). These results further proved that ROS generation was a natural result of mitochondrial damage rather than a causative factor.

In conclusion, these findings demonstrate that HPL exerts antifungal activity *via* a multifaceted mechanism. Briefly, HPL can selectively penetrate the fungal cell wall, destroy the plasma membrane, and lead to organelle damage including mitochondrial dysfunction. Although ROS accumulation was also observed in fungal cells, it did not serve as the principal cause of cell death. Notably, HPL uptake was shown to be energy-independent, bypassing active transport and thus potentially evading classical resistance pathways such as efflux pumps and stress responses. This comprehensive mechanism highlights HPL as a promising class of antifungal agents with a novel mode of action distinct from traditional antifungal drugs such as azoles or echinocandins.

Selective Antifungal Mechanism

The selective antifungal activity of HPLs can be explained by their cellular entry, which is governed by their physicochemical properties (Scheme 1).

Particle size is critical for the entry of HPLs into fungal cells. A previous study indicated that molecules with a hydrodynamic diameter (D_h) below 6 nm can passively traverse the *Candida* cell wall.^[18] Additionally, our previous work showed

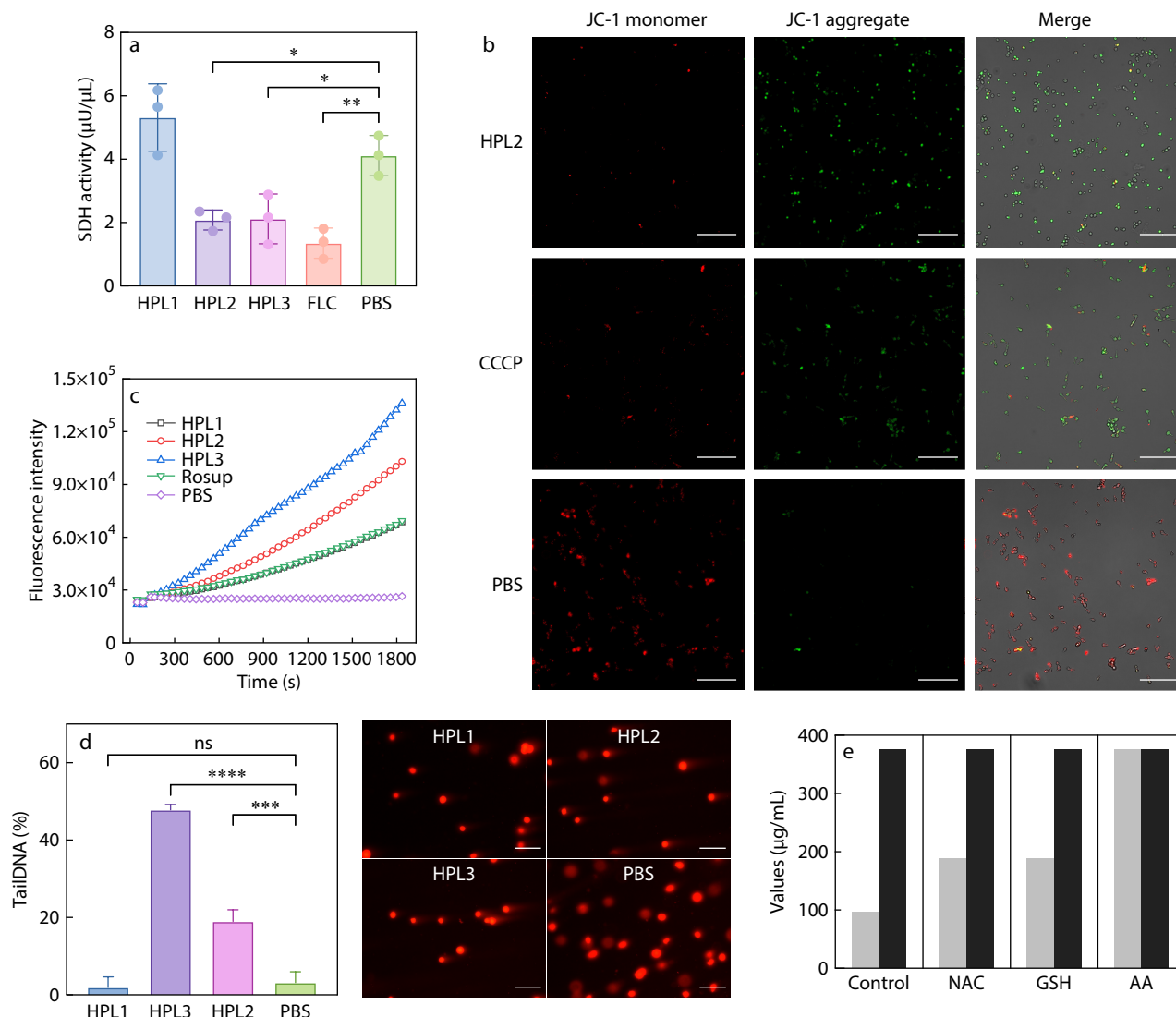
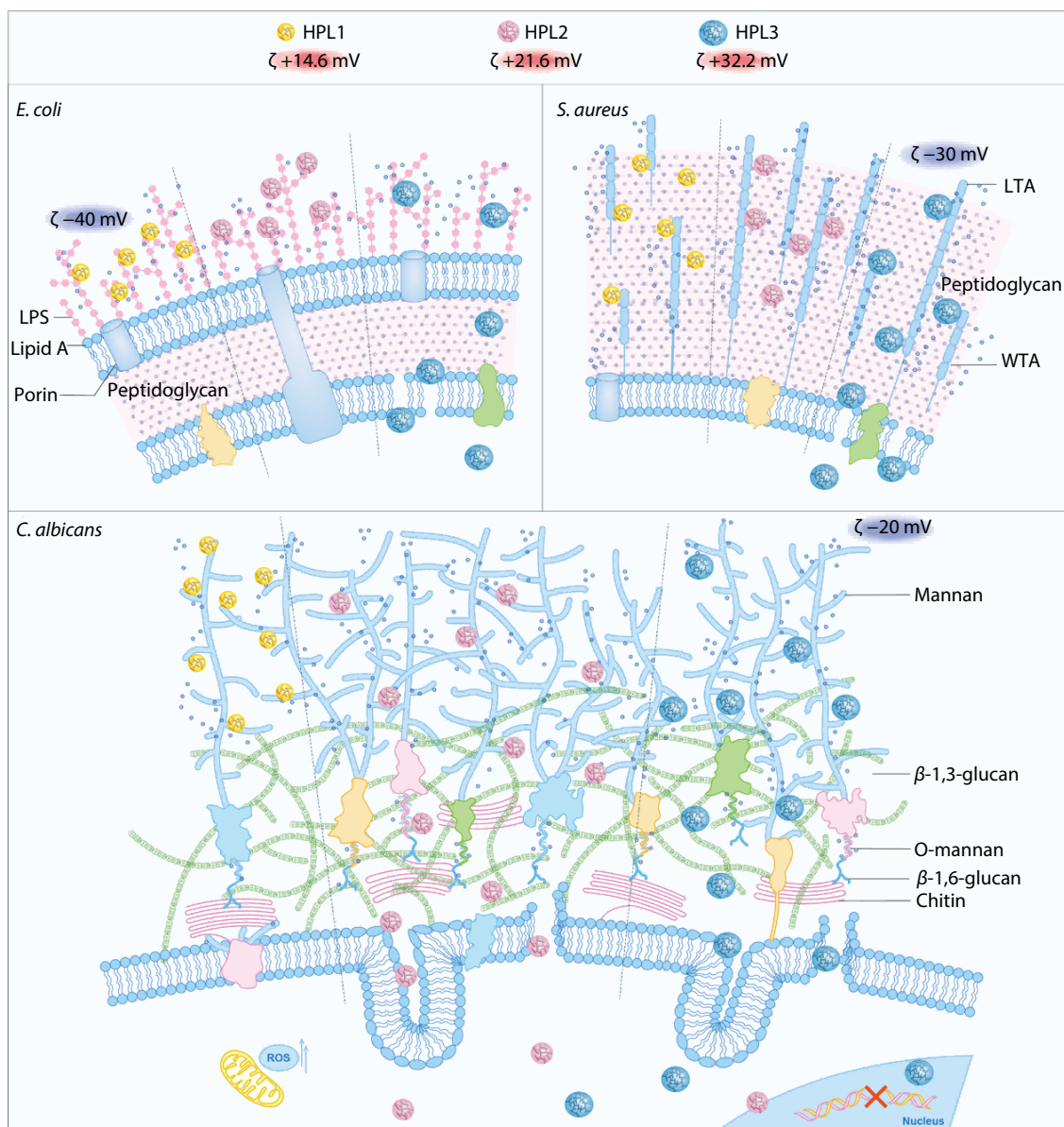


Fig. 4 Mitochondrial-related antifungal mechanisms study. (a) Mitochondrial function (SDH activity) of *C. albicans* treated with HPLs and FLC; (b) Confocal images of *C. albicans* treated with HPL2 and CCCP, using JC-1 as an indicator of the mitochondrial membrane potential. Scales bars represent 30 μm . (c) ROS accumulation monitoring in *C. albicans* treated with HPLs or Rosup (positive control), measured with DCFH-DA; (d) The DNA damage in *C. albicans* treated with HPLs, assessed by the comet assay. Data are presented as mean $\pm$ SD. Scale bars: 100 μm . (e) Effects of ROS scavengers on MIC and MFC of HPL2 against *C. albicans*. The gray columns represent the MIC values and the dark columns represent the MFC values. The test was performed with three repeats. Since the MIC or MFC values in each three repeats were same, there were no error bars provided.

that HPLs with increasing D_h values (about 3.5–13 nm) exhibited diminished antifungal efficacy, and lost the activity with D_h around 13 nm, with a MIC value $>1500 \mu\text{g/mL}$.^[20] Since the D_h values of HPL1–HPL3 in this study were at around 1.68, 2.88 and 4.36 nm (Fig. 1b), respectively, passive diffusion into *C. albicans* cells is possible for each HPL.

Cationic antimicrobial agents function by first attaching to the cell surface, then concentrating at the surface and reaching a critical local concentration, which induces membrane perturbation and damage. Previous studies have suggested that a charge density threshold is required for cationic antimicrobial agents to induce cell death.^[41] For a cationic antimicrobial agent with a lower positive charge density, a higher concentration is required to reach the critical local concentration, resulting in an increased minimum inhibitory concentra-

tion (MIC). As the MIC value increased to a certain level, this agent would no longer be considered to have antimicrobial activity. The initial electrostatic interactions between HPLs and pathogen cells were measured by ITC experiments (Fig. S4 in ESI). The ΔH in HPL/pathogen changed, proving the electrostatic binding of positive HPL onto the negatively charged bacterial and fungal cell surfaces. Among the three tested HPLs, HPL1, with the lowest zeta potential (+14.6 mV), had high MIC values ($>1500 \mu\text{g/mL}$) against all test species, including bacteria and fungi. HPL3, with the highest zeta potential (+32.2 mV), displayed broad-spectrum antimicrobial activity with MIC values less than $375 \mu\text{g/mL}$ against all test species (Figs. 1c and 1d). The selective antifungal activities of HPL2 could not be explained by the charge density difference and electrostatic interactions; however, they could be



Scheme 1 Summary of HPLs targeting bacteria or fungi. HPL1 (yellow particles) could not pass through the bacterial or fungal cell envelope. HPL2 (pink particle) could selectively penetrate the cell wall, disrupt the cell membrane, accelerate the ROS accumulation and damage DNA, but get stuck in the cell envelope of *E. coli* or *S. aureus*. HPL3 (blue particle) could penetrate both the bacteria and fungi cell envelopes.

considered from the perspective of the structural variations of microbial cells. The outer membrane of gram-negative bacteria and thick peptidoglycan layer of gram-positive bacteria limit the migration of antimicrobial agents to the cell membrane.^[42] The fungal cell wall is composed of β -glucans, chitin, and mannoproteins, forming a porous polysaccharide network that provides a comparatively lower barrier for HPL2 to penetrate the cell wall to reach the cell membrane. TEM analysis (Fig. 2b, Fig. S3 in ESI) and the cell wall integrity experiment measured by NPN dye (Fig. 3b) proved that HPL penetrated the cell wall of *C. albicans* without destroying it.

The interaction between HPLs and microbial plasma membranes was verified using cytoplasmic membrane depolarization experiments (Fig. 3a, Fig. S5 in ESI). HPL1 did not induce membrane depolarization in either *C. albicans* or *S. aureus*, in-

dicating that HPL1 is not in contact with the cell membrane. With the increase in positive charges, both HPL2 and HPL3 induced membrane depolarization in *C. albicans*, whereas only HPL3 affected *S. aureus*. The entrance of HPLs into the cells was further verified by a live/dead staining assay. There was no observable PI uptake in the HPL1-treated pathogens. PI uptake increased in HPL2- and HPL3-treated *C. albicans* cells, but was only observed in bacterial cells exposed to HPL3 (Fig. 3c), further proving the selective membrane-destroying effect of HPL2.

In conclusion, increasing the zeta potential of HPL could lower the working inhibition concentration to kill microbial cells. Although there is a difference in zeta potential between bacteria and fungi, the main factor influencing the selective antifungal activity of HPL2 is the different architecture of the

cell envelope in bacteria and fungi.

In vivo Safety Assessment

The acute oral toxicity of HPLs was evaluated in mice based on the Technical Standard for Disinfection of China (2002 edition) with some modifications. Mice (6–8 weeks) were orally gavaged with HPLs at a single dose of 5000 mg/kg body weight and monitored for 14 days (Fig. 5a). All mice survived, with an estimated $LD_{50} > 5000$ mg/kg, classifying HPL as practically non-toxic according to the guidelines. Throughout the observation period, the HPL-treated group exhibited normal behaviors, including physical activity, feeding, grooming, and defecation. No clinical signs of distress or toxicity were noted. The body weight of the treated mice increased steadily over 14 days, similar to the trend observed in the saline control group, suggesting no

adverse effects on health or metabolism (Fig. 5b). At the end of the experimental period, six mice were randomly selected from each group and serum inflammatory markers were quantified by ELISA. The levels of pro-inflammatory cytokines, including TNF- α and IL-6, were not significantly different between the HPL-treated and control groups (Fig. 5c), indicating that systemic inflammatory exposure did not induce systemic inflammatory responses. Histopathological evaluation of the major organs (liver, spleen, kidney, ileum, and colon) revealed no tissue damage, including inflammatory cell infiltration, edema, necrosis, or architectural disruption (Fig. 5d). These findings demonstrate that HPLs exhibit low systemic toxicity and are well tolerated even at high doses, supporting a favorable safety profile for potential therapeutic or prophylactic applications.

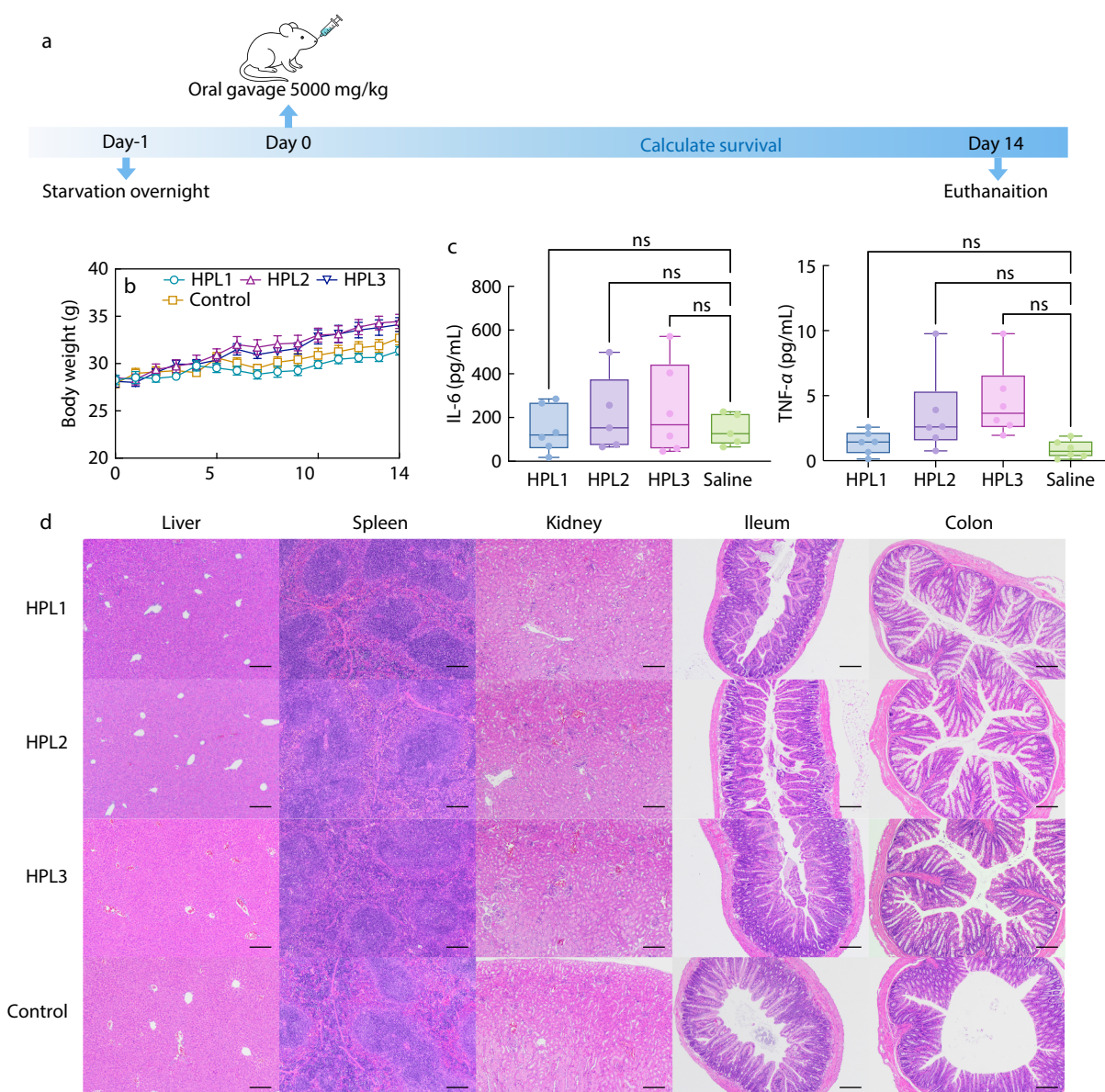


Fig. 5 The *in vivo* safety evaluation. (a) Schematic diagram of the acute oral toxicity model; (b) The body weight change in the acute oral toxicity model ($n=6$); (c) The inflammatory factors of IL-6 and TNF- α in mice serum ($n=6$); (d) The histological study of the main organs stained by H&E. Scale bars: 200 μ m.

In vivo Therapeutic Model

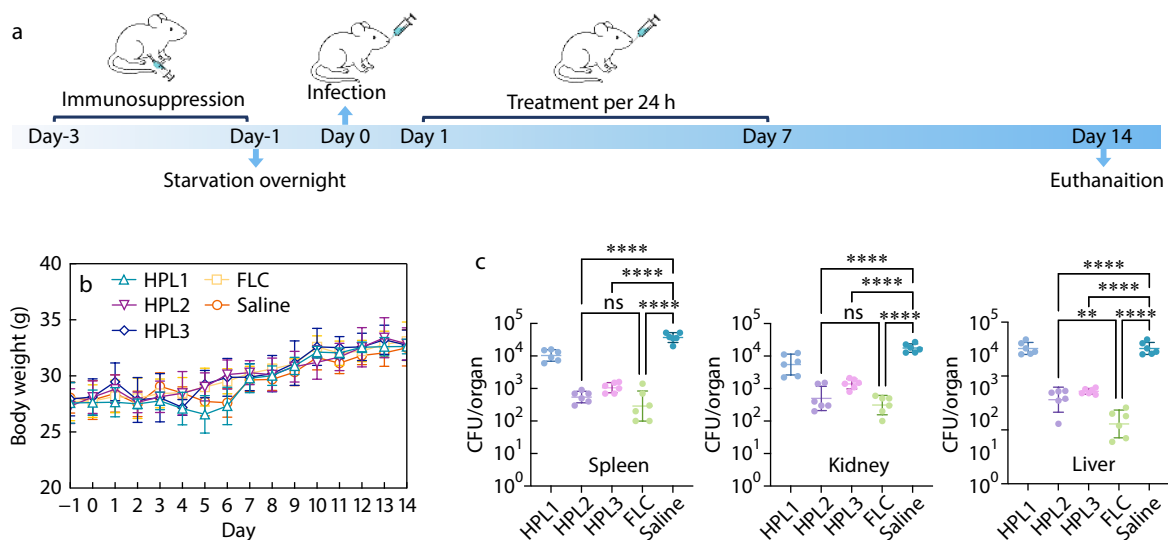
To evaluate the *in vivo* antifungal efficacy of HPLs, a gastrointestinal invasive infection model of *C. albicans* was established in immunosuppressed mice, mimicking clinical invasive candidiasis with high mortality, especially in immunocompromised patients.^[43–45] Mice were pretreated with immunosuppressants and then gavaged with 200 μ L of *C. albicans* suspension (about 10^8 CFU/mL). After 24 h, the mice were randomly divided into five groups ($n=8$) and treated once daily by oral gavage with HPL1, HPL2, HPL3, or FLC solution for seven consecutive days. The saline-treated group served as the infected control. The mice were monitored for an additional seven days (Fig. 6a). Throughout the 14 days, survival status and body weight were recorded daily. Mice treated with HPL2, HPL3, or FLC exhibited continuous weight gain, indicating infection control and health improvements. The saline and HPL1 groups showed initial weight loss in the first 6 days, with recovery after day 7, likely due to the combined effects of infection and gavage. (Fig. 6b). At the end of the experiment, the fungal burdens in the liver, spleen, and kidneys were quantified. The HPL2, HPL3, and FLC groups had significantly reduced fungal loads in all three organs. The saline and HPL1 groups showed high colony burdens, confirming the establishment of the invasive infection model (Fig. 6c). Notably, no statistically significant difference in fungal burden was observed between the HPL2 and FLC groups, indicating that HPL2 was as effective as FLC in reducing systemic fungal dissemination via the gastrointestinal route. Histological examination (H&E staining) of the major organs showed prominent inflammatory infiltration in the saline and HPL1 groups, but only mild changes were observed in the HPL2, HPL3, and FLC groups (Fig. 6d), further supporting the therapeutic efficacy of HPL2 and HPL3.

Given the importance of gut microbiota in host metabolism and immune function and the known disruptive effects of conventional antifungals such as FLC on intestinal microbial communities,^[46] the impact of HPLs on gut microbiota composition was further examined. At the end of the treatment period, fecal samples were collected for microbiome analysis using 16S rRNA sequencing. The untreated

healthy group was included as an additional negative control group. As shown in Fig. S6 (in ESI), the alpha diversity indices showed no significant differences among the groups, indicating that the overall microbial richness and evenness were not substantially altered. Beta diversity is shown as a heatmap of the sample distance (Fig. 6e). Compared to the control group, significant differences were observed in the FLC and saline groups, whereas slight differences were observed in the HPL groups.

Community composition analysis revealed differences at the phylum level between these groups. In the control group, the average relative abundance of *Bacteroidetes* was 61.2%. This decreased to 47.9% in the FLC group and 41.3% in the saline group, while the HPL2 group had a relative abundance of 54.9%. The relative abundance of the phylum *Firmicutes* was 29.9% in the control group, whereas it increased to 35.9% and 41.0% in the HPL2 and FLC groups, respectively. These two phyla are key indicators of gut health and metabolic function, indicating that HPL2 maintained the microbial composition more effectively than FLC. Notably, the *Pseudomonadota* phylum relative abundance of the phylum *Pseudomonadota* increased from 2.6% in the control group to 6.7% and 11.3% in the FLC and saline groups, respectively, indicating dysbiosis of the microbiota in the FLC and untreated saline groups, whereas the abundance of the HPL2 group was 3.1% (Fig. 6f, Fig. S7 in ESI). The community heatmap displays the distribution of the most dominant species in all samples across the different samples (Fig. 6g). Compared to the control group, the *Muribaculaceae* family in HPL1 and the saline group showed a decrease, which can produce short-chain fatty acids and play an important role in gut microbial balance in mice.^[47] Moreover, the *Lachnospiraceae* in the FLC group increased, indicating metabolic disease in mice.^[48]

Collectively, the *in vivo* results demonstrated that HPL exerted potent antifungal activity against gastrointestinal invasive *C. albicans* infection, with therapeutic efficiency comparable to that of FLC. HPL can significantly reduce fungal burden in major organs, alleviate tissue inflammation, and promote recovery in infected mice. In addition to antifungal effi-



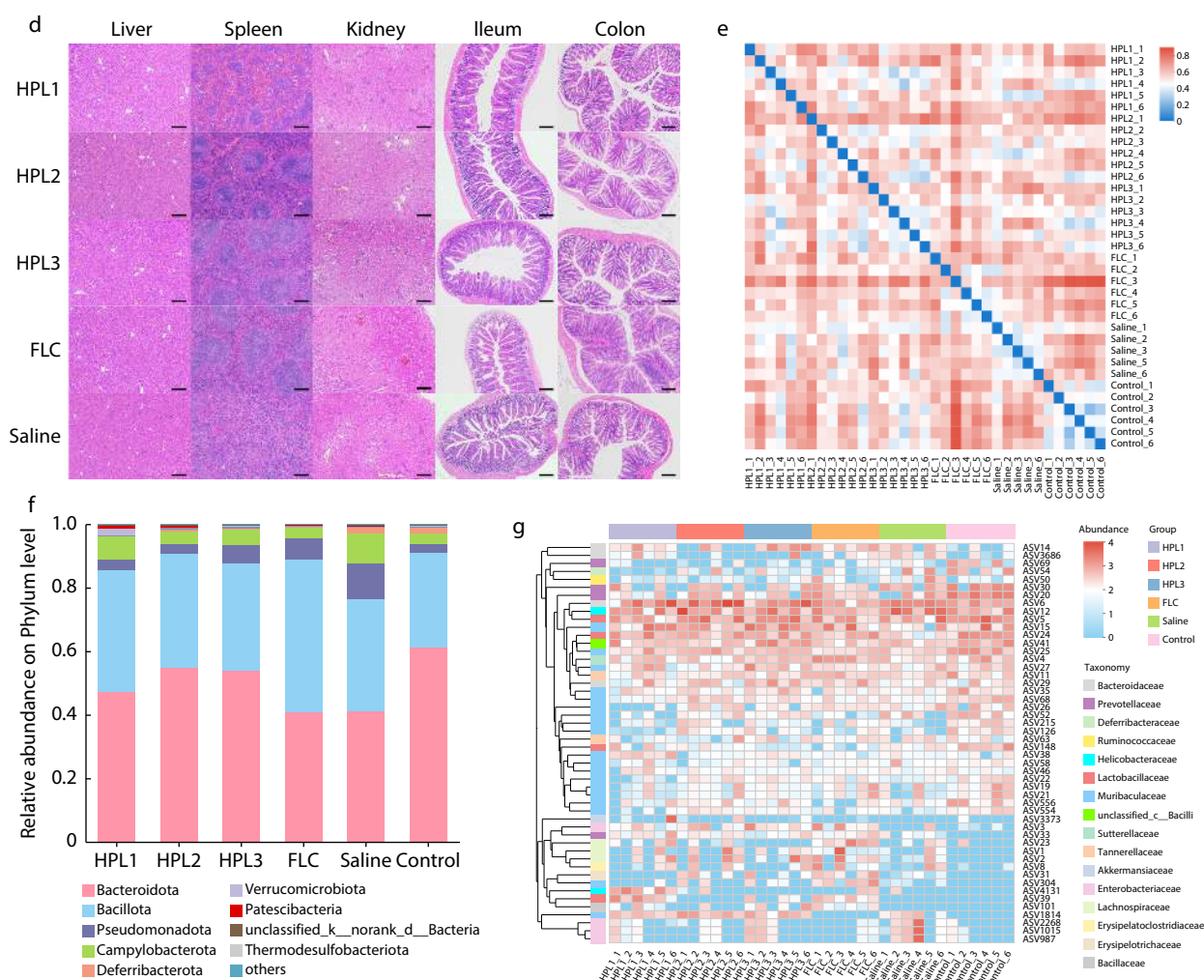


Fig. 6 A gastrointestinal invasive Candidiasis mouse model. (a) Schematic diagram of the *in vivo* antifungal study using an invasive gastrointestinal mouse model; (b) The mice body weights during the infection and treatment period. Data are presented as mean $\pm$ SD. (c) The fungal burden in different organs of mice that were treated with HPLs, FLC, or saline. $n=6$ biologically independent animals. (d) Histological images of organs from infected mice, with H&E staining after antifungal treatment. Scale bars: 200 μ m. (e) The sample distance on the ASV level based on weighted unifrac; (f) The community composition analysis of the relative abundance of bacteria at the phylum level; (g) Heatmap at the ASV level displaying the distribution of the top dominant species in all samples across different samples.

cacy, HPL maintained the gut microbiota balance better than FLC. These findings suggest that HPL2 not only effectively controls systemic fungal infection, but also minimizes gut dysbiosis, highlighting its potential as a microbiota-compatible antifungal candidate.

CONCLUSIONS

In summary, three hyperbranched polylysines (HPLs) were synthesized at the kilogram scale, leading to distinct antimicrobial properties. HPL1, which has the lowest molecular weight, showed negligible activity against bacteria and fungi. HPL2, with a moderate molecular weight, zeta potential (+21.6 mV), and hydrodynamic size ($D_h=2.88$ nm), showed potent activity against *C. albicans* with an MIC value of 96 μ g/mL. HPL2 is also effective against other clinically relevant *Candida* species, including *C. krusei*, *C. parapsilosis*, *C. tropicalis*, and *C. glabrata*. In contrast, HPL3, which has the highest molecular weight,

demonstrated broad-spectrum antimicrobial activity. Resistance development assays further indicated that neither HPL2 nor HPL3 induces resistance in *C. albicans*.

The molecular weight-dependent antimicrobial activities of the HPLs were mainly attributed to differences in charge density. Although all HPLs could interact with pathogen surfaces, only HPL2 and HPL3 could depolarize the *C. albicans* plasma membrane, and only HPL3 could depolarize the *S. aureus* plasma membrane. Live/dead staining and SEM confirmed that HPL2 selectively disrupted the fungal cell membrane integrity. In addition, NPN uptake and TEM analyses showed that HPL2 could penetrate rather than destroy the cell wall, potentially reducing the risk of resistance. Furthermore, ROS accumulation, reduced SDH activity, and impaired mitochondrial membrane potential indicated that HPLs induced oxidative stress and mitochondrial dysfunction in *C. albicans*.

In vivo biosafety evaluation revealed that HPLs were well tolerated, with acute oral toxicity assays classifying them as

practically nontoxic ($LD_{50} > 5000$ mg/kg). In a murine infection model, HPL2 significantly reduced fungal burden while preserving the gut microbial balance. Collectively, these findings highlight HPL2 as a promising selective antifungal candidate with favorable efficacy and safety.

Conflict of Interests

The authors declare no interest conflict.

Electronic Supplementary Information

Electronic supplementary information (ESI) is available free of charge in the online version of this article at <http://doi.org/10.1007/s10118-026-3721-7>.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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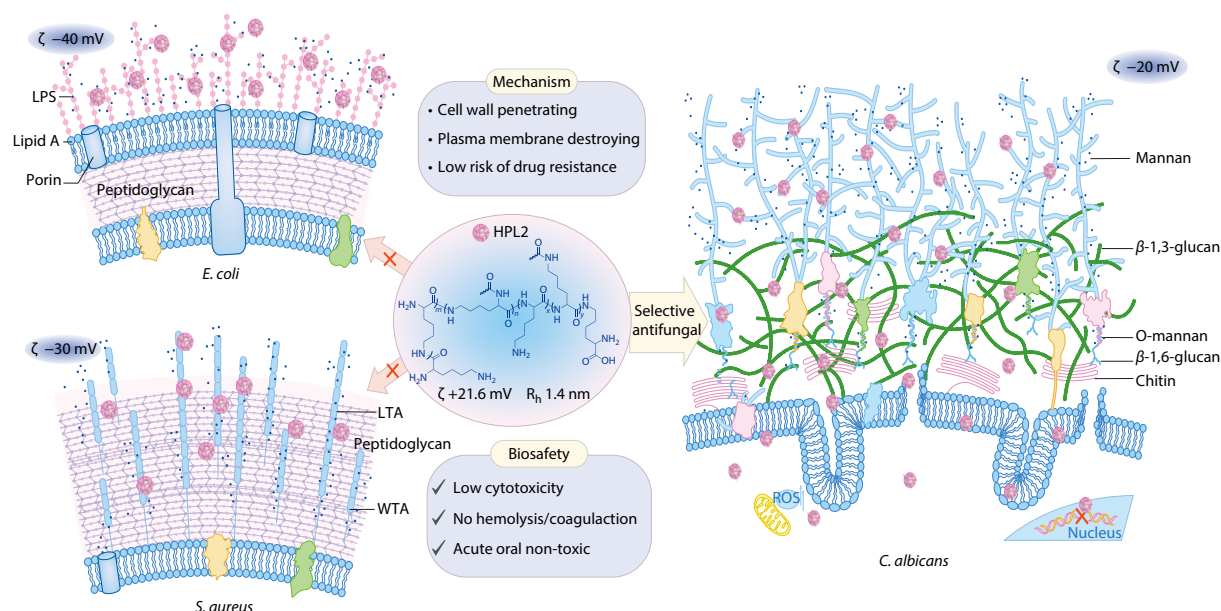
Graphical Abstract

Selective Antifungal Activity of Hyperbranched Polylysine Mediated by Charge-dependent Membrane Interactions

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This study introduces a hyperbranched polylysine, HPL2, which exerts its selective antifungal effect through membrane disruption via electrostatic interactions, shows excellent biocompatibility and minimal effects on the host gut microbiota.



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