Phospholipid/protein co-mediated assembly of Cu₂O nanoparticles for specific inhibition of growth and biofilm formation of pathogenic fungi

Liping Peng[†], Henan Wei[†], Lei Tian, Jiachun Xu, Mingchun Li^{*} and Qilin Yu^{*}

ABSTRACT As the increasing number of the individuals suffering from AIDs, chemotherapy, and radiotherapy, pathogenic fungi, which may rapidly grow and invade the host tissues in these immune-compromised patients, is becoming great threat to human health. In this study, we constructed a novel fungal pathogen-responsive assembly of cuprous oxide (Cu₂O) nanoparticles (NPs) for specific targeting and inhibiting growth and biofilm formation of the representative fungal pathogen, *Candida albicans* (*C. albicans*). This assembly was formed by coating the initial Cu₂O NPs with both phosphatidylethanolamine (PE) and bovine serum albumin (BSA), followed by hydrophobic/electrostatic interaction-driven formation of the Cu₂O-PE-BSA microaggregates. The formed microaggregates could be induced for disassembly by the fungal pathogen *C. albicans*, leading to close binding of the NPs to the cell wall of the pathogen. Both confocal microscopy and viability assays showed that the assembly strongly inhibited growth and biofilm formation of the pathogen, but had extreme low toxicity to mammalian cells. *In vivo* mouse wound model further revealed that the assembly had high capacity of healing the fungus-infected wounds and reduced the fungal burden of the wound tissues. This study sheds a novel light on facile development of pathogen-responsive nano-assemblies for efficient and safe antifungal therapy.

Keywords: Cu₂O nanoparticle, assembly, fungal pathogen, biofilm, antifungal therapy

INTRODUCTION

As the increasing population of immunocompromised individuals, such as those suffering from human immunodeficiency virus (HIV) infection, chemotherapy, radiotherapy, and organ transplantation, the infections by

opportunistic fungal pathogens are becoming severe and global issues. These infections are attributed to the defect of immune response in these patients against the pathogens [1–3]. The fungal infections frequently contribute to hospital-acquired infection-related death [4]. Especially, the common fungal pathogens, e.g., *Candida albicans* (*C. albicans*), *Cryptococcus neoformans*, and *Aspergillus fumigatus*, frequently cause not only superficial infections, but also life-threatening systemic infections that severely damage diverse organs and disturb the circulatory system [5–7]. For example, *C. albicans* could secrete both lipase and protease to disrupt the mammalian cell membrane, leading to severe death of host cells [8]. Antifungal therapy is critical for saving the lives of fungus-infected people. However, unlike antibacterial therapy that is supported by abundant antibacterial agents (i.e., antibiotics), antifungal therapy only possesses very limited kinds of drugs, mainly including azoles, echinocandins, and polyenes [9–12]. Owing to the increasing emergence of drug-resistant fungal pathogens, it is very urgent to develop novel antifungal agents for clinical application [13–15].

Cuprous oxide (Cu₂O) nanoparticles (NPs) have attracted great attention due to their wide applications in photocatalysts, electronics, optics, sensors, and biomedicine [16–19]. Especially, recent studies revealed that Cu₂O NPs exhibited strong antifungal efficiency, which is associated with the release of copper ions and induction of reactive species oxygen (ROS), followed by cell wall/membrane disruption and DNA damage [20–22]. Due to their excellent antifungal activity, these NPs have been developed as fungicidal agents in agriculture to fight against common plant fungal pathogens (e.g., *Phy-*

Key Laboratory of Molecular Microbiology and Technology, Ministry of Education, Department of Microbiology, College of Life Sciences, Nankai University, Tianjin 300071, China

[†] These authors contributed equally to this paper.

^{*} Corresponding authors (emails: nklimingchun@163.com (Li M); yuqilin@mail.nankai.edu.cn (Yu Q))

tophthora capsici, *Botrytis cinerea*, and *Fusarium oxysporum*) [23–25]. However, Cu_2O NPs have considerable toxicity to the mammalian cells [26,27], limiting their wide biomedical applications. In our approach, we aimed to design Cu_2O NP-based platforms to reduce the cytotoxicity of these NPs for application in antifungal therapy.

Stimuli-responsive assemblies of NPs are becoming a hot topic of materials science and chemistry [28,29]. By introducing assembly-driving forces (e.g., hydrophobic interaction, electrostatic interaction, hydrogen bond, host-guest complexation, and coordination) and regulation groups responding to diverse environmental stimuli (e.g., light, magnetic field, heating, and pH), NPs could self-assemble to form nano-/micro-aggregates [30–35]. In biomedical and environmental fields, formation of these assemblies may endow the NPs with specific targeting to aimed cells, increased biosafety, and improved physicochemical properties (e.g., magnetic resonance imaging, magnetic collection, and drug delivery) [36–40]. It is anticipated that construction of NP assemblies is a promising strategy to broaden the application of bioactive NPs.

In this study, we constructed the facile assembly of Cu_2O NPs to remarkably reduce their toxicity for antifungal therapy (Scheme 1). By non-covalently coating the Cu_2O NPs with phosphatidylethanolamine (PE) and bovine serum albumin (BSA), the NPs could self-assemble to form the Cu_2O -PE-BSA microaggregates driven by hydrophobic and electrostatic interactions. The assembly could sensitively respond to the pathogenic fungal cells

for disassembly, leading to close attachment of NPs on the surface of the fungal cells. Further antifungal experiments revealed that the assembly exhibited strong activity to kill the fungal pathogens, and to inhibit fungal hyphal growth and biofilm formation. This study provides a facile strategy of constructing NP assemblies for improving NP biocompatibility and developing novel nano-assemblies in biomedical application.

EXPERIMENTAL SECTION

Materials

$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, poly(vinyl pyrrolidone) (PVP, weight-average molecular weight $M_w \approx 55$ kD), *L*-ascorbic acid (ASA), PE, BSA, fluorescein isothiocyanate (FITC), Calcofluor White, and propidium iodide (PI) were purchased from Sigma, USA. RPMI-1640 medium was purchased from Gibco, USA. The CCK-8 assay kit was purchased from Dojindo, Japan. Other reagents were obtained from Aladdin, China.

Synthesis of Cu_2O NPs

The Cu_2O NPs were synthesized by the ASA-reducing method [41,42]. $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (10 mmol) was dissolved in 100 mL distilled water. PVP (200 mg) was thoroughly dissolved in 20 mL distilled water, and then mixed with the $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ solution. After magnetic stirring for 10 min, 5 mL NaOH solution (200 mmol L^{-1}) was added into the solution under stirring, followed by further stirring for 10 min. Twenty milliliter of ASA solution

Scheme 1 (a) Illustration of construction of Cu_2O -PE-BSA assembly and (b) the assembly-mediated inhibition of growth and biofilm formation of the pathogenic fungal cells.

(500 mmol L⁻¹, dissolved in distilled water) was slowly added into the mixture, generating the yellow Cu₂O NPs. The NPs were collected by centrifugation, washed with distilled water and anhydrous ethanol for three times, and dried at room temperature under the nitrogen flow.

Characterizations of Cu₂O NPs and Cu₂O-PE-BSA

The morphology of NPs was characterized by transmission electron microscopy (TEM; FEI Tecnai T12, USA). The hydration diameters and Zeta potentials of these NPs in distilled water were measured by a Zeta potential analyzer (ZetaPLAS, Brookhaven, USA) at room temperature. The surface organic groups of the NPs were characterized by Fourier transformed infrared spectroscopy (FT-IR; Bio-Rad, FTS6000, USA).

Assays of fungal growth inhibition

The pathogenic fungus *C. albicans* was cultured in liquid RPMI-1640 medium, which contains a variety of inducing factors that can activate expression of specific genes involved in hyphal development [43,44]. The *C. albicans* strains, i.e., SC5314, BWP17, and NKFG1 (expressing Hwp1-GFP under control of the *HWPI* promoter) [45], were cultured in YPD medium at 30°C with shaking overnight, harvested by centrifugation, washed and re-suspended in the RPMI-1640 medium supplemented with 80 µg mL⁻¹ of uridine to an initial optical density at 600 nm (OD₆₀₀) of 0.1. Cu₂O NPs or Cu₂O-PE-BSA were then added into the cell suspensions to the concentrations from 0 to 160 mg L⁻¹. The suspensions were cultured at 37°C with shaking for 4 h. The cells were then harvested, stained by PI (5 mg L⁻¹) for 5 min, and observed by fluorescence microscopy (Zeiss, Germany). The dead (PI-positive) cells, total cells, and hyphal cells were counted, and the percentages of dead cells and hyphal cells were calculated. At least 20 observing fields were observed and counted.

Disassembly of Cu₂O-PE-BSA

To evaluate the disassembly of Cu₂O-PE-BSA induced by *C. albicans* cells, the Cu₂O-PE-BSA assembly was added into the suspension of *C. albicans* cells pre-cultured in RPMI-1640 medium. The mixture was incubated at 37°C for 4 h, followed by staining of the cells with Calcofluor White (10 mg L⁻¹) and PI. The cells were then observed by confocal microscopy (Olympus, Japan).

Biofilm formation

Fungal biofilms were formed in polystyrene microtiter plates as described previously [46]. Briefly, the *C. albicans*

cells (NKFG1) were suspended in RPMI-1640 medium containing different concentrations of Cu₂O NPs or Cu₂O-PE-BSA with the OD₆₀₀ of 0.1. An aliquot of the suspension (100 µL) was then added into 96-well flat-bottom microplates. The plates were statically incubated at 37°C for 24 h. After being washed with phosphate buffered saline (PBS), the biomass of the fungal biofilms was evaluated by XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) assays. The biofilms were also formed in confocal dishes, stained with PI, and the different layers of the biofilms were observed by confocal microscopy.

Viability assays of mammalian cells

The mammalian 293T epithelial cells and the RAW264.7 macrophages were obtained from the Cell Resource Center, Chinese Academy of Medical Sciences. The cells were suspended in RPMI-1640 medium containing different concentrations of Cu₂O NPs or Cu₂O-PE-BSA, and added into 96-well microplates. The microplates were cultured for 48 h, and cell viability was evaluated by the CCK-8 assay kit.

Mouse skin wound model

To assess the therapeutic efficacy of Cu₂O NPs and Cu₂O-PE-BSA against *C. albicans* infection, the 6-week-old female BALB/c mice were used to construct the mouse skin wound model. The hair on the mouse back was cut, and a skin wound was generated by surgical scissors for each mouse. Overnight cultured *C. albicans* were suspended in PBS to 1×10⁸ cells per mL. The cell suspension (100 µL) was dropped on the wound. The Cu₂O NPs or Cu₂O-PE-BSA solution (200 µL, 40 mg L⁻¹) were smeared on the wound each day. After treatment for 7 days, the wound area and the fungal burden of the wound tissues were measured. The animal experiments were approved by the Animal Care and Use Committee at Nankai University.

Statistical analysis

Each experiment was performed in triplicate. The data were described as mean ± standard deviation (SD). All statistical analyses were performed using ANOVA test (*P* < 0.05) by the SPSS software (Version 22, IBM, USA).

RESULTS AND DISCUSSION

Characterization of the synthesized Cu₂O NPs and Cu₂O-PE-BSA

The initial Cu₂O NPs were synthesized by the ASA-reducing method [31,32], and then coated by PE and BSA

to obtain Cu_2O -PE-BSA. TEM observation revealed that the Cu_2O NPs had round morphology with the size of 100–150 nm (Fig. 1a). In contrast, Cu_2O -PE-BSA drastically aggregated to form clusters (Fig. 1a). Zeta potential and dynamic light scattering (DLS) analyses further showed that Cu_2O -PE-BSA had an obvious increase in Zeta potential from -32 to -9 mV (Fig. 1b), and exhibited a remarkable higher hydration diameter than Cu_2O (900–2000 nm versus 100–200 nm, Fig. 1c). In addition, Cu_2O -PE with no further coating by BSA, or Cu_2O -BSA without pre-coating with PE, showed much smaller sizes than Cu_2O -PE-BSA (Fig. S1), indicating that both PE and BSA are required for the formation of the assembly. FT-IR analysis showed the presence of $-\text{NH}_2$, $-\text{CH}_2-$, $-\text{CO}-\text{NH}-$, $-\text{COO}-$, and $\text{P}=\text{O}$ groups on Cu_2O -PE-BSA as compared with Cu_2O (Fig. 1d), suggesting successful PE/BSA coating on the Cu_2O NPs. These results indicate that PE/BSA coating resulted in assembly of Cu_2O -PE-BSA to form microaggregates.

Disassembly of the Cu_2O -PE-BSA microaggregates induced by pathogenic fungal cells

Owing to the presence of PE and BSA on the surface of Cu_2O -PE-BSA, the Cu_2O -PE-BSA microaggregates may respond to pathogenic fungal pathogens that produce lipase and protease [8]. To confirm this, the FITC-tagged

microaggregates were added into the dispersion of the pathogenic fungus *C. albicans* for further incubation. Confocal microscopy revealed that the untreated Cu_2O -PE-BSA had particle morphology with the size of 1000–2000 nm (Fig. 2, upper). In contrast, when incubated with the fungal cells, Cu_2O -PE-BSA strongly bind to the cell wall surface of the fungal cells, leading to drastic disassembly of the aggregates, forming a equally distributed shell on the cell wall of fungal cells (Fig. 2, middle). This result indicated that the pathogenic fungal cells may induce disassembly of the microaggregates, leading to close attachment of the NPs to the fungal cells.

Inhibition of fungal growth by the Cu_2O -PE-BSA assembly

Cu_2O NPs can release copper ions that bind the groups of microbial proteins, such as $-\text{SH}$, $-\text{NH}_2$, $-\text{COOH}$ and $-\text{OH}$, causing denaturation of the proteins and pathogen death [47]. Here, we tested the effects of Cu_2O NPs and Cu_2O -PE-BSA on the death and growth of *C. albicans* cells. PI staining showed that the NPs at the concentrations from 20 to 160 mg L^{-1} caused remarkable death of fungal cells (Fig. 3a). For example, Cu_2O NPs and Cu_2O -PE-BSA killed $> 85\%$ and $> 55\%$ cells at 40 mg L^{-1} , and 99% and $> 95\%$ cells at 160 mg L^{-1} (Fig. 2, bottom; Fig. 3a), respectively. ROS assays further revealed that both Cu_2O NPs and Cu_2O -PE-BSA induced drastic in-

Figure 1 Characterizations of the synthesized Cu_2O NPs and Cu_2O -PE-BSA. (a) TEM images. (b) Zeta potential. (c) Size distribution. (d) FT-IR spectra.

Figure 2 Pathogenic fungus-induced disassembly of the Cu_2O -PE-BSA microaggregates. Note that the Cu_2O -PE-BSA alone had aggregation with microparticle morphology (top), while the presence of the pathogenic *C. albicans* cells strongly adsorbed the particles and led to their disassembly (middle). The group of *C. albicans* alone (bottom) exhibited no autofluorescence on the surface.

Figure 3 Fungal death and inhibition of hyphal growth by Cu_2O NPs and Cu_2O -PE-BSA. (a) Fungal death induced by Cu_2O NPs and Cu_2O -PE-BSA at different concentrations. (b) Percent of hyphal cells under treatment of the agents. (c) Microscopy images of the fungal cells under the hypha-inducing condition with 40 mg L⁻¹ of the agents. * indicates significant difference between the control group (0) and the treated group ($P < 0.05$).

crease in intracellular ROS levels (Fig. S2), indicating that the fungicidal activities of Cu_2O NPs and Cu_2O -PE-BSA are associated with ROS accumulation. Moreover, in-

ductively coupled plasma (ICP) assays showed that both Cu_2O NPs and Cu_2O -PE-BSA (40 mg L⁻¹) released 1.6–1.9 mg L⁻¹ of Cu^+ to the medium (Fig. S3), and Cu^+ at

this releasing level also caused remarkable intracellular ROS accumulation and cell death (Fig. S4), indicating that Cu^+ release and ROS production contribute to anti-fungal activity of Cu_2O NPs and Cu_2O -PE-BSA.

Hyphal growth of *C. albicans* is required for the pathogen to invade into the host tissues and hence involve in its virulence [48]. Therefore, inhibition of this process is becoming a strategy fighting against its infections [49]. The percent of hyphal cells under the treatment of the NPs and assembly was then evaluated. Both Cu_2O NPs and Cu_2O -PE-BSA could strongly inhibit the hyphal growth (Fig. 3b, c), with the percent of hyphal cells decreasing to 20%–30% at 40 mg L^{-1} , and to 3%–6% at 160 mg L^{-1} (Fig. 3b). Statistical analysis further revealed that Cu_2O -PE-BSA and Cu_2O NPs had a half maximal inhibitory concentration (IC_{50}) value of $< 30 \text{ mg L}^{-1}$ against all of the tested strains, including SC5314, BWP17, and NKFG1 (Table 1). In addition, the assemblies also led to severe death of the yeast-type *C. albicans* cells (Fig. S5) and another important clinical fungal pathogen, *Cryptococcus neoformans* (Fig. S6). These results indicated that the Cu_2O -PE-BSA assembly, similar to Cu_2O NPs, could efficiently kill the fungal pathogens and inhibit hyphal and yeast growth of these pathogens.

Inhibition of fungal biofilm formation by the Cu_2O -PE-BSA assembly

Biofilm formation plays an important role in colonization and drug resistance of fungal pathogens [50,51]. We then evaluated the effect of the assembly on fungal biofilm formation. Confocal microscopy showed that the control group had thick biofilms composed of dense hyphal fungal cells, with only few dead cells in the biofilms (Fig. 4a). In contrast, the group of Cu_2O -PE-BSA assembly, similar to the group of Cu_2O NPs, exhibited specially thin biofilms, with most of the cells dead at the observed layers (Fig. 4a). XTT assays further revealed that both kinds of NPs drastically reduced biomass of the biofilms, leading to decrease in the biomass to $< 40\%$ of the control biomass (Fig. 4b), with the IC_{50} values against biofilms of $< 35 \text{ mg L}^{-1}$ (Table 1). These results revealed that the assembly could strongly inhibit fungal biofilm

formation and kill the fungal cells.

Biofilm formation is determined by specific expression of biofilm-related genes, such as the cell wall adhesion-encoding gene *HWPI* [52]. We thus used green fluorescent protein (GFP) quantification assays of Hwp1-GFP, whose expression level is controlled by the initial *HWPI* promoter in the strain NKFG1 [45]. Consistent to the results of biofilm formation assays, both the Cu_2O -PE-BSA assembly and the Cu_2O NPs strongly reduced the expression of *HWPI* to almost the half level of the control (Fig. 4c), which may be attributed to the inhibition effect of the nanocomposites on the activation of the hypha-inducing signaling pathways. This result indicated that the impairment of biofilm formation by the assembly is associated with decreased expression of biofilm-related genes, followed by reduction of adhesion required for the biofilm development.

Reduced toxicity of the Cu_2O -PE-BSA assembly to mammalian cells

It is necessary to evaluate the cytotoxicity of the antimicrobial agents to mammalian cells for biomedical application. Herein, two mammalian cell types commonly interacting with the fungal pathogens, including the epithelial cells (293T) and the macrophages (RAW264.7) were used for cytotoxicity evaluation. CCK-8 assays showed that Cu_2O NPs strongly impaired the viability of both kinds of mammalian cells, leading to $> 50\%$ reduction of viability at 40 mg L^{-1} and $> 80\%$ at 160 mg L^{-1} (Fig. 5a), indicating high cytotoxicity of Cu_2O NPs. Similarly, both Cu_2O -PE and Cu_2O -BSA, which failed to form assembly, exhibited high toxicity to both kinds of mammalian cells, leading to $> 40\%$ reduction of viability at 40 mg L^{-1} (Fig. S7). In contrast, the Cu_2O -PE-BSA assembly showed only slight toxicity to these cells, with the viability $> 85\%$ at 40 mg L^{-1} and $> 50\%$ even at 160 mg L^{-1} (Fig. 5b). Therefore, the formation of assemblies remarkably reduced the toxicity of the Cu_2O NPs to the mammalian cells. This attenuation of the toxicity is most likely attributed to PE/BSA coating of the Cu_2O NPs that prevents the release of Cu^+ or their contact with the mammalian cells.

Table 1 IC_{50} of Cu_2O NPs and Cu_2O -PE-BSA against *C. albicans* growth and biofilm formation

Strain	IC_{50} against growth (mg L^{-1})		IC_{50} against biofilm (mg L^{-1})	
	Cu_2O NPs	Cu_2O -PE-BSA	Cu_2O NPs	Cu_2O -PE-BSA
SC5314	< 20	24.02	34.21	33.91
BWP17	< 20	25.35	< 20	13.72
NKFG1	< 20	20.16	< 20	18.67

Figure 4 Inhibition of fungal biofilm formation by Cu₂O NPs and Cu₂O-PE-BSA. (a) Confocal images of biofilms at different layers from the bottom to the top (0–40 μm). (b) Biomass of the fungal biofilms evaluated by XTT assays. (c) Expression of *HWP1* in the treated biofilms revealed by Hwp1-GFP fluorescence quantification. * indicates significant difference between the control group (0) and the nanoparticle-treated group ($P < 0.05$).

Figure 5 Impairment of mammalian cell viability by Cu₂O NPs and Cu₂O-PE-BSA. (a) Viability of 293T cells. (b) Viability of RAW264.7 cells. * indicates significant difference between the two groups ($P < 0.05$).

***In vivo* antifungal efficacy of the assembly**

The *in vivo* antifungal capacity of the assembly was evaluated using a mouse skin wound model. In this model, the mice were firstly injured at the back skin, infected by *C. albicans* cells, followed by treatment of the

NPs or the assembly (Fig. 6a). After treatment for 7 days, the control mice had the wound healing rate of < 50%, indicating slow healing of the wounds suffering from the fungal infection. In contrast, while Cu₂O NPs slightly increased the wound healing rate (65%), the Cu₂O-PE-

Figure 6 Wound healing and reduction of fungal cells in the mouse wounds by Cu₂O NPs and Cu₂O-PE-BSA. (a) Illustration of the mouse skin wound model. (b) Wound healing rate of the treated mouse wounds. (c) Fungal burden in the wounds. * indicates significant difference between the groups ($P < 0.05$).

BSA assembly strongly enhanced wound healing, with the healing rate reaching $> 95\%$ (Fig. 6b). Moreover, fungal burden assays showed that the assembly led to much lower levels of fungal cells in the wound tissues than Cu₂O NPs (Fig. 6c). The higher fungal burden in the Cu₂O-treated wounds than in the assembly-treated wounds could be explained by the higher toxicity of Cu₂O NPs to the immune cells (e.g., the macrophages, Fig. 5c), leading to partial reduction of antifungal capacity of these immune cells.

CONCLUSIONS

In conclusion, this study constructed the facile pathogen-responsive assembly of Cu₂O NPs for specific inhibition of growth and biofilm formation of pathogenic fungi. By coating the Cu₂O NPs with both PE and BSA, these NPs could self-assemble to form the Cu₂O-PE-BSA microaggregates. Owing to the presence of the substrates of lipase and protease (i.e., PE and BSA), the Cu₂O-PE-BSA microaggregates could sensitively respond to the pathogenic fungal cells secreting these enzymes for disassembly. The pathogen-induced disassembly further led to close interaction between the Cu₂O NPs and the pathogens, followed by strong inhibition of growth and biofilm formation of the pathogens, and acceleration of healing of the fungus-infected wounds *in vivo*. Moreover, this assembly showed drastically reduced toxicity to

mammalian cells as compared with the naked Cu₂O NPs. This study sheds a novel light on the construction of pathogen-responsive nano-assemblies for efficient and safe antifungal therapy.

Received 9 June 2020; accepted 6 July 2020;
published online 16 October 2020

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Acknowledgements This work was supported by the National Natural Science Foundation of China (31870139 and 81873961), the Natural Science Foundation of Tianjin (19JCZDJC33800), the National Training Program of Innovation and Entrepreneurship for Undergraduates (201810055105), and the Fundamental Research for the Central Universities.

Author contributions Peng L, Wei H, and Yu Q designed and performed the experiments, and wrote the paper; Tian L and Xu J performed the data analysis; Yu Q and Li M supervised this study. All authors contributed to the general discussion and revision of the manuscript.

Conflict of interest The authors declare that they have no conflict of interest.

Supplementary information Supporting data are available in the online version of the paper.

Liping Peng is currently a PhD student at the Department of Microbiology, College of Life Sciences, Nankai University. Her research focuses on the development of antimicrobial nanosystems for treatment of fungal/bacterial infections, together with investigation of the virulence factors of clinical pathogens.

Henan Wei is currently an undergraduate at the Department of Microbiology, College of Life Sciences, Nankai University. Her research focuses on the design of antifungal nanoplatforms for efficient anti-*Candida* therapy.

Qilin Yu received his PhD degree in 2013 from the College of Life Sciences, Nankai University. In 2017, he was appointed associated professor in Nankai University. In 2019, he started his visiting scholar research in Prof. Jeffrey Zink's Lab, Department of Chemistry and Biochemistry, University of California, Los Angeles. His research interests focus on the design and application of nanotechnology-based self-assembly of biological systems.

磷脂/蛋白介导的氧化亚铜纳米粒子组装体对致病真菌生长及生物被膜的抑制作用

彭丽萍[†], 卫贺南[†], 田雷[†], 许佳春^{*}, 李明春^{*}, 喻其林^{*}

摘要 随着艾滋病感染、化疗、放疗等免疫缺陷人群的日益增加, 病原真菌感染已成为人类健康的巨大威胁。本文构建了一种新型的病原真菌响应性氧化亚铜纳米粒子组装体, 用于特异性靶向并抑制病原真菌的生长与生物被膜形成。首先通过在氧化亚铜纳米粒子表面包覆磷脂酰乙醇胺及牛血清白蛋白, 继而包覆分子通过疏水及静电作用驱动纳米粒子形成微米组装体。形成的组装体能够在病原真菌(白念珠菌)的诱导下进行解组装, 导致纳米粒子与病原真菌的细胞壁紧密结合。共聚焦显微观察及细胞活性检测表明, 组装体能够显著抑制病原真菌的生长及生物被膜形成, 且对哺乳动物细胞表现出极低的毒性。体内小鼠伤口感染模型进一步显示组装体能够促进病原真菌感染伤口的愈合, 并降低伤口组织的真菌负载量。该研究为病原菌响应性纳米组装体的开发及其在抗真菌感染治疗中的高效与安全应用提供了新的思路。