Supporting information for

Matrix Assisted Antibacterial Activity of Polymer Conjugates with Pendant Antibiotics, Bioactive and Biopassive Moieties

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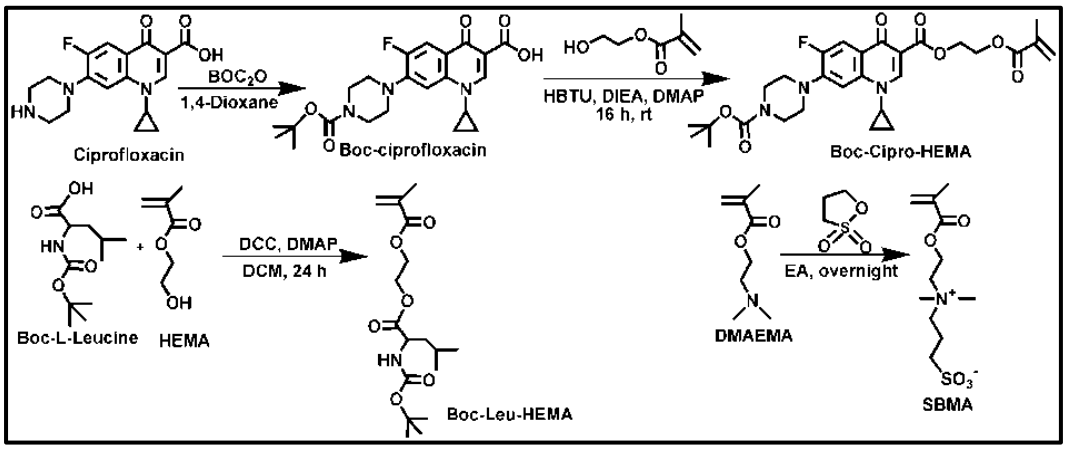
Materials and methods

Materials. Ciprofloxacin (98 %) was obtained from Alfa Aesar. Boc-L-leucine (Boc-L-Leu-OH, 99%), 4-dimethylaminopyridine (DMAP, 99%), dicyclohexylcarbodiimide (DCC, 99%), 2-hydroxyethyl methacrylate (HEMA, 97%), *N*,*N*-diisopropylethylamine (DIEA, 99%), anhydrous methanol (MeOH, 99.9%), 1,3-propane sultone, 2-(dimethylamino)ethyl methacrylate (DMAEMA) and phosphate buffered saline (PBS) tablets were purchased from Sigma-Aldrich. Di-*tert*-butyl pyrocarbonate (BOC_2O) was obtained from Spectrochem, India. Trifluoroacetic acid (TFA, 99.5%), [(*O*-benzotriazole-1-yl)-*N*,*N*,*N*,*N*-tetramethyluroniumhexafluoro phosphate] extrapure (HBTU) and trypticase soy broth (TSB) were received from Sisco Research Laboratories Pvt. Ltd., India. 2,2′-Azobisisobutyronitrile (AIBN, Sigma-Aldrich, 98%) was recrystallized twice from methanol. $CDCl_3$ (99.8% D) and D_2O (99% D) were purchased from

Cambridge Isotope Laboratories, Inc., USA for NMR study. The Boc-L-leucine methacryloyloxyethyl ester (Boc-Leu-HEMA) monomer (Scheme S1),[1] and 4-cyano-4-(dodecylsulfanylthiocarbonyl)sulfanylpentanoic acid (CDP)[2] as chain transfer agent (CTA) were synthesized by previously reported procedure. Zwitterionic sulphabetaine methacrylate (SBMA) was synthesized (Scheme S1) as reported in the literature[3] and characterized by ^{1}H NMR spectroscopy (Fig. S1). The solvents such as hexanes (mixture of isomers), acetone, ethyl acetate (EA), dichloromethane (DCM) etc. were purified by standard procedures. Agar agar, tryptone, sodium chloride (NaCl), sodium phosphate dibasic (Na_2HPO_4•$7H_2O$), potassium phosphate monobasic (KH_2PO_4), ammonium chloride (NH_4Cl), glucose, magnesium sulfate ($MgSO_4$), calcium chloride ($CaCl_2$) and yeast extract were obtained from Merck, India. Petriplates were obtained from Tarsons Products Pvt. Ltd., India. Milli-Q filtered water was used to prepare solutions and autoclaved before using. The bacterial strains used for the experimental purpose were *Escherichia coli* (*E. coli*), *Bacillus subtilis* (*B. subtilis*), *Staphylococcus aureus* (*S. aureus*), pathogenic *Vibrio alginolyticus* (*V. alginolyticus*), and *Vibrio chemaguriensis* Iso1 (*V.* chemaguriens). 4',6-Diamidino-2-phenylindole (DAPI) (Amresco, USA) and fluorescein-5-isothiocyanate (F143) (Thermo Fisher Scientific) were used for live and treated bacterial cells staining.

Instrumentation. NMR spectra were acquired in a Bruker Avance^{III} 500 MHz spectrometer at 25 ^{o}C. Positive mode electrospray ionization mass spectrometry (ESI-MS) was carried out on a Q-Tof Micro YA263 high resolution (Waters Corporation) mass spectrometer. Optical density measurements of bacterial cultures with and without polymer at 600 nm (OD_{600}) were performed by UV-Vis spectrophotometer (Hitachi U2900, Japan). DAPI, F143 stained *V.*

chemaguriensis cells were examined by epifluorescence microscopy (Olympus). Hemoglobin release was measured by UV-visible plate reader (Spectra Max 190).



Scheme S1 Synthesis of Boc protected ciprofloxacin, leucine and sulphabetaine methacrylate monomers (Boc-Cipro-HEMA, Boc-Leu-HEMA and SBMA).

Synthesis of Boc-ciprofloxacin hydroxyethylmethacrylate (Boc-Cipro-HEMA). First, the Boc group protection of ciprofloxacin was performed to prepare Boc-ciprofloxacin following an earlier reported method (Scheme S1).[4] Then, Boc-ciprofloxacin (10.35 g, 24.00 mmol), HBTU (22.80 g, 0.16 mol) and DMAP (292 mg, 120 mmol) were taken in a round bottom flask in 500 mL of DCM and cooled to 0 ^{o}C. DIEA (21 mL, 0.12 mol) was added to the reaction mixture followed by addition of HEMA (11.7 g, 90.0 mmol) and stirred for 10 min. The solution was stirred at room temperature for 16 h. Then, the reaction mixture was washed with brine solution (2 × 200 mL) and the organic phase was dried over anhydrous sodium sulphate. After evaporation of the solvent, the product was precipitated in diethyl ether and then purified by column chromatography using 10 % ethyl acetate in hexanes as mobile phase (yield 40 %): ^{1}H NMR (Fig. S2) and ESI-MS spectrum (Fig. S3).

Copolymer synthesis. A typical copolymerization procedure was as follows: Boc-Leu-HEMA (40.0 mg, 0.09 mmol), Boc-Cipro-HEMA (127.0 mg, 0.29 mmol), SBMA (33.0 mg, 0.07 mmol), CDP (3.8 mg, 9.3 µmol), AIBN (0.3 mg, 1.8 µmol; 0.3 g stock solution of 5.0 mg AIBN in 4.5 g DMF), MeOH (1.0 g) and a magnetic stir bar were sealed in a 20 mL septa-sealed glass vial. The reaction mixture was purged with dry nitrogen for 20 min, and was placed in a preheated reactor at 65 ± 1 °C. The polymerization reaction was stopped after 18 h by cooling the vial in an ice-water bath. Approximately 2.0 mL MeOH was added to the vial and the mixture was precipitated into hexanes. The polymer (P(Boc-Leu-HEMA-*co*-Boc-Cipro-HEMA-*co*-SBMA), PLCS) was reprecipitated 5 times from MeOH/hexanes and dried under vacuum at 40 ^{o}C for 6 h to obtain a yellow material. Similarly, another three copolymers; P(Boc-Leu-HEMA-*co*-SBMA) (PLS), P(Boc-Cipro-HEMA)-*co*-SBMA) (PCS) and P(Boc-Leu-HEMA-*co*-Boc-Cipro-HEMA) (PLC) were synthesized following the above mentioned procedure. The copolymers are designated as PLCS, PLS, PCS, and PLC where P, L, C and S represent copolymer, Boc-Leu-HEMA, Boc-Cipro-HEMA and SBMA, respectively.

Synthesis of P($^{+}$H$_3$N-Leu-HEMA). Boc-Leu-HEMA was homopolymerized using CDP as CTA and AIBN as initiator following previously reported procedure (molecular weight from NMR ($M_{n,NMR}$) = 5500 g/mol, theoretical molecular weight ($M_{n,theo}$) = 5900 g/mol).[5] The deprotection of Boc group using TFA lead to the desired P($^{+}$H$_3$N-Leu-HEMA) for control antibacterial study.

Determination of molecular weight by UV-visible spectroscopy ($M_{n,UV}$). 3.1 mg of CDP (molecular weight (MW) = 403.497 g/mol) was dissolved in 3 mL of DMSO and from this solution 100 µL was taken out and was added in 3 mL of DMSO. UV-visible spectrophotometer (path length = 1 cm) was used to determine absorbance at 311 nm, which was 1.04. From this

result, the molar absorptivity (ε) was calculated as 12,589 M^{-1} cm^{-1}. The same process was repeated for three times. The average ε = 12,587 M^{-1} cm^{-1} was used for the determination of $M_{n,UV}$ values for PLS, PCS, PLC, and PLCS from the corresponding absorbance values at 311 nm (average of three measurements) of known amounts of the polymers in DMSO.[6]

Deprotection of Boc groups. 2.0 mL TFA was added to the solution containing 0.3 g of polymer in 1.0 mL DCM in a 20 mL glass vial. The solution was stirred for 2 h at room temperature, precipitated four times in hexanes from acetone solutions, and finally dried under vacuum at 40 ºC for 8 h. Boc deprotected polymers are represented as DPLS, DPCS, DPLC and DPLCS, where D stands for Boc deprotection.

Antibacterial activity: zone of inhibition *via* plate well method. Antibacterial activity by zone of inhibition (ZOI) technique was determined against widely studied Gram negative *E. coli* and a novel species of *Vibrio*, namely, *V. chemaguriensis* (potentially pathogenic due to presence of pathogenic genes) and another widely studied Gram positive bacterium, *S. aureus*. Luria-Bertani medium (LB) was prepared by adding 1.0 g tryptone (Merck, India), 1.0 g NaCl (Merck, India), 0.5 g yeast extract (Merck, India) and 2.0 g agar agar (Merck, India) in 100 mL de-ionized (DI) water were weighed and autoclaved for culturing *E. coli* and *B. subtilis*. LB medium was similarly prepared in salinity 11.5 seawater to culture *V. chemaguriensis.* Sterile LB agar plates were prepared by pouring the medium into petri dishes to completely cover the bottom of the dish. 100 µL of inoculum of each type of bacterium mentioned above was spread on the surface homogeneously. The plates were allowed to dry for 10 min. Wells of 0.1 cm radius were created in the agar plates to which polymer solutions of DPLC, DPLS, DPCS and DPLCS at different concentrations (200, 400, 600 and 800 µg/mL) were added. A plate with bacteria but no polymer served as negative control for the experiment. The agar plates were incubated at 37 ºC

for 12 h. The diameter of the zone up to which the polymer prevents the bacterial growth was measured to determine ZOI.[5] Each experiment was performed in duplicates.

Antibacterial assay: bacterial growth experiment in LB broth. Polymer antibacterial activity was investigated against *E. coli*, *B. subtilis*, *V. alginolyticus* and *V. chemaguriensis* in LB broth medium. Cultures were grown till OD_{600} 0.5 and used as starter culture for the growth experiments. The experiment was set with one control to serve as blank (50 mL of only medium), one negative control without presence of polymer (500 µL of overnight culture of bacteria in LB broth) and two experimental set-ups (500 µL culture + 50 mL medium + 200 µg/mL of polymer). Cells were incubated at 37 ^{o}C at 180 rpm.

Antibacterial assay: pH dependent broth microdilution method. Glycerol stocks of *V. chemaguriensis* cells were inoculated into LB broth and allowed to grow overnight at 37 ^{o}C under shaking conditions. This overnight culture (200 µL, OD_{600} = 0.5) was diluted with fresh LB broth of salinity 11.5 (10 mL) prepared at three different pH (6.5, 8.0 and 9.0). At each pH, four experimental tubes comprising of one control (no polymer, no bacterium); one negative control (no polymer) and two treatment tubes (bacterium treated with polymers). The cultures were then incubated at 37 ^{o}C for 18 h under shaking condition. The experiments were performed in duplicates. Cell growth was monitored by recording OD_{600} over a 4 hour time span. *Vibrio chemaguriensis* has a doubling time of ~11 minutes. Hence 4 hours was sufficient time to observe the influence of the polymer on cell growth. Antibacterial properties were further explored against *V. chemaguriensis* at three different pH (6.5, 8.0 and 9.0). Cells were subjected to growth in presence of the polymer in LB broth (salinity 11.5) for 12 hours. To check for consistency of antibacterial properties of the polymer, the experiment was also performed in TSB medium which is specifically used to prompt biofilm formation by bacteria. Cells were grown

overnight at pH 6.0, 7.0, 8.0 and 9.0 in presence of 200 µg/mL polymer solution. The bacterial culture was allowed to grow for 48 h. The planktonic cells were removed by washing twice with autoclaved saline water. The attached cells were stained for 30 min with 0.1% aqueous crystal violet. The attached crystal violet was eluted using 95% ethanol and absorption was measured at 570 nm. The effect of the polymer on cell morphology was explored using field emission scanning electron microscopy (FESEM; Carl Zeiss-Sigma, Germany) and transmission electron microscopy (TEM-2100Plus, Jeol, USA). Staining of treated cell with F143 and DAPI were performed by the method reported in our earlier work.[7]

Dual matrix growth experiment in LB medium. Glycerol stocks of *V. chemaguriensis* cells were inoculated into LB broth and allowed to grow overnight at 37 ^{o}C under shaking conditions. This overnight culture (200 µL, OD_{600} = 0.5) was diluted with fresh LB broth (10 mL). Sterile LB agar plates were prepared by pouring the medium into petri dishes to half cover the area of the petri dish and another half portion was filled with 10 mL LB broth. 100 µL of inoculum of *V. chemaguriensis* were spread on the LB agar surface homogeneously and the plates were allowed to dry for 10 min. 100 µL of culture was also added to inoculate the LB broth. Then, 200 µg/mL of DPLCS was added to both solid agar and liquid broth incubated at 37 ^{o}C for 18 h with 180 rpm shaking. The negative control plate was prepared without polymer addition.

Gram staining. Gram staining was performed with cells collected from the solid and liquid portion of each plate (control and treated) after 18 h incubation. Standard protocol[8] for Gram staining was followed and cells were visualized by optical microscopy under 400X magnification.

Hemolytic assay. Plasma free blood corpuscles were prepared by centrifugation of citrated whole chicken blood and washed three times with PBS at 4000 rpm, for 10 min at 4 °C.[9] 50 µL of blood corpuscles suspended in PBS at 10% (v/v) was mixed with DPLCS solutions with several final concentrations of 100, 200, 500, 1000 and 5000 µg/mL in an autoclaved 0.5 mL microcentrifuge tube and incubated for 1 h at 37 °C. After incubation, each microcentrifuge tube was centrifuged at 4000 rpm for 10 min, and absorbance of the supernatants was measured at 540 nm to check hemoglobin release using a UV-visible plate reader. Hemoglobin release in PBS and in 0.1% (v/v) Triton X-100 were used as negative (0% release) and positive control (100% release), respectively. Percentage of hemolysis was determined as $(A - A_0)/(A_{total} - A_0) \times 100$, where A is the absorbance of the test well, A_0 is the absorbance of the negative control, and A_{total} is the absorbance of 100% hemolysis well, all at 540 nm. Hemolysis was plotted as a function of polymer concentration and the HC_{50} was defined as the polymer concentration, which causes 50% hemolysis relative to the positive control. Since hemolysis did not reach 50 % up even at highest polymer concentration tested (5000 µg/mL), we could not determine the HC_{50} value. Each experiment was performed in triplicates.

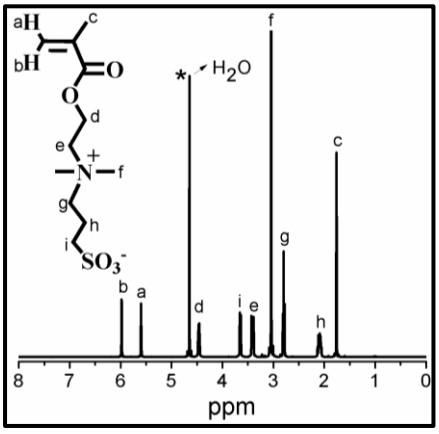


Fig. S1 ^{1}H NMR spectrum of SBMA in D_2O.

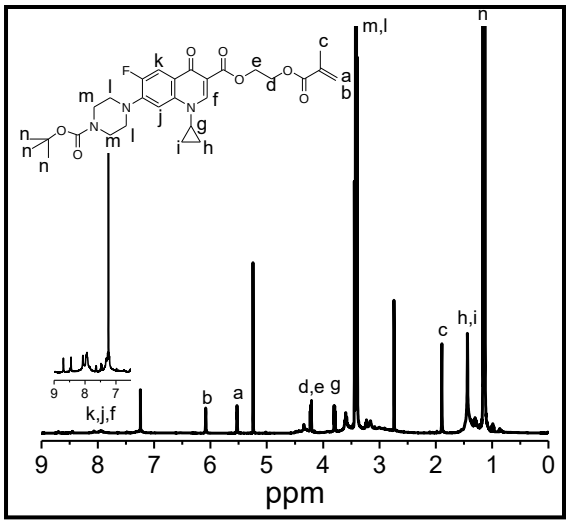


Fig. S2 ^{1}H NMR spectrum of Boc-Cipro-HEMA in $CDCl_3$ (magnification 30X for the peak position 7.0-9.0 ppm).

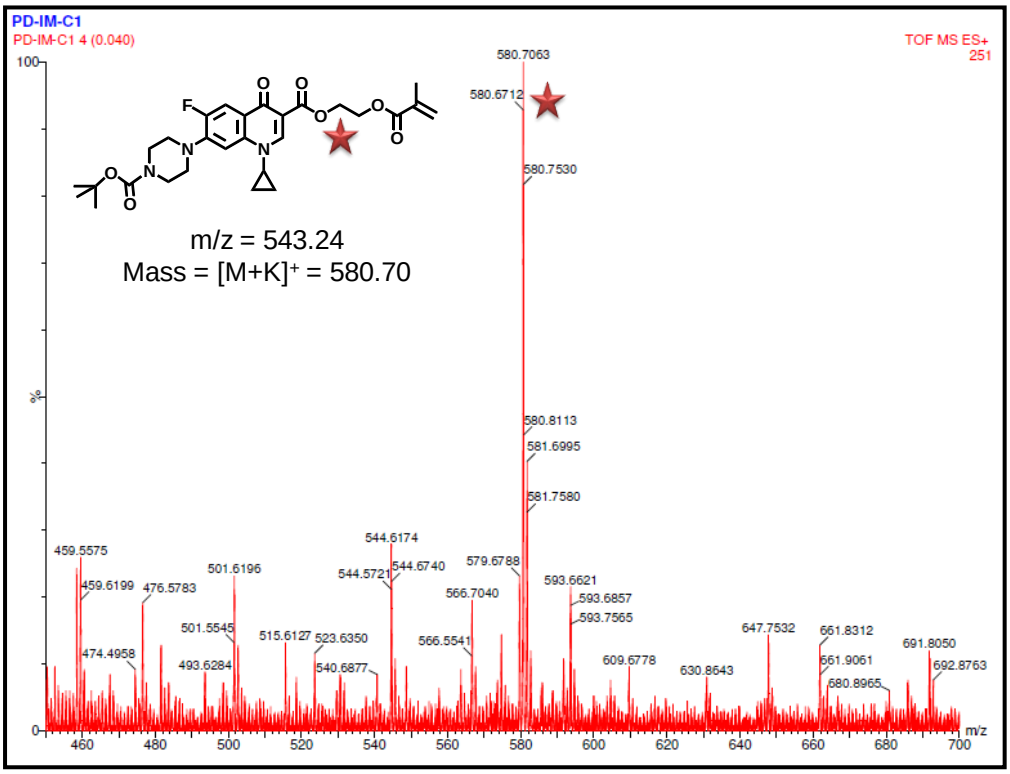


Fig. S3 ESI-MS spectrum of Boc-Cipro-HEMA (calculated *m*/*z* for $[M+K]^+$: 582.24; observed *m*/*z*: 580.70).

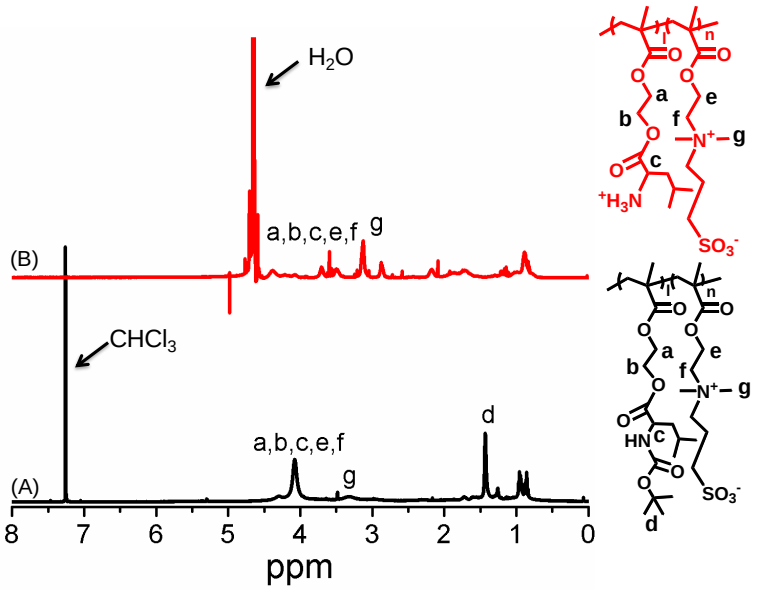


Fig. S4 ^{1}H NMR spectra of PLS in $CDCl_3$ (A) and DPLS in D_2O (B).

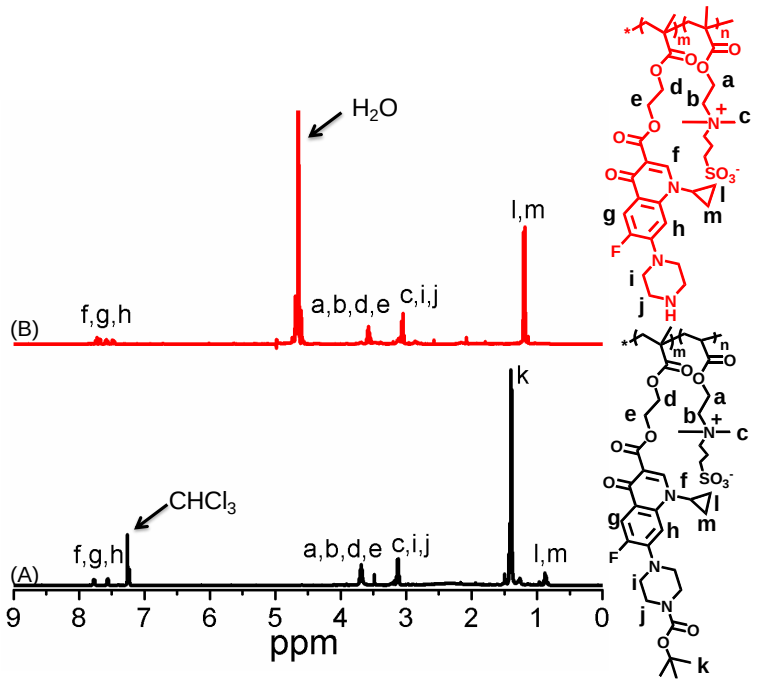


Fig. S5 ^{1}H NMR spectra of PCS in $CDCl_3$ (A) and DPCS in D_2O (B).

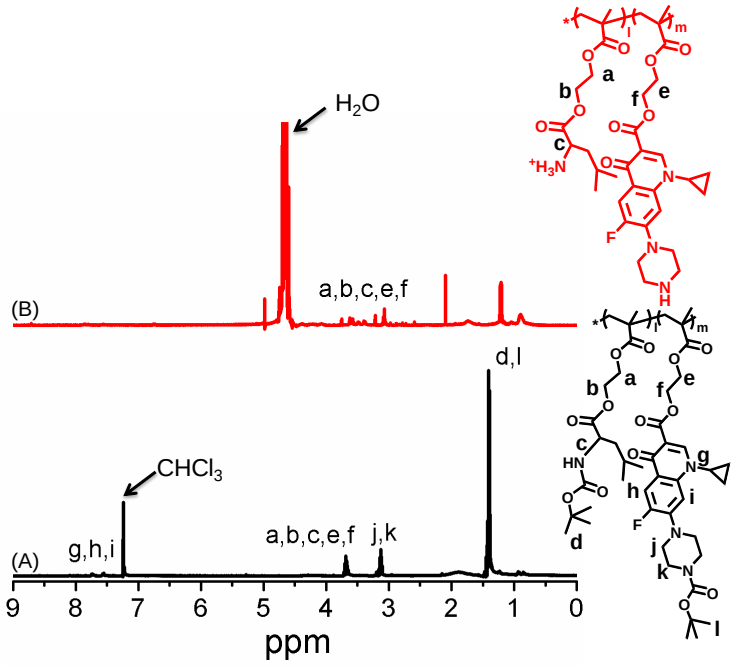


Fig. S6 ^{1}H NMR spectra of PLC in $CDCl_3$ (A) and DPLC in D_2O (B).

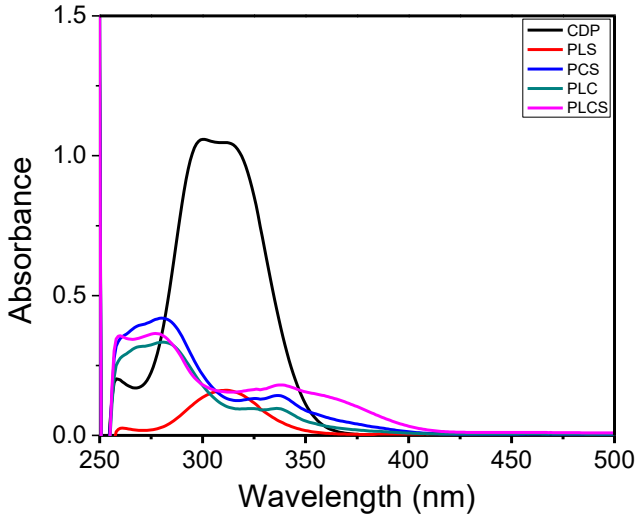


Fig. S7 UV-visible absorbance spectra of CDP, PLS, PCS, PLC and PLCS in DMSO.

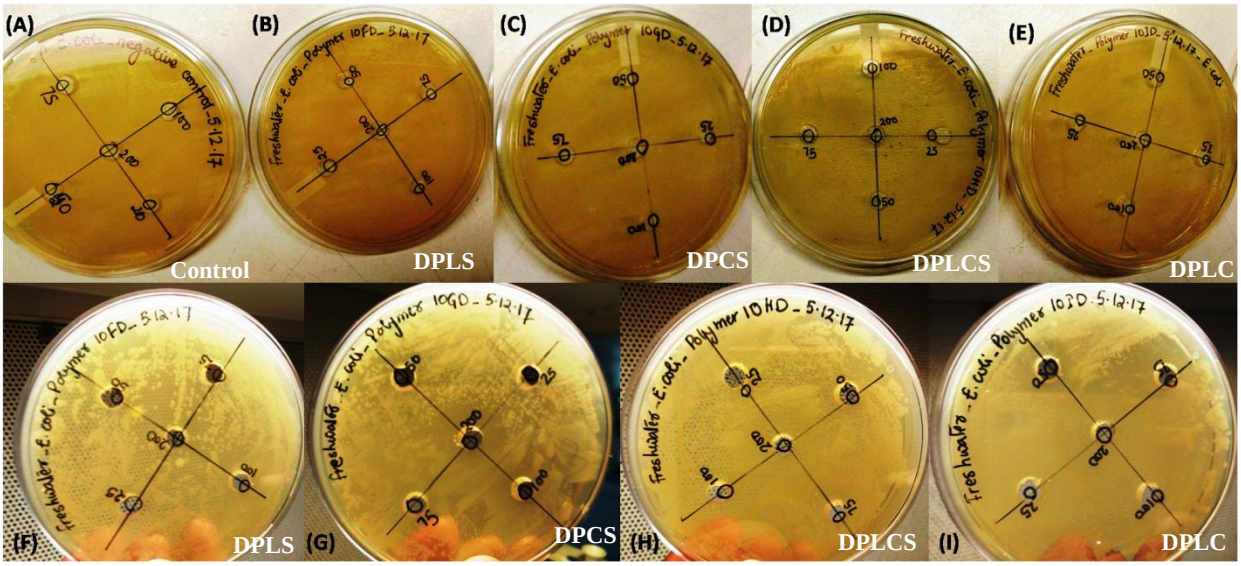


Fig. S8 Zone of inhibition against *E. coli*: (A) control (without polymer); treatment with (B) and (F) DPLS, (C) and (G) DPCS, (D) and (H) DPLCS, (E) and (I) DPLC at lower concentrations (up to 100 µg/mL).

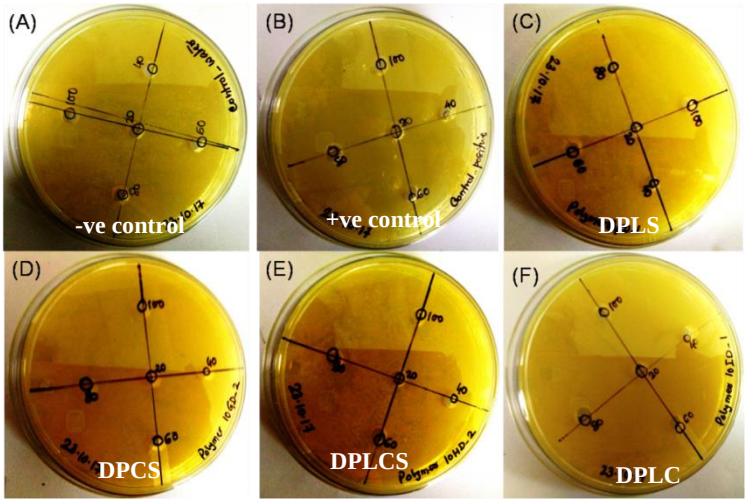


Fig. S9 Zone of inhibition against *V. chemaguriensis*: (A) water control (negative), (B) control [without polymer, positive]; treatment with (C) DPLS, (D) DPCS, (E) DPLCS and (F) DPLC at lower concentrations (up to 100 µg/mL).

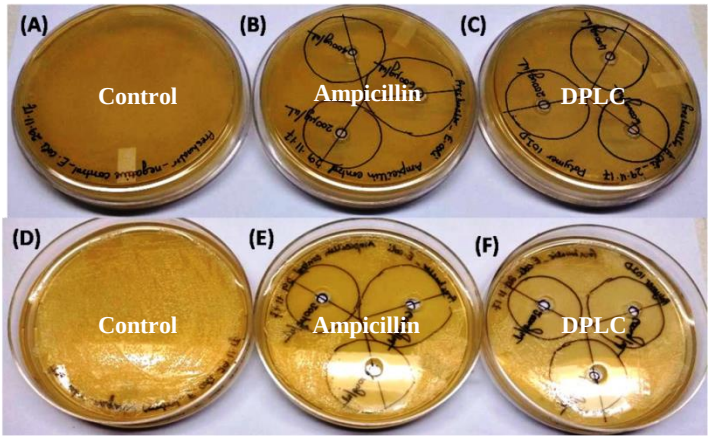


Fig. S10 Zoomed plate view of zone of inhibition experiment against *E. coli*: (A) and (D) control (without polymer); treatment with (B) and (E) ampicillin (well-known antibiotic control), (C) and (F) DPLC at 200, 400 and 600 µg/mL.

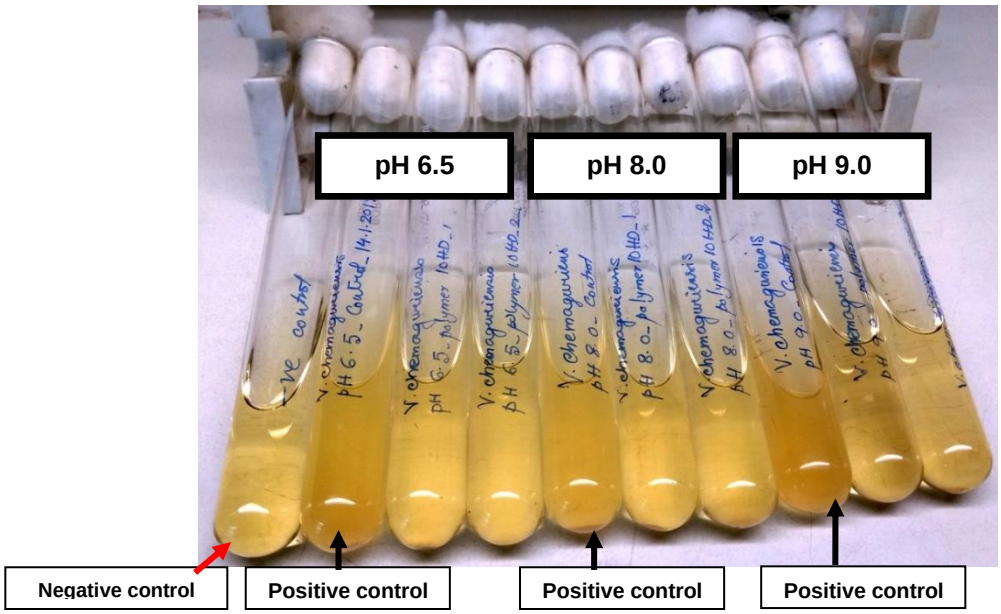


Fig. S11 pH dependent growth experimental setup in LB broth medium; only LB medium was taken as negative control, *V. chemaguriensis* culture in LB medium at salinity 11.5 was taken as positive control, treatment with DPLCS with duplicate experimental set at pH 6.5, pH 8.0 and pH 9.0 with salinity 11.5.

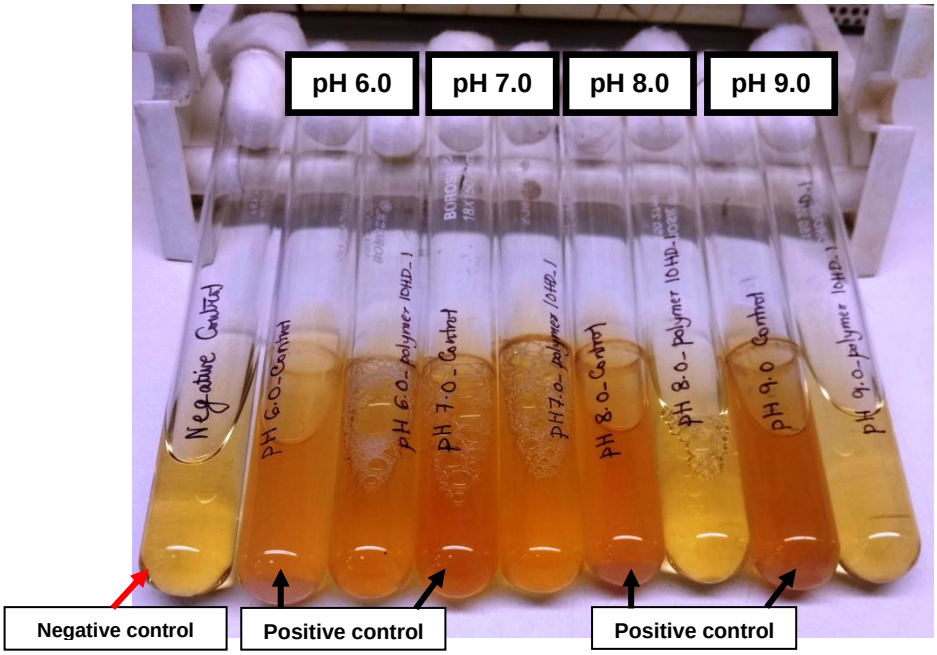


Fig. S12 pH dependent growth experimental setup in TSB medium; only TSB medium was taken as negative control, *V. chemaguriensis* culture in LB medium at salinity 11.5 was taken as positive control, treatment with DPLCS with duplicate experimental set at pH 6.0, pH 7.0, pH 8.0 and pH 9.0 with salinity 11.5.

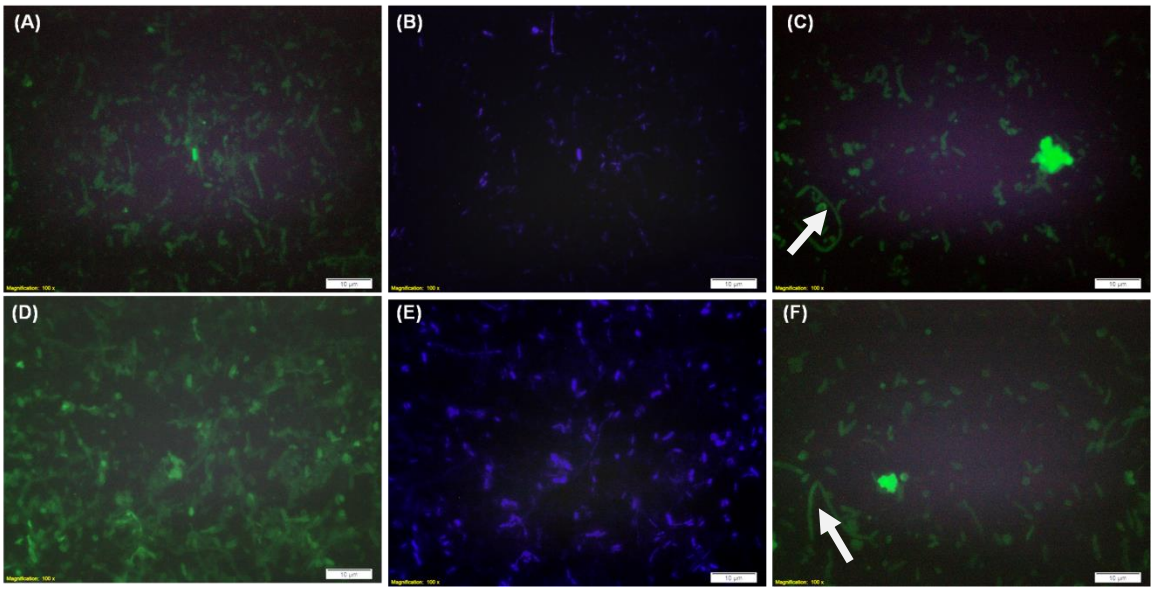


Fig. S13 Epifluorescence microscopy images of *V. chemaguriensis* cells at pH 6.5 (A) and (B) control staining with F143 and DAPI respectively, (C) after treatment with DPLCS and staining with F143; at pH 8.0 (D) and (E) control staining with F143 and DAPI respectively; (F) after treatment with DPLCS and staining with F143 at 100X resolution. White arrow indicates cell elongation.

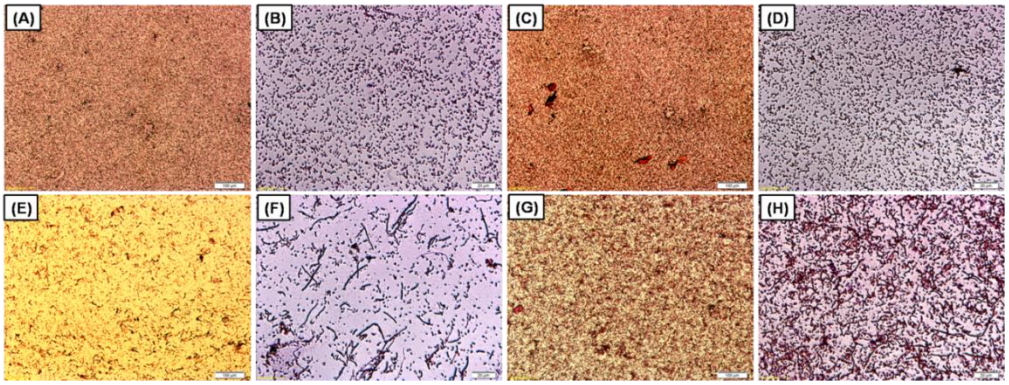


Fig. S14 Optical microscopic images of *V. chemaguriensis* cells following Gram staining: from LB agar control at (A) 100X, (B) 400X; from LB broth control at (C) 100X, (D) 400X resolution; after treatment with DPLCS from LB agar portion at (E) 100X, (F) 400X resolution; from LB broth portion at (G) 100X, (H) 400X resolution.

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