Electronic Supplementary Material (ESI) for Biomaterials Science.

Electronic Supplementary Information

Quaternary Ammonium Substituted Pullulan Accelerates Wound Healing and Disinfects *Staphylococcus aureus* Infected Wounds in Mouse Through an Atypical 'Non-Pore Forming' Pathway of Bacterial Membrane Disruption

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Materials

Pullulan (P) (Mol Wt: 200,000 g/mol) was procured from TCI Co. Ltd. 2,7-dichlorofluoroscein diacetate, propidium iodide, cell proliferation assay kit II (XTT), Bradford reagent, glutaraldehyde, deuterium oxide (D_2O) and glycidyl trimethyl ammonium chloride (GTMAC) were purchased from Sigma-Aldrich. 5,5-dithio-bis(2-

nitrobenzoic acid), 5 (and 6)-carboxyfluorescein diacetate succinimidyl ester and 3,3′-Dipropylthiadicarbocyanine iodide were obtained from TCI Co. Ltd. Ethanol and Luria−Bertani broth were purchased from Merck. Rhodamine-Phalloidin, 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA), DMEM culture medium, Fetal bovine serum (FBS) and penicillin−streptomycin (Pen Strep) were obtained from Gibco (Thermo Scientific). HEPES buffer was obtained from SRL Pvt. Ltd.

In vitro cytotoxicity of CP derivatives

HEK-293A cells (Invitrogen) were grown overnight at a density of 10,000 cells/well in 96-well plates at 37 °C and under 5% CO_2 in an incubator. Increasing concentrations of P & CP (0.25 – 8 µg/mL) in DMEM supplemented with 10% FBS were added to the adhered cells and treated for 24 h. Cells which did not receive any P or CP treatment served as controls. XTT reagent was added to each well after treatment and incubated for 2 h in a CO_2 incubator. Absorbance at 490 and 690 nm was measured for each well using a plate reader (Tecan Infinite M200 Pro). The background absorbance was minimized by subtracting the absorbance at 690 nm from that of 490 nm. % cell viability was calculated using the following equation:

$$\%\ Cell\ viability = \frac{Absorbance\ of\ treated\ cells}{Absorbance\ of\ untreated\ cells} \times 100$$

The morphology of the HEK-293A cells after treatment with CP derivatives was also visualized using fluorescence microscopy. For this, HEK-293A cells (2×10^4 cells) were grown on poly-L-lysine coated coverslips in 1 mL DMEM medium containing 10% FBS for overnight. The media was then replaced with 1 mL of fresh media containing 4 µg/mL pullulan & CP derivatives and the adhered cells were incubated for 12 h. Untreated cells served as controls. Next, the coverslips were washed with PBS and the cells were fixed with 4% formaldehyde for 15 mins. Fixed cells were washed with PBS and

permeabilized with 0.1% Triton-X-100 for 10 min followed by washing with PBS. The cells were then stained with Rhodamine-Phalloidin as per manufacturer's protocol for 30 min in dark at room temperature and washed with PBS to remove excess stain. The stained cells were air dried and mounted on a clean glass slide using mounting media with DAPI. All samples were observed with a fluorescence microscope at excitation wavelengths of 540 nm and 405 nm for Rhodamine and DAPI, respectively.

Hemolysis assay

1 mL blood was collected from a healthy donor, suspended in PBS buffer and centrifuged (2000 rpm, 10 min) to separate red blood cells. The precipitated RBCs was washed with PBS until a clear supernatant is achieved. Next, the RBCs were resuspended in PBS at a volume concentration of 2%. Pullulan, CP-1, CP-2, CP-3 & CP-4 were added into 2% RBCs suspension at different test concentrations (0.25-64 μg/mL). RBCs treated with Triton-X-100 and PBS served as the positive and negative controls, respectively. All the suspensions were incubated at 37 °C for 1 h and then centrifuged at 2000 rpm for 10 min. 100 μL supernatant was collected in 96-well plates and the absorbance of the supernatant was measured at 545 nm. The hemolysis ratio was calculated using the following equation:

$$\text{Hemolysis ratio } (\%) = \frac{\text{OD}_{\text{test}} - \text{OD}_{\text{neg}}}{\text{OD}_{\text{pos}} - \text{OD}_{\text{neg}}} \times 100$$

where OD_{test}, OD_{neg}, and OD_{pos} are the absorbance at 545 nm of sample, negative control, and positive control, respectively.

Assays for Studying Membrane Integrity

(1) cFDA/SE leakage assay:

The assay was performed as per the method reported in our previous study[1]. Briefly, bacterial cells were washed and suspended in sterile PBS, stained with 5 (and 6)-

carboxyfluorescein diacetate succinimidyl ester (cFDA-SE) dye for 20 min at 37 °C. The stained cells were washed with PBS to remove excess dye molecules and further resuspended in PBS containing increasing concentrations of CP-4 (0.5-8 µg/mL) and treated for 6 h at 37 °C and 180 rpm. Following treatment, the cells were pelleted down and the supernatants were collected. The fluorescence of the supernatants was measured at an excitation wavelength of 488 nm and emission wavelength of 518 nm in a spectrofluorometer. Control cells were stained with the dye and incubated under similar conditions in the absence of CP-4. Fluorescence measurements were taken for three independent samples.

(2) PI uptake assay:

The assay was performed as per the method reported in our previous study[1]. Briefly, bacterial cells were first treated with increasing concentrations of CP-4 (0.5-8 µg/mL) for 6 h at 37 °C and 180 rpm, and then stained with 30 µM propidium iodide (PI) dye for 30 min at 37 °C. After washing with PBS to remove excess PI molecules, the fluorescence of the stained cells were measured at an excitation wavelength of 535 nm and emission wavelength of 617 nm. The fluorescence measurements were recorded after the fluorescence obtained from control samples was subtracted. Fluorescence measurements were taken for three independent samples.

(3) cFDA/SE-PI dual stain fluorescence microscopy assay:

The assay was performed as per the method reported in our previous study[1]. Briefly, bacterial cells were treated with increasing concentrations of CP-4 (0.5-8 µg/mL) for 6 h at 37 °C and 180 rpm, and then stained with cFDA/SE and PI as mentioned earlier. After washing with PBS to remove excess dye molecules, stained cells were fixed in 2.5% glutaraldehyde, drop-casted on a clean glass slide, airdried, and observed under a fluorescence microscope equipped with a blue and green laser. Fluorescence images of the cells were recorded.

(4) Protein leakage assay:

E. coli and *S. aureus* cells (10^6 CFU/mL) were treated with increasing concentrations of CP-4 (0.5-8 µg/mL) for 6 h at 37 °C and 180 rpm. Following treatment, the cells were pelleted down at 5000 rpm for 5 min, and the cell-free supernatant was collected. The concentration of leaked proteins in the supernatant was measured using standard Bradford assay.

Surface morphology characterization of bacteria

Surface morphology of CP-4 treated *E. coli* and *S. aureus* cells was studied by SEM. Bacteria were treated with 4 µg/mL CP-4 for 4 h at 37 °C and 180 rpm. The cells were washed, fixed with 2.5% glutaraldehyde for 1 h at 4 °C and subsequently subjected to a series of ethanol gradient dehydration (20%, 50%, 70%, 90%, 100%). The dehydrated cells were then drop casted on a cleaned silicon wafer, airdried, sputter-coated with gold, and observed under SEM. Control cells, without CP-4 treatment, were also processed in the same manner.

Measurement of Intracellular ROS Production

Intracellular ROS was measured using standard 2,7-dichlorofluoroscein diacetate (DCFH-DA) assay. 10^6 CFU/mL of *E. coli* and *S. aureus* cells were treated with increasing concentrations of CP for 6 h at 37 °C and 200 rpm. Following treatment, both control and treated cells were washed with PBS and incubated with 100 µM DCFH-DA probe for 30 min in dark at 37 °C. The green fluorescence originating from the oxidative cleavage of DCFH-DA to DCF was measured in a microplate reader (Tecan Infinite M200 Pro) with an excitation wavelength of 485 nm and emission wavelength of 528 nm. Cells treated with H_2O_2 served as positive control.

Measurement of Intracellular Glutathione (GSH) Activity

The activity of intracellular GSH was determined using standard Ellman's assay as widely reported in literature. Then 10^6 CFU/mL of *E. coli* and *S. aureus* cells were treated with increasing concentrations of CP for 6 h at 37 °C and 200 rpm. Following treatment, both control and treated cells were centrifuged at 5000 rpm for 5 min, washed with PBS, and lysed. The lysed cells were further centrifuged, and the clear supernatant was collected. The supernatant was mixed with 50 mM Tris-HCl and 100 mM 5,5-dithiobis(2-nitrobenzoic acid) (DTNB) and incubated for 30 min in dark at 37 °C. The absorbance of the resulting solution was measured at 412 nm using a UV−visible spectrometer. The percentage loss of glutathione was calculated as

$$\left(1 - \frac{OD@412\ nm\ of\ treated}{OD@412\ nm\ of\ control}\right) \times 100$$

Antibiofilm assay

S. aureus were grown in 96-well microtiter plates for 48 h to form biofilm. Following growth, the mature biofilms were washed gently with 1×-PBS twice to remove loosely attached and non-adherent cells. The biofilms were treated with increasing concentrations of CP-4 (32 – 1024 µg/mL) for 24 h at 37 °C in a moist and static environment conditions. After treatment, the biofilms were carefully washed with PBS to remove planktonic cells. The total biomass of the treated biofilms was quantified using crystal violet (CV) staining. The treated and untreated biofilms were stained with 100 µL of 0.1% CV (w/v) for 10 mins and then washed with PBS thrice to remove the unbound stain. The CV-stained biofilms were air-dried for 30 mins, following which 200 µL of 33% acetic acid solution was added to each well and incubated for 15 min for solubilizing the CV stain. 150 µL of the eluate were taken in a separate 96-well plate and its absorbance was measured at 590 nm using a microplate reader (Tecan Infinite M200 Pro). The viability of the biofilms was measured using XTT assay according to the manufacturer's

protocol. Briefly, the treated and untreated biofilms were incubated with 100 µL XTT reagent for 1 h and the absorbance of each well was measured at 490 nm using a microplate reader (Tecan Infinite M200 Pro). The effect of CP-4 on biofilm biomass and viability was calculated with respect to an untreated sample (control) and expressed in terms of % Biofilm biomass and % Biofilm viability using the following equations:

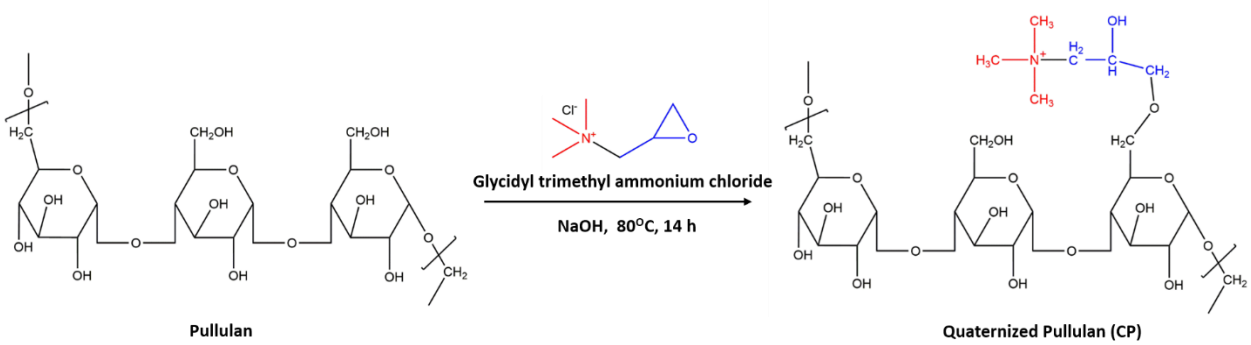
$$\%\ Biofilm\ biomass = \frac{Absorbance\ of\ treated\ @\ 590\ nm}{Absorbance\ of\ untreated\ @\ 590\ nm} \times 100$$

$$\%\ Biofilm\ viability = \frac{Absorbance\ of\ treated\ @\ 490\ nm}{Absorbance\ of\ untreated\ @\ 490\ nm} \times 100$$

For fluorescence imaging of biofilms, *S aureus* biofilms were grown on tissue culture glass coverslips. Following CP-4 treatment, the biofilms were washed twice with 1× PBS and then stained with 50 µL of 25 µM cFDA-SE dye for 30 mins at 37°C. The samples were washed twice with 1× PBS, and visualized under a fluorescence microscope. SEM analysis of the biofilms were carried out by first fixing the treated and untreated biofilms with 2.5% glutaraldehyde for 1 h at 4°C followed by dehydrating the samples using an ethanol gradient (20%, 50%, 70% and 100%). The fixed and dehydrated samples were then air dried overnight, sputter coated with gold and visualized using SEM.

Statistical analysis

The values for all experiments are expressed as mean ± SD of three individual experiments. The data were analyzed using Student's t-test, and statistically significant values are denoted by *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.



Scheme S1: Scheme showing quaternization of pullulan using GTMAC in NaOH aqueous solution.

Table S1. Reaction parameters for synthesis of quaternized pullulan derivatives

Sample ID	Pullulan (g)	GTMAC (g)	5M NaOH (mL)	Water (mL)
CP-1	0.75	7.5	1.6	41.63
CP-2	0.75	10	1.6	39.45
CP-3	0.75	13	1.6	37.5
CP-4	0.75	15	1.6	35

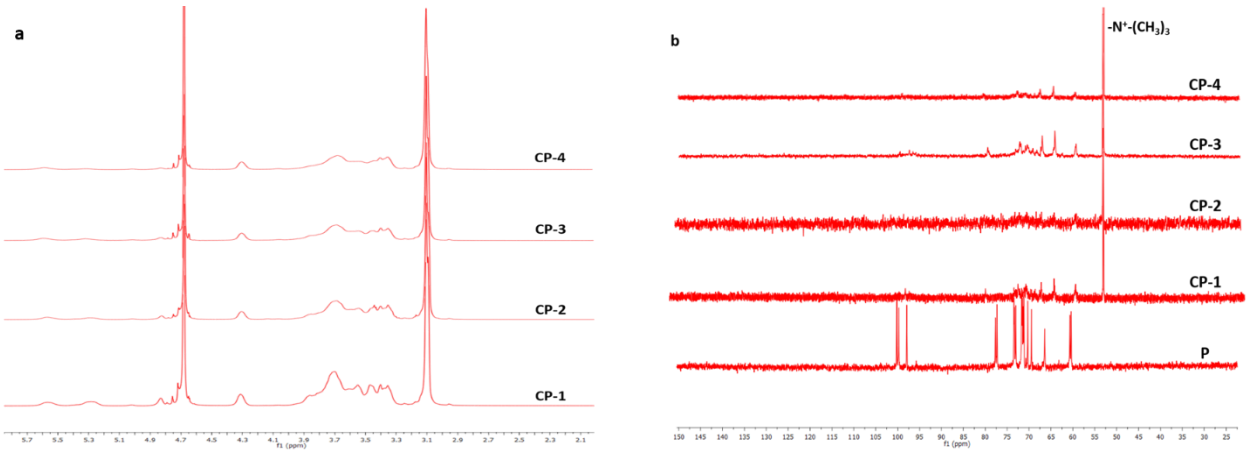


Figure S1. (a) ^{1}H-NMR & (b) ^{13}C-NMR spectra of CP derivatives.

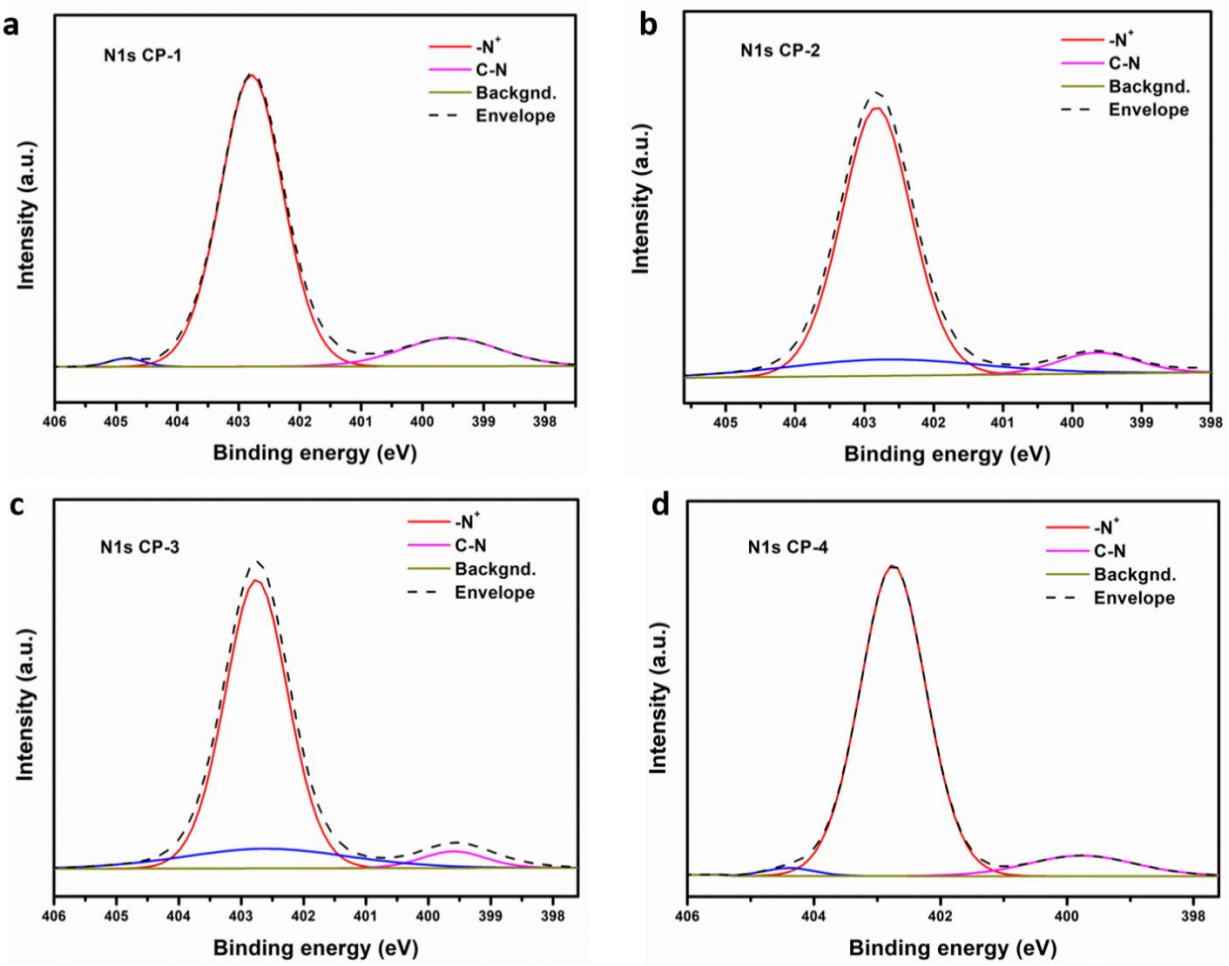


Figure S2. N1s high resolution spectra of (a) CP-1, (b) CP-2, (c) CP-3 and (d) CP-4.

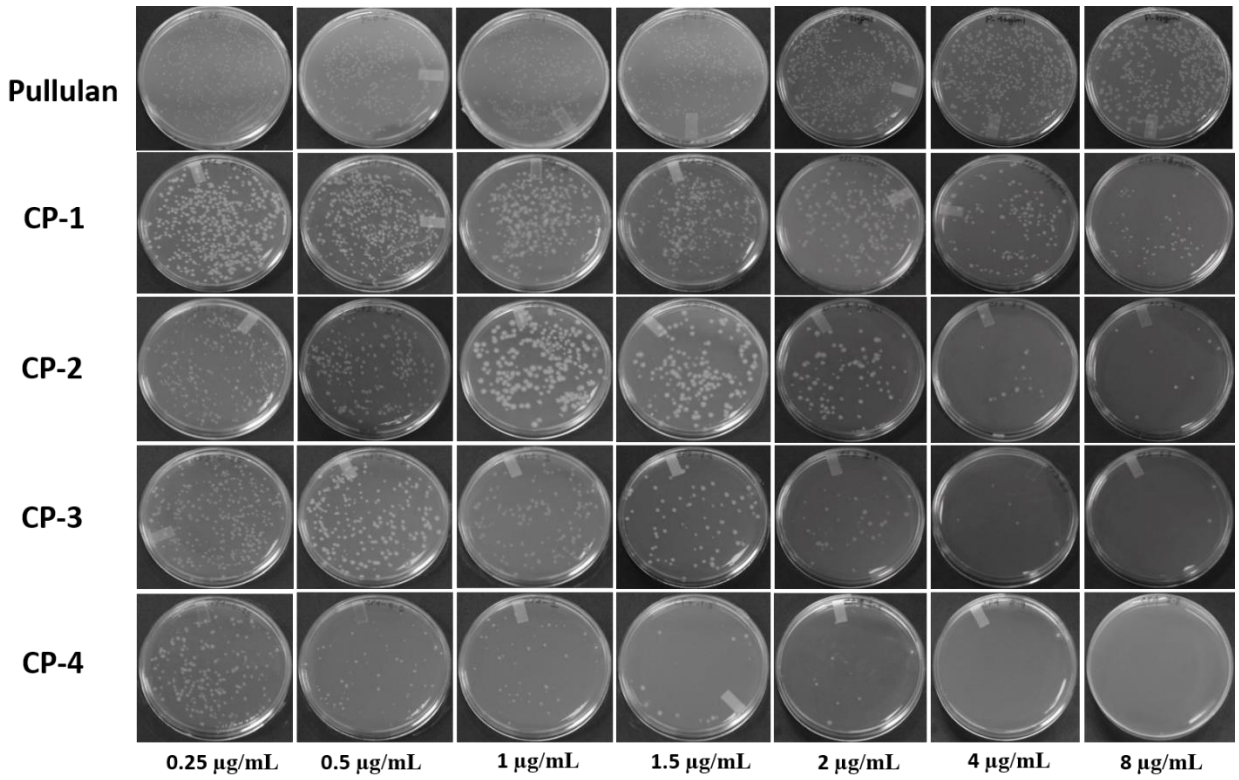


Figure S3. Concentration dependent antibacterial activity of CP derivatives against *E coli* treated for 12 h.

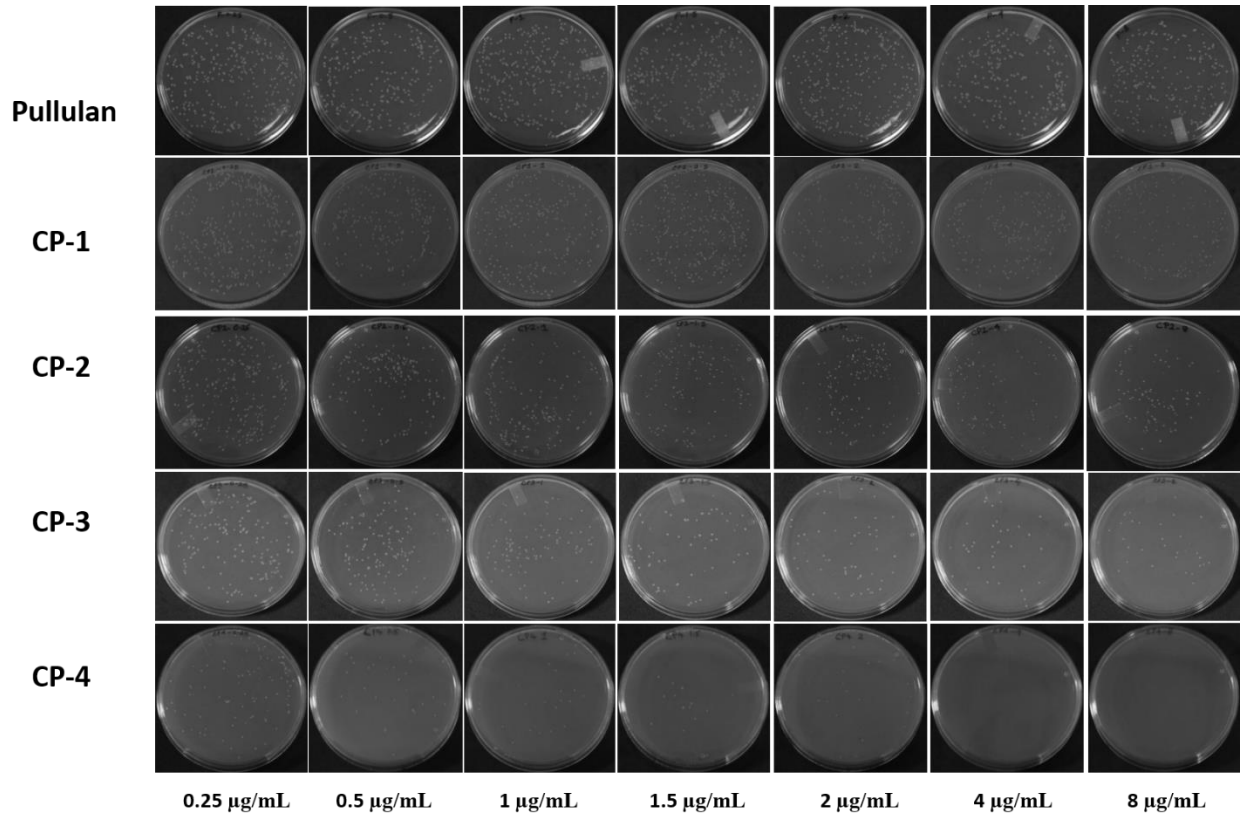


Figure S4. Concentration dependent antibacterial activity of CP derivatives against *S aureus* treated for 12 h.

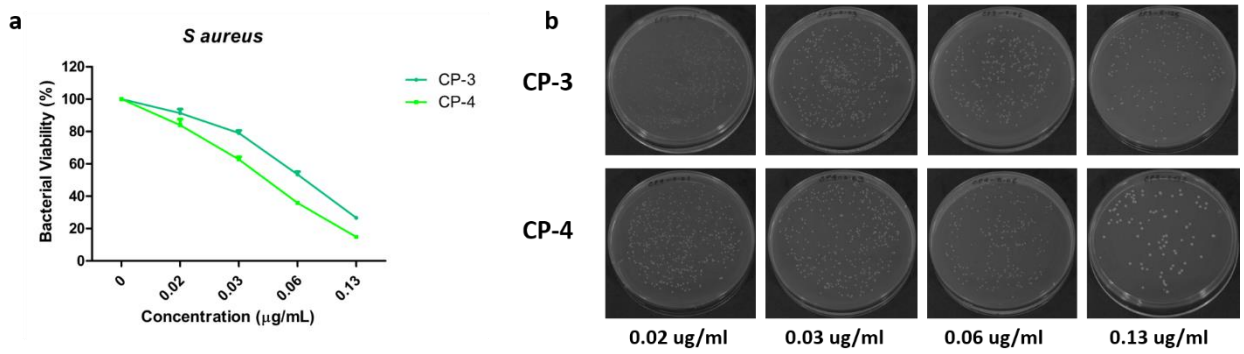


Figure S5. Concentration dependent antibacterial activity of CP-3 & CP-4 against *S aureus* treated for 12 h at concentrations ranging from 0.02-0.13 µg/mL.

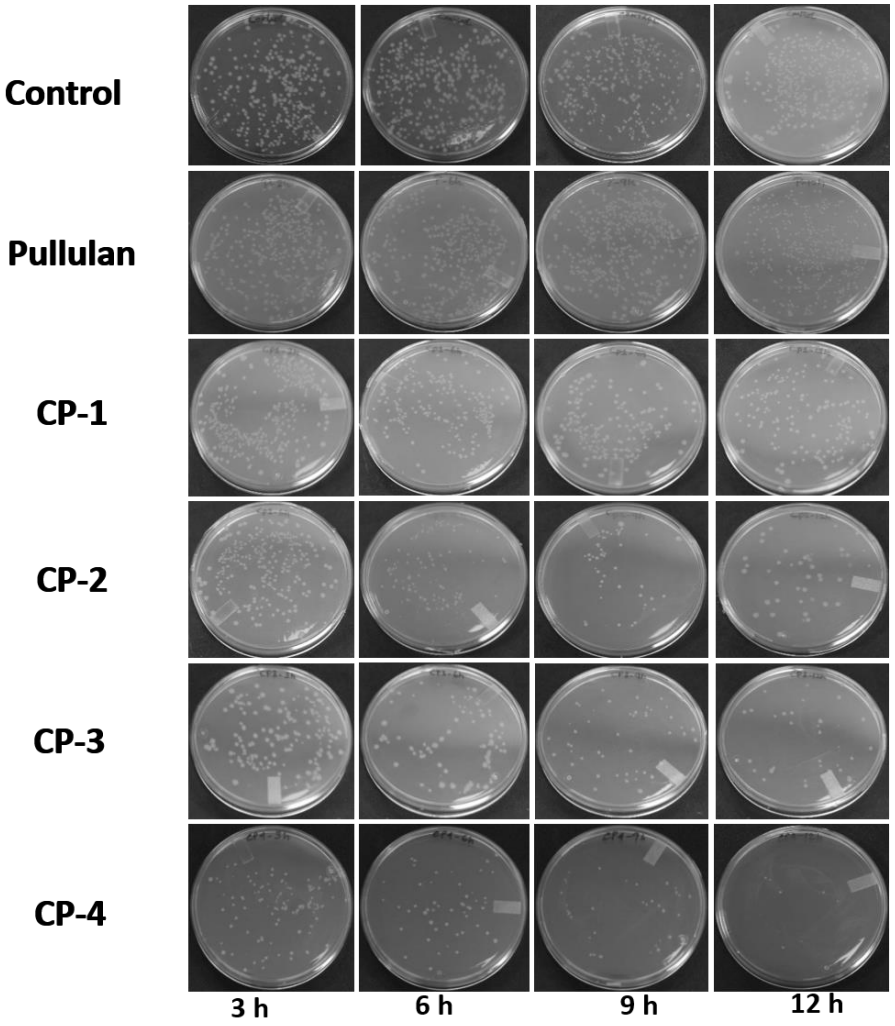


Figure S6. Time dependent antibacterial activity of CP derivatives (2 µg/mL) against *E coli* treated for 3h, 6h, 9h & 12 h.

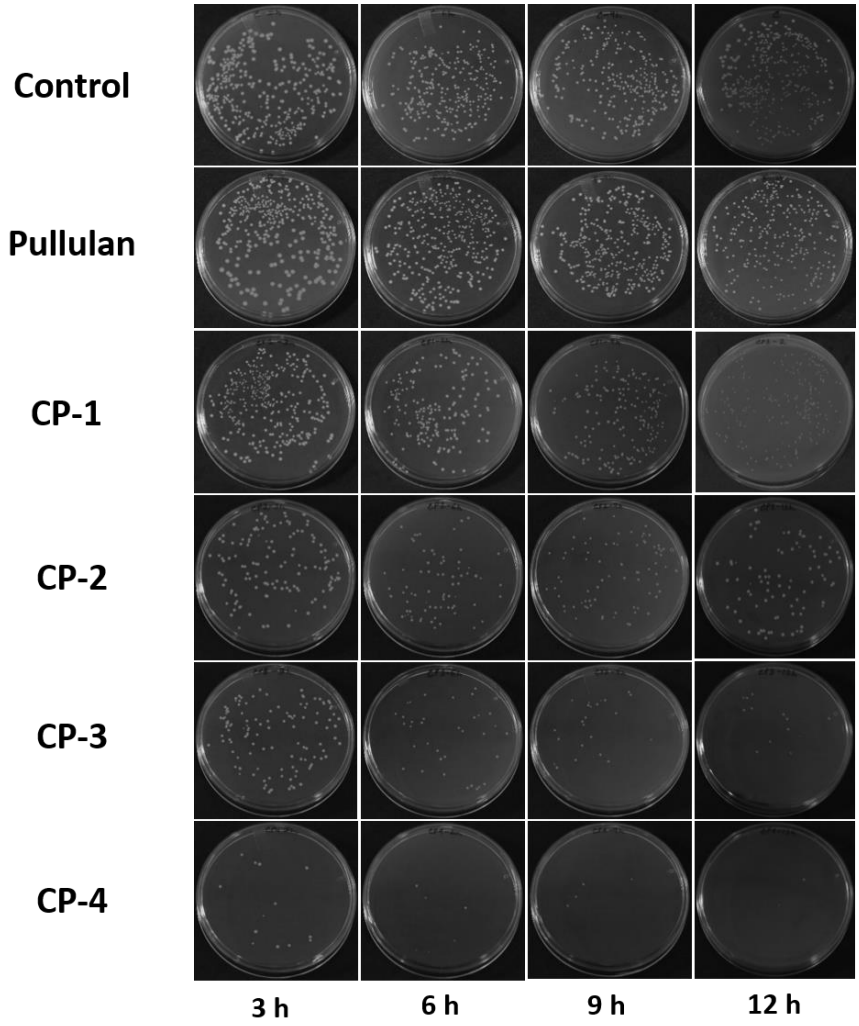


Figure S7. Time dependent antibacterial activity of CP derivatives (2 µg/mL) against *S aureus* treated for 3h, 6h, 9h & 12 h.

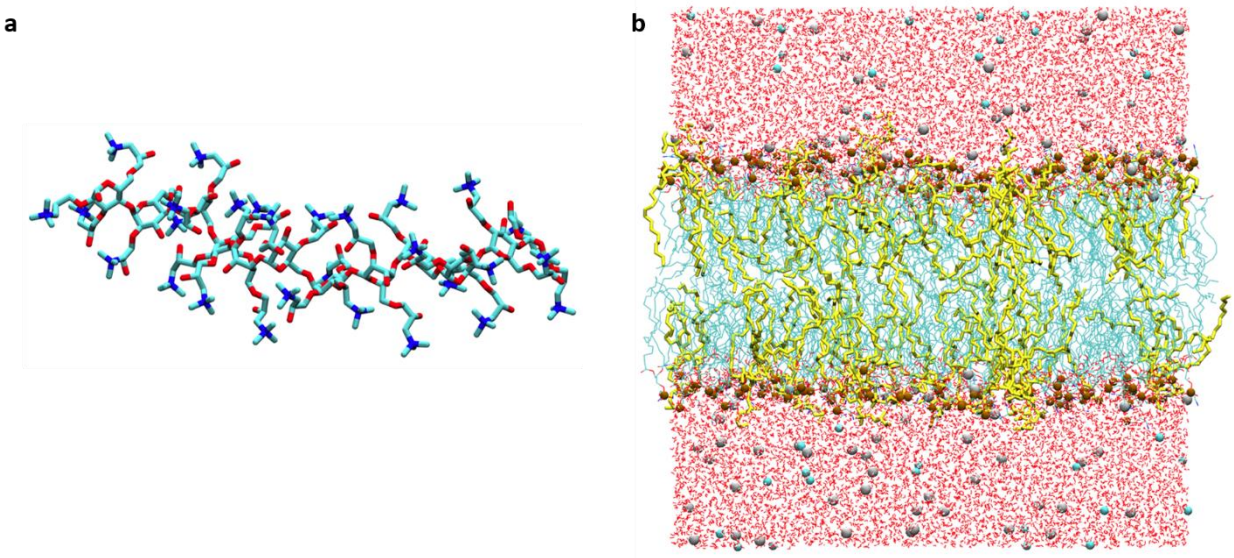


Figure S8. (a) Representation of CP-4 tetramer. Nitrogen and oxygen atoms are shown in blue and red, respectively. **(b)** Snapshot of DOPE/DOPG bacterial membrane with DOPE shown in cyan, DOPG in yellow, water molecules in red and sodium and chloride ions as grey & blue spheres, respectively.

Table S2. Composition of the simulated bacterial membrane-tetramer systems

System	DOPE/DOPG	Water	Na^+/Cl^-	Tetramers
Pure	200/50	15000	85/35	-
CP4-tet-1	200/50	32382	86/64	1
CP4-tet-2	200/50	32404	64/70	2
CP4-tet-3	200/50	32029	63/97	3
CP4-tet-4	200/50	32029	63/125	4
CP4-tet-6	200/50	31664	62/180	6

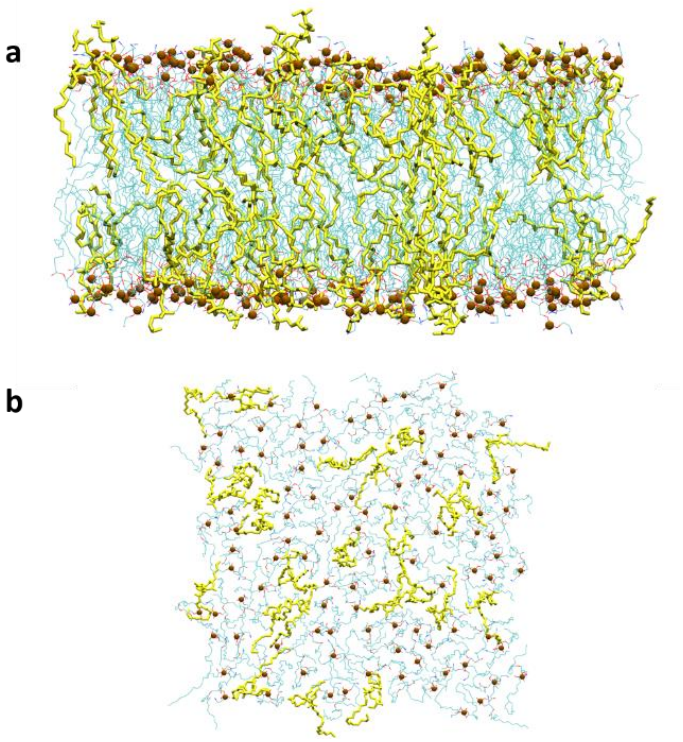


Figure S9. (a) Side view & **(b)** top view of untreated bilayer.

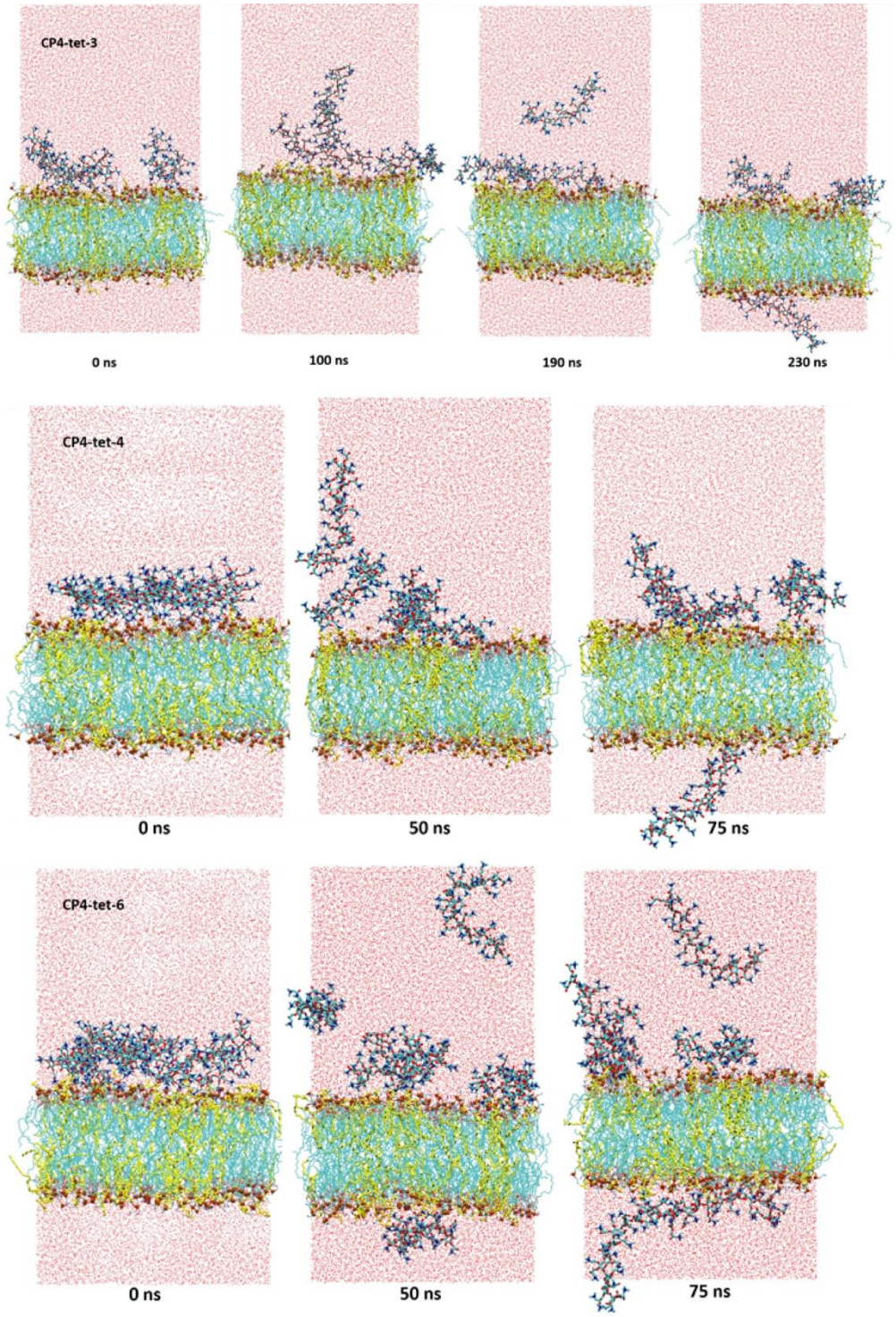


Figure S10. Snapshots showing the interaction of 3, 4 & 6 CP-4 tetramers with the bacterial membrane.

Table S3. Average number of H-bond between various constituent pairs of the systems studied.

System	PG-PG /PG lipid	PE-PE /PE lipid	PE-PG /PG lipid	PG-water /PG lipid	PE-water /PE lipid	PE-tet/tet molecule	PG-tet/tet molecule
pure	0.61	0.51	0.66	6.85	5.94		
tet-1	0.63	0.50	0.65	6.66	5.87	1.34	1.41
tet-2	0.66	0.52	0.63	6.48	5.85	1.11	1.34

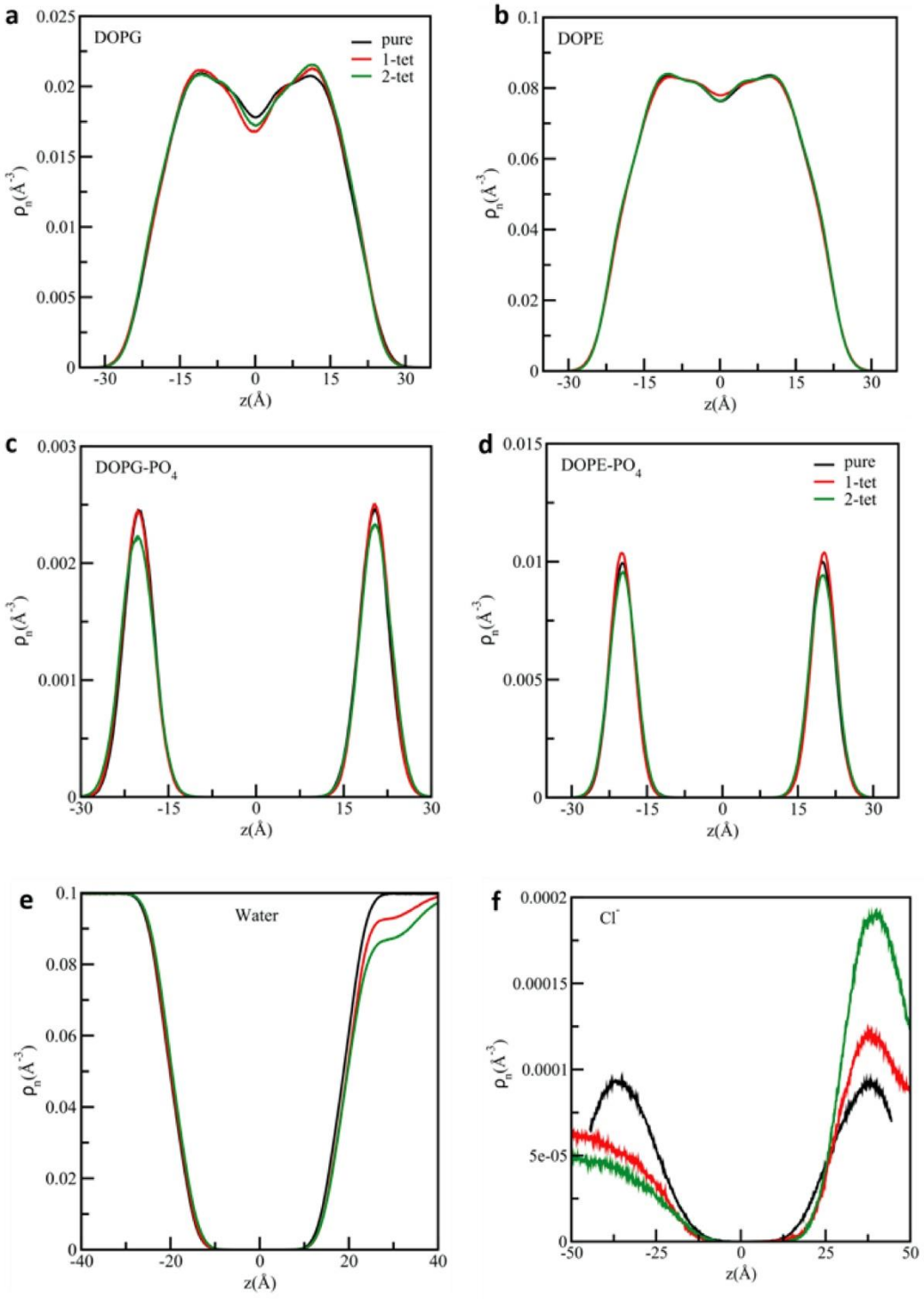


Figure S11. Average number density plots for various constituent species (a) DOPG lipids, (b) DOPE lipids, (c) DOPG-phosphate, (d) DOPE-phosphate (e) water (f) chloride ion at varying concentrations of tetramers.

Table S4. Average Area per lipid (Å^2), thickness (Å), and surface roughness (Å) at varying concentrations of tetramer.

System	APL (Å^2)	Thickness (Å)	Roughness (Å)	
			Upper Leaflet	Lower Leaflet
pure	64.1 ± 1.1	39.8	2.2	2.2
tet-1	63.8 ± 1.4	40.3	2.3	2.4
tet-2	64.0 ± 0.8	40.3	2.5	2.6

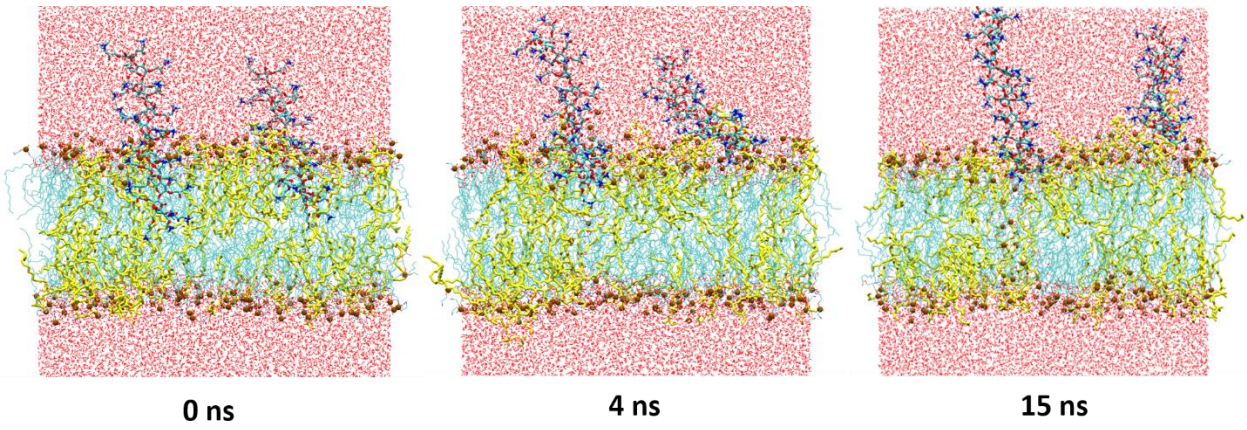


Figure S12. Snapshots showing the interactions of two half-inserted CP-4 tetramers within the bacterial membrane.

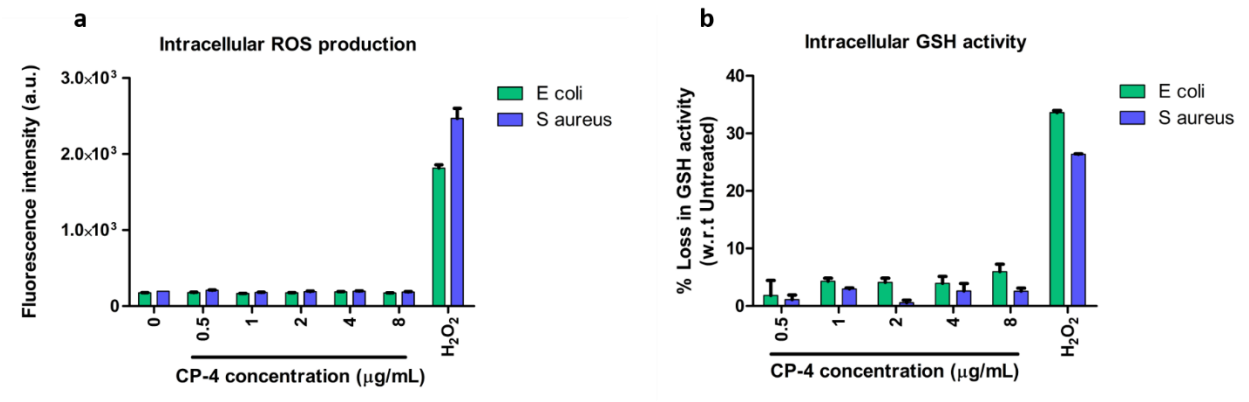


Figure S13. (a) Intracellular ROS production & **(b)** GSH activity in CP-4 treated *E coli* and *S aureus*.

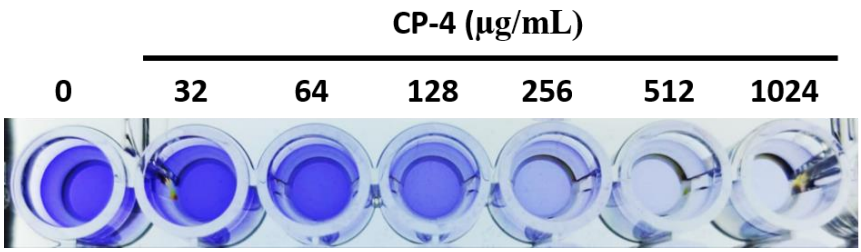


Figure S14. Crystal violet staining of CP-4 treated mature *S aureus* biofilms.

Reference:

1. Roy, S.; Mondal, A.; Yadav, V.; Sarkar, A.; Banerjee, R.; Sanpui, P.; Jaiswal, A., Mechanistic insight into the antibacterial activity of chitosan exfoliated MoS2 nanosheets: membrane damage, metabolic inactivation, and oxidative stress. *ACS Appl. Bio Mater.* **2019,** *2* (7), 2738-2755.