Near-infrared-triggered synergistic therapy with Ru-based nanocomposite hydrogel for eradicating multidrug-resistant wound infections

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Materials and method

Synthesis of Ru NPs

First, 0.5 g of cetyltrimethylammonium bromide (CTAB) was weighed and dissolved in 46 mL of deionized water. The solution was stirred at room temperature for 2 h, after which 12.9 mg of NaOH was gradually added. The mixture was then heated to 80 °C under continuous stirring. Subsequently, 32.6 mg of $RuCl_3$ was weighed, dissolved in deionized water, and slowly introduced into the aforementioned solution. The reaction was allowed to proceed at 80°C for 2 h. Then, 1.0 mL $NaBH_4$ (1.0 M) was added dropwise to the mixture. After an additional 2 h of stirring, the reaction solution was centrifuged and sequentially washed with acetone, hydrochloric acid, ethanol, and deionized water. Finally, the obtained product was dried under vacuum and designated as Ru NPs.

Synthesis of BBR@Ru NPs-PEI

The Ru NPs synthesized previously were dispersed in methanol. Berberine (BBR) was then added at a concentration ratio of 1:3 (Ru NPs: BBR) and dissolved via ultrasonication. The mixture was stirred at room temperature for 24 h to ensure the

loading of BBR onto the Ru NPs. Upon completion of the reaction, the product was isolated by centrifugation and sequentially washed with methanol and deionized water to obtain the BBR@Ru NPs.

Subsequently, polyethyleneimine (PEI) solution (5.0 mg/mL) was added to the suspension of BBR@Ru NPs. The mixture was stirred and subjected to ultrasonication for 2 h to achieve uniform dispersion. Finally, the resulting product was collected by centrifugation, washed thoroughly with deionized water, and dried under vacuum to yield the BBR@Ru NPs-PEI.

Synthesis of BBR@Ru NPs-PEI hydrogel (RBP hydrogel)

Briefly, 0.5 g of carboxymethyl chitosan (CMCS) was dissolved in 10 mL of deionized water. Subsequently, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) solutions were added sequentially to the CMCS solution. The BBR@Ru NPs-PEI was then introduced into the mixture and stirred thoroughly to ensure homogeneity. The reaction was allowed to proceed at room temperature for 30 min, resulting in the formation of the BBR@Ru NPs-PEI hydrogel (RBP hydrogel).

Analysis of RBP hydrogel photothermal property and photostability

Briefly, RBP hydrogels at varying concentrations (30 μg/mL, 60 μg/mL, 120 μg/mL) were exposed to 808 nm NIR light at different power densities (0.5 W/cm^2, 1.0 W/cm^2, and 1.5 W/cm^2) for 5 min. The temperature changes during this period were recorded to evaluate the photothermal performance. Additionally, the photothermal stability of the RBP hydrogel was investigated by subjecting it to 10 on-off cycles of

808 nm laser irradiation.

For the measurements of photothermal conversion efficiency, the RBP hydrogel aqueous solution (120 μg/mL) was irradiated by 808 nm laser. The temperature changes of the solution within 5 min were recorded by Fluke infrared thermal imager (recorded once every 60 s). The photothermal conversion efficiency (η) was calculated by Equation (1).

$$\eta = \frac{hA(T_{max} - T_{surr}) - Q_{dis}}{I(1 - 10^{-A\lambda})} \quad (1)$$

T_{surr} and T_{max} respectively represent the ambient temperature and the equilibrium temperature that RBP hydrogel can reach after being irradiated by laser. Aλ indicates the absorbance of RBP hydrogel at 808 nm. I represents the laser power, and Q_{dis} indicates the heat loss. Therefore, in Equation (1), only hA is unknown. Subsequently, we calculate hA using Equations (2) and (3).

$$\theta = \frac{T - T_{surr}}{T_{max} - T_{surr}} \quad (2)$$

$$\tau = \frac{m_p c_p}{hA} \quad (3)$$

θ, τ, c_p and m_p are dimensionless constants, the time constant of the sample, the specific heat capacity of water, and the mass of the RBP hydrogel solution respectively. Through Equation (4), we can calculate the time constant (τ).

$$\tau = \frac{t}{-ln(\theta)} \quad (4)$$

Based on the above equation, the photothermal conversion efficiency (PCE) of RBP hydrogel can be calculated.

In vitro antibacterial activity of RBP hydrogel

The *in vitro* antibacterial activity of the RBP hydrogel against Methicillin-resistant *Staphylococcus aureus* (MRSA) and *Pseudomonas aeruginosa* (*P. aeruginosa*) was evaluated using the coating plate method. Briefly, bacterial suspensions were incubated with RBP hydrogels at varying concentrations (30, 60, and 120 μg/mL) for 1 h, with sterile PBS solution serving as the control. For the illumination group, the samples were irradiated with an 808 nm laser (1.0 W/cm^2) for 5 min. Subsequently, 50 μL from each reaction solution was spread onto LB agar plates. After overnight incubation at 37°C, the colonies on each plate were counted to determine bacterial survival rates..

To further assess bacterial viability, the bacterial solutions were stained with propidium iodide (PI) and 4',6-diamidino-2-phenylindole (DAPI) for live/dead staining, while ROS levels were detected using 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA). Additionally, the protein content of each group was quantified using a BCA protein concentration assay kit (Jiancheng Biotechnology Co., Nanjing, China), and nucleic acid content was measured using a DNA and RNA assay kit (TIANGEN BIOTECH, Beijing).

In vitro biofilm removal assays

MRSA and *P. aeruginosa* (OD 0.6-0.8) were inoculated into a 96-well plate and cultured at 37°C for 48 h to allow biofilm formation. The biofilms were treated with RBP hydrogel, either with or without NIR irradiation (808 nm, 1.0 W/cm^2, 10 min). After treatment, the supernatant was removed and the biofilms were fixed with 99% methanol for 15 min. Subsequently, they were stained with 0.2% crystal violet for 30

min. After washing three times with PBS to remove unbound dye, the crystal violet retained by the biofilm was dissolved in ethanol. The absorbance of the eluted dye was measured at 570 nm to quantify the residual biofilm biomass.

Additionally, the treated biofilms were visualized using laser confocal microscopy. Briefly, biofilms subjected to different treatments were fixed with 4% glutaraldehyde overnight. After discarding the supernatant and washing with PBS, the biofilms were stained with SYBR Green I for 30 min in the dark. The biofilm morphology of each group was then observed using confocal laser scanning microscopy (Leica TCS SP8).

To further investigate the effects of the treatments, total RNA was extracted from bacteria following different experimental conditions. The relative expression levels of virulence factors were analyzed by reverse transcription quantitative polymerase chain reaction (RT-qPCR) using the primers listed in Table S1.

In vivo wound healing experiment

A mouse wound model of MRSA infection was established to evaluate the *in vivo* antibacterial efficacy of the RBP hydrogel. Under sterile conditions, a wound with a diameter of approximately 8.0 mm was created on the dorsal skin of BALB/c mice (6–8 weeks old, 18–22 g, female). Subsequently, 100 μL of MRSA suspension (10^7 CFU/mL) was subcutaneously injected into the wound site and allowed to incubate for 24 h. The mice were then randomly divided into four groups: PBS-$NIR_{(+)}$, PBS-$NIR_{(-)}$, RBP hydrogel-$NIR_{(-)}$, RBP hydrogel-$NIR_{(+)}$. The NIR irradiation groups were exposed to an 808 nm laser (1.0 W/cm^2) for 30 s.

On days 0, 1, 3, 5, 7, and 9 of treatment, wound areas were photographed and the body weights of the mice were recorded. Bacterial colonies from the wound sites were collected, smeared, and quantified to assess bacterial load. On day 9, all mice were euthanized, and tissues from the heart, liver, spleen, lung, kidney, and epithelial regions were harvested for hematoxylin and eosin (H&E) staining and Masson's trichrome staining to evaluate tissue morphology and collagen deposition. Additionally, blood samples were also collected for biochemical analysis. All the animal tests were approved by the animal care and experiment committee of Tianjin University of Science & Technology.

Results

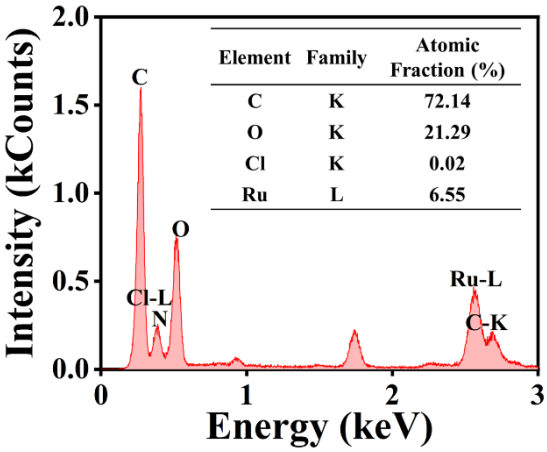


Fig. S1. EDS analysis of BBR@Ru NPs-PEI.

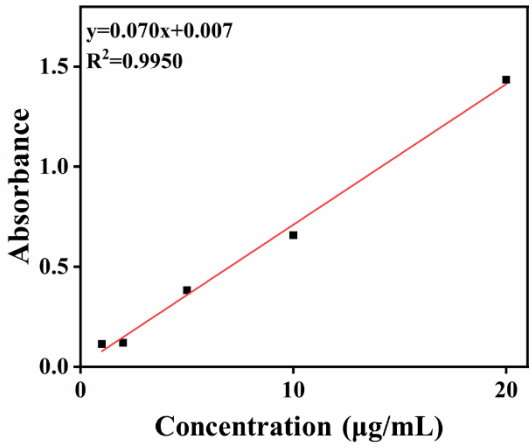


Fig. S2. The UV-Vis absorption of BBR at different concentrations.

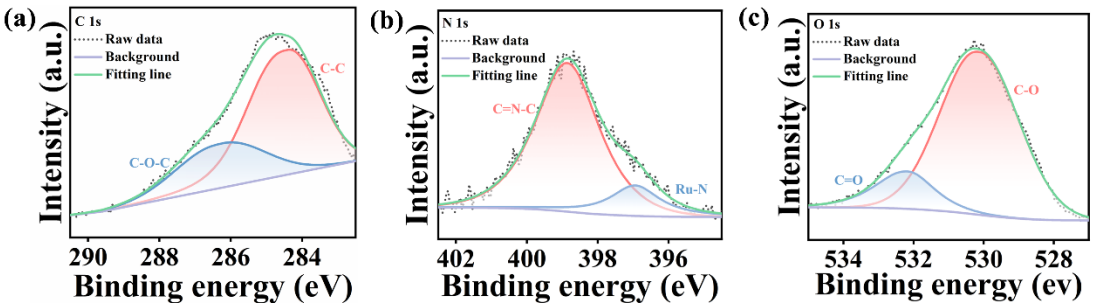
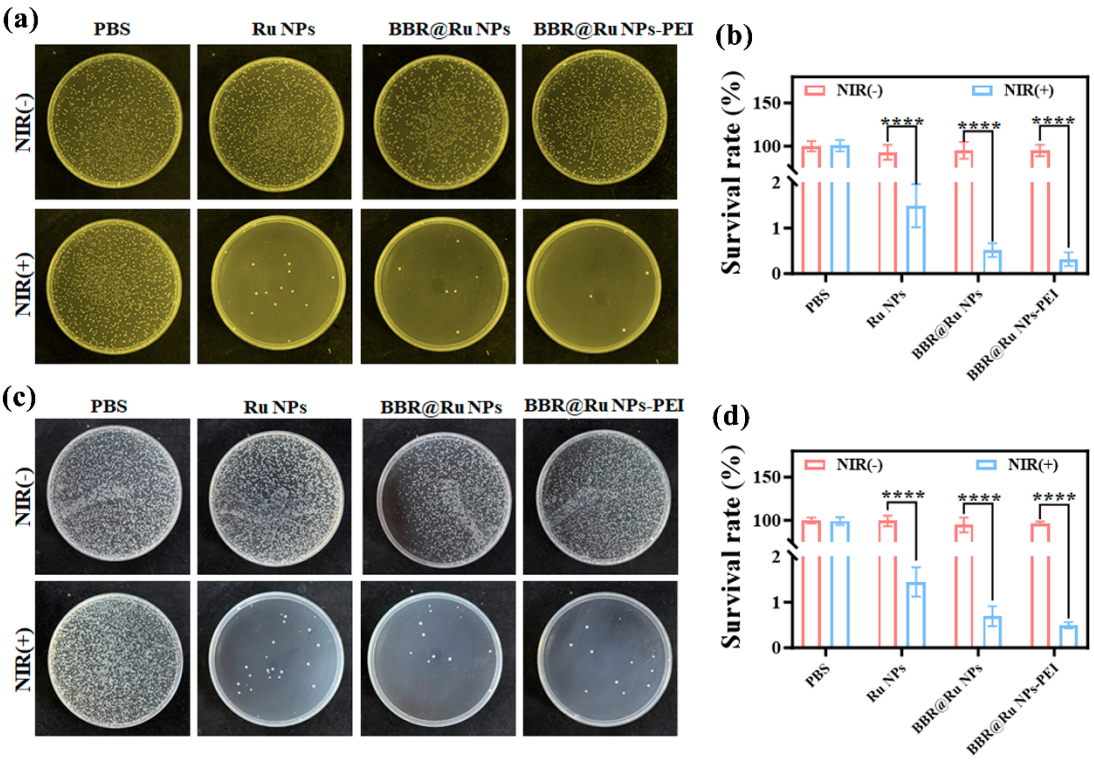


Fig. S3. High-resolution XPS spectra of C 1s (a), N 1s (b) and O 1s (c) orbitals.



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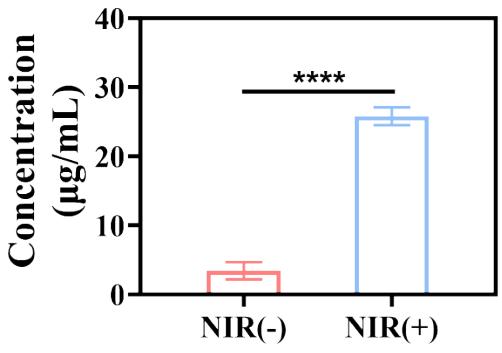
126 **Fig. S4.** MRSA (a) and *P. aeruginosa* (c) colony images after treatment with Ru NPs,

127 BBR@Ru NPs or BBR@Ru NPs-PEI (120 μg/mL). The survival rate of MRSA (b) and

128 *P. aeruginosa* (d) (n=3). Error bars represent means ± SD. Differences between groups

129 were tested using one-way ANOVA followed by Tukey's multiple comparisons test.

130 ****$p < 0.0001$.



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132 **Fig. S5.** Determination of BBR Release (n=3). Error bars represent means ± SD.

133 Differences between groups were tested using one-way ANOVA followed by Tukey's

134 multiple comparisons test. ****$p < 0.0001$.

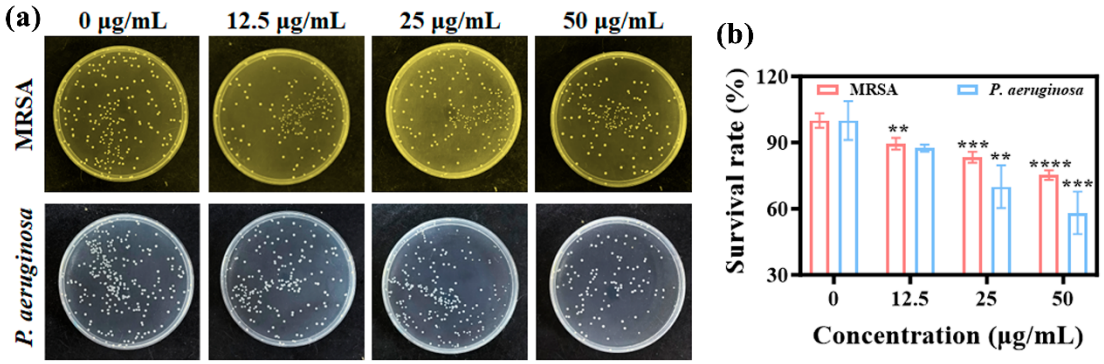

Fig. S6. (a) MRSA and *P. aeruginosa* colony images after treatment with BBR at different concentrations (0-50 μg/mL). (b) The survival rate of MRSA and *P. aeruginosa* (n=3). Error bars represent means ± SD. Differences between groups were tested using one-way ANOVA followed by Tukey's multiple comparisons test. **p < 0.01, ***p < 0.001, ****p < 0.0001.

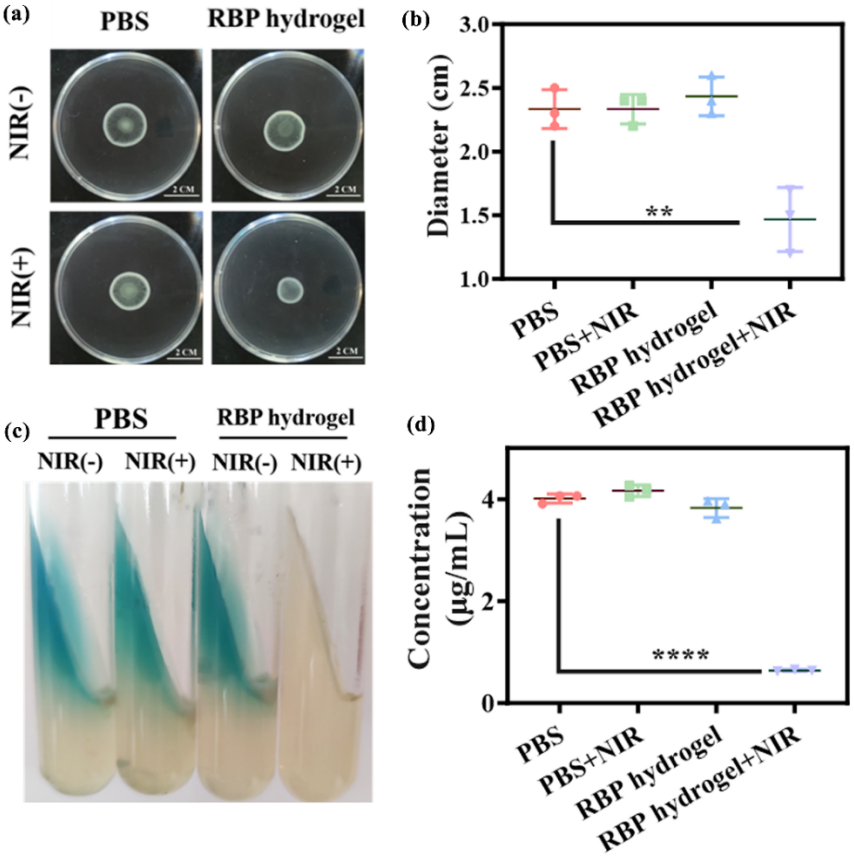

Fig. S7. (a) Swarming motility and (b) corresponding colony diameters of *P. aeruginosa* following different treatments (n=3). Pyocyanin secretion (c) and its

corresponding quantification (d) following different treatments (n=3). Error bars represent means ± SD. Differences between groups were tested using one-way ANOVA followed by Tukey's multiple comparisons test. **p < 0.01, ****p < 0.0001.

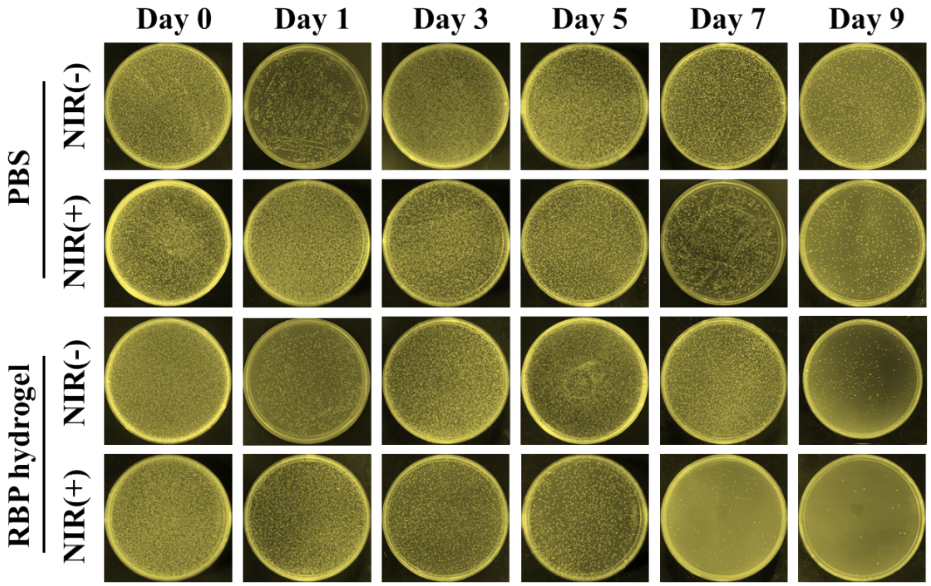

Fig. S8. Bacterial colonization in wound of mice after different treatments.

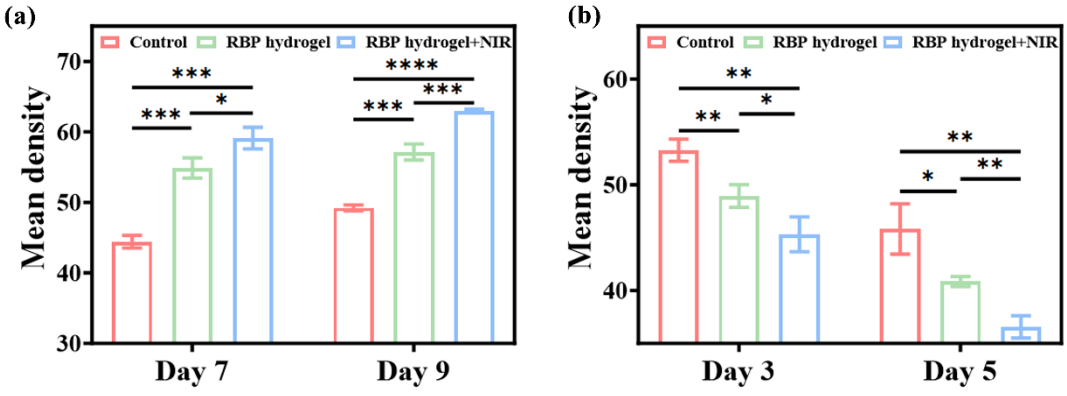

Fig. S9. Quantification of IL-6 (a) and VEGF (b) fluorescence intensity (n=3). Error bars represent means ± SD. Differences between groups were tested using one-way ANOVA followed by Tukey's multiple comparisons test. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.