Supporting information

Stimuli-responsive drug delivery, antioxidant and photothermal antibacterial hydrogel wound dressing for MRSA-infected pressure ulcer repair

Materials and methods

Materials

Hyaluronic acid (Mn=1300000–1400000 Da) was purchased from Bloomage Freda Biopharm Co., Ltd., dopamine hydrochloride, 1-hydroxybenzotriazole (HoBT), 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC), N-Hydroxysuccinimide (NHS), quercetin, and 3-aminophenylboronic acid (PBA) were purchased from Macklin, China.

Synthesis of 3-aminophenylboronic acid modified HA (HA-PBA) and quercetin-loaded HA-PBA (HA-PBA-Q)

HA-PBA was synthesized according to the previous study [1], as shown in Figure S1a. 1 mmol PBA was added to 0.5 % HA solution (1 mmol), and 1 mmol 1-hydroxybenzotriazole (HOBT) and 1 mmol EDC were dissolved in DMSO/H_2O mixture with a volume ratio of 1. The mixture was then added into the HA and PBA solution. The pH was adjusted to about 4.75 for 4 h, and subsequently was adjusted to 7.5 to terminate the reaction. The obtained solution was dialyzed for three days and lyophilized for preservation.

HA-PBA-Q was synthesized by further modifying HA-PBA with quercetin. 0.5 mmol quercetin solution was added to the HA-PBA reaction solution and the pH was adjusted to 7.4 to let them react overnight. The product was dialyzed for 3 days and freeze-dried

for further usage.

Synthesis of dopamine modified HA (HA-DA) and polydopamine (PDA)

HA-DA was synthesized according to the literature [2] (Figure S1b). 1 g HA powder was dissolved in deionized water to obtain 0.5 % HA solution and adjust its pH to about 5.5 with 1 M HCl solution. Then, 575 mg EDC and 345 mg NHS were added. After fully dissolved and stirred for 20 min, 569 mg DA was added, and the pH of the reaction solution was maintained at 5.5 for 3 h. The reaction was carried out a nitrogen atmosphere. The obtained solution was dialyzed for three days against distilled water to remove unreacted chemicals, freeze-dried, and stored in darkness.

PDA was synthesized using the methods reported previously [3]. 180 mg DA was dissolved in 90 mL dd water, followed by the addition of 400 μL NaOH solution (1 M), stirring vigorously at 50 ^{o}C for 3 h. With the reaction going on, the solution turned from shadow yellow to dark brown. The product was collected by centrifuging the solution at 10,000 rpm for 10 min and washed with DD water for 3 times. The final product was obtained by drying in the vacuum oven.

Preparation and characterization of HA-DA/HA-PBA (HD/HP) hydrogel

HA-PBA was dissolved in PBS (pH 7.4) to obtain a 2% precursor solution A. HA-DA is dissolved in deionized water to form a 2% precursor solution B. The pH of each precursor solution was adjusted to about 7.4 before mixing. Firstly, HD/ HP hydrogels were prepared using HA-DA and HA-PBA with different grafting ratio of DA and PBA on HA and named HD/HP-0.6, HD/HP-1.0, HD/HP-1.2, HD/HP-1.6 hydrogels according to the ratio of catechol group to phenylboronic acid group (0.6, 1.0, 1.2 and

1.6). Hydrogels were then optimized by incorporating PDA to prepare HD/HP-X/PDA hydrogels, incorporating quercetin to obtain HD/HP-X-Q hydrogels, and incorporating both PDA and quercetin to obtain HD/HP-X/PDA-Q hydrogels (Figure 1), where X represents the ratio of catechol group to phenylboronic acid group.

Effects of different concentrations of quercetin on the viability of fibroblasts

67.6 mg of quercetin was dissolved in 1 mL of dimethyl sulfoxide (DMSO) to form a stock solution of 200 mM. The stock solution was diluted with DMEM medium containing 10 % fetal bovine serum to 200 μM, 100 μM, 50 μM, 25 μM, 10 μM, 5 μM, 2.5 μM, 1 μM, 0.5 μM, and 0.1 μM, and the final concentration of DMSO was not more than 0.1%. L929 cells were seeded in 96-well plates and cultured with the above diluents for 24 h. The cells were incubated with AlamarBlue® solution according to the vendor's protocol for 4 h, and then the cell viability was measured by a microplate reader.

Stimuli-responsive properties of the HD/HP hydrogels

The hydrogels were stained with rhodamine dye, and then the hydrogels were injected into pH 7.4 PBS, pH 5.5 PBS, and 3 % H_2O_2 solution using 18 G needles. The status of the hydrogels after 30 min and 60 min in different media and the diffusion degrees of rhodamine dye were observed.

Mechanical properties, swelling ratio, injectability, and self-healing properties of hydrogels

The mechanical properties of hydrogels including modulus, fracture point and self-healing behavior were measured using a TA rheometer (DHR-2). A 500 μL hydrogel is

placed at a height of 1000 μm between 20 mm parallel plates and sealed with silicone oil to prevent water evaporation. At 37 °C, the modulus of these hydrogels was measured by time-sweeping test at a constant strain of 1% and a frequency of 1 rad/s. The swelling ratio of the hydrogel was measured by the weight changes before and after soaking in PBS. Detailly, 500 μL of the hydrogel was weighed and placed in PBS (pH 7.4) and placed in a shaker at 37 °C, 100 rpm. The hydrogel was taken out and weighed within the specified time and the swelling ratio was calculated according to the following formula:

$SR\ (\%) = (w_t - w_0)/ w_0 \times 100\%$,

Where: SR-swelling ratio /%; w_t – the weight of hydrogel after swelling (mg); w_0-initial weight of hydrogel (mg).

The hydrogel was injected into a plate with an 18 G needle to form different letters (XJTU) to show its injectability.

The breaking point of the hydrogel was measured by recording the value of the critical strain region using the strain amplitude scanning method (γ from 1 % to 2000 %). The self-healing property of hydrogels was confirmed via macroscopic and quantitative ways. For the macroscopic display, a hydrogel was cut into two pieces and then placed together to testify its self-healing behavior, or two hydrogels were placed together to observe whether self-healing occurs; the quantitative test was performed using a TA rotary rheometer with a parallel plate geometry (20 mm diameter) at a fixed angular frequency of 1 rad/s and 25 °C. After loading the hydrogel sample and setting a 1 mm measuring gap, the sample was allowed to equilibrate for 3 min to release residual

stresses. An alternating strain scanning test was then performed using 1 % strain (within the linear viscoelastic region, below the breaking strain) and 1300 % strain (above the breaking strain) for cyclic measurements. Each strain level was applied for an interval of 60 s, and the sequence was repeated for 5 cycles. Storage and loss moduli were recorded continuously throughout the test.

Blood compatibility and cytocompatibility of hydrogels

500 μL of hydrogel was prepared in a 24-well plate, using the same volume of PBS solution and 0.1 % Triton X-100 as negative and positive controls, respectively, and the plate was placed in a 37 °C shaker to preheat for 0.5 h. The obtained red blood cell diluent with heparin sodium anticoagulant was added to the plate (500 μL per well). After incubation at 37 °C for 1 h, the red blood cells in each well were gently blown up and transferred to a new centrifuge tube, centrifuged at 116 × g for 10 min, and then 100 μL of liquid was taken from the supernatant and added to a 96-well tissue culture plate. The absorbance of the solution was read at 540 nm using a microplate reader. Hemolysis ratio is used to evaluate the blood compatibility of the material. Hemolysis ratio is calculated as:

$HR\ (\%) = [(A_p\text{-}A_b)/(A_t\text{-}A_b)] \times 100\%$

HR-hemolysis ratio /%; A_p - the absorbance value of experimental group; A_t -Triton X-100 (positive control) group's absorbance value; A_b -PBS (negative control) group's absorbance value.

The cytotoxicity of the hydrogel was evaluated by culturing L929 cells with the extract of the hydrogel. The sterilized hydrogel was immersed in DMEM medium at 37 °C for

24 h to obtain different concentrations of hydrogel extract solution (10 mg/mL, 5 mg/mL and 2.5 mg/mL). First, L929 cells were seeded on a 96-well plate at the density of 10,000 cells/well. Then, the culture medium was replaced with the above different concentrations of hydrogel extract. After 24 h of culture, the cells were incubated with 10 % AlamarBlue® solution for 4 h and the fluorescent intensity at 560 nm excitation wavelength and 600 nm emission wavelength was read by a microplate reader. In this study, cells cultured in growth medium (DMEM with 10% fetal bovine serum and 1% penicillin/streptomycin) on TCP were used as a control group. Each group has 6 repetitions.

Stimuli-responsive drug release behavior and antioxidant performance of hydrogels

The stimuli-responsive drug release behavior of the hydrogel is tested by placing 200 μL of the hydrogel in 4 mL of PBS (pH 7.4), PBS (pH 6.0), and 1.5% H_2O_2 solution, respectively, and incubating at 37 °C, shaking at a speed of 100 rpm. UV analysis was taken according to the specified time intervals. The measured results were accumulated to obtain the cumulative release amount.

The antioxidant properties of hydrogels were tested according to the method of DPPH scavenging provided by previous literature [4]. The hydrogel (300 μL) was placed in a 5 mL centrifuge tube and 1.8 mL ethanol was added to the hydrogel. The system was placed in a shaker at 37 °C. 200 μL 1 mM DPPH ethanol solution was added to each tube. All centrifuge tubes were placed in darkness and incubated at 37 °C for 30 min. After incubation, the supernatant of each sample was obtained and the absorbance of

the supernatant at 517 nm was measured by UV-vis spectrometer. The continuous antioxidant capacity of the hydrogel is carried out by continuously scavenging DPPH free radicals. Firstly, the hydrogel (300 μL) was placed in a 5 mL centrifuge tube, 1.8 mL ethanol and 200 μL 1 mM DPPH ethanol solution was added, and then incubated in the dark at 37 °C for 30 min, and then all the liquid was used for UV test. Then, 1.8 mL of ethanol and 200 μL of 1 mM DPPH ethanol solution were added and incubated in the dark at 37 °C for 30 min, then all the liquid was used for UV test. Repeat the above steps and the accumulated DPPH scavenging ratio was calculated. The time points for testing were set as 0.5 h, 1 h, 1.5 h, 2 h, 2.5 h, 3 h, 3.5 h, 4 h, 5 h, 6 h, 7 h, 8 h, 9 h and 10 h. The antioxidant properties of the hydrogel can be measured using the DPPH scavenging ratio formula:

DPPH scavenging ratio (%) = $(A_b\text{-}A_h)/A_b$*100%,

A_b represents the absorbance of the blank group (DPPH with ethanol); A_h represents the absorbance of the samples (DPPH with ethanol and samples).

In vitro quercetin (Q) release under the conditions estimating in vivo NIR irradiation has been performed by expose the HD/HP-1.0/PDA-Q hydrogel to NIR irradiation (1.4 W/cm^2) for 0, 1, and 10 min respectively. Afterwards, the hydrogel was put in the PBS solution (pH 7.4, 25 °C) to measure the release of the quercetin. And an untreated hydrogel was used as a blank control.

Photothermal and photothermal antibacterial performance of hydrogel

The hydrogel was placed on a glass slide, irradiated with NIR light with a power density of 1.4 W / cm^2 and the temperature was recorded using a thermal imager (30 s interval,

600 s in total) to obtain a hydrogel temperature-time curve. In addition, NIR irradiation at different power densities (1.0 W/cm^2, 1.4 W/cm^2 and 1.8 W/cm^2) was used to test the photothermal adjustability of hydrogels containing PDA. The NIR irradiation power density was calculated using the method below: Use a calibrated optical power meter to measure the total output power (P, in watts) of the 808 nm laser at the target position, then determine the spot area (A, in cm^2) at the same position. Finally, calculate the optical density (power density) as OD = P / A (W/cm^2).

The photothermal antibacterial properties of hydrogels were tested by the photothermal antibacterial experiments against *E. coli* and MRSA. Firstly, 10 μL (10^8 CFU mL^{-1}) bacterial suspension (*E. coli* or MRSA) was added to 200 μL hydrogel, and then irradiated with 808 nm near-infrared laser at 1.4 W/cm^2 for 0, 1, 3, 5, and 10 min, respectively. The bacteria on the hydrogel were resuspended and seeded on agar plates, and cultured overnight at 37 °C. At the same time, 10 μL bacterial suspension (10^8 CFU mL^{-1}) was added to 200 μL PBS as a negative control, and also exposed to 808 nm NIR (1.4 W/cm^2) for the same time. The colony-forming units on agar plates were calculated. The antibacterial ratio was used to evaluate the photothermal antibacterial ability of the hydrogel.

Inhibition ratio (%) = (number of control bacteria-number of surviving bacteria after photothermal treatment)/number of control bacteria × 100 %.

Hydrogel's effect on infected pressure ulcer wound's repair

A mouse model of pressure ulcer was established by the ischemia-reperfusion method[5, 6]. Kunming mice (5 ~ 6 weeks, 30-35 g, female) were randomly divided

into 3 groups, each group contained 6 mice (n=6), anesthetized with 10 % chloral hydrate solution (0.33 g/kg), shaved off the back hair, and disinfected with 75 % ethanol. Marks on both sides of the back midline of the mouse were made to ensure that each animal's modeling position is consistent. Gently pull the skin up between two round strong magnets (diameter of 10 mm, thickness of 3 mm, average weight of 1.5 g, 1000 g magnetic source) (Castle Rock, Co.). The back skin of the mice was clamped by two magnets and formed a 2.0 mm skin bridge. Each animal was kept separately in a non-magnetic plastic cage to ensure freedom of food, water and activity. The magnet was fixed for 16 h for ischemia, and then was released for 8 h for reperfusion. This was one cycle and two cycles were performed. On the second day after the end of the two cycles, the mice were euthanized and samples of the wound at the modeling site and the skin around the wound were collected. After fixation in 4 % paraformaldehyde solution, hematoxylin-eosin (H&E) staining was performed to observe the histological morphology of the pressure ulcer wound.

The successful pressure ulcer animals were modeled for the next infection wound formation. Firstly, Kunming mice (female, 30-35 g) were anesthetized by intraperitoneal injection of 10 % chloral hydrate solution (0.33 g/kg), and 10 μL MRSA (10^8 CFU / mL) bacterial suspension was injected on the pressure ulcer wound to establish an infection model. Subsequently, hydrogel materials were applied to the wounds of each group and fixed with Tegaderm™ transparent dressing (3M Healthcare Company, USA). The photothermal group was irradiated with a near-infrared laser at a power of 1.4 W/cm^2 for 10 min. In this experiment, the wounds of mice treated with

Tegaderm^{TM} transparent skin dressing alone were used as the blank control group. On the 3rd, 7th and 14th day of the experiment, the mice were euthanized, the wound condition was photographed and the wound area was measured by the same researcher who has performed the treatments. The hydrogel was replaced each time the wound size was measured, and the Tegaderm dressing in the control group was also replaced accordingly. During the replacement procedure, no additional wound care was performed. The data were processed by Image J calculation. Wound healing rate (%) was calculated according to the following formulation:

Wound healing rate (%) = (area (0 days) -area (n days))/area (0 days) × 100%,

At the settled time, the mice were euthanized, and the pressure ulcer wound was cut off together with the surrounding normal skin. The obtained skin tissue was fixed with 4 % paraformaldehyde solution, and the wound samples were histologically tested. H&E staining (Biyuntian, China) was used to evaluate the properties of the healed skin tissue; CD31 (Yike Bio, China) for evaluating new blood vessels [7-9]; CD68 (Yike bio, China) is used to stain macrophages to assess the level of inflammation of the wound [10]. All skin sections are photographed and analyzed using a microscope (Olympus IX53, Japan).

Statistical analysis

The data obtained were tested by one-way analysis of variance, and expressed as mean ± standard error, *p indicated significant difference (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

Results

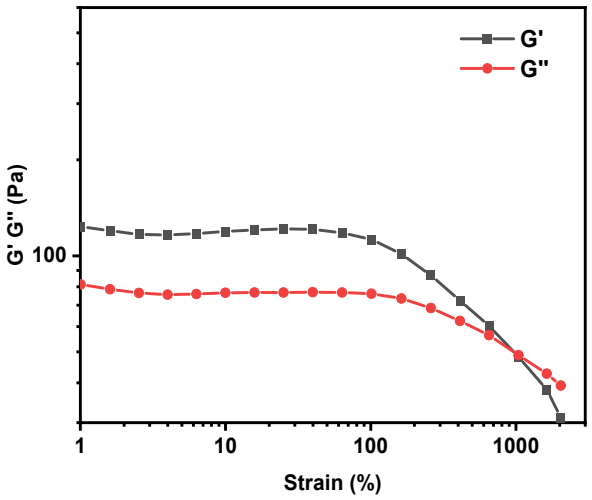

Figure S1 The critical strain point (the intersection of G' and G" curves) of the critical state of solid and fluid that was obtained by strain amplitude sweeping experiment.

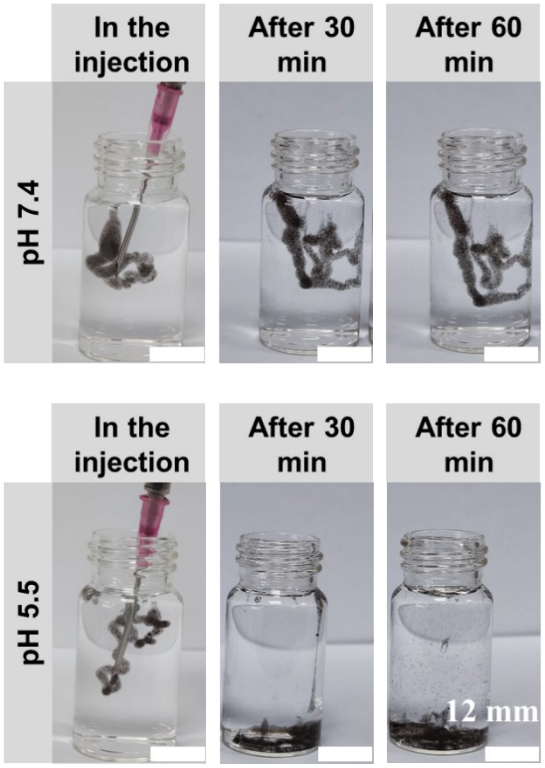

Figure S2 pH-responsive behavior of the HD/HP-1.0/PDA hydrogel.

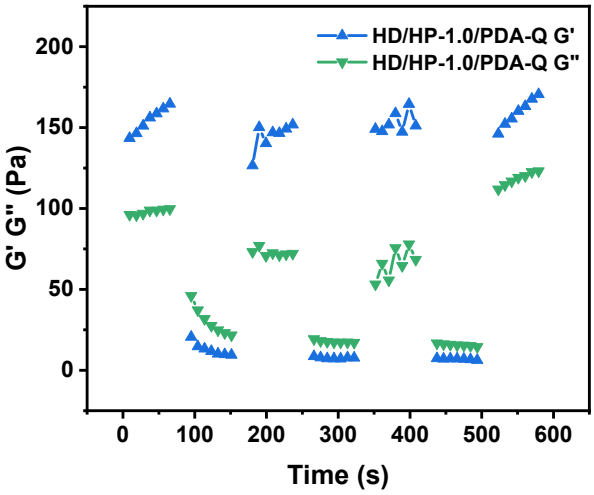

Figure S3 Self-healing behavior of HD/HP-1.0/PDA-Q hydrogel confirmed by the rheological testing.

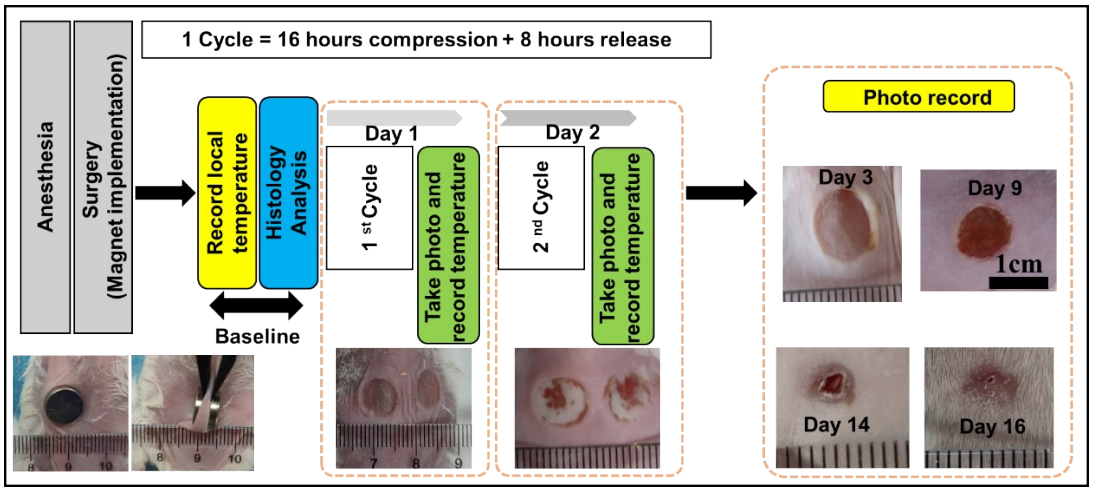

Figure S4 Process of the establishment of pressure ulcer mouse model.

Table S1 Skin temperature at different time points during the pressure ulcer model establishment

Normal skin (pretreatment), baseline	Immediately following I/R	After I/R 30 min
34.9 ± 0.1 ℃ (n = 7)	33.9 ± 0.2 ℃ (n = 7)	36.3 ± 0.2 ℃ (n = 7)

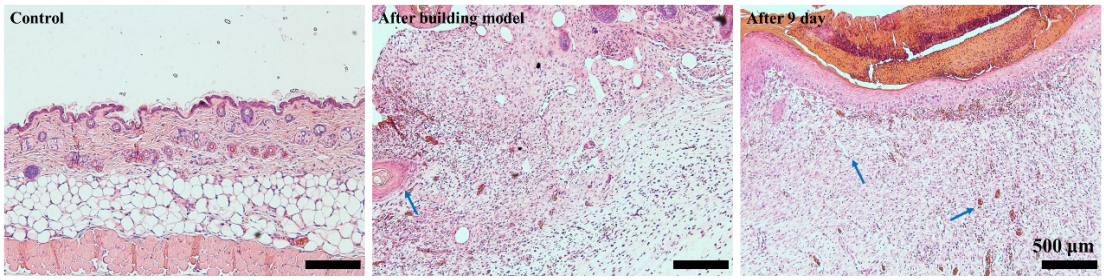

Figure S5 H&E staining of the normal skin and the pressure ulcer skin immediately after the model building and 9 days after model building.

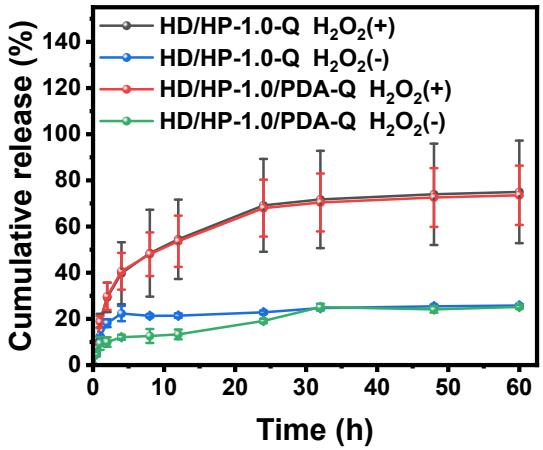

Figure S6 Cumulative release of quercetin (Q) from HD/HP-1.0-Q and HD/HP-1.0/PDA-Q hydrogels when treated with 500 μM H_2O_2.

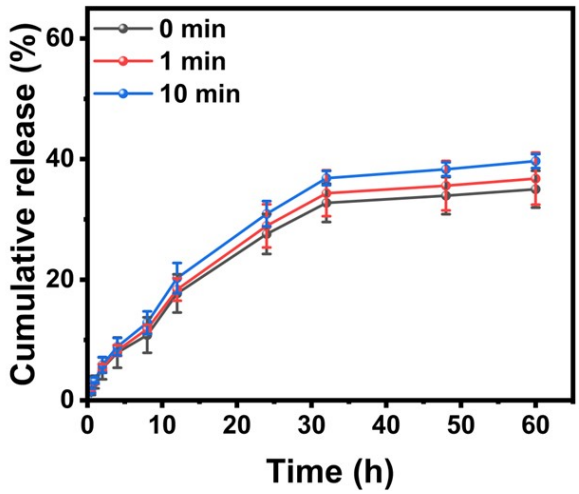

Figure S7 Cumulative release of quercetin (Q) from the HD/HP-1.0/PDA-Q hydrogels when treated with NIR irradiation for different durations.

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