Supplementary Information

ZIF-8 Coated ZnO with pH-Responsive Natamycin Releasing Boosting Antifungal and Anti-Inflammation Activity to Treat Fungal Keratitis

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1. Materials and Method:

1.1 Materials

Ethanol, $Zn(CH_3COO)_2 \cdot 2H_2O$, Sodium hydroxide (NaOH), N,N-dimethylformamide (DMF), 2-Methylimidazole, and sodium citrate were purchased from Macklin (Shanghai, China). Hexadecyl trimethyl ammonium bromide (CTAB) was obtained from Aladdin (Shanghai, China). NATA was purchased from MCE (NJ, USA). Human corneal epithelial cells (HCECs) gained from the Xiamen University were immortalized cells. RAW 264.7 cells gained from Chinese Academy of Sciences in Shanghai. *A. fumigatus* was provided by China General Microbiological Culture Collection Center.

1.2 Synthesis of NATA@ZnO-Z

1.2.1 ZnO-Z nanospheres synthesis. 2 mmol of 2-methylimidazole was dissolved in 30 mL of DMF and subsequently warmed up to 75°C in advance. Then, 10 mL of a mixture containing 0.25 mmol of ZnO nanoparticles and 0.4 mL of CTAB, all dissolved in DMF, was introduced into the preheated solution while being stirred magnetically at 75°C for a duration of 3 h. The resulting white precipitate was harvested through centrifugation, rinsed multiple times with water, and subsequently dried at 60°C for a full night.

1.2.2 NATA@ZnO-Z synthesis. 2 mmol of 2-methylimidazole was dissolved in 30 mL of DMF and subsequently warmed up to 75°C in advance. Afterwards, 10 mL of a mixture containing 0.25 mmol of ZnO nanoparticles, 100 mg of NATA, and 0.4 mL of CTAB, all dissolved in DMF, was introduced into the preheated solution while being stirred magnetically at 75°C for a duration of 3 h. The resulting yellow precipitate was harvested through centrifugation, rinsed multiple times with water, and subsequently dried at 60°C for a full night.

1.2.3 NATA@ZIF-8 nanospheres synthesis

2 mmol of 2-mim was dissolved in 15 mL of DMF, and then the solution was preheated at 70°C. Subsequently, 5 mL of the system consisting of 0.25 mmol

of $Zn(CH_3COO)_2 \cdot 2H_2O$, 100 mg of NATA and 0.2 mL of CTAB in DMF solution were added to the preheated solution under magnetic stirring at 70°C for 2 h. The yellow precipitate was collected by centrifugation, washed several times with water, and then dried at 60°C overnight.

1.3 Characterizations

The structure and composition were characterized by X-ray diffractometer (XRD; Bruker D8 Advance, Bruker AXS,Germany) over the 2θ scanning range of 0–80˚. Fourier transform infrared spectroscopy (FTIR) spectrometer (Thermo Scientific, USA) was used to measure the transmittance spectra of the samples over a wavelength range of 500 to 4000 cm^{-1}. Ultraviolet-visible (UV–Vis) spectroscopy was used to measure the absorption peaks of the samples. The morphologies and composition of the samples were characterized by scanning electron microscopy (SEM; JEOL, JSM-7001F,10 kV) and transmission electron microscopy (TEM; JEOL, JEM-2100F,200 kV).

1.4 Drug loading and release experiment

1.4.1 Drug loading determination. The drug loading was measured based on a previous study [30]. In the process of preparing NATA@ZnO-Z, the yellow precipitate and supernatant were separated by centrifugation at 14000 g for 15 min. The supernatant was gathered, and its absorbance at a wavelength of 304 nm was determined through UV–Vis spectroscopy. Based on the NATA standard curve at 304 nm (Fig. S3) [13], the supernatant's drug content was computed, enabling the analysis of the drug loading efficiency of ZnO-Z.

The equation for calculating the drug encapsulation efficiency (DEE) is given below:

$$\text{DEE (\%)} = \frac{(\text{Total drug} - \text{Remaining drug})}{(\text{Total drug})} \times 100\ \%$$

The equation for calculating the drug loading efficiency (DLE) is given below:

$$\text{DLE}(\%) = \frac{(\text{Total drug} - \text{Remaining drug})}{(\text{weight of NATA@ZnO - Z})} \times 100\,\%$$

1.4.2 Drug release experiment. A dialysis bag containing 2 mg of NATA@ZnO-Z was diluted with 1 mL of PBS and subsequently submerged in 50 mL of PBS solution (pH= 6.0, 6.5, 7.0, or 7.5) at room temperature.The solution was continuously stirred using a magnetic agitator. Every 2 hours, 2 mL of solution was removed from the medium and replaced with 2 mL of fresh PBS. The absorbance of the samples was then measured at a wavelength of 304 nm. The formula utilized for calculating the drug release rate was derived from a previous study [13].

1.4.3 Nuclear Magnetic Resonance Spectroscopy (NMR). A dialysis bag containing 2 mg of NATA@ZnO-Z was diluted with 1 mL of distilled water and subsequently submerged in 50 mL of distilled water at room temperature. The solution was continuously stirred using a magnetic agitator for 24h. Obtain the sample by freeze-drying the solution. The ^{1}HNMR of samples was recorded by nuclear magnetic resonance spectroscopy (Advance 400, Bruker, Germany). Dissolve the sample with DMSO-d6 for hydrogen spectrum detection.

1.5 Determination of antifungal efficiency

1.5.1 Minimum inhibitory concentration (MIC). 1×10^5 CFU/mL *A. fumigatus* conidia were co-cultured with ZnO-Z, NATA, NATA@ZIF-8 and NATA@ZnO-Z with a concentration gradient on a 96-well plates for 48 h at 37°C. The mycelial density was detected at 540 nm by spectrophotometry at 12, 24, 36 and 48h.

1.5.2 In vitro anti-biofilms assay. *A. fumigatus* conidium (1×10^5 CFU/mL) were cultured in 12-well plates for 24h to form fungal biofilms. The fungal biofilms were incubated with 5 μg/ml ZnO-Z, NATA and NATA@ZnO-Z for 24 h. Then, biofilms were stained by 0.1% crystal violet solution or Aniline blue for 15 min. Ethanol was used to decolorize the crystal violet stained biofilm

and was detected OD values by microplate reader at 570 nm. Images of Aniline blue staining were captured by fluorescence microscope (Nikon, Tokyo, Japan,100x), and the fluorescence intensity was estimated using Image J.

1.5.3 Ultrastructural observation of A. fumigatus. *A. fumigatus* hyphae samples in PBS, NATA (5 μg/ml) and NATA@ZnO-Z (5 μg/ml) groups were fixed with 2.5% glutaraldehyde, and observed by SEM and TEM.

1.6 Determination of reactive oxygen species (ROS) in mycelia

Intracellular ROS accumulation in mycelia was evaluated using a REDOX sensitive fluorescence probe (DCFH-DA) [31]. *A. fumigatus* conidium suspension ($1x10^5$ /mL) was cultured in 12-well plates for 24h, and co-cultured with PBS, ZnO-Z (5 μg/ml), NATA (5 μg/ml), NATA@ZIF-8 (5 μg/ml) and NATA@ZnO-Z (5 μg/ml) for 1h. Then DCFH-DA (10 μmol/L) was added to each well. After incubation for 20 min in dark, the images were captured by fluorescence microscope and the fluorescence intensity was analyzed.

1.7 Determination of DNA Damage

The DNA damage of the *A. fumigatus* incubated with nanoparticles was investigated by measuring the concentration of DNA. The *A. fumigatus* hyphae were collected, and then treated with 5 μg/mL of ZnO-Z , NATA , NATA@ZIF-8 and NATA@ZnO-Z. A control sample was treated with PBS. After treatment for 6 h, DNA was extracted from all cells using a kit manufactured by Shenggong in China. Subsequently, the concentration of the extracted DNA was measured using a spectrophotometer.

1.8 Antioxidant activity assays

The superoxide dismutase (SOD), Catalase (CAT), and malonaldehyd (MDA) content examined were performed 6h after *A. fumigatus* hyphae incubated with 5 μg/ml NATA@ZnO-Z. Activity of the different enzymes was measured

by the detection kits according to the manufacturer's instructions. Sabouraud medium treatment was as control.

1.9 Biocompatibility evaluation of NATA@ZnO-Z in vitro and in vivo

1.9.1 Cell viability assay. Human corneal epithelial cells (HCECs) and RAW 264.7 cells were incubated in 96-well plates and co-cultured with different concentration of ZnO-Z , NATA and NATA@ZnO-Z in 100 μL medium for 24 h. Then, cells were treated with 10 μL of CCK-8 for 2 h. The absorbance was measured by a spectrophotometry at 450 nm.

Scratch wound assay. HCECs ($3x10^5$/mL) were seeded in 6-well plates until they reached about 90% confluence. 200 uL pipette tips were used to scrape several parallel lines on the cell layer. Followed with gently rinsing with sterile PBS. The medium without FBS but with ZnO-Z (5 μg/ml) , NATA (5 μg/ml) and NATA@ZnO-Z (5 μg/ml) were added to each group for 24 hours. Images were captured by microscope (Axio Vert; Germany, 100×).

1.9.2 Ocular toxicology studies (Draize eye test). The right eye of mouse were treated with PBS, ZnO-Z (5 μL, 100 μg/mL), NATA (5 μL, 5 % w/v) or NATA@ZnO-Z (5 μL, 100 μg/mL) 4 times a day for five days. The left eye managed with PBS was used as control. Corneal fluorescein staining (CFS) was assessed by microscopy of slit-lamp under a lamp of cobalt blue at 1, 3, and 5 days after local corneal administration. According to a previous publication, ocular changes are graded by a scoring criteria including corneal opacity score (0-4), iritis score (0-2) and conjunctival redness or swelling score (0-3). The total score was the sum of all scores obtained from the cornea, iris and conjunctivae [32]. Futhermore, H&E staining of the retina was carried out to verify the toxicity of nanoparticles *in vivo.*

1.10 Assessment of the polarization of RAW 264.7 cells

RAW 264.7 cells were co-cultured with inactivated *A. fumigatus* hyphae and different concentration of ZnO-Z, NATA, NATA@ZIF-8 and NATA@ZnO-Z in

1000 μL DMEM for 8 h. The expression mRNA level of TNF-α, IL-1β, IL-6 in RAW 264.7 cells were detected by PCR experiments (n = 3/group). The primers sequence of mice and fungi can be found in Table S1.

RAW 264.7 cells were incubated with CD86-PE, CD206-FITC and F4/80-PE/Cy7 antibodies. Then the proportion of F4/80+ CD86+ and F4/80+ CD206+ cells were detected by the flow cytometer (Beckman Coulter, USA) and analysed by FlowJo software.

1.11 Establishment and treatment of FK Model

Processing is in accordance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research, and the program was approved by the Medical Ethics Committee of the Affiliated Hospital of Qingdao University. Female C57BL/6 mice, aged 6 to 8 weeks and weighing between 20 and 30 grams, were anesthetized with 8% chloral hydrate. 5 μL of 1×10^8 CFU/mL *A. fumigatus* hyphae were used to infect the left eyes after corneal epithelium of mice were sacrificed. Topical treatment of ZnO-Z (100 μg/mL), NATA@ZnO-Z (100 μg/mL), or NAT (5 % w/v) started at 1 day post-infection (p.i.) and lasted 5 days with 4 applications per day. Corneas were detached entirely at 3 days p.i. and prepared for RT-PCR, immunofluorescence and HE staining. The progression of keratitis in mice was recorded with a camera under a slit lamp and scored clinically. Clinical scores were examined based on 12 point scoring system.The thickness of corneal opacity, surface regularity, and area of opacity were examined and marked from 0 to 4.

1.12 Assessment of the anti-inflammatory properties of NATA@ZnO-Z in mouse FK

The corneas (n = 3/group) of mice were separated at 3 days p.i. The protein levels of TNF-α, IL-1β and IL-6 in the mice corneas were detected by ELISA,

and the mRNA levels were detected by PCR experiments (n = 3/group). The primers used for PCR are exhibited in Table S1.

Neutrophils in FK corneas (n = 3/group) were incubated with Ly6G-PE, and CD11b-FITC antibodies. Then the proportion of neutrophils were investigated by the flow cytometer (Beckman Coulter, USA) and analysed by FlowJo software.

Mice eyeballs (n = 3/group) at 3 days p.i. were colleted and frozen. The frozen eyeballs were cut into 8 μm thick corneal slices. The explicit processes including blocked with goat serum and incubated with neutrophil marker antibody were implemented based on the prior approach [33]. Images were captured using a fluorescent microscope (400×). Futhermore, corneal slices were stained by HE to observe the changes including infiltration of neutrophil in corneas and edema of the cornea (n = 3/group).

1.13 Examination of the therapeutic efficacy of NATA@ZnO-Z on murine FK

Plate count was employed to quantify the number of visible fungal colonies on the plates, which could indicate viable fungi surviving on the cornea. Treated mice corneas at 3 days p.i. (n = 3/group) were homogenized and diluted in sterile PBS. 50 uL dilution was plated onto Sabouraud dextrose agar mediums for 24 hours. The number of viable fungal colonies grown on the plate was used to determine the fungal burden in the corneas.

2. Figures:

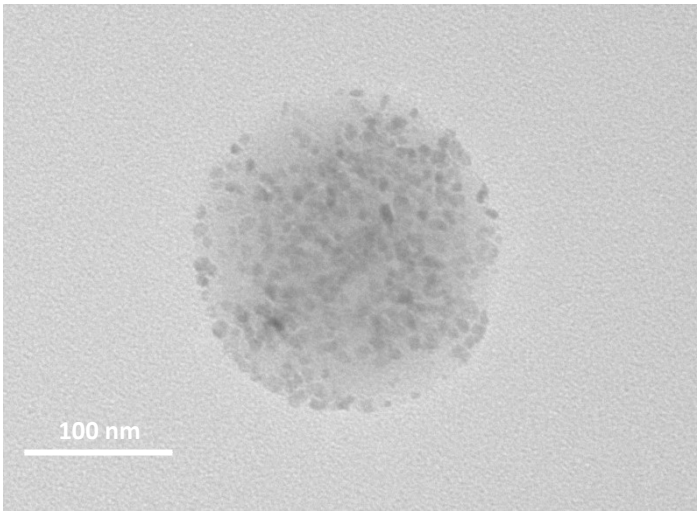

Fig. S1. TEM images of zno nanoparticels.

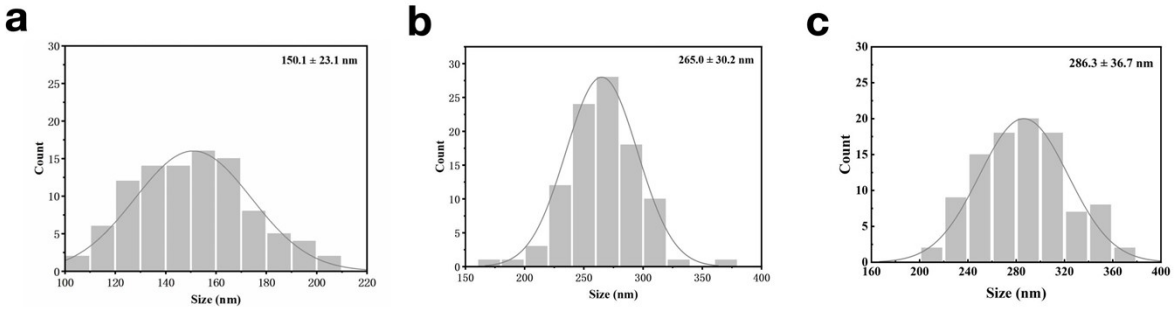

Fig. S2. Size distribution of ZnO (a), ZnO-Z (b), and NATA@ZnO-Z(c).

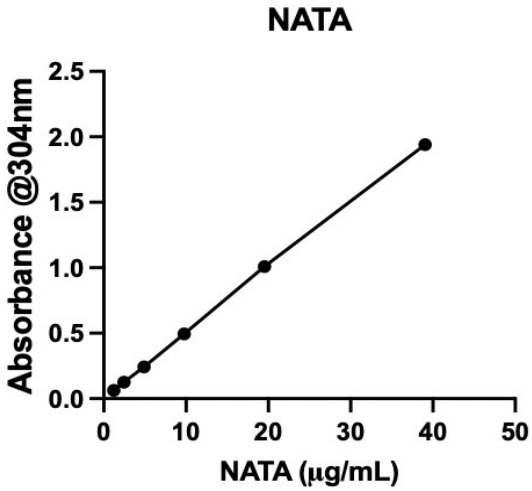

Fig. S3. NATA standard curve measured at 304 nm.

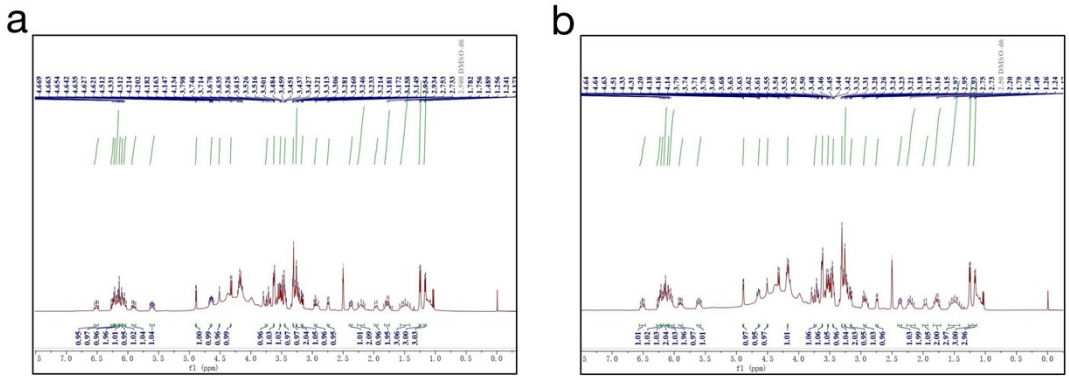

Fig. S4. ^{1}H-NMR spectra of NATA standard (a) and NATA relased from ZnO-Z (b).

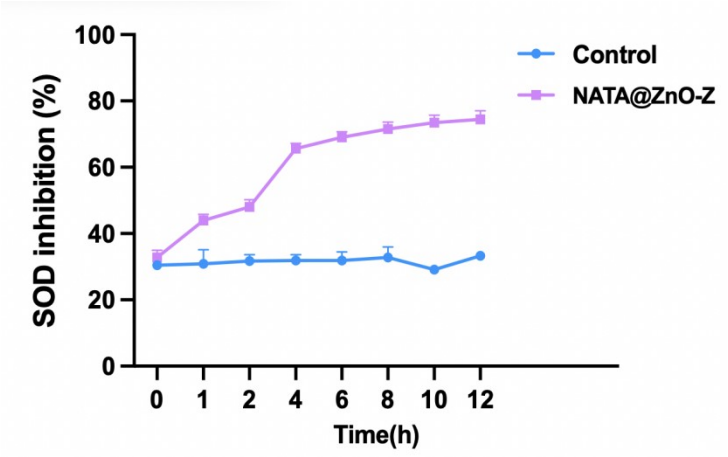

Fig. S5. SOD inhibition rate of *A. fumigatus* after treated with 5 μg/mL NATA@ZnO-Z for 12h.

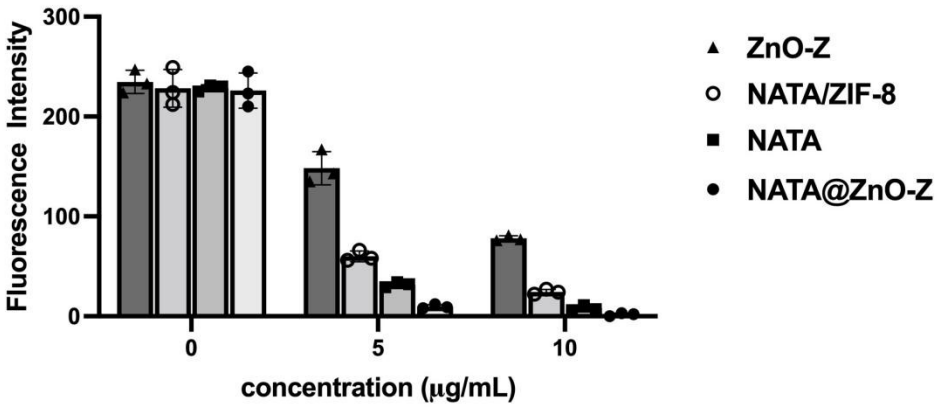

Fig. S6. Quantitative analysis of *A. fumigatus* hyphae stained by CFW.

Fig. S7. Biocompatibility evaluation of NATA and ZnO-Z. HCECs and RAW 264.7 cells activity at different concentrations of NATA (a, c) and ZnO-Z (b, d).

Table S1. Primers used for RT-PCR.

	Gene	GenBank no.	Primer sequence (5'-3')
mouse primers	β-actin	NM_007393.5	F: GATTACTGCTCTGGCTCCTAGC
			R: GACTCATCGTACTCCTGCTTGC
	IL-1β	NM_008361.4	F: CGCAGCAGCACATCAACAAGAGC
			R:TGTCCTCATCCTGGAAGGTCCACG
	IL-6	NM_001314054.1	F: TGATGGATGCTACCAAACTGGA
			R: TGTGACTCCAGCTTATCTCTTGG
	TNF-α	NM_013693.2	F: ACCCTCACACTCAGATCATCTT
			R: GGTTGTCTTTGAGATCCATGC
	18s rRNA		F: CTTAAATAGCCCGGTCCGCATT
			R: CATCACAGACCTGTTATTGCCG
A. fumigatus primers	ChsF	XM_742271.1	F: GCCTGGTTTACGTGTTGTGGA
			R: CATGGCCCTTGTCCTGTTCTC
	GelA	XM_744160.1	F: ACTCGGTCGACAACTCCAAGA
			R: GCGGTTGATGGAGTACTTGGG
	Ags 1	NW_020290899.1	F: TCTTCCGTGCTTGCGTTATCC
			R: CGGTCATCTTCCAGGTGTTGG