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Supporting Information

Micellar Chemotherapeutic Platform Based on a Bifunctional Salicaldehyde Amphiphile Delivers a "Combo-Effect" for Heightened Killing of MRSA

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EXPERIMENTAL SECTION

1.0 Minimum Inhibitory Concentration (MIC) and Minimum Killing Concentration (MKC) of C1, Rifampicin and Vancomycin

The MIC and MKC of C1, rifampicin and vancomycin against the target bacterial strains was determined by a standard microtitre dilution method as described previously. [1]

2.0 Field Emission-Scanning Electron Microscope (FESEM) Analysis

Overnight grown cells of *S. aureus* MTCC 96 and *E. coli* MTCC 433 were collected by centrifugation, washed twice with sterile PBS and resuspended in the same. The cells were then treated with either 40 µM of C1 in case of *S. aureus* MTCC 96 or 80 µM of C1 in case of *E. coli* MTCC 433 for 12 h at 37°C. Untreated cells were also incubated in sterile PBS under the same conditions as control samples. Following incubation, untreated as well as treated cells were collected by centrifugation, washed with sterile PBS and sterile MilliQ water and finally suspended in sterile MilliQ water. A 10 µL aliquot of each sample was spotted on separate aluminium foil covered glass stub and air dried in the laminar hood. The samples were then mounted on a carbon tape covered metal stub and gold (Au) coating was done twice. Finally the samples were analyzed in a field emission scanning electron microscope (Zeiss Sigma, USA) at 2.0-3.0 Kv.

3.0 Membrane-Directed Activity of C1

The experiments conducted to determine the membrane-directed activity of C1 consisted of the following:

3.1. cFDA-SE Leakage Assay

Cells of *S. aureus* MRSA 100 were labelled with cFDA-SE following a standard protocol. [1] Various concentrations of C1 (10 µM, 20 µM, 40 µM 80 µM and 160 µM) was then added to separate sets of cFDA-SE labelled *S. aureus* MRSA 100 cells and incubated at 37^{o}C and 180 rpm. At intermittent periods of incubation (3 h and 6 h), cells were harvested by centrifugation and leakage of carboxyfluorescein from the cells was determined by measuring fluorescence of the cell free supernatant at an excitation wavelength of 488 nm and emission wavelength of 518 nm in a spectrofluorimeter (FluoroMax-4, HORIBA). The fluorescence measurements were recorded after subtracting the fluorescence of effluxed dye from control

samples. Fluorescence measurements were recorded from three independent experimental samples.

3.2. Fluorescence Microscope Analysis

Cells of *S. aureus* MTCC 96 suspended in sterile PBS (approximately 10^6 CFU/mL) were treated with C1 (10 μM and 20 μM each) at 37°C and 180 rpm for 6 h. In case of control sample, DMSO was added to the cells and incubated under the same conditions. Subsequently, cells were washed twice with sterile PBS to remove unbound amphiphile and labelled with cFDA-SE and PI in separate sets as described earlier. [2] A 10 μL aliquot of the stained sample was spotted on a clean glass slide, air dried and observed under fluorescence microscope (Eclipse Ti-U, Nikon, USA) with a filter that allowed blue light excitation at 445 nm - 495 nm for cFDA-SE and green light excitation at 495 nm - 570 nm in case of PI stained cells. The images of the cells were recorded.

3.3. Membrane Depolarization Assay

The membrane depolarization of *S. aureus* MTCC 96 cells in the presence of 5.0 μM, 10 μM and 15 μM C1 was ascertained by the standard $DiSC_3(5)$ based assay. [1] Cells treated with valinomycin (30 μM) were used as positive control. Fluorescence measurements were taken for three independent experimental samples.

3.4. Outer Membrane Permeabilization Assay

The outer membrane permeabilization of *E. coli* MTCC 433 cells in the presence of 5.0 μM, 10 μM and 15 μM C1 was ascertained by following a standard NPN assay. [3] Cells treated with polymyxin B (1.0 μg/mL) were used as positive control. Fluorescence measurements were taken for three independent experimental samples.

4.0. Bactericidal Activity of C1 in Physiological Fluids and Cytotoxic Effect

Varying concentrations of C1 (5.0 μM, 10 μM, 15 μM and 20 μM) were added separately to target cells of *S. aureus* MTCC 96 and *E. coli* MTCC 433 (6.0 $\log_{10}$ CFU/mL) suspended in simulated body fluid (SBF) and incubated at 37°C and 180 rpm. The composition of SBF was as reported earlier. [4] In a separate experiment, target cells of the gastro-intestinal pathogen *L. monocytogenes* Scott A (6.0 $\log_{10}$ CFU/mL) were suspended in either simulated intestinal fluid (SIF) or simulated gastric fluid (SGF) and treated with varying concentrations of C1 (5.0 μM, 10 μM, 15 μM and 20 μM) at 37°C and 180 rpm. The composition of SIF and

SGF are as described earlier. [5] For all the treated samples, viable cell numbers (log_{10} CFU/ mL) were determined at regular intervals by serial dilution and plating. The viable cell count was also determined at the specified intervals for cells suspended in the respective physiological fluids alone.

The cytotoxic effect of C1 was ascertained on model human embryonic kidney cells (HEK 293) by a standard MTT assay. [3] The tested amphiphile concentrations were equivalent to various multiples of the MIC (20 µM - 320 µM) of C1 against *S. aureus* MRSA 100. The MTT assay was performed in six sets for each tested concentration.

5.0 Bactericidal Efficacy of Rifampicin and Vancomycin in Combination with C1

In a sterile microtitre plate, 100 µL of BHI media was incorporated with either rifampicin (1.22 µM and 2.43 µM) or vancomycin (0.35 µM and 0.69 µM) in combination with varying concentrations of C1 (5.0 µM, 10 µM and 20 µM). To each well, 10 µL aliquot of *S. aureus* MRSA 100 cell suspension (10^6 CFU suspended in BHI media) was inoculated. The cells were incubated at 37^{o}C and 180 rpm for 12 h. Bacterial cell growth was estimated by measuring absorbance at 600 nm in a microtitre plate reader (Infinite M200, TECAN, Switzerland) and expressed as percentage growth inhibition compared to untreated cells (cells grown in the absence of antibiotics and C1). In separate sets, the effect of varying concentrations of the antibiotic or C1 alone on the growth of target bacteria was also ascertained. For every sample, three independent experiments were performed, each having three replicas. Data analysis and calculation of standard deviation was performed with Microsoft Excel 2010 (Microsoft Corporation, USA).

6.0. Effect of C1 on MRSA Biofilm

S. aureus MRSA 100 biofilm was grown in BHI media supplemented with 0.25% glucose in sterile 96 well microtiter plate in presence of varying concentrations of C1 (10 µM - 640 µM) and incubated for 24 h in a static and humid chamber at 37^{o}C. Following incubation for 24 h, media from the wells was removed, the wells were washed with sterile PBS to remove non-adherent bacterial cells. Subsequently, the metabolic activity of biofilm cells was estimated by performing an MTT assay, [6] and the minimum biofilm inhibition concentration ($MBIC_{50}$) for C1 was determined as the concentration, which resulted in 50% reduction in biofilm metabolic activity as compared to untreated control sample. In another experiment, biofilm of *S. aureus* MRSA 100 was grown in sterile 96 well microtitre plate by following a standard protocol as described earlier. [7] Following 24 h of biofilm growth in static and humid

condition at 37°C, the spent media from the microtitre plate wells was gently aspirated and the wells were washed once with sterile MilliQ water (200 µL) to remove non-adherent bacteria. The pre-grown biofilm was then treated with C1 (10 µM - 640 µM) for 24 h. Untreated biofilm was also incubated under the same conditions as control. Following incubation for 24 h, media from the wells was removed, the wells were washed with sterile PBS to remove non-adherent bacterial cells. Subsequently, the metabolic activity of biofilm cells was estimated by performing an MTT assay. The minimum biofilm eradication concentration ($MBEC_{50}$) for C1 was determined as the concentration, which resulted in 50% reduction in biofilm metabolic activity as compared to untreated control sample. All the experiments were performed in three independent sets and every set consisted of three replicates. Data analysis and calculation of standard deviation was performed with Microsoft Excel 2010 (Microsoft Corporation, USA).

In a separate experiment, *S. aureus* MRSA 100 biofilm was grown in BHI media supplemented with 0.25% glucose in sterile 96 well microtiter plate in presence of varying concentrations of C1 (20 µM, 40 µM, 80 µM and 160 µM) and incubated for 24 h in a static and humid chamber at 37°C. Following incubation for 24 h, media from the wells was removed, the wells were washed with sterile PBS to remove non-adherent bacterial cells. Subsequently, untreated biofilm samples (control) as well as C1-treated biofilm samples were subjected separately to cFDA-SE and Congo red staining by following a standard procedure. [7]

7.0. Atomic Force Microscope Analysis of C1

Glass cover slips (18 mm x 18 mm) were surface sterilized with ethanol. A 10 µL aliquot of C1 (40 µM in water) was drop casted on the cover slip and the sample was allowed to dry overnight. Atomic force microscope (AFM) images of the sample was then captured with an Agilent 5500 AFM (Agilent Technologies, Chandler, AZ, USA). Cantilevers made of silicon nitride were used having a resonant frequency of ca. 150 to 250 kHz. Images were acquired in intermittent contact mode, with 3.0 µm x 3.0 µm size at a scan rate of 0.5-1.0 line/s. Analysis of the topographic images of the surface was conducted by using the WSxM v5.0 Develop 6.5 image viewer software. [8]

8.0. Interaction of C1 with Bacteria

The binding of C1 onto *S. aureus* MTCC 96 cells was determined by Dynamic Light Scattering (DLS) technique. Initially the bacterial cells were grown overnight in BHI media at 37 ^{o}C and 180 rpm. The cells were harvested and washed with sterile PBS thrice and then diluted in the same to around 10^6 cells. The cell suspension was then incubated with varying concentration of C1 (10 µM, 20 µM and 40 µM) in separate sets for 30 minutes at room temperature. The cells were then separated by centrifugation at 8000 rpm for 3 minutes. The supernatant, which constitutes the residual C1 in solution was then subjected to particle size estimation by DLS (Zeta Sizer, Malvern, UK). In a separate set, varying concentrations of C1 alone (10 µM, 20 µM and 40 µM) were also subjected to particle size estimation by DLS. The DLS experiments were performed in three independent sets and every set consisted of multiple replicates.

9.0 Bactericidal Activity and Cytotoxicity of C1 Micelle

The bactericidal activity of C1 and $C1_M$ (20 µM - 80 µM each) was tested against *S. aureus* MRSA 100 by following a standard procedure and generating a time-kill curve. [3] The viable cell count was also determined at the specified intervals for cells suspended in PBS as well as cells treated with DMSO alone. The cytotoxic effect of $C1_M$ was ascertained on HEK 293 cells by a standard MTT assay. [1] The concentrations of $C1_M$ was in the range of 20 µM - 320 µM.

10.0. Characterization of $C1_M$, $C1_M$-R and $C1_M$-V

The absorbance spectra of $C1_M$, $C1_M$-R and $C1_M$-V was recorded in a spectrophotometer (Lambda 25, Perkin-Elmer) in scanning mode from 200 nm to 600 nm. Absorbance measurements were acquired from three independent experimental samples. In a separate experiment, particle size estimation of $C1_M$, $C1_M$-R and $C1_M$-V was accomplished by DLS (Zeta Sizer, Malvern, UK). In these experiments, the concentration of $C1_M$ was 79 µM and the concentration of rifampicin and vancomycin in the loaded micelle were 2.9 µM and 0.2 µM, respectively. The DLS experiments were performed in three independent sets and every set consisted of multiple replicates. In another experiment, a 10 µL aliquot of $C1_M$, $C1_M$-R and $C1_M$-V were analyzed in a field emission scanning electron microscope (Zeiss Sigma, USA) at 2.0 Kv.

11.0. *In Vitro* Release Kinetics and Cytotoxicity of Antibiotic-loaded C1 Micelle

An aliquot of antibiotic-loaded C1 micelles (corresponding to a final concentration of 2.26 μM vancomycin and 14 μM rifampicin) were dispersed in separate sets in 1.0 mL each of SBF (pH 7.4) and SIF (pH 8.0). The samples were incubated in an orbital shaker at 180 rpm and 37°C. Samples were withdrawn at regular intervals (0 h, 3 h, 9 h, 12 h, 24 h and 48 h) and centrifuged at 8,000 rpm for 3 min. The supernatant from the samples were transferred into a fresh microcentrifuge tube and the absorbance spectra of the solutions were measured from 250 nm to 750 nm in a spectrophotometer (Perkin-Elmer). A previously generated calibration curve for vancomycin and rifampicin was used to quantify the release of the antibiotics at various time periods and expressed as % cumulative release. All the experiments were performed in three independent sets and every set consisted of three replicates.

The cytotoxic effect of $C1_M$-R and $C1_M$-V was ascertained on HEK 293 cells by a standard MTT assay. [1] The concentrations of C1 present in $C1_M$-R and $C1_M$-V were 32 μM, 65 μM and 130 μM. The corresponding concentrations of rifampicin in $C1_M$-R were 1.21 μM, 2.43 μM and 4.86 μM. The corresponding concentrations of vancomycin in $C1_M$-V were 0.69 μM, 1.38 μM and 2.76 μM.

References

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RESULTS

Characterization of C1:

^{1}H NMR [400 MHz, Chloroform-d1, TMS, J (Hz), δ (ppm)]: 7.26 - 6.76 (4H, m), 5.06 (1H, broad s), 3.99 (1H, s), 2.66 (2H, t), 1.53 (2H, m), 1.37 - 1.21 (20H, m), 0.88 (3H, t), ^{13}C NMR [150 MHz, Chloroform-d1, TMS, δ (ppm)]: 158.58, 128.80, 128.37, 122.83, 119.07, 116.54, 52.94, 42.97, 32.11, 31.12, 29.82, 29.78, 29.74, 29.66, 20.34, 27.34, 22.88, 14.31. ESI-MS (positive mode, *m/z*) Calculated for $C_{19}H_{33}NO$: 292.2562. Found: 292.2657 [(M + H+)].

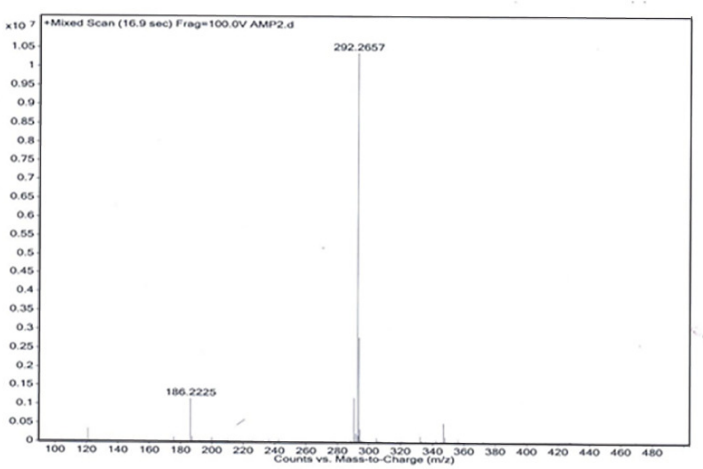

Figure S1. ESI-MS of C1 performed in positive mode.

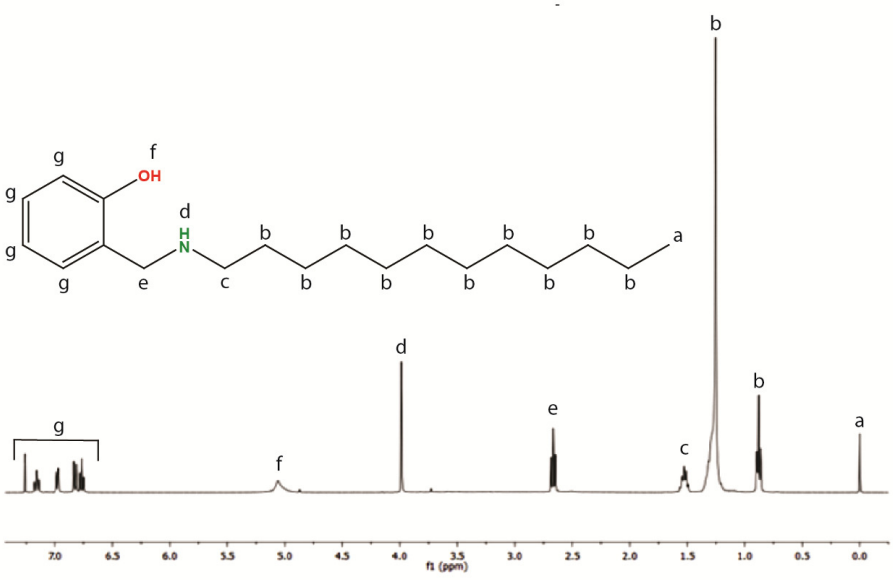

Figure S2. ^{1}H NMR spectra of C1 in $CDCl_3$ solution.

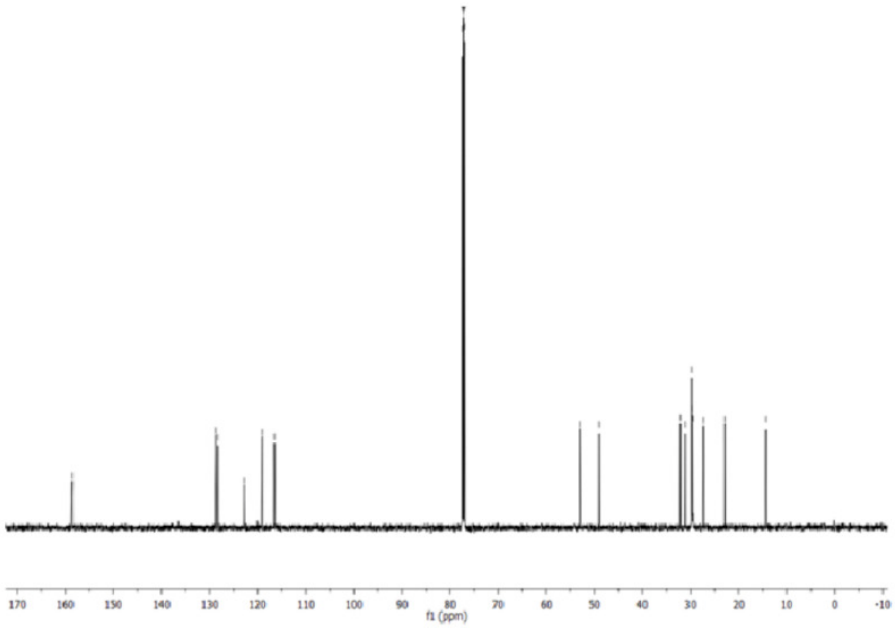

Figure S3. ^{13}C NMR spectra of C1 in $CDCl_3$ solution.

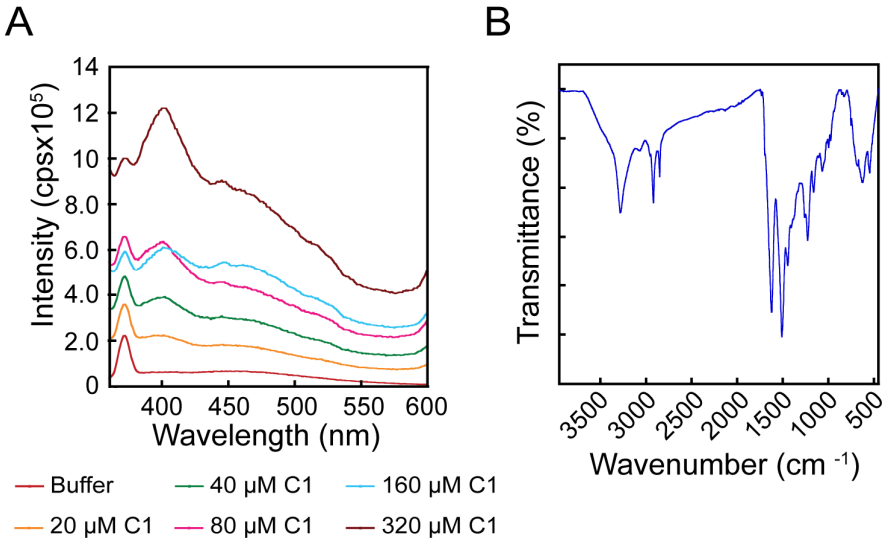

Figure S4. (A) Fluorescence emission spectra and (B) FTIR spectra of C1.

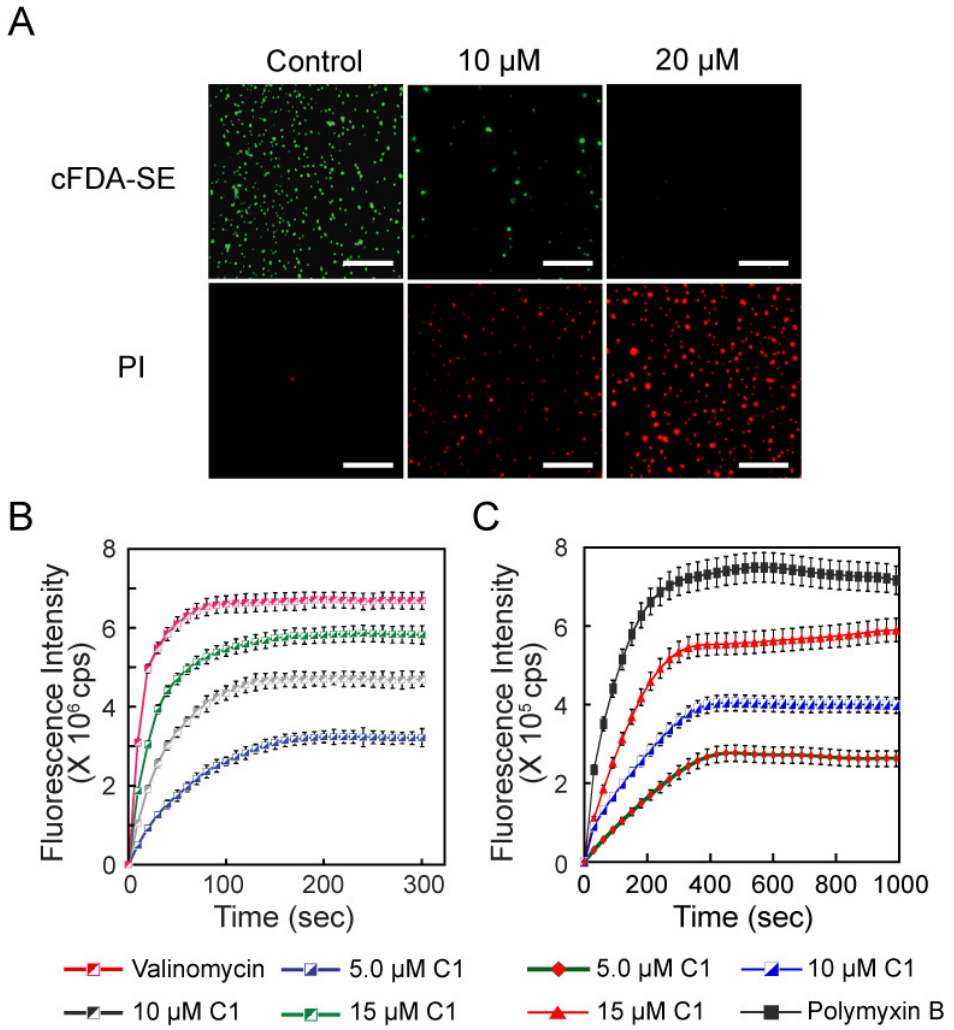

Figure S5. (A) Fluorescence microscope-based live/dead assay with C1-treated *S. aureus* MTCC 96 using cFDA-SE and PI. Scale bar for the images is 100 μm. (B) DiSC3(5)-based membrane depolarization assay on C1-treated *S. aureus* MTCC 96. Cells treated with 30 μM valinomycin was used as a positive control. (C) NPN uptake assay to ascertain membrane permeabilization in *E. coli* MTCC 433 cells treated with C1. Cells treated with 1.0 μg/mL polymyxin B was used as a positive control.

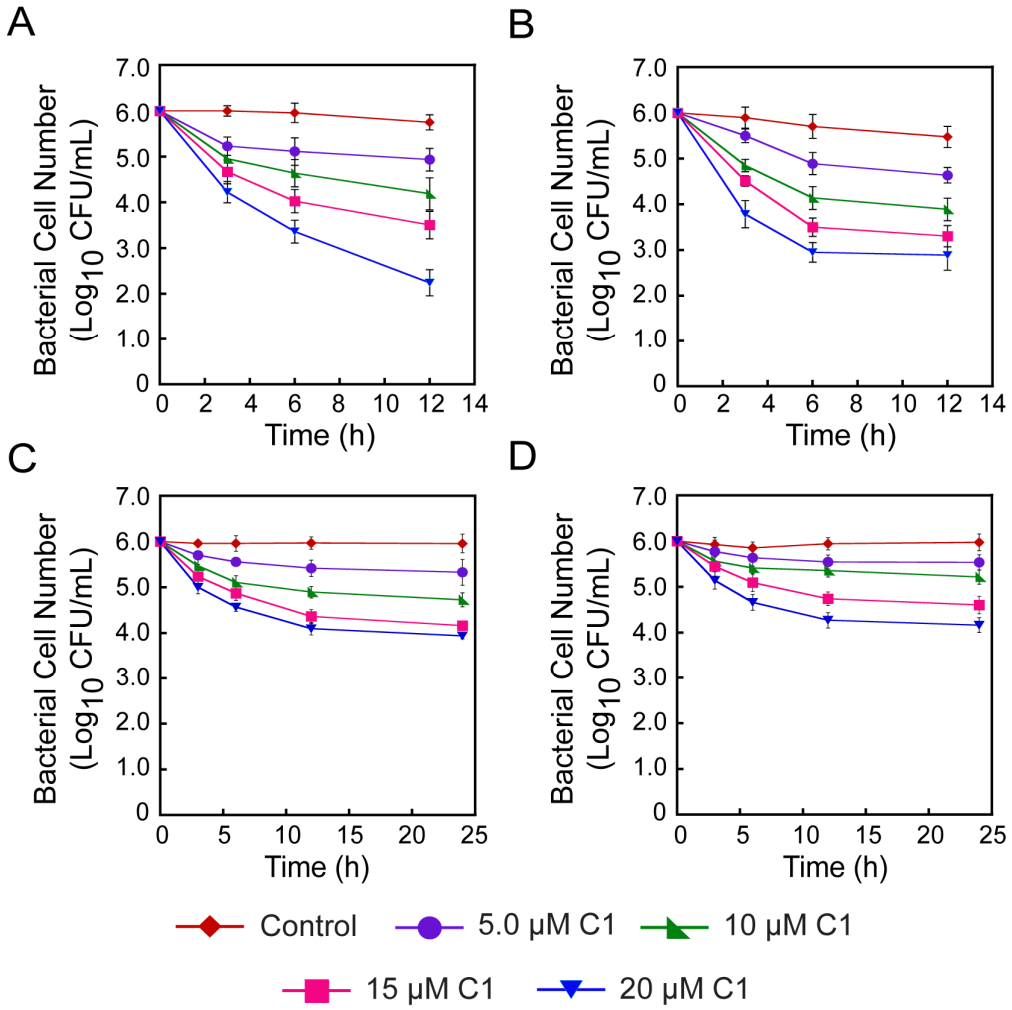

Figure S6. Time-kill curves of C1 against (A) *S. aureus* MTCC 96 in simulated body fluid, (B) E. *coli* MTCC 433 in simulated body fluid, (C) *L. monocytogenes* Scott A in simulated intestinal fluid and (D) *L. monocytogenes* Scott A in simulated gastric fluid.

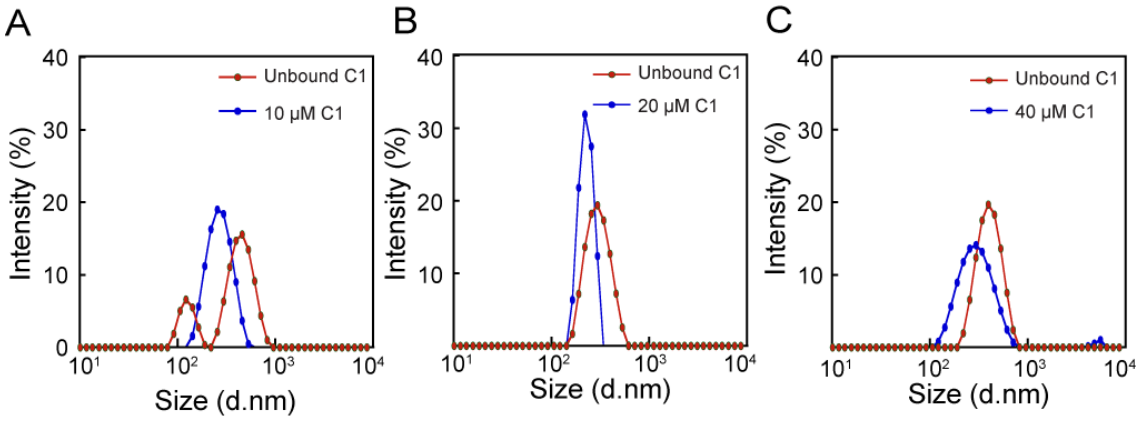

Figure S7. (A-C) Particle size distribution of varying concentrations of C1 and unbound C1 following interaction with *S. aureus* MRSA 100 in PBS. C1 was interacted with *S. aureus* MRSA 100 cells in PBS for 30 minutes.

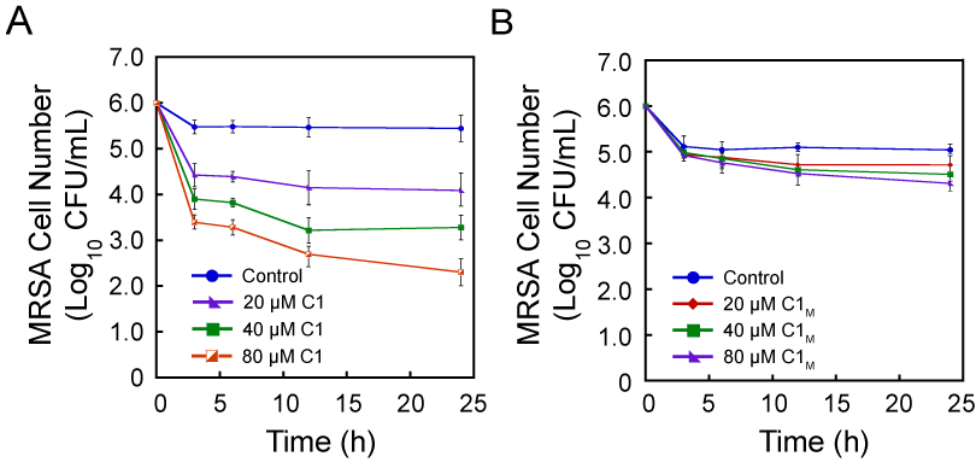

Figure S8. Time-kill curves of (A) C1 and (B) $C1_M$ against *S. aureus* MRSA 100 in PBS.

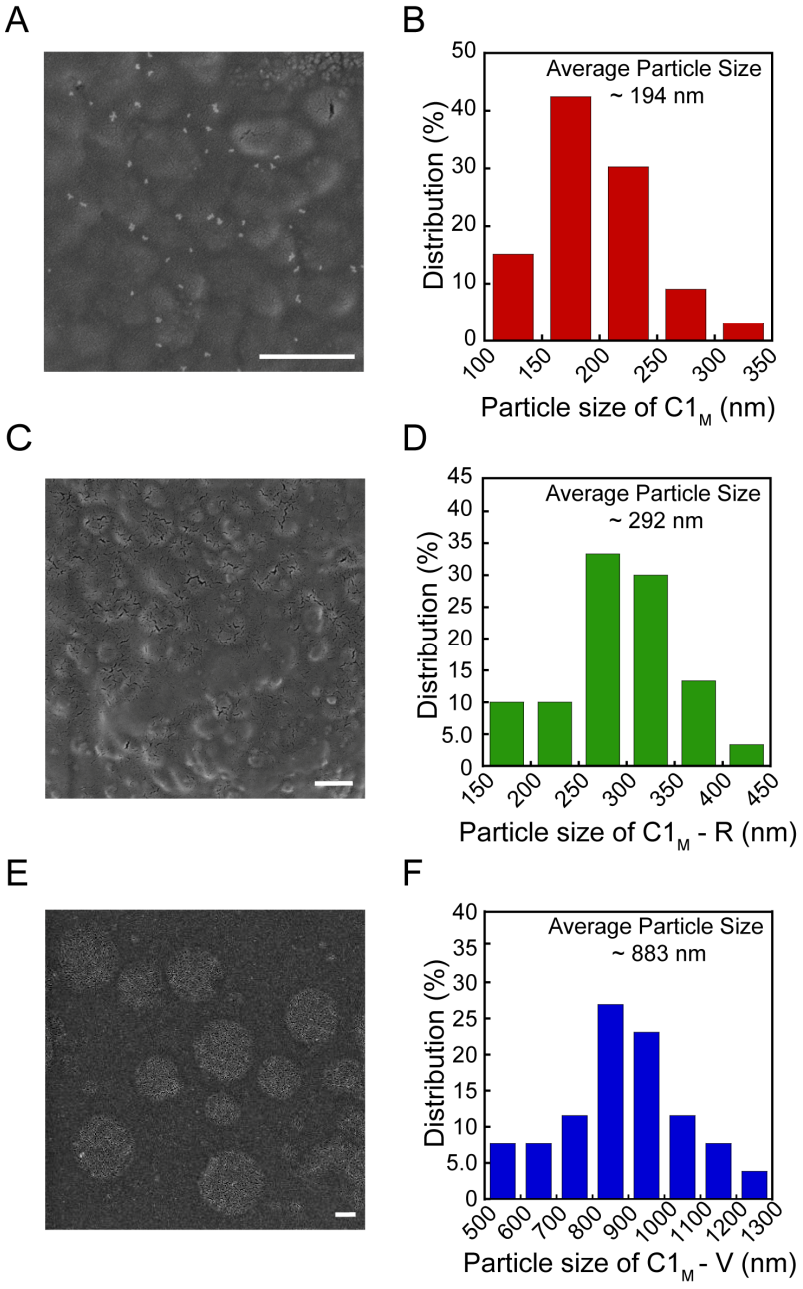

Figure S9. (A, C and E) FESEM image $C1_M$, $C1_M$-R and $C1_M$-V. Scale bar for the image is 500 nm. (B, D and F) Particle size distribution of $C1_M$, $C1_M$-R and $C1_M$-V determined by ImageJ software.

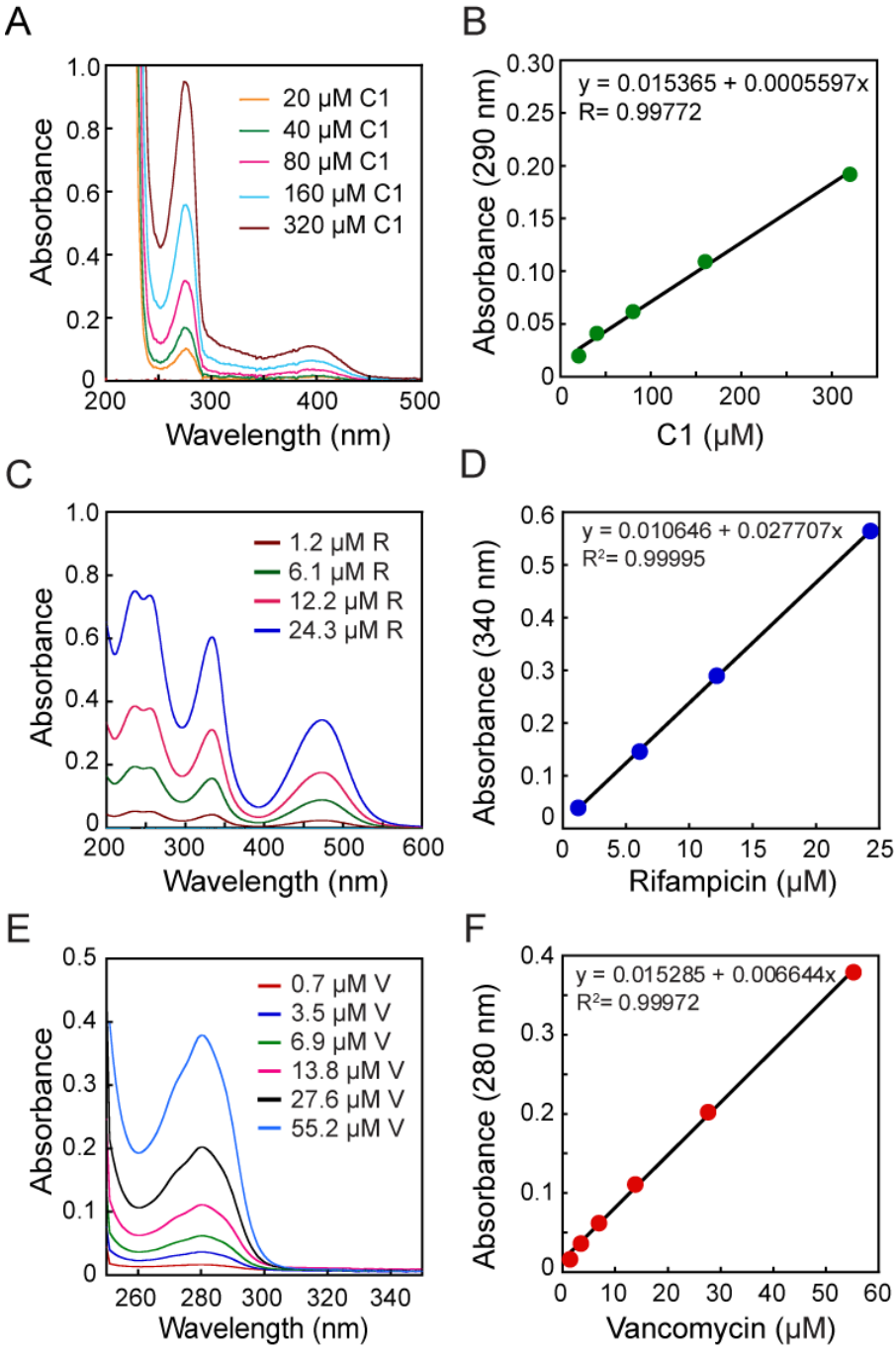

Figure S10. UV-visible absorption spectra of varying concentrations of (A) C1, (C) rifampicin (R) and (E) vancomycin (V). Calibration plots for (B) C1, (D) rifampicin and (F) vancomycin generated by plotting the respective absorbance maxima against concentration.

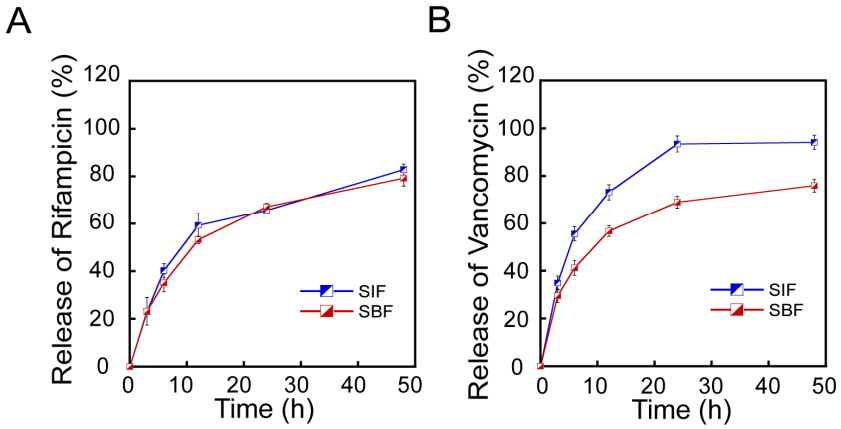

Figure S11. *In vitro* release kinetics of (A) rifampicin and (B) vancomycin from loaded $C1_M$ incubated in simulated body fluid (SBF) (pH 7.4) and simulated intestinal fluid (SIF) (pH 8.0).

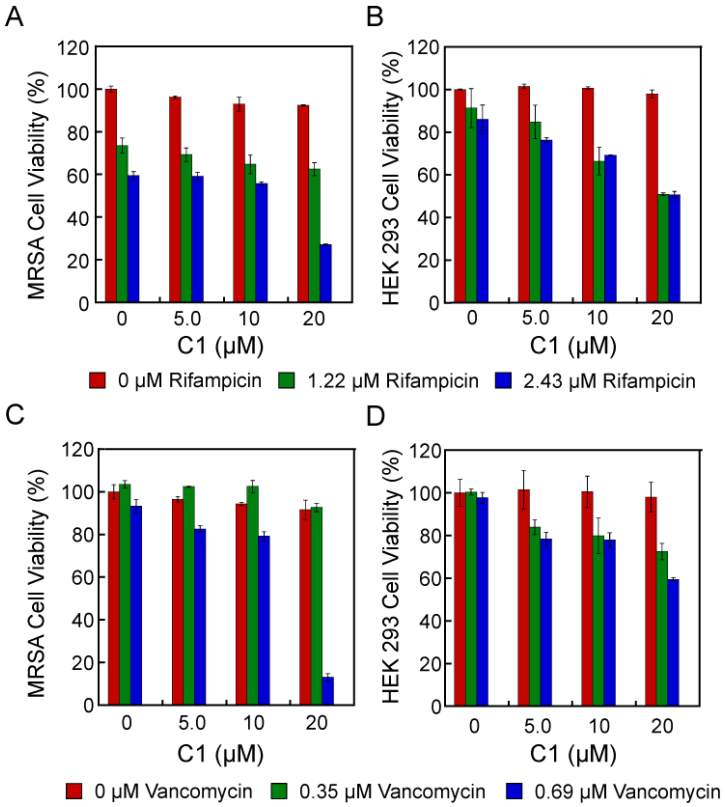

Figure S12. (A) Antibacterial activity of C1 in combination with rifampicin against MRSA strain *S. aureus* 100. (B) MTT assay to ascertain the effect of C1 in combination with rifampicin on HEK 293 cells. Each data point represents mean ± standard deviation from six independent samples. (C) Antibacterial activity of C1 in combination with vancomycin against MRSA strain *S. aureus* 100. (D) MTT assay to ascertain the effect of C1 in combination with vancomycin on HEK 293 cells. Each data point represents mean ± standard deviation from six independent samples.

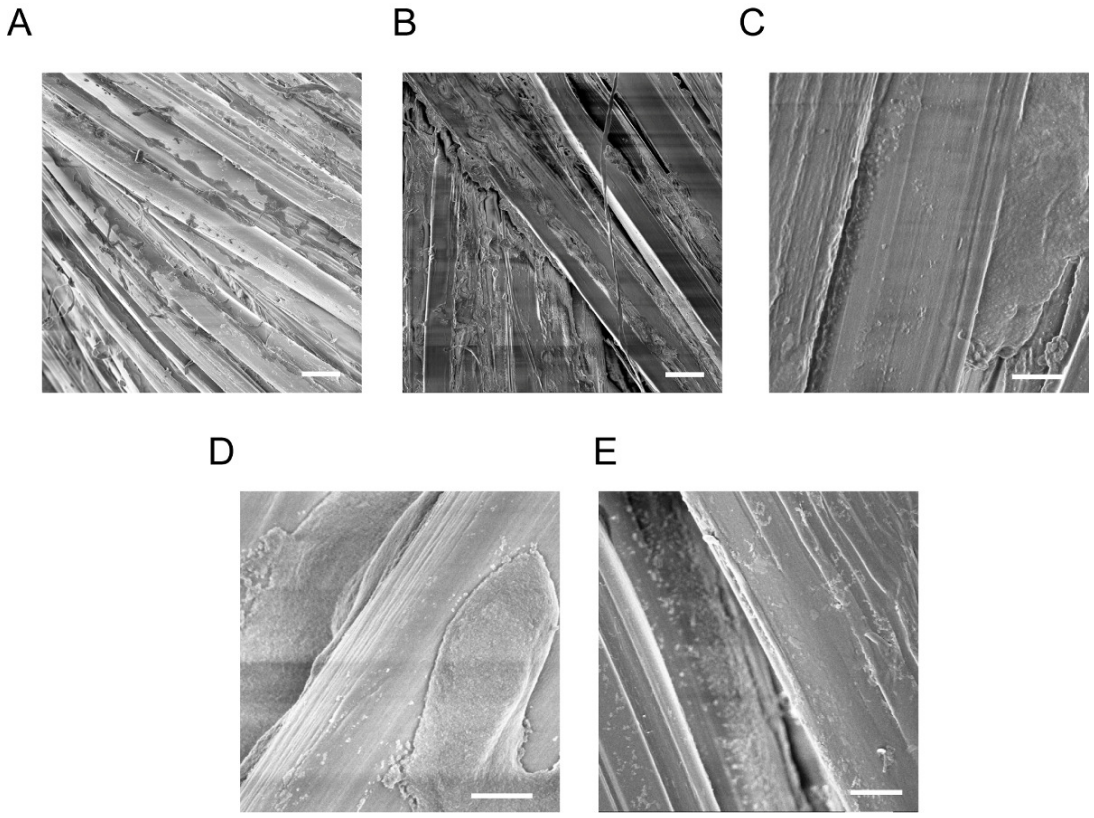

Figure S13. FESEM images of (A) Non-coated silk suture and silk sutures coated with (B) C1, (C) $C1_M$, (D) $C1_M$-R and (E) $C1_M$-V. Scale bar for the images is 2.0 µm.

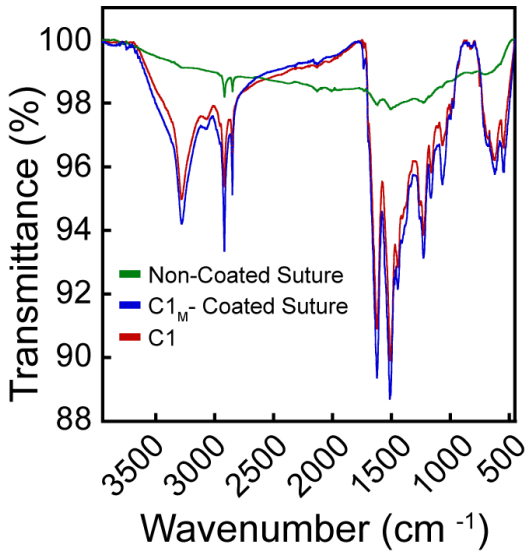

Figure S14. ATR-FTIR of non-coated silk suture, $C1_M$ coated silk suture and C1 alone.

Table S1. Area per molecule at varying concentrations of C1.

C1 (μM)	Number of Molecules ($\times 10^{18}$)	Molecular Area ($Å^2$)	Area per Molecule ($Å^2$) $\times 10^{-18}$
5.0	3.0115	40.3723	13.40
10	6.023	26.039	4.32
20	12.046	21.4873	1.78
50	30.115	18.885	0.62

Table S2. MIC and MKC of C1 against various target bacteria.

Sl. No.	Target Bacteria	MIC	MKC
1.	*S. aureus* MTCC 96	20 μM	40 μM
2.	*L. monocytogenes* Scott A	40 μM	80 μM
3.	*P. aeruginosa* MTCC 2488	80 μM	160 μM
4.	*E. coli* MTCC 433	40 μM	80 μM
5.	*S. aureus* MRSA 100*#	40 μM	80 μM
6.	*S. aureus* MSSA 104*	20 μM	40 μM

* MRSA and MSSA strains were provided by Prof. Benu Dhawan (AIIMS, New Delhi, India) and Prof. Kasturi Mukhopadhyay (JNU, New Delhi, India)

MIC of rifampicin and vancomycin against *S. aureus* MRSA 100 was 4.86 μM and 1.38 μM, respectively.

Table S3. Amount of $C1_M$, $C1_M$-R and $C1_M$-V coated on surgical silk suture.

Sample	Amount of species coated per unit length of suture (mg/cm)
$C1_M$ coated suture	0.177
$C1_M$-R coated suture	0.210
$C1_M$-V coated suture	0.187