Supplementary Information for:

A pH-responsive hydrogel with potent antibacterial activity against both aerobic and anaerobic pathogens

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This supplement includes:
Materials and Methods (Page S2-S9)
Supplementary Figure 1 to 4 and Table 1 (Page S10-S14)
**Materials.**

Amine terminated and ethylenediamine-cored generation 1 (G1) polyamidoamine (PAMAM) dendrimer was purchased from Dendritech, Inc. (Midland, USA). Ornidazole was purchased from Macklin, Inc. (Shanghai, China). Tobramycin was purchased from Meilun, Inc. (Dalian, China). Dextran (450-650 kDa) was purchased from Sigma-Aldrich (St. Louis, USA). O-phthalaldehyde, 3-mercaptopropionic acid, boric acid, hydroxylamine hydrochloride, potassium carbonate, sodium periodate, sodium chloride, sodium dihydrogen phosphate and disodium phosphate were purchased from Aladdin Reagent (Shanghai, China). Tryptone, Yeast extract, Mueller-Hinton broth (MHB) and tryptic soy broth (TSB) were purchased from Oxoid, Inc. (Basingstoke, UK). Defibrinated Sheep Blood was purchased from YuanMu Bio. (Shanghai, China). Agar, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sangon Biotech (Shanghai, China). Live/dead Baclight™ Bacterial Viability Kits (L13152) was purchased from ThermoFisher (Carlsbad, USA). AnaeroPouch™-Anaero was purchased from Mitsubishi Gas Chemical Company Inc., (Tokyo, Japan). *Staphylococcus aureus* (*S. aureus*, USA 300), *Pseudomonas aeruginosa* (*P. aeruginosa*, PAO1), and *Escherichia coli* (*E. coli*, DH5α) were obtained from ATCC. *Clostridium sporogenes* (*C. sporogenes*, ATCC 11437), and *Bac aeroides fragilis* (*B. fragilis*, ATCC 25285) were obtained from FuXiang Bio. Co. Ltd (Shanghai, China).

**Synthesis and characterization of G1-orni and dextran aldehyde.**

To synthesis the G1-orni conjugation, 250 mg G1 (0.17 mM, 100 mg/mL in dimethyl
sulfoxide) was added in a vial, and 153.6 mg ornidazole (0.7 mM, 50 mg/mL in dimethyl sulfoxide) was then added dropwise into the G1 solution, followed with the addition of 115.8 mg K$_2$CO$_3$ (0.84 mM). The mixture was stirred gently at 60 °C for two days, and then the solvent was removed by freeze-drying. The dried products were dissolved in 2 mL methanol, and precipitated in ethyl ether, after filtration, the product was dried overnight in a vacuum drying oven, and obtained as brown powders. $^1$H NMR and $^{13}$C NMR spectra of the products was performed on a 500 MHz NMR spectrometer (Varian Instrument, USA) at 298.2 ± 0.1 K. The molecular weight was determined by Mass Spectrometry (ABI 4800 plus, USA).

The synthesis of dextran aldehyde (Dex-CHO) was performed as previously described.\(^1\) Generally, 8 mL sodium periodate (0.5 M, 107 mg/mL) was added dropwise into the dextran solution (1 g, 10% w/v), and the mixture was stirred gently in dark for 4 h at room temperature. The reaction was then stopped by the addition of ethylene glycol (10% v/v) and stirred for another 1 h. The solution was fully dialyzed against deionized water, and then concentrated into 50 mg/mL with the ultrafiltration centrifuge tube (6000 r/min, 30 min). The solution was stored under 4 °C and nitrogen atmosphere before use. The oxidation degree of dextran was measured to be 50% by a colorimetric hydroxylamine titration method.\(^2\)

**Preparation and characterization of the antibacterial hydrogels.**

Generally, the antibacterial hydrogel was prepared by mixing the G1-orni solution (150 µL, 200 mg/mL) with tobramycin solution (50 µL, 50 mg/mL), and then added with Dex-CHO aqueous solution (300 µL, 50% oxidation degree, 50 mg/mL), the gel was
formed within minutes after mixing. The rheology study was performed at 25 °C on a rheometer (TA Instrument, USA). The time-dependent rheology study was conducted at 1% strain and 10 rad/s. The shear thinning study was conducted at 1% strain, and the angular frequency was swept from 0.1 rad/s to 628 rad/s. For the viscosity-based thixotropy property assay, the gel breaking and recovery was performed at a constant 1% strain, and the shear rate was set at an alternative of 2 s⁻¹ and 20 s⁻¹, the measurement was performed for three recycles. The continuous step strain measurement was also performed to support the thixotropy property. The strain was set at an alternative of 1% and 200% with a constant angular frequency of 10 rad/s, the breakage and recovery process were repeated for three times. For self-healing property, 500 μL G1-orni/tobramycin/Dex-CHO hydrogel was prepared as described above in a ring shape, and then the gel was placed statically at room temperature to observe the self-healing behavior. In a separate study, a few pieces of gel blocks were attached together to construct gels with different shapes.

**In vitro drug release studies.**

500 μL G1-orni/tobramycin/Dex-CHO hydrogel (5.38% G1-orni, 0.45% tobramycin) were prepared as described above and transferred into a dialysis bag (molecular weight cut off of 3500 Da), which was immersed in 50 mL buffer solution (pH 7.4 or 5.0) and stirred continuously at room temperature. At scheduled time intervals, 3 mL samples were collected. To determine the concentration of released G1-orni in the buffer solution, the absorbance of collected samples at 315 nm was recorded for colorimetric analysis. The residual samples were derivatized by o-phthalaldehyde as described in
the previous study\textsuperscript{1}, and the absorbance of the obtained samples at 333 nm were recorded. The calibration curve for G1-orni is \(y_1 = 4.8441x_1 + 0.0306\) (\(R^2 = 0.999\), \(x_1\) is the concentration of G1-orni (mg/mL), and \(y_1\) is the absorbance at 315 nm). Besides, G1-orni was also derivatized using o-phthalaldehyde and the corresponding calibration curve of G1-orni is \(y_2 = 1.6973x_2 + 0.1684\) (\(R^2 = 0.999\), \(x_2\) is the concentration of G1-orni and equals to \(x_1\), and \(y_2\) is the absorbance of G1-orni at 333 nm after derivatization). Then, the aminoglycoside concentration in the collected samples was calculated by subtracting the absorbance of derivatized G1-orni at 333 nm. The calibration curve for tobramycin using the o-phthalaldehyde derivatization method is \(y_3 = 0.9305x_3 + 0.0297\) (\(R^2 = 0.997\), \(x_3\) is the concentration of tobramycin, and \(y\) is the absorbance of tobramycin at 333 nm after derivatization). Three repeats were conducted for each sample. The acid-responsiveness was also supported by immersion of the complex hydrogel (500 \(\mu\)L) in 1 mL of phosphate buffers (pH 7.4 and pH 5.0) for 0.5 h, and the mechanical modulus was studied using a rheological measurement which was conducted at 1% strain and 10 rad/s angular frequency.

**Cell culture, cytotoxicity and hemolysis evaluation.**

A mouse embryonic fibroblast cell line, NIH 3T3 cell, was obtained from ATCC and employed to evaluate the cytotoxicity of the hydrogels. Generally, the cells were seeded in a 96-well plate at a cell density of \(10^4\) per well, and cultured in the DMEM supplemented with 1% penicillin, 1% streptomycin and 10% heat-inactivated FBS, and then incubated under a humidified 5% CO\(_2\) atmosphere at 37 °C. 200 \(\mu\)L of G1-orni/tobramycin/Dex-CHO hydrogel was immersed in 2 mL DMEM without FBS for
24 h, and the extract was collected and supplemented with 10% FBS before incubation with the cells. The extract solutions were added to the wells at various concentrations, and the cells were cultured for another 24 h. The cell viability was analyzed by a standard MTT method.

Hemolytic activity was evaluated using the red blood cell (RBC) suspension of the 8-week old Balb/c mouse. Briefly, 1 mL of the RBC suspension (2%) was added into the centrifuge tube with 20 μL hydrogel and treated for 1 h at 37 °C. Simultaneously, the RBC suspension was treated with Triton X-100 (0.5%) and PBS (pH 7.4) as the positive control and negative control, respectively. Then, the mixture was centrifuged at 2000 r/min for 5 min, and the supernatant was collected and transferred a 96-well plate to observe the absorption at 540 nm. The suspension treated with Triton X-100 was set as 100% hemolysis.

**Bacteria culture.**

*S. aureus* was freshly prepared by inoculating a single colony from a tryptic soy agar (TSA) plate in 5 mL sterile TSB medium. *P. aeruginosa* was prepared by isolating the single colony from a Luria-Bertani (LB) plate and suspend in 5 mL LB medium. For anaerobic strains, *C. sporogenes* was inoculated on the GIM plate to grow into single colony, which was suspended in 4 mL GIM medium and plated in a 96-well plate for culture. *B. fragilis* strain was single cloned on the TSA plate supplemented with 5% defibrinated sheep blood, and suspended all the colonies with 4 mL TSB medium and then plated in a 96-well plate, cultured at 37 °C. It should be mentioned that the anaerobic culture was performed in the AnaeroPouch (Mitsubishi, Japan). The
inoculated bacteria suspension was incubated overnight and the optical density of suspension at 600 nm (OD$_{600}$) was measured to quantify the count of bacteria. For all the assays, the bacteria were allowed to grown to the logarithmic phase before use (OD$_{600}$ = 0.6-0.8). To observe the pH changes during bacterial growth, bromothymol blue was added in the culture media as an indicator. Generally, *E. coli* was used as a presentation of aerobic bacteria and diluted to $10^6$ CFU/mL for incubation. 1 mL of the bacteria suspension was taken out for pH measurement every half an hour, and then added with 3 µL bromothymol blue (1%) for observation. Monitoring of *B. fragilis* was started after incubation for 12 h due to the slow growth. The bacteria suspension was divided into several repeats, and each one was taken out for pH measurement at determined intervals. Besides, bromothymol blue was also added for color observation.

**Minimum inhibition concentration (MIC) determination.**

The MIC concentrations against all the strains were determined by a two-fold serial dilution method in 96-well plates, and the bacteria were cultured in the MHB medium. Generally, the bacteria were collected from medium by centrifugation, washed with sterile PBS, and resuspended in MHB at $1.0 \times 10^4$ CFU/mL before use. The drug solution was added to the first column of well in a 96-well plate at a concentration of 2.74 µM for tobramycin, and 5.84 µM for ornidazole and G1-orni, and then the drug solutions was two-fold serial diluted. 10 µL of the drug solution was added to a new plate, and 90 µL of bacteria suspension was seeded into each well. The wells containing MHB only were used as the blank control, and the wells containing bacteria without any antibacterial agent were used as the positive control. Three repeats were performed.
for each drug. The bacteria were incubated at 37 °C for 16 h for *S. aureus* and *P. aeruginosa*, and 48 h for *C. sporogenes* and *B. fragilis*. The optical density of the suspension at 600 nm was recorded, and the lowest concentration in which well the bacteria solution was clear and transparent was set as the MIC value.

**In vitro antibacterial activity of the hydrogels.**

To assess the antibacterial activities of the hydrogels, 50 μL G1-orni/tobramycin/Dex-CHO hydrogel (5.38% G1-orni, 0.45% tobramycin) was directly prepared in the 96-well plate. The G1-orni/Dex-CHO hydrogel and tobramycin/Dex-CHO hydrogel with the equal drug contents were prepared as control hydrogels. The bacteria culture at logarithmic phase was diluted to $1.0 \times 10^6$ CFU/mL, and 100 μL of the suspension was added to each well. After 24 h incubation, the live bacteria counts were assessed by a plate counting method. Generally, the bacteria suspension was gradient diluted ($10^1$ to $10^8$), and then 10 μL of the diluted suspension was seeded on a TSA plate (GIM plate for *C. sporogenes*) and incubated at 37 °C overnight to get a single colony. The survival bacteria can be determined from the number of colonies in the plate. Drug-free bacteria suspension and bacteria-free medium were also set as controls.

The data were presented as mean ± SD, and the differences between two groups were analyzed using a minitab two-sample t-test. *$P < 0.05$ was recognized as statistically significant different, and $P > 0.05$ was recognized as no significant different (N.S.).

**Live-dead staining.**

20 μL of the bacteria suspensions were carefully transferred to wells after treatment with different hydrogels as described above, and 20 μL of Live/Dead dye solution (6
µM SYTO 9 and 30 µM propidium iodide) was added into the wells and incubated for 15 min. Then, appropriate amount (5-10 µL) of the bacteria were mounted on the glass slides and visualized using a fluorescent microscopy (Olympus, Japan). SYTO, a green fluorescent dye that can penetrate across the membranes of live cells was used to label the live bacteria, and propidium iodide, a red fluorescent dye that can only penetrate across damaged cell membranes, was used to stain dead bacteria.

**Scanning electron microscope (SEM) characterization.**

The morphologies of *P. aeruginosa* and *B. fragilis* treated separately with the G1-orni/Dex-CHO, the tobramycin/Dex-CHO, and the G1-orni/tobramycin/Dex-CHO hydrogels were observed by SEM. Briefly, after treatment with the hydrogels as described above, the bacteria were collected by centrifugation (2500 r/min, 5min) and resuspended using 1 mL PBS. Then, 20 µL of the bacteria suspension was added on a piece of sterilized cover glass, and then fixed with 5 mL glutaraldehyde (2.5%) overnight at room temperature, and carefully rinsed with PBS for three times. Then, the bacteria were further fixed with osmic acid for 1 h, and dehydrated with ethanol solutions of gradient concentrations (30%, 50%, 75%, 90%, and 100%, 10 min for each concentration), and dried by supercritical CO₂. Finally, the samples were sputter-coated with a layer of gold and imaged by SEM (FEI sirion 200, USA).

**References**

Figure S1. $^1$H NMR spectrum of the synthesized G1-orni. The protons of G1-orni were assigned and labeled on the chemical structure.
Figure S2. $^{13}$C NMR spectrum of the synthesized G1-orni. The carbons on G1-orni were assigned and labeled on the chemical structure.
Figure S3. pH conditions of the medium during B. fragilis growth. The insert is the image of the bacteria suspensions taken at 6, 12, 24, and 32 h, respectively. Bromothymol blue was used as a pH indicator.
Figure S4. Fluorescence images of the bacteria by live-dead staining: a) *P. aeruginosa* treated by the tobramycin gel, b) *B. fragilis* treated by the G1-orni gel. Scale bar, 200 µm.
Table S1. MIC values of tobramycin, ornidazole, and G1-orni against *S. aureus*, *P. aeruginosa*, *C. sporogenes*, and *B. fragilis*, respectively.

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<th>MIC</th>
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<th>Anaerobic Bacteria</th>
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<td></td>
<td><em>S. aureus</em></td>
<td><em>P. aeruginosa</em></td>
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<td>Tob</td>
<td>8.5 nM</td>
<td>2.1 nM</td>
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<td>Orni</td>
<td>&gt;0.58 μM</td>
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<td>G1-orni</td>
<td>&gt;0.58 μM</td>
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