Supporting Information:

Gram-Selective Antibacterial Activity of Mixed-Charge 2D-MoS_2

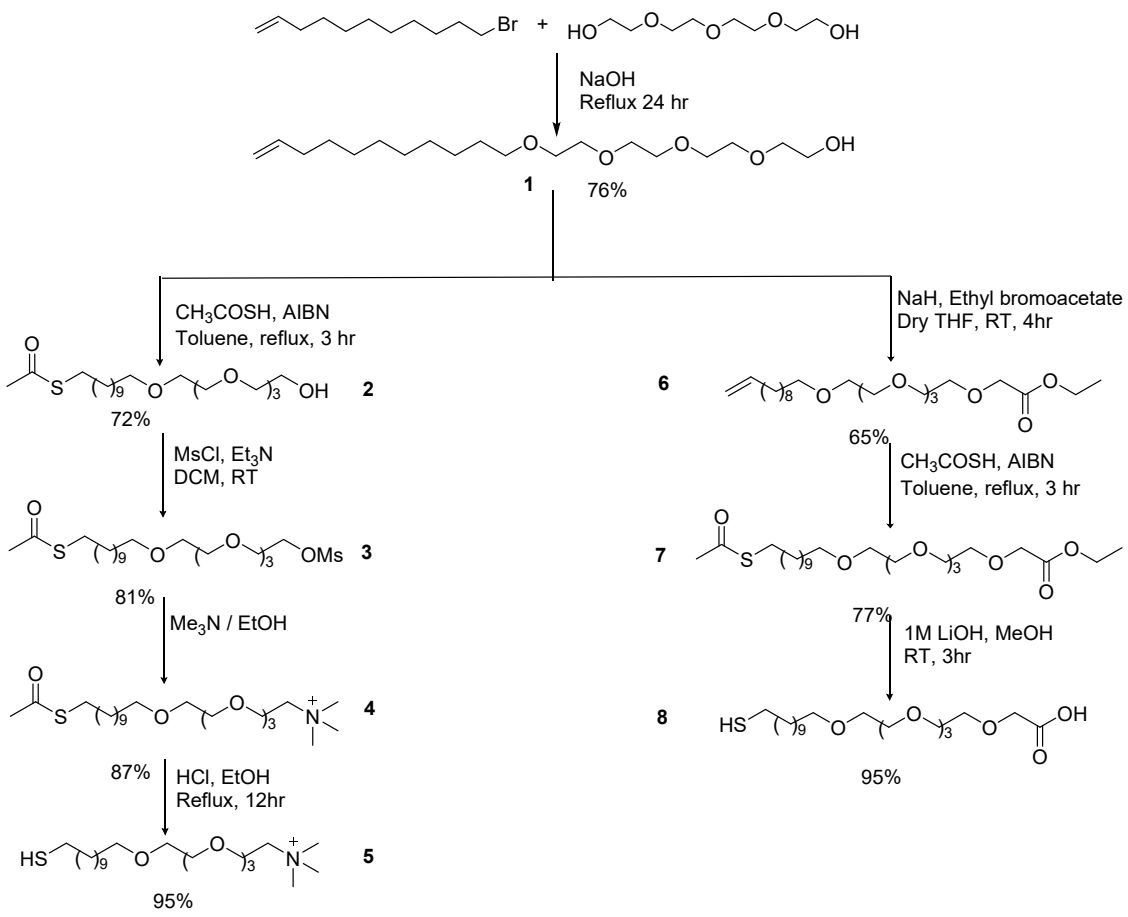
Jagabandhu Sahoo,[a] Mrinmoy De[a]

[a]Department of Organic Chemistry, Indian Institute of Science, Bangalore- 560012, India.

Email : md@iisc.ac.in

EXPERIMENTAL SECTION:

1. Materials and Instrumentation: Molybdenum disulphide (MoS_2), n-Butyl lithium, 11-bromo undecane, Tetraethyleneglycol, Sodium hydride (NaH), Sodium hydroxide (NaOH), Azobisisobutyronitrile (AIBN), Thioacetic acid, Ethyl bromoacetate, Methane sulfonylchloride (MsCl), Trimethyl amine (Me_3N), Lithium hydroxide (LiOH), DiSC3(5) (3,3'-Dipropylthiadicarbocyanine Iodide), DTNB (5,5′-dithiobis (2-nitrobenzoic acid)), Glutathione, Luria broth media (LB, HiMedia) were purchased from commercial sources (Sigma Aldrich and SRL India Pvt. Ltd.) and used without further purification. NMR spectra of all products were recorded using TMS (Me_4Si) as internal standard at 400 MHz. NMR data are reported in the following manner: chemical shift value in δ (ppm) relative to tetramethylsilane (δ 0, s), multiplicity (s = singlet, d = doublet, dd = double doublet, t = triplet, q = quartet, br = broad, m = multiplet), coupling constants (Hz) and integration. Fluorescence was measured by Thermo Scientific Varioskan Flash Multimode Reader. The AFM image was recorded using JPK instrument and the height was measured by using JPK software. SEM images were recorded by Ultra55 FE-SEM Karl Zeiss EDS instrument. The Zeta potential of all the samples were recorded by Malvern Zetasizer Nano instrument. The concentration of Mo in functionalized samples were determined by using quadrupole inductively coupled plasma mass spectrometer (ICP-MS). The optical density of bacterial solution was measured Thermo Fisher Scientific Microplate Reader.



1. Ligand Synthesis:

Synthesis of ligand 1 (Positive ligand):

A mixture of sodium hydroxide (21.4 mmol) and tetraethyleneglycol (106.5 mmol) was subjected to stir for 15 min at 100^0c under Argon atmosphere. Then 11-bromoundec-1-ene (21.4 mmol) was added slowly to the reaction mixture and refluxed for 24h. After the completion of reaction, the reaction mixture was cooled to room temperature and the product was extracted by repeated washing with hexane containing 5% ethyl acetate mixture. After evaporation of solvent, the mixture of the monoalkylated and dialkylated was subjected and the monoalkylated product was purified by column chromatography with yield of 75%.

1.1 Synthesis of Compound 2:

The monoalkylated product 1 (5.77 mmol) was taken in a double neck round bottom flask and dissolved in 10 ml of dry toluene under argon atmosphere. Then, 0.3 equivalent of AIBN was added followed by the addition of thioacetic acid (11.4 mmol) and refluxed for 3h at 110^0C. After 3h, the reaction mixture was cooled to room temperature and transferred to separating funnel with ethyl acetate. Then, the product mixture was washed with saturated sodium

bicarbonate twice and organic part was dried over anhydrous sodium sulphate and concentrated by rotary evaporator. The crude mixture was purified by column chromatography and confirmed by ^{1}H- NMR with yield 70%.

1.2 Synthesis of Compound 3:

The compound 2 (4.5 mmol) was taken in a RB with 20 ml of DCM and kept in stirring at 0^0C. After 10 min. triethylamine was added to the reaction mixture and stirred for another 15 min. Then 6.5 mmol methane sulfonyl chloride was added and stirred at room temperature for overnight. After the completion of reaction, the crude was dissolved in ethyl acetate and washed twice with dil. HCl (0.1M) and three times with saturated sodium bicarbonate solution. Then, the organic layer was dried using anhydrous sodium sulfate, concentrated by rotary evaporator and purified by column chromatography. The product was confirmed by ^{1}H- NMR with yield 80%.

1.3 Synthesis of Compound 4:

The compound 3 was dissolved in 15 ml of Ethanol followed by the addition of trimethylamine. The reaction mixture was stirred under argon atmosphere until the disappearance of reactant spot. The reaction was monitored by checking TLC at regular interval and if required then extra trimethylamine was added till the reactant spot vanished. Then after completion of reaction the product was separated by solvent separation method in hexane and ether (1:1) and kept in refrigerator for overnight. The precipitated product was collected, and product was confirmed by ^{1}H- NMR with a yield of 90%.

1.4 Synthesis of Compound 5:

The compound 4 (3.75 mmol) was dissolved in 20ml of EtOH. Then, 1ml of 0.1 M HCl was added and refluxed for overnight under argon atmosphere. After that the solvent was evaporated and the product was obtained by trituration three times with hexane: diethylether (1:1). The pure product obtained from precipitation was characterized by ^{1}H- NMR.

^{1}H-NMR (400 MHz, $CDCl_3$): δ 3.965 (b, 2H, -O-CH_2-CH_2-N-), 3.761 (b, 2H, - O-CH_2-CH_2-N(CH_3)$_3$), 3.683-3.559 (m, 12H, -O-CH_2-CH_2-O-), 3.322 (s, 9H, -N+ (CH_3)$_3$), 3.459-3.425 (t, 2H, -CH_2-CH_2-O-), 2.553- 2.498 (q, 2H, -CH_2-SH), 1.626-1.553 (m, 4H, -CH_2-), 1.274 (m, 15H, -CH_2).

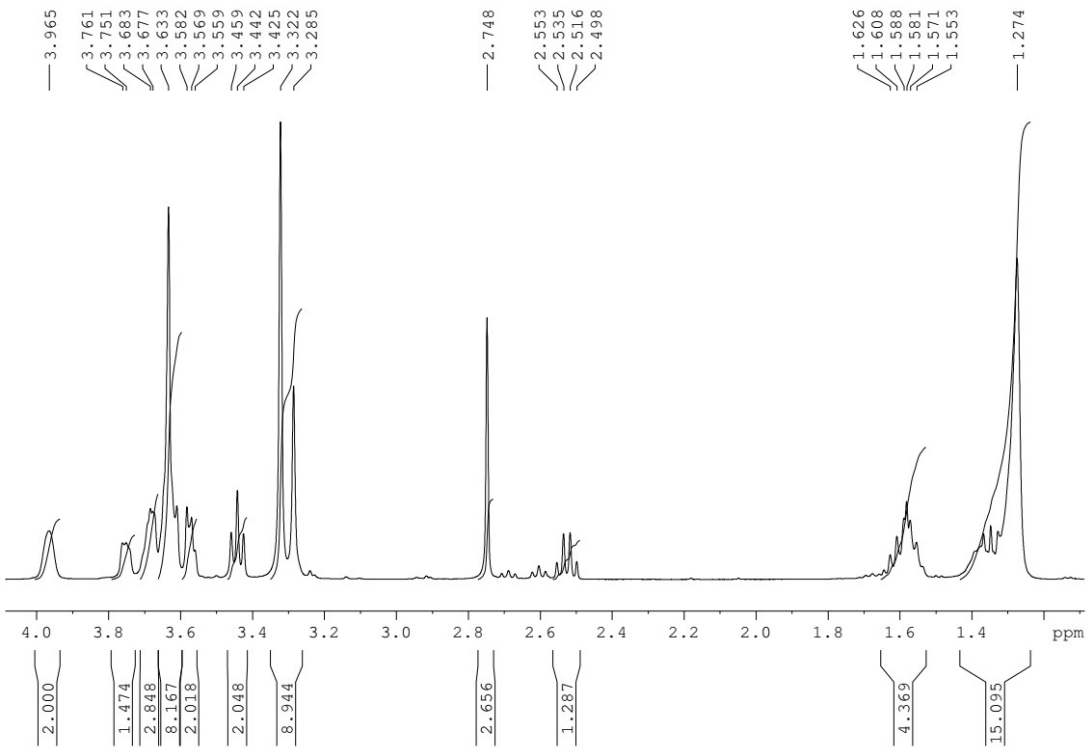


Figure S1. NMR spectra of positive charge ligand

2. Synthesis of Negative ligand:

2.1 Synthesis of Compound 6:

Sodium hydride (7.21 mmol) was taken in a RB and dry THF was added at 0^0C under argon atmosphere. Subsequently, the compound 1 (2.89 mmol) was dissolved in dry THF and added to the Sodium hydride solution and kept stirring. After 15 min, ethylbromoacetate (7.21 mmol) was added slowly to the reaction mixture at 0^0C under argon atmosphere. The reaction was kept stirring for 4h and then quenched few drops of water. The crude product was concentrated by rotatory evaporator and washed using ethylacetate and brine solution. After washing the organic part was dried over sodium sulfate and purified by column chromatography. The product was confirmed by ^{1}H- NMR with a yield of 60%.

2.2 Synthesis of Compound 7:

In a double neck round bottom flask, AIBN (0.597 mmol) was dissolved on 5 mL of toluene under nitrogen atmosphere followed by the addition of thioacetic acid (5.97 mmol). Subsequently, compound 6 dissolved in 5 mL of toluene was added and the reaction mixture was refluxed for 4h at 110 ºC. After reflux, the reaction mixture was cooled to room temperature. Then, the toluene was concentrated from reaction mixture by using rota

evaporator and washed with ethyl acetate and saturated sodium bicarbonate solution for three times. The organic layer was dehydrated over anhydrous sodium sulfate and concentrated by rotary evaporator. The product was separated through column chromatography with 77% yield. The desired compound was confirmed by H^1 NMR.

2.3 Synthesis of Compound 8:

The compound 7 was taken in a round bottom flask and dissolved in 10 mL of MeOH. Then, 4 mL of LiOH (1M) aq. Solution was added and allowed to stir at room temperature for 3h. After the completion of reaction, the reaction mixture was cooled down to 0ºC and acidified to pH- 2 by adding 1M HCl solution. Then, the solvent was evaporated and redissolved in ethyl acetate and washed thrice with brine solution. After that, the organic layer was concentrated using rotary evaporator and the product was purified by column chromatography. The purified product was confirmed by H^1 NMR. We observed quantitative yield of the reaction.

^{1}H- NMR (400 MHz, $CDCl_3$): δ 4.171 (s, 2H, -O-CH_2-CO-), 3.772- 3.5999 (m, 16H, -O-CH_2-CH_2-O-), 3.483- 3.449 (t, 2H, -CH_2-CH_2-O-), 2.705- 2.669 (t, 2H, -CH_2-S-S-), 2.539- 2.520 (q, 2H, -CH_2-SH), 1.675- 1.566 (m, 4H, -CH_2-), 1.278 (s, 15H, -CH_2).

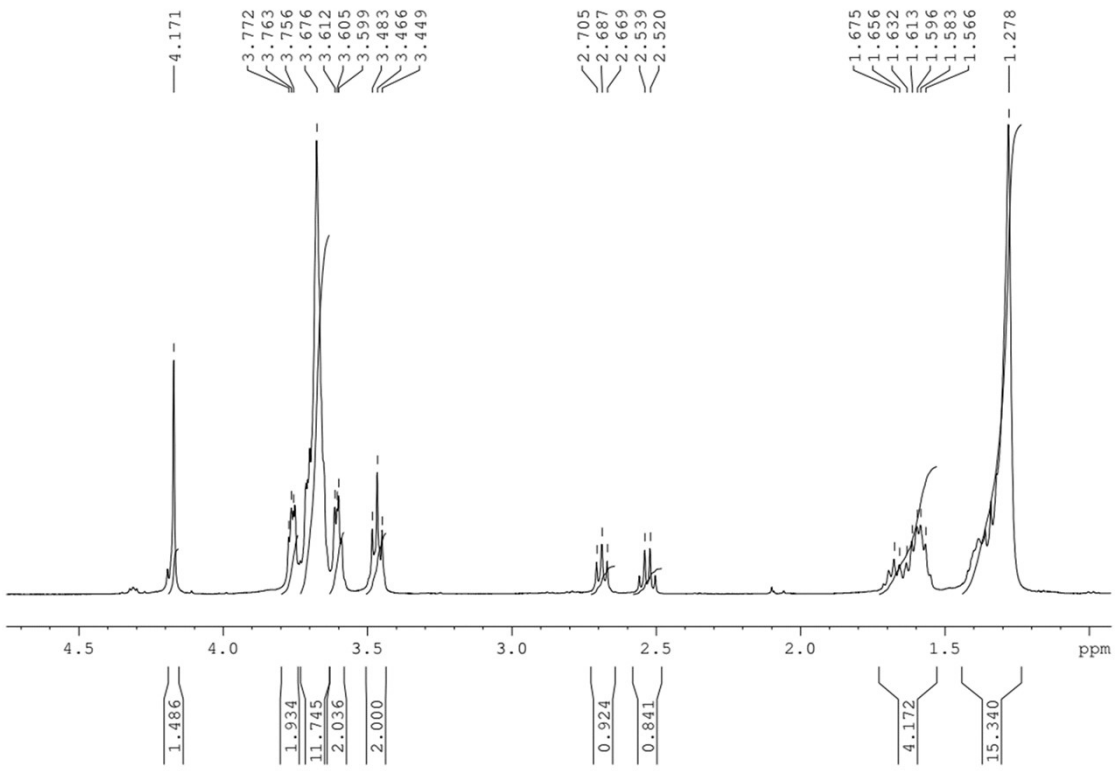


Figure S2: NMR spectra of negative charge ligand

3. Characterization of functionalized 2D-MoS_2:

3.1. IR and Raman spectra of functionalized and chemically exfoliated MoS_2:

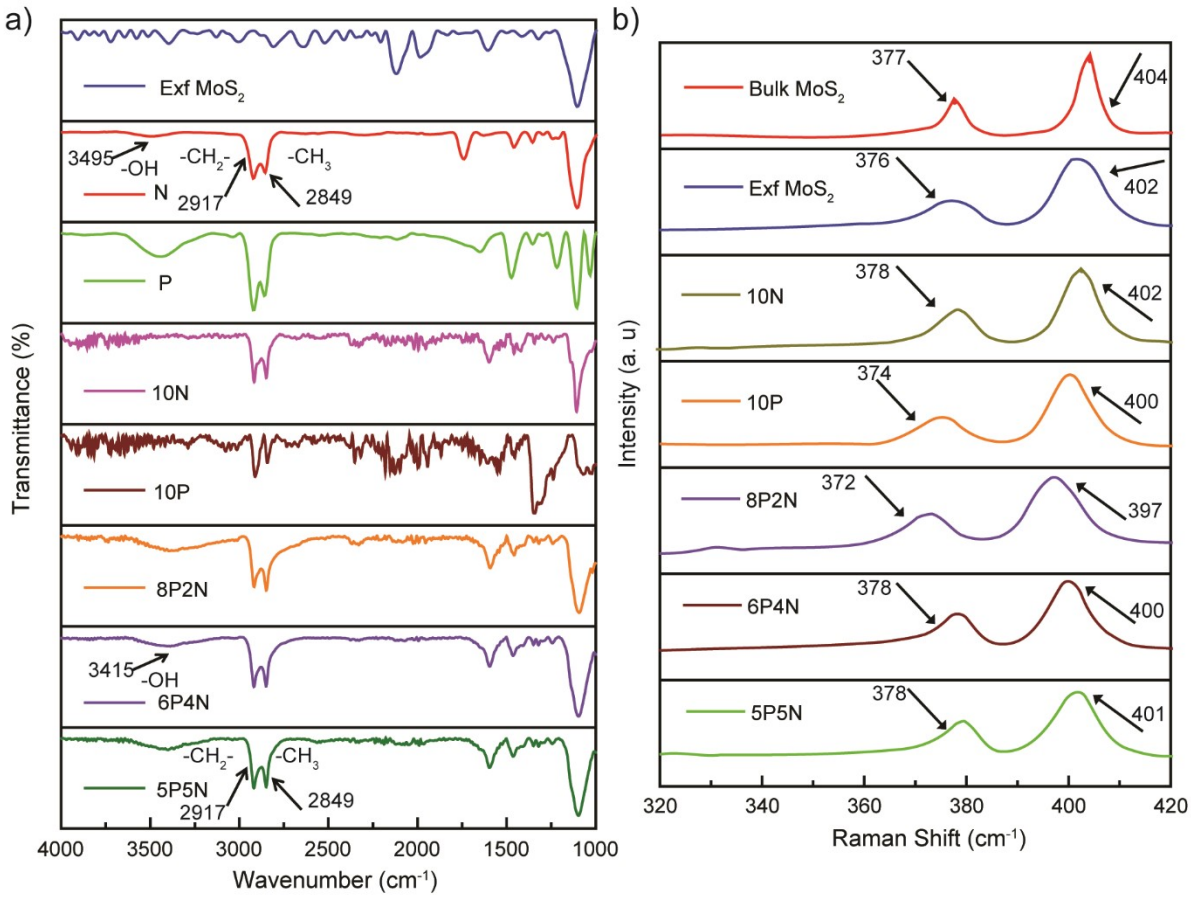


Figure S3. IR (a) and Raman (b) spectra of mixed charge functionalized 2D MoS_2.

4. Functionalization of MoS_2 nanosheets:

The molar ratios of positive and negative thiol ligands (100:0, 80:20, 60:40, 50:50, and 0:100) were taken in 20 mL glass vials. Then, 8 mL of distilled water was added to dissolve ligand and subsequently 2 mL of ce- MoS_2 (2mg/mL) was mixed to make up total 10 mL solution. Further, the solution was stirred for 24h with ultrasonication at regular interval. After 24h completion, the solution was subjected to dialysis for 24h using dialysis membrane (MW-10,000 cut off) to remove non-functionalized ligands. The distilled water was changed during dialysis at every 2hr interval. Then, the pH of func- MoS_2 was adjusted to 7.4 using 1M NaOH and stored at 4^{o}C for further use.

5. Zeta Potential of mixed charge MoS_2:

The zeta potential of mixed charge functionalized MoS_2 were measured Malvern Zetasizer Nano, UK; in 5 mM sodium phosphate buffer (PBS), pH 7.4 to confirm surface functionalization.

6. Determination of concentration of functionalized Ce-MoS_2 using inductively coupled plasma mass spectrometry (ICP- MS):

ICP-MS samples were prepared by taking 20 µL of functionalized MoS_2 solution in a 15 mL tube and 100 µL of concentrated HNO_3 was added into it. Then, the solution was kept for digestion at 100^0C for 4hr. After that, the volume of the solution was made up to 5mL by adding DI water. Then, the external standards were prepared by dissolving required amounts of ammonium heptamolybdate $(NH_4)_6Mo_7O_{24}.4H_2O$ in DI water with maintaining 2% HNO_3 concentrations. Before measurement, the accuracy of external standards was confirmed by ICP-MS by linear regression fitting of ICP-MS values of external standards (R^2>99.99). After measuring Mo concentration of the sample solutions, the value was multiplied by dilution factor to get the exact concentration of stock solutions.

7. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) determination of mixed charge functionalized MoS_2:

The antibacterial activity of mixed charge MoS_2 was tested by considering *Methicillin Resistant Staphylococcus aureus* (MRSA, USA300) as Gram-positive and *Pseudomonas aeruginosa* as Gram-negative bacteria. The freeze-dried bacteria were revived on nutrient agar plates. Few colonies of the bacteria were cultured Luria broth media (LB, HiMedia – 20 g/L) overnight for 10 -12 h to get primary culture. Further, the secondary culture was sub-cultured by taking 50 µL from primary culture in 5mL of fresh LB media until it reaches the mid-log phase (A_{600nm}~0.3). For experiments, the optical density (OD) was adjusted to A_{600nm}=0.01 ($10^6/10^7$ bacterial/mL).

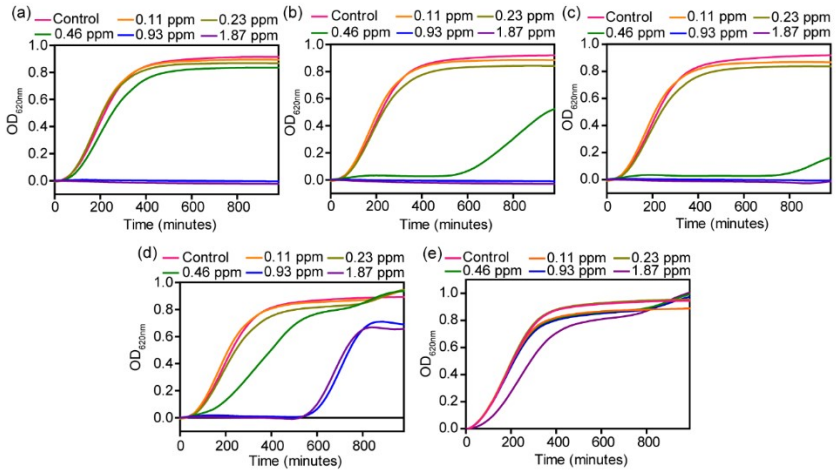


Figure S4. Kinetic growth curve of functionalized MoS_2 against MRSA (a) 10P, (b) 8P2N, (c) 6P4N, (d) 5P5N, (e) 10N.

The minimum inhibitory concentration of mixed charge MoS_2 was estimated by using microbroth dilution method in 96- well plates. The phosphate saline buffer (PBS) was used for serial dilution of the working concentrations. The 100 µL bacterial suspension with A_{600nm} =0.01 was added to 100 µL of working concentration prepared in 96- well plate. The bacterial growth curve was monitored by using micro-plate reader (Eppendorf AF2200) equipped with shaker at 37^0C over the period of 15h. The kinetic cycle optical density at 600 nm taken at 10 min interval followed by shaking. The minimum concentration at which the growth curve is 95% decline was designated as minimum inhibitory concentration (MIC).

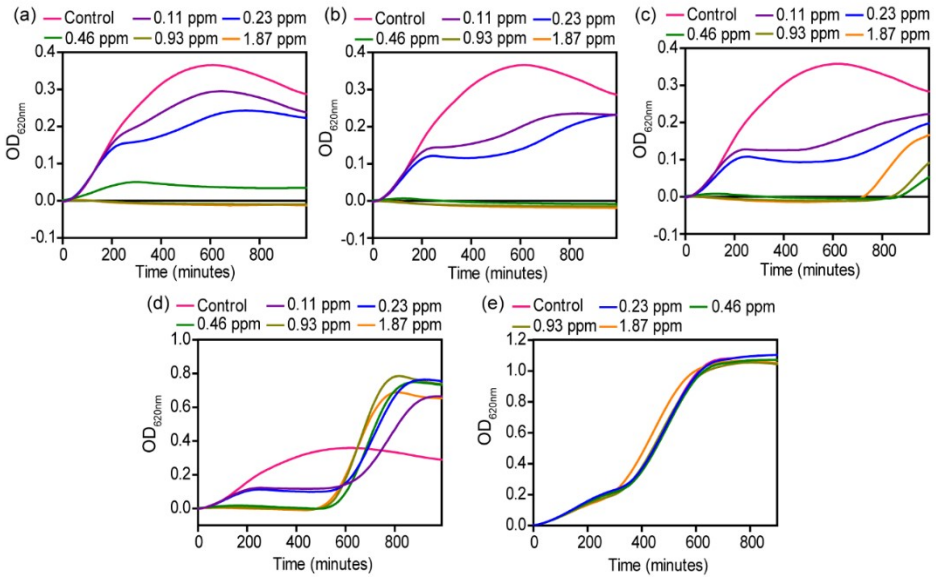


Figure S5. Kinetic growth curve of functionalized MoS_2 against PA (a) 10P, (b) 8P2N, (c) 6P4N, (d) 5P5N, (e) 10N.

For minimum bactericidal concentration, the same plate after MIC determination was incubated for another 5h at 37^0C and then the bacterial solution from treated wells was streaked on nutrient agar plate. The minimum concentration at which there is no colony formation was designated as minimum bactericidal concentration (MBC).

8. Estimation of oxidative stress:

The oxidative stress induced by mixed charge MoS_2 was estimated by Ellman's assay. In this method, 10 times of MIC value was taken for all mixed charge MoS_2. Then, the glutathione (GSH, 0.4mM) dissolved in bicarbonate buffer (50mM, pH- 8.6) was added. The negative control was considered without MoS_2 whereas positive control contains 10mM H_2O_2. All the solutions were incubated at 37^0 C and wrapped with aluminium foils to avoid photochemical oxidation. From the stock, 100 µL of the solution at different time intervals (15 min, 30 min, 60 min, 120 min) was added to 100 µL of 2 mM 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB, SRL Chem) in 50 mM TRIS-HCl (pH 8.3, SRL Chem) buffer taken in a 96-well plate. Then, the absorbance was measured immediately at 412 nm using UV- Vis spectrometer by plate reader. The percentage loss of glutathione was calculated as,

$$\%\ loss = \left(1 - \frac{absorbance\ at\ 412\ nm\ of\ the\ sample\ at\ a\ particular\ time}{absorbance\ at\ 412\ nm\ of\ negative\ control\ at\ 0\ min}\right) \times 100$$

9. Estimation of Intracellular ROS generation:

The mid-log phase bacteria ($A_{600nm} \approx 0.5$) were harvested and washed thoroughly with PBS buffer. Then, the bacteria were resuspended and diluted with PBS to adjust A_{600nm}=0.2. Further, 100 µL of bacterial suspension was added to 100 µL of mixed charge MoS_2 with concentration of 10×MIC value and incubated for 45 min at 37^0C. After that, the solution was incubated with DCFDA (2.5 mM) dye for 30 min and then centrifuged to remove the excess dye. The bacteria were further washed and redispersed in PBS. The bacteria suspension was subjected on glass slide for visualizing by fluorescence microscope (Exc~485 nm/ Emi~535 nm).

10. Membrane depolarization assay:

The membrane depolarization quantification was performed by harvesting the MRSA and PA bacteria at mid- log phase culture ($A_{600nm} \approx 0.3$) and centrifuged at 5000 rpm for 5 min. Then, the pellet was washed with mixture of glucose (5 mM) and HEPES buffer (5 mM, pH 7.2) in 1:1 ratio. Again, the washed pellet was resuspended in the mixture of glucose (5 mM), HEPES buffer (5 mM), and KCl solution (100 mM) in 1:1:1 ratio. Then, 100 µL of bacterial suspension was added into a 96-w ell plate containing 2 µL of 5 mM DISC3 dye (3,3′-dipropylthiadicarbocyanine iodide, TCI Chemicals) followed by incubation for 30 min. After that, 100 µL of 10×MIC concentration of mixed charge MoS_2 was added into the same plate containing bacterial suspension and DISC3. After addition, the fluorescence was measured with an excitation wavelength of 622 nm and emission wavelength of 670 nm, for 2hr with lag time 10 min. An increase in fluorescence indicates the membrane depolarization of bacteria.

11. Outer membrane permeabilization assay:

The PA bacteria were harvested to $A_{600nm} \approx 0.2$ by centrifugation at 6000 rpm for 5min. Then, the bacteria pellet was redispersed in PBS (pH- 7.4) and washed for three times by centrifugation. The final bacteria were resuspended in PBS and 100 µL of taken in 96- well plate. Then, 20 µL of NPN (1mg/mL, 95% Ethanol) with 80 µL of functionalized MoS_2 were added into above bacterial solution. The fluorescence intensity was determined by fluorescence spectrophotometer. The excitation wavelength was 350 nm, and the emission wavelength was 420 nm.

12. SEM sample preparation:

The MRSA and PA was culture in LB media and harvested in mid log phase (A_{600nm}≈ 0.3). Then, the bacteria solution was centrifuged at 5000 rpm for 6 min to get the bacteria pellet. After that, the pellet was dispersed in PBS and washed by centrifugation. Then, the pellet again redispersed in PBS and treated with the mentioned dosage of functionalized MoS_2 for 2h. After incubation, it was centrifuged at 5000 rpm for 5 min and fixed with 3% glutaraldehyde for another 1h. After fixation, it was further centrifuged at 5000 rpm for 5 min and dehydrated using ethanol gradient (20%, 30%, 40%, 50%, 70% and 90% ethanol). Then, redispersed in 100% ethanol and drop- casted on silicon wafer. Before imaging in SEM, it was sputtered with gold.