# $H_2S$-Releasing Amphiphilic Dipeptide Hydrogels Are Potent *S. aureus* Biofilm Disruptors

Yun Qian,[a‡] Afnan Altamimi,[b,c‡] Shaina Yates Alston,[b] Santu Sarkar,[b] Matthew Cochran,[b] Mingjun Zhou,[a] Nicole Levi-Polyachenko[b*] and John B. Matson [a*]

a. Department of Chemistry, Macromolecules Innovation Institute, and Virginia Tech Center for Drug Discovery, Virginia Tech, Blacksburg, VA 24061.
b. Department of Plastic and Reconstructive Surgery, Wake Forest School of Medicine, Winston Salem, NC, USA. Email:
c. King Saud University, College of Medicine, Department of Surgery, Riyadh, Saudi Arabia.

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**Synthesis of dipeptides**

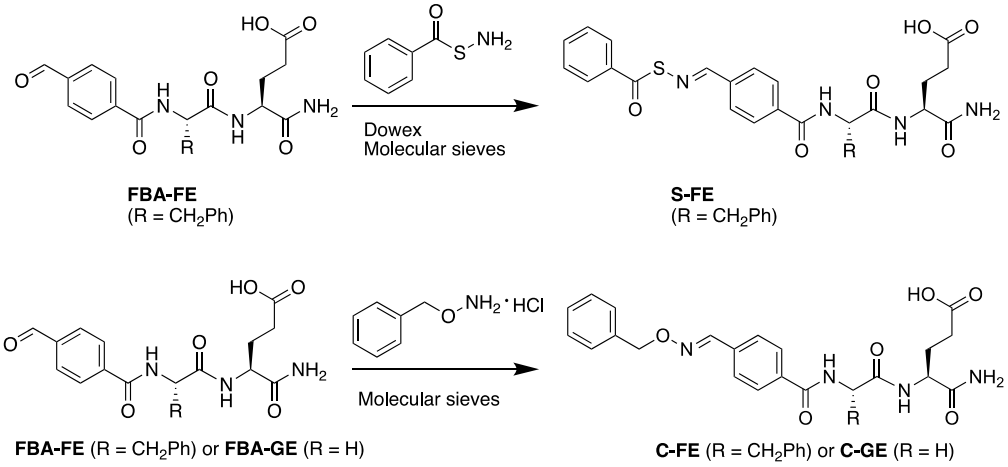
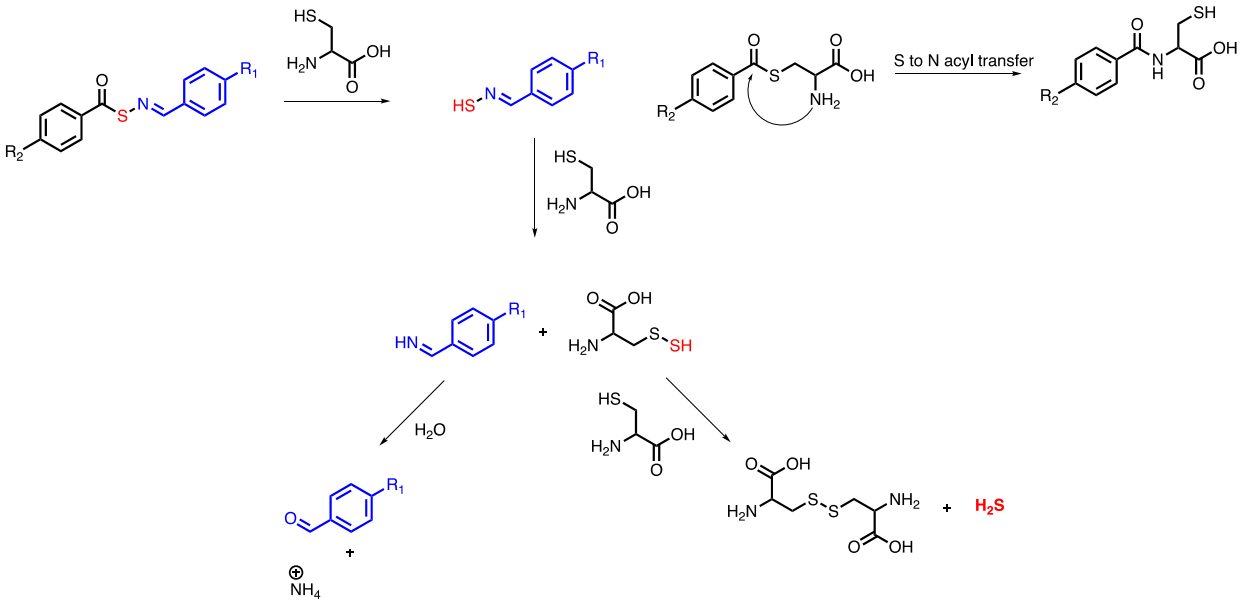



**Figure S1.** Synthesis of S-FE, C-FE and C-GE dipeptides.

The aldehyde-containing FBA-FE (FBA = 4-formylbenzoic acid) dipeptide was synthesized via solid phase peptide synthesis following published procedures for similar FBA peptides.[1, 2] After purification, the peptide was treated with 0.01 M HCl and lyophilized to remove residual trifluoroacetic acid (TFA). SATO-containing S-FE was prepared by mixing FBA-FE (100 mg, 1 equiv), *S*-benzoylthiohydroxylamine (72 mg, 2 equiv) and Dowex (20 mg) in 500 µL DMSO. After 3 h, the peptide was isolated and purified as described previously for similar SATO

peptides.[1,2] Oxime-containing C-FE was produced by adding 96 mg (1 equiv) of FBA-FE and 56 mg (2 equiv) of *O*-benzylhydroxylamine hydrochloride into 500 μL DMSO. After 3 h, the peptide was isolated and purified as described previously for similar peptides.[1,2] Oxime-containing C-GE was prepared by the same method as C-FE. FE dipeptide was synthesized by solid phase peptide synthesis directly. All dipeptides were purified by HPLC using pure ACN and milliQ water as mobile phase, with a flow rate of 10 mL/min and a gradient change of ACN from 30% to 90% over 25 min, as noted in previously published procedures for similar peptides.[2]




**Scheme S1.** Proposed mechanism of cysteine-triggered $H_2S$ release from SATOs.

**Critical aggregation concentration**

The critical aggregation concentrations of dipeptides were determined by the Nile Red assay, following a published method.[1] In brief, a 1 mg/mL Nile Red stock solution was prepared in acetone and then diluted in DI water to a final concentration of 0.01 mg/mL. The 0.01 mg/mL Nile Red solution was used in all dissolving and diluting operations to keep Nile Red concentration consistent in all samples. Next, a series of peptide solutions, with concentrations of 4, 3, 2, 1, 0.5, 0.25, 0.1, 0.01, 0.001, 0.0001 mg/mL, were prepared with the 0.01 mg/mL Nile Red solution. Samples of dilution series were transferred into a 96-well plate and analyzed by fluorescence spectroscopy. The excitation wavelength was set at 550 nm and the emission wavelength was 648 nm.

**Table S1.** CAC of dipeptides

| | CAC (mg/mL) | CAC (mM) |
|---|---|---|
| S-FE | 0.3±0.1 | 0.5±0.2 |
| C-FE | 0.3±0.1 | 0.5±0.2 |
| C-GE | 0.5±0.0 | 1.0±0.1 |
| FE | 0.2±0.1 | 0.7±0.3 |
| FBA-FE | 0.4±0.0 | 1.0±0.1 |

**Circular dichroism spectroscopy**

Circular dichroism (CD) was used to determine the possible β-sheet interaction in the self-assembling process. For unassembled dipeptide solutions, 0.1 mM dipeptide solutions were prepared and sonicated before the testing. Self-assembled dipeptides were prepared by adding 2 μL of dipeptide hydrogels (36 mM) to 718 μL DI water, and CD spectra were acquired immediately after dilution. CD spectra are shown in Figure S2.

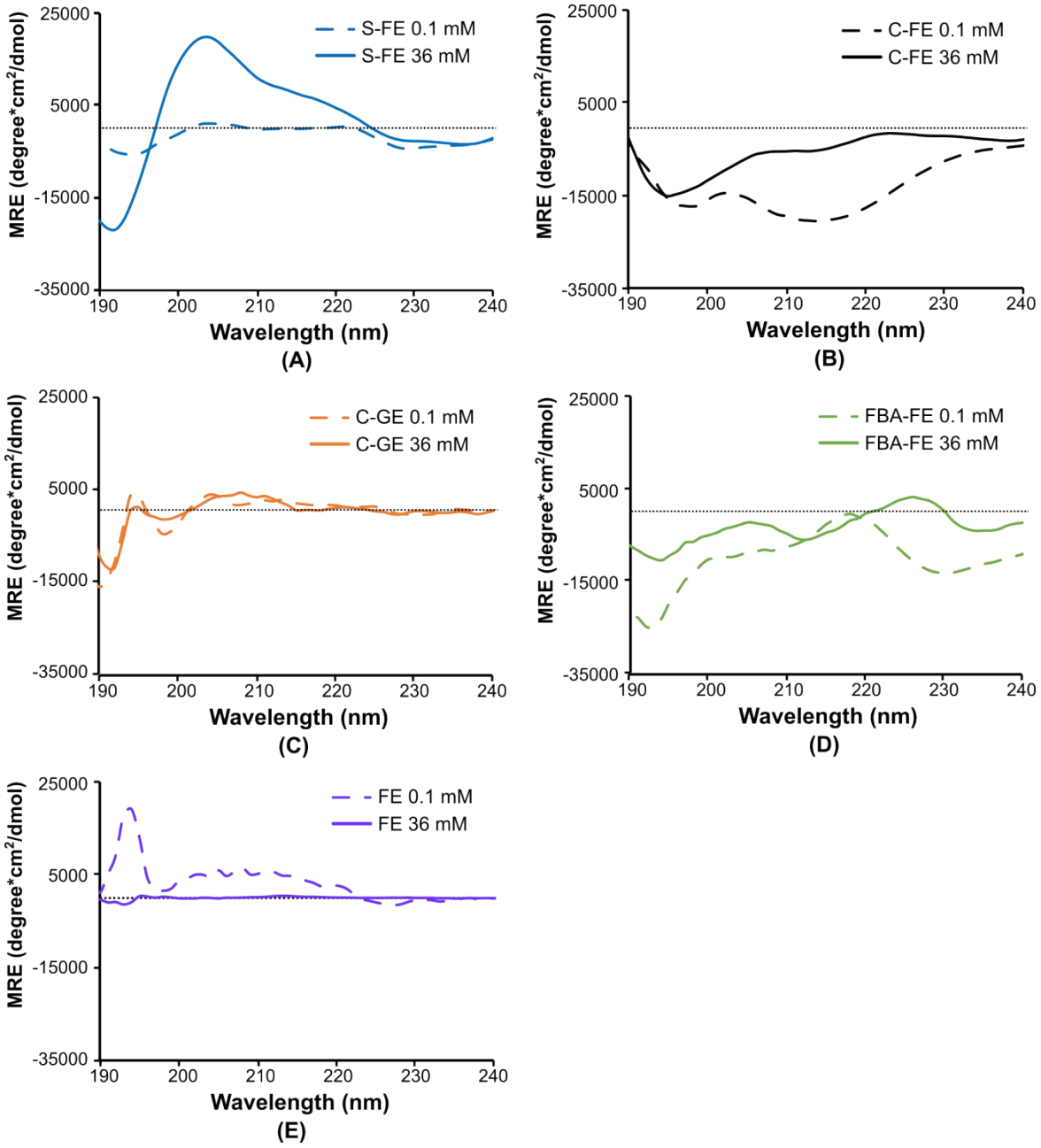



**Figure S2.** Circular dichroism spectra of (A) S-FE, (B) C-FE, (C) C-GE, (D) FBA-FE, (E) FE in $H_2O$.

**Rheology of hydrogels**

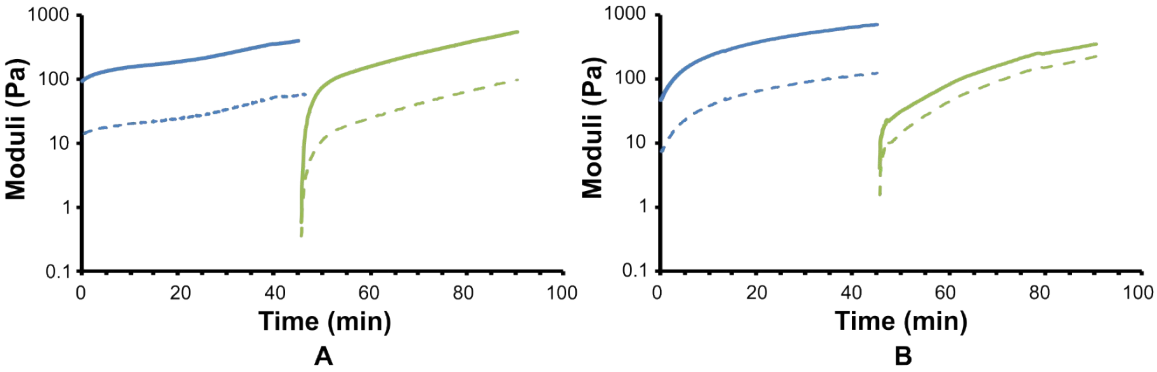



**Figure S3.** Rheology results of (A) S-FE and (B) C-FE hydrogels. Time-sweeps were set up under 0.5% strain and 1 Hz frequency for 45 min. A 30 s steady shear operation (500 /s) was applied between two time-sweeps to disrupt the hydrogel structures. Solid curves: storage moduli; dash curves: loss moduli.

**Biological assays to determine the antimicrobial effects of S-FE**

We tested the effect of cysteine on the growth of *Staphylococcus aureus* (ATCC 12600, Xen29) bioluminescent pathogenic bacteria. The Xen29 bacteria frozen stock solution was used to grow an overnight culture in Nutrient broth1 (NB1) at 37 ˚C. 1440 µL of the cysteine solution (500 mM) was added to a stock bacterial culture with a concentration $1x10^6$ colony forming units per mL (CFU/mL). A control group was prepared by adding 1440 µL of PBS (1X) to NB1 bacterial broth and making the final bacterial concentration 1 $x10^6$ CFU/mL. Samples were incubated in an orbital shaker at 37 ºC for 24 h with a shaking speed of 166 rpm. Serial dilution was carried out, and bacterial solutions were plated on NB1 plates. Finally, all plates were incubated to form colonies, and the number of CFUs were enumerated.

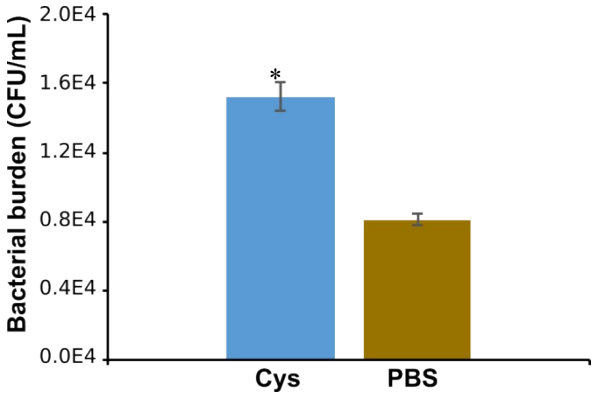



**Figure S4.** Bacterial burdens of bacteria with or without Cys treatment. $10^6$ CFU/mL Xen29 with or without 500 mM Cys was cultured in NB1 broth for 24 h at 37 ºC, with a shaking speed of 166 rpm. Bacterial burdens were measured by a serial dilution assay using NB1 plates. Mean values were calculated from three trials with error bars indicating standard errors. Tukey-Kramer HSD test was performed with n=3 and $p < 0.05$.

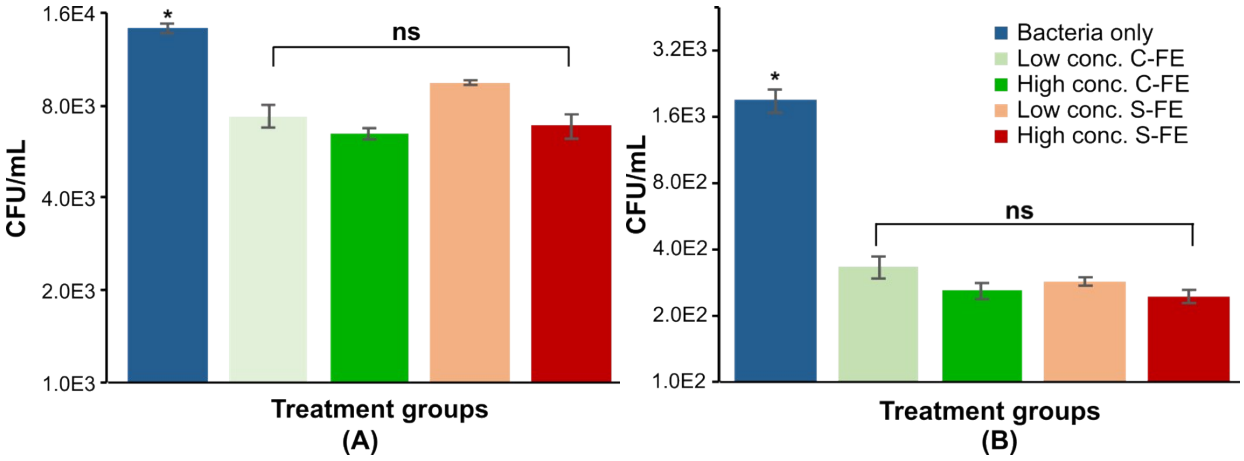
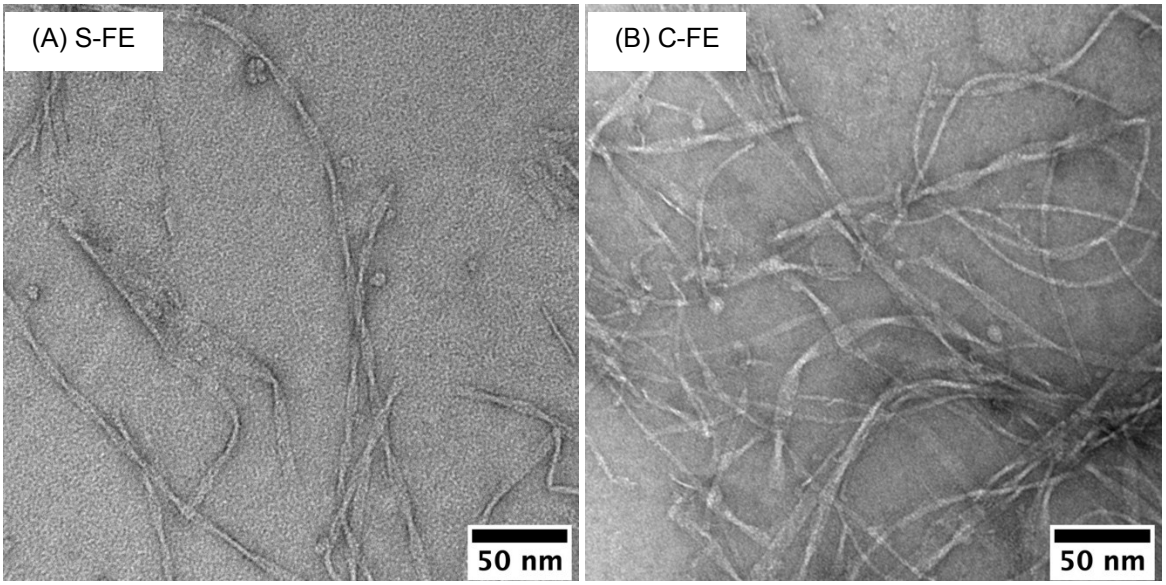



**Figure S5**. Bacterial burdens after treatment of Xen29 with dipeptide hydrogels. (A) Bactericidal data; (B) Bacteriostatic data. Peptide samples (2 mg) were dissolved in pH 6.0 PBS (1X) at a concentration of 36 mM with 14.4 μL of 500 mM Cys. The resulting solutions were then mixed with Xen bacterial culture (20 μL, $1x10^8$ CFU/mL), NB1 broth (280 μL) and PBS (1X) to prepare different concentration groups with total volume of 400 μL: high concentration (100 μL, 9 mM final peptide concentration) and low concentration (30 μL, 2.7 mM final peptide concentration). There are significant differences between groups treated with S-FE and C-FE compared to the bacteria only groups. Mean values from three replicates are presented, with error bars indicating standard errors. An ANOVA and Tukey-Kramer HSD test were performed with n=3 and $p < 0.05$. * indicates $p < 0.05$ with respect to the four treatment groups; ns indicates no significant differences among the four treatment groups.

**TEM images of self-assembled dipeptides**



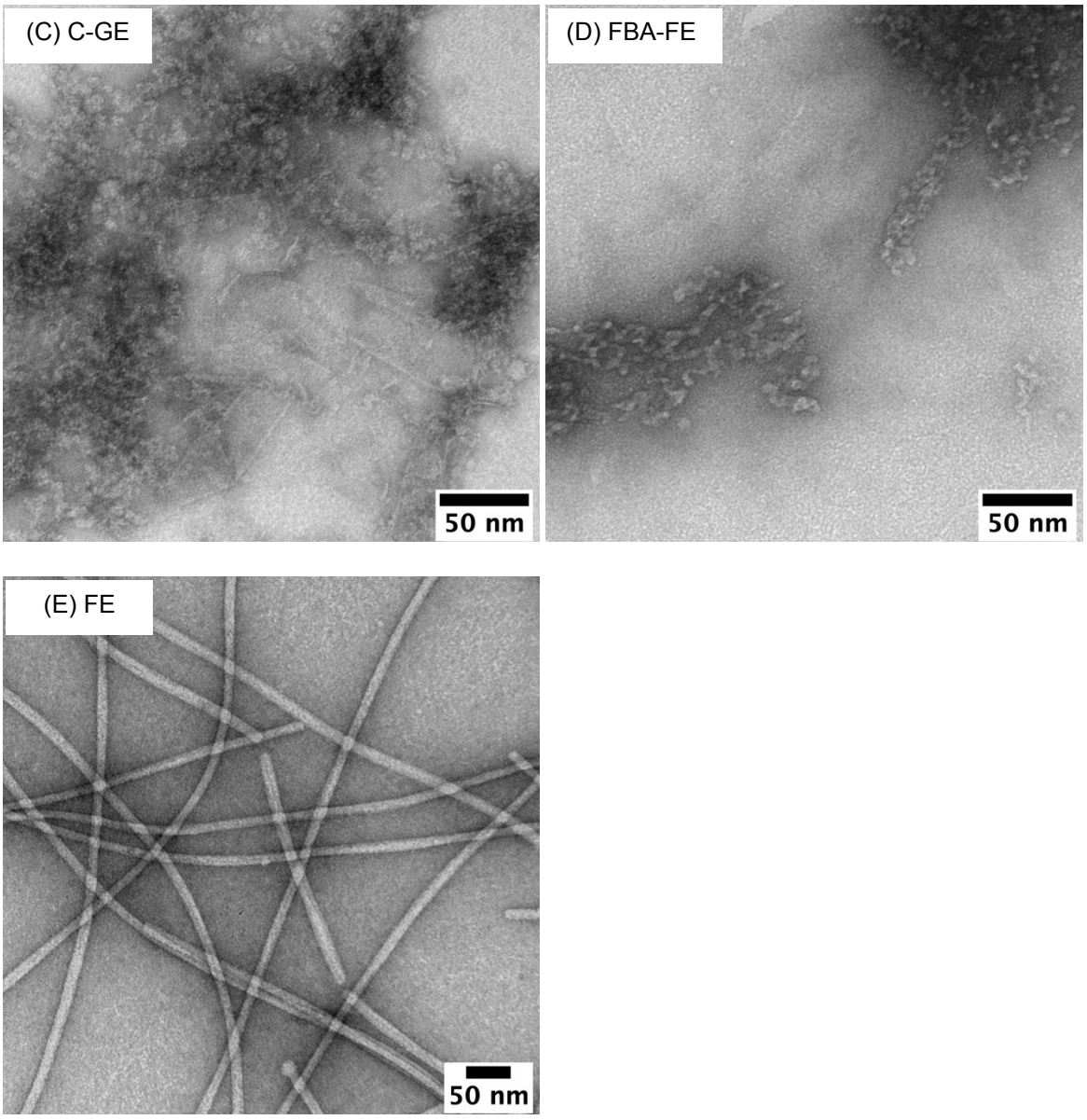



**Figure S6.** TEM images (stained with 2 wt% uranyl acetate) of (A) S-FE, (B) C-FE, (C) C-GE, (D) FBA-FE, and (E) FE.

**SEM images of dipeptide treated bacterial biofilms**

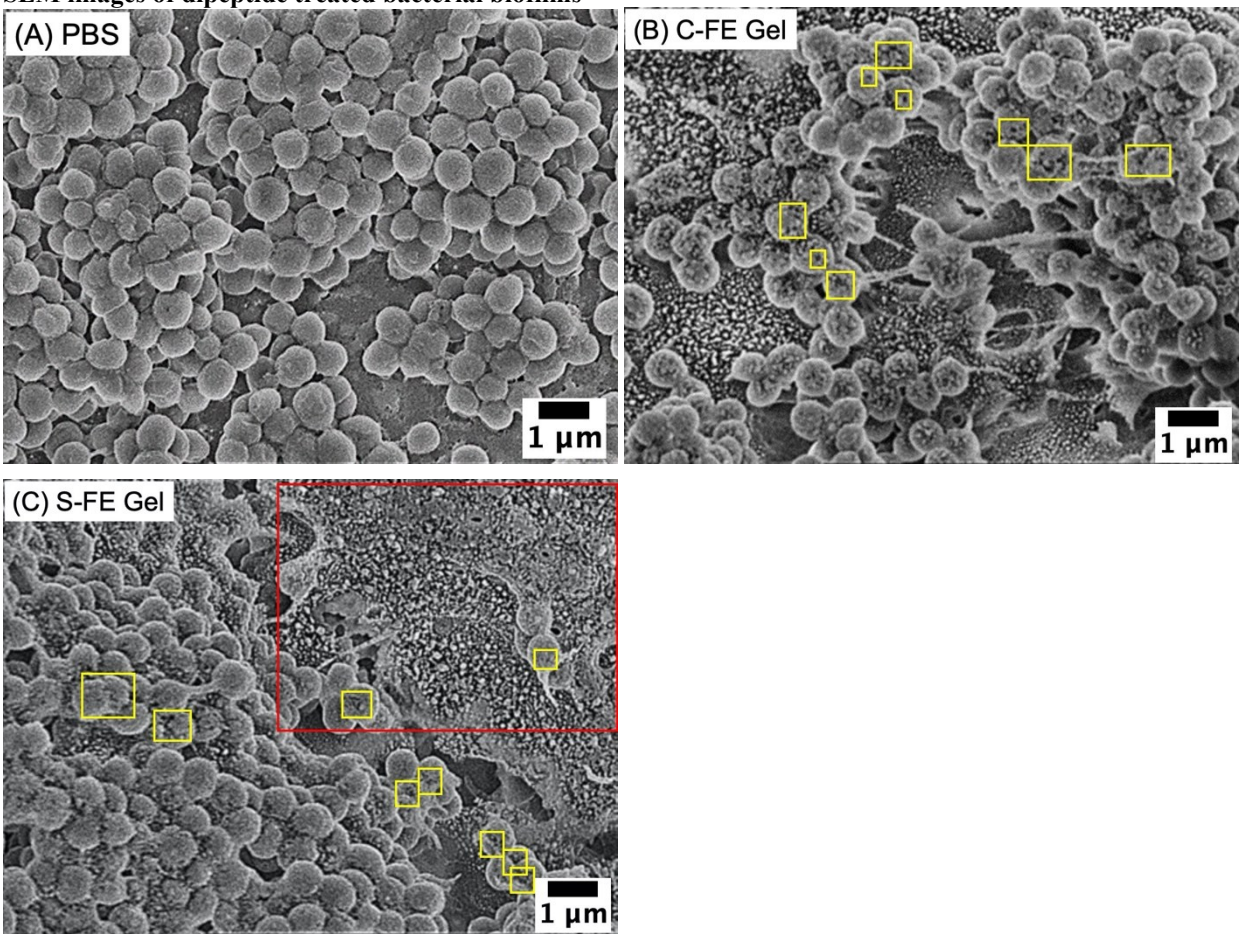



**Figure S7.** SEM images of established biofilms treated with PBS, C-FE or S-FE and imaged at 10,000X magnification. Yellow boxes indicate the holes and rough surfaces of bacteria, while the red box shows the salt crystal area.

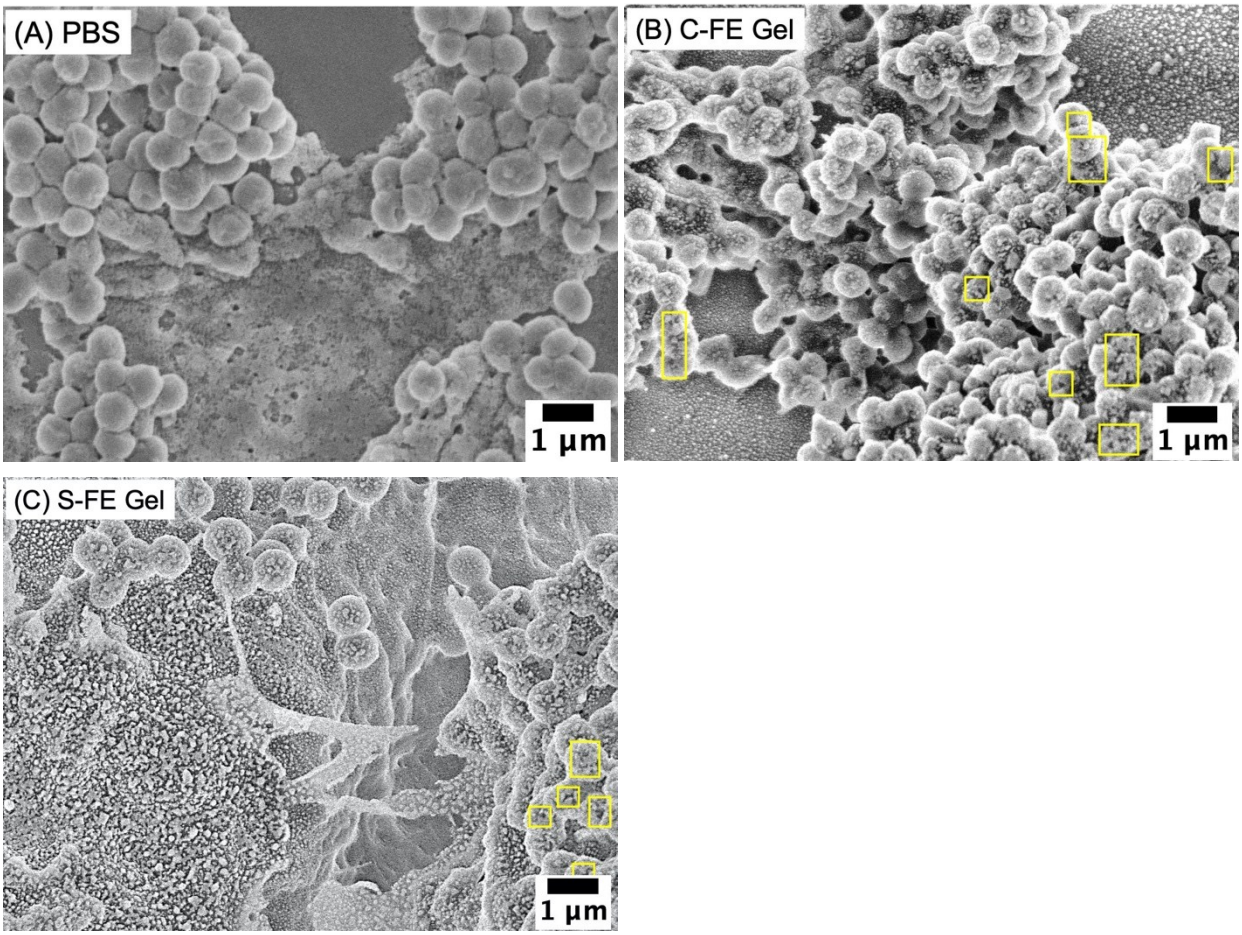



**Figure S8.** SEM of biofilms treated prophylactically with PBS, C-FE or S-FE and imaged at 10,000 magnification. Yellow boxes indicated the holes and rough surfaces of bacteria.

1 J. M. Carter, Y. Qian, J. C. Foster and J. B. Matson, *Chem. Commun.*, 2015, **51**, 13131-13134.
2 K. Kaur, Y. Qian and J. B. Matson, in *Biomaterials for Tissue Engineering*, Springer, 2018, pp. 193-208.