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Matrix assisted antibacterial activity of polymer conjugates with pendant antibiotics, and bioactive and biopassive moieties†

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Antibacterial activity against a series of potentially pathogenic bacterial strains was evaluated in both liquid and solid matrices using ciprofloxacin (a well-known fluoroquinolone antibiotic) based polymeric systems with pendant cationic charge and zwitterionic units. The ciprofloxacin containing polymeric architecture was designed to incorporate bioactive killing properties of cationic moieties, whereas biopassive activity was regulated by the bacterial cell repelling capacity of the zwitterionic group. *tert*-Butyl carbamate (Boc)-leucine hydroxyethylmethacrylate (Boc-Leu-HEMA), Boc-ciprofloxacin hydroxyethylmethacrylate (Boc-Cipro-HEMA) and zwitterionic sulphabetaine methacrylate (SBMA) are copolymerized, followed by deprotection of Boc groups from the copolymer under acidic conditions. Boc deprotection resulted in water soluble polymers with cationic charge from the leucine moiety and retained the antibiotics in their active original form. The antibacterial activity of the polymeric system was evaluated against several bacterial species, namely, *Escherichia coli* (*E. coli*), *Bacillus subtilis* (*B. subtilis*), *Staphylococcus aureus* (*S. aureus*), *Vibrio alginolyticus* (*V. alginolyticus*), and *Vibrio chemaguriensis* Iso1 (*V. chemaguriensis*), in both liquid and solid matrices. The copolymer displayed significant bactericidal efficacy against non-biofilm forming *E. coli* and *B. subtilis* in both liquid and solid matrices. However, the antibacterial effect on biofilm forming *V. chemaguriensis* was comparatively less prominent in the liquid matrix. Optical microscope images of Gram staining revealed an enhanced surface area of individual cells and chain formation in cells of *V. chemaguriensis*. The non-cytotoxic profile of the polymer towards mammalian red blood cells (RBCs) and enhanced bactericidal effect in the physiological to basic pH (7.0–9.5) range against potentially pathogenic *V. chemaguriensis* using a multitude of microscopic techniques hinted toward the need to develop new antibiotics to combat possible clinical infections in the near future.

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Introduction

Rapidly developing bacterial resistance toward treatment with conventional antibiotics has generated an urgent need to develop new drugs with novel antibacterial mechanisms.[1,2] In light of the potential hazardous effects of the rapidly developing multidrug resistance (MDR) property in bacteria against a wide range of broad spectrum antibiotics,[3] combating such infections poses a serious challenge to modern medical chemistry and resulting epidemiology.[4,5] Conventional antibiotics often mediate their antibacterial activities by modifying the cell's genetic machinery or by targeting the synthesis of bacterial cell walls. Bacterial cells often modify the antibiotic 'common target site', produce specific inhibitory enzymes to break down the drugs or design improved efflux pumps to acquire resistance against known antibiotics.[6] New drug development and approval are highly time consuming and cost intensive. Therefore, there are limited reports on new types of antibiotics.[7,8] Recently, functionalization and derivatization of existing antibiotics have been shown to be a promising alternative pathway to overcome some of these challenges.[9,10] Efficient derivatization of antibiotics with polymeric materials can lead to the development of polymer antibiotic conjugates (PACs) with customized antibiotic properties such as toxicity, solubility and activity.[11,12] The bioactive killing efficacy of these PACs is due to interference with bacterial DNA

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† Electronic supplementary information (ESI) available: Detailed experimental procedure, synthetic scheme for various monomers, their characterization by NMR and electrospray ionization mass spectrometry (ESI-MS), UV-visible absorbance spectra of CDP, PLS, PLS, PLC and PLCS in DMSO, ZOI experiment against several bacteria, pH dependent growth experimental setup in LB broth medium, epifluorescence and optical microscopic images of *V. chemaguriensis* cells following Gram staining, DAPI and F143 as noted in the manuscript. See DOI: 10.1039/c9tb00328b

replication and transcription through inhibition of DNA gyrase/topoisomerase II and DNA topoisomerase IV similar to conventional antibiotics.[13]

Antimicrobial peptides (AMPs) offer a promising alternative to conventional antidotes due to their broad-spectrum antibacterial activities as well as anti-inflammatory properties and ability to kill multidrug resistant (MDR) bacteria,[14,15] through cellular disintegration *via* electrostatic and hydrophobic interactions. This mode of action is different from genetic regulation by antibiotics.[12] For example, several AMPs such as indolicidin, LL-37 and human lactoferrin-derived peptides can bind to bacterial endotoxins including negatively charged hydrophobic lipopolysaccharide (LPS), which is a major component of the outer membrane of Gram negative bacterial cells.[16,17] LPS rapidly self-assembles in aqueous solutions leading to aggregate formation, and AMPs, being amphiphilic molecules,[18,19] are reported to interact with these LPSs cumulatively, resulting in their dissociation thereby causing unmanageable and detrimental inflammation.[20,21] Complicated multistep synthesis, high manufacturing costs and easy proteolytic degradation caused several difficulties in large scale commercial production of AMPs.[22] Hence, biocompatible cationic amphiphilic polymeric materials mimicking the properties of AMPs with potent antibacterial activity and low toxicity can be explored as favourable alternatives to AMPs.[23,24]

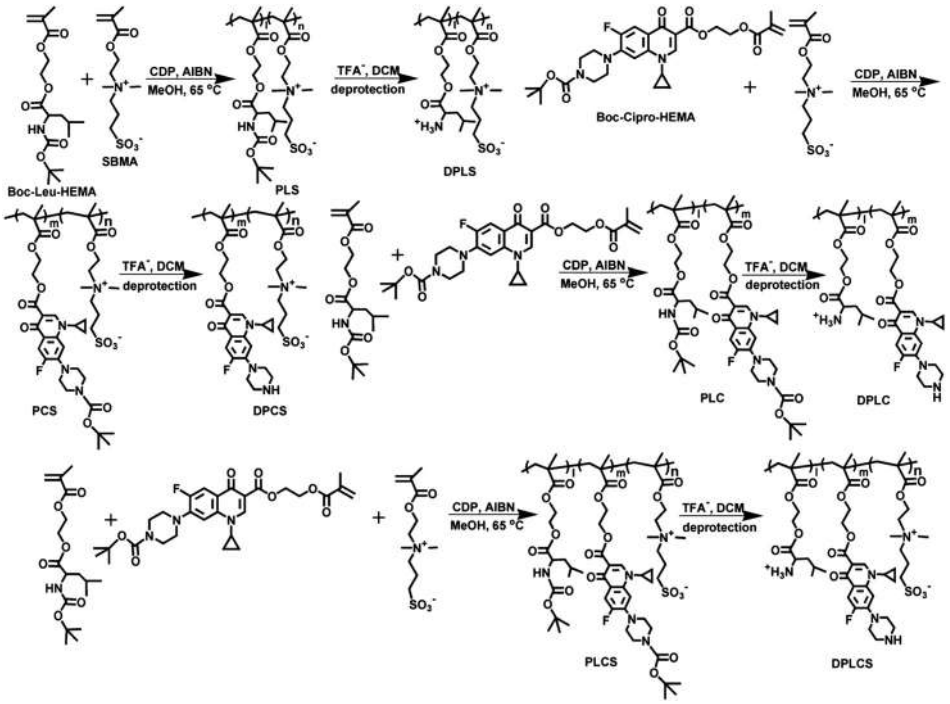
Along with active infections, biofilm formation by selective pathogenic bacteria on colonisable surfaces of medical devices such as catheters and pace-makers further increases risks resulting from nosocomial infections.[25] Biofilm formation can be diminished by the introduction of polyethylene oxide (PEO) or polyethylene glycol (PEG) brushes in the form of hydrophilic neutral or zwitterionic polymeric moieties to antibacterial agents. Such moieties repel negatively charged bacterial cells from adhering to the surfaces of medical devices and are essentially only 'biopassive' antibacterial agents.[26] As opposed to 'bioactive bactericidal agents' that disrupt membrane integrity and in turn biofilm formation by pathogenic bacteria, biopassive agents do not kill pathogens.[27,28] This property brings a new dimension to amphiphilic cationic biocides to act as promising new treatment alternatives against negatively charged biofilm forming pathogenic bacteria. Recently, major attention has been paid toward antibacterial agents integrated with both coupled bioactive and biopassive moieties. Though several promising antibacterial surface switching methods have been expanded *via* a ''kill-and-release'' strategy including bioactive biocidal activity and biopassive antifouling mediated by regulating physiological parameters,[29,30] the field is yet to be explored thoroughly to establish a deeper understanding of antibacterial mechanisms against fast emerging MDR bacterial strains globally including in India.

In recent years, ciprofloxacin has attracted special attention in the discovery of new antibiotic derivatives for the treatment of bacterial infection.[31] Such ciprofloxacin derivatives have been found to possess the ability to interfere with biofilm formations.[32] Though these drug moieties can effectively kill pathogenic bacteria by numerous bactericidal actions,[31] lower water solubility in physiological and slightly basic environments remains a constraint to their widespread application as effective antibacterial agents. Interestingly, coupling with charged antibacterial moieties not only enhances solubility, but also renders the drug effective against a wide variety of pathogens.[9,10] Herein, a water soluble ciprofloxacin based PAC was synthesized with added cationic charge originating from the side-chain leucine moiety and zwitterionic sulphabetaine pendants. Antibacterial activity against several bacteria such as non-biofilm forming *Escherichia coli* (*E. coli*), *Bacillus subtilis* (*B. subtilis*), *Staphylococcus aureus* (*S. aureus*), pathogenic *Vibrio alginolyticus* (*V. alginolyticus*), and biofilm forming as well as potentially pathogenic *Vibrio chemaguriensis* Iso1 (*V. chemaguriensis*) was extensively studied in both liquid and solid matrices. Antibacterial properties were further explored against *V. chemaguriensis* at different pH using a multitude of optical microscopy approaches.

Results and discussion

Design, synthesis and characterization of polymers

To incorporate AMPs mimicking the cationic charge,[33] ciprofloxacin antibiotic[27,34] and zwitterionic component[35] in a single system, *tert*-butyl carbamate (Boc)-leucine hydroxyethylmethacrylate (Boc-Leu-HEMA), Boc-ciprofloxacin hydroxyethylmethacrylate (Boc-Cipro-HEMA) and zwitterionic sulphabetaine methacrylate (SBMA) were copolymerized *via* the reversible addition–fragmentation chain transfer (RAFT) technique in methanol (MeOH) at 65 °C using 2,2′-azobisisobutyronitrile (AIBN) as a radical initiator and 4-cyano-4-(dodecylsulfanylthiocarbonyl)-sulfanylpentanoic acid (CDP) as a chain transfer agent (CTA) (Scheme 1) at a constant monomer to CTA to AIBN ratio of [Boc-Leu-HEMA + Boc-Cipro-HEMA + SBMA]/[CDP]/[AIBN] = 50 : 1 : 0.2 with a monomer feed composition of [SBMA]: [Boc-Leu-HEMA]:[Boc-Cipro-HEMA] = 12.5 : 12.5 : 25. Three other polymers were also synthesized from the copolymerization of Boc-Leu-HEMA and SBMA; Boc-Cipro-HEMA and SBMA; and Boc-Leu-HEMA and Boc-Cipro-HEMA, respectively, having cationic and antibiofilm; antibiotic and antibiofilm; and cationic and antibiotic functionalities for the control study. 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. The characterization results for these polymerization reactions are shown in Table 1. Polymers were analyzed *via* ^{1}H NMR spectroscopy (Fig. 1 and Fig. S4–S6, ESI†), where signals from different monomer units were observed. The theoretical molecular weight ($M_{n,theo}$) values were determined by using the formula: $M_{n,theo}$ = ((([monomer]/[CDP] × average molecular weight (MW) of monomer × conversion (Conv.)) + MW of CDP). From UV-visible spectroscopy, molecular weight ($M_{n,UV}$) values were determined and Table 1 shows close agreement between the $M_{n,theo}$ and $M_{n,UV}$ values. The copolymers demonstrated the expected absorption for the trithiocarbonate moiety at λ_{max} = 307 nm, indicating retention of the



Scheme 1 Synthesis of ciprofloxacin, leucine and SBMA based linear random copolymers by RAFT polymerization, followed by deprotection of the Boc groups.

Table 1 Results obtained by the synthesis of copolymers *via* RAFT polymerization at 65 °C in MeOH for 18 h under various reaction conditions

Polymer	[M]/[CDP]/[I][e]	[SBMA] : [Boc-Leu-HEMA] : [Boc-Cipro-HEMA]	Conv.[f] (%)	$M_{n,theo}$[g] (g mol^{-1})	$M_{n,UV}$[h] (g mol^{-1})
PLS[a]	25/1/0.2	50 : 50 : 0	84	6800	8300
PCS[b]	38/1/0.2	33 : 0 : 67	82	14 500	14 800
PLC[c]	25/1/0.2	0 : 50 : 50	85	19 100	18 900
PLCS[d]	50/1/0.2	25 : 25 : 50	87	18 900	17 500

[a] [Monomer (M)] = [Boc-Leu-HEMA + SBMA]. [b] [M] = [Boc-Cipro-HEMA + SBMA]. [c] [M] = [Boc-Leu-HEMA + Boc-Cipro-HEMA]. [d] [M] = [Boc-Leu-HEMA + Boc-Cipro-HEMA + SBMA]. [e] [I] = [AIBN]. [f] Calculated gravimetrically. [g] $M_{n,theo}$ = ((([M]/[CDP] × average molecular weight (MW) of monomer × Conv.) + MW of CDP). [h] $M_{n,UV}$ = Determined by UV-visible spectrophotometry.

ω-trithiocarbonate group of CDP during RAFT polymerization (Fig. S7, ESI†).[36]

Cationic charges were incorporated into the above polymers by removing Boc groups from pendent leucine moieties in the presence of trifluoroacetic acid (TFA) at room temperature to generate polymers with primary ammonium ($-NH_3^+$) salts (Scheme 1). Boc deprotected polymers are represented as DPLS, DPCS, DPLC and DPLCS, where D stands for Boc deprotection. ^{1}H NMR spectroscopy confirmed the disappearance of Boc proton signals at around 1.40–1.47 ppm (Fig. 1 and Fig. S4–S6, ESI†). The introduction of cationic charge was proved by our group by the measurement of zeta potential.[37,38] Boc groups were also removed from the ciprofloxacin moiety upon TFA treatment revealing its original antibiotic form.[39] All four deprotected polymers are readily soluble in aqueous medium, thus making them physiologically and biologically significant.[40] Antibacterial efficacy of these polymers in terms of bioactive and biopassive routes against several biofilm forming and non-biofilm forming pathogenic bacterial strains was further studied in the following section.

In vitro antibacterial activity in the solid matrix

Initially, bactericidal efficacy of DPLS, DPCS, DPLC and DPLCS against several pathogenic bacterial strains was investigated in solid Luria-Bertani (LB) agar medium *via* the zone of inhibition (ZOI) experiment. Non-biofilm forming *E. coli* and *S. aureus*, and biofilm forming *V. chemaguriensis* were chosen for the experiment. Among them, *E. coli* and *Vibrio* are Gram negative bacteria and *S. aureus* is a Gram positive bacterium. DPLS, DPCS and DPLC are composed of cationic + zwitterionic antibiofilm,[35] antibiotic + zwitterionic biofilm inhibiting, and cationic + antibiotic properties respectively, whereas all these three properties are integrated in DPLCS. Well-plate ZOI experiments were performed at concentrations up to 800 μg mL^{-1} by incubating the culture plates at 37 °C for 12 h. No bactericidal effect was observed up to 100 μg mL^{-1} for all the four polymers (Fig. S8 and S9, ESI†). However, treatment of DPCS, DPLC and DPLCS at concentrations ≥200 μg mL^{-1} resulted in considerable inhibition of bacterial growth resulting in ZOI formation. Generally, the diameter of the ZOI increased

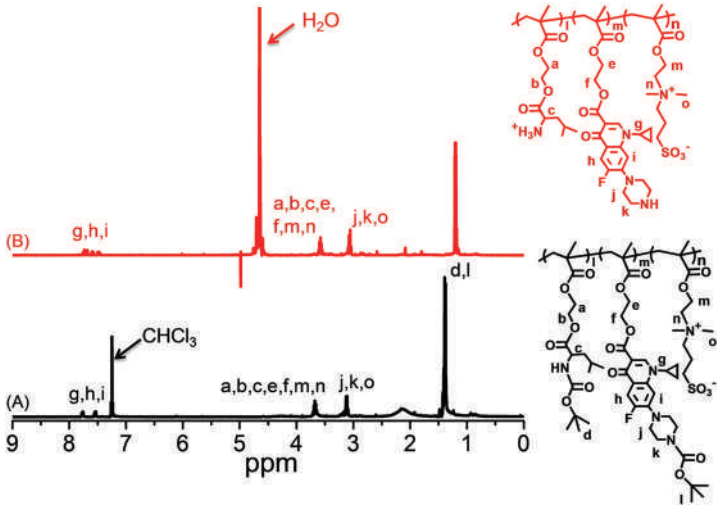


Fig. 1 ^{1}H NMR spectra of (A) PLCS in $CDCl_3$ and (B) DPLCS in D_2O.

with increasing concentrations.[41] These three polymers were effective on all three strains: *E. coli*, *S. aureus* and *V. chemaguriensis*. Interestingly, DPLS consisting of both cationic and zwitterionic antibiofilm functionalities did not exhibit any bactericidal effect in the solid matrix (Fig. 2–4) against biofilm and non-biofilm forming bacteria. This indicates that killing action by the used polymers may not be mediated by electrostatic interactions between the cationic moiety of the polymer and the negatively charged bacterial cell wall that result in the disruption of the cell wall. Hence, these polymers may not specifically be following the AMP mimicking pathways reported previously.[42] This was further confirmed by the non-bactericidal effect of only cationic leucine based polymer leucine hydroxyethylmethacrylate ($P(H_3N^+$-Leu-HEMA)) up to 600 μg mL^{-1} concentration (Fig. 2). The bactericidal property of this polymer against *E. coli* has been demonstrated previously.[43] The differential bactericidal activity demonstrated by DPCS, DPLC, DPLCS and DPLS indicated the predominant effect of the ciprofloxacin active component on killing the pathogenic bacteria, as this drug based repeating unit was the common fragment of these three polymers with the ciprofloxacin units covalently attached to the polymeric side chain through an ester linkage, which eventually undergoes hydrolysis.[44]

The wider ZOI area indicated greater inhibition efficiency for DPLC over DPLCS and DPCS against pathogenic non-biofilm

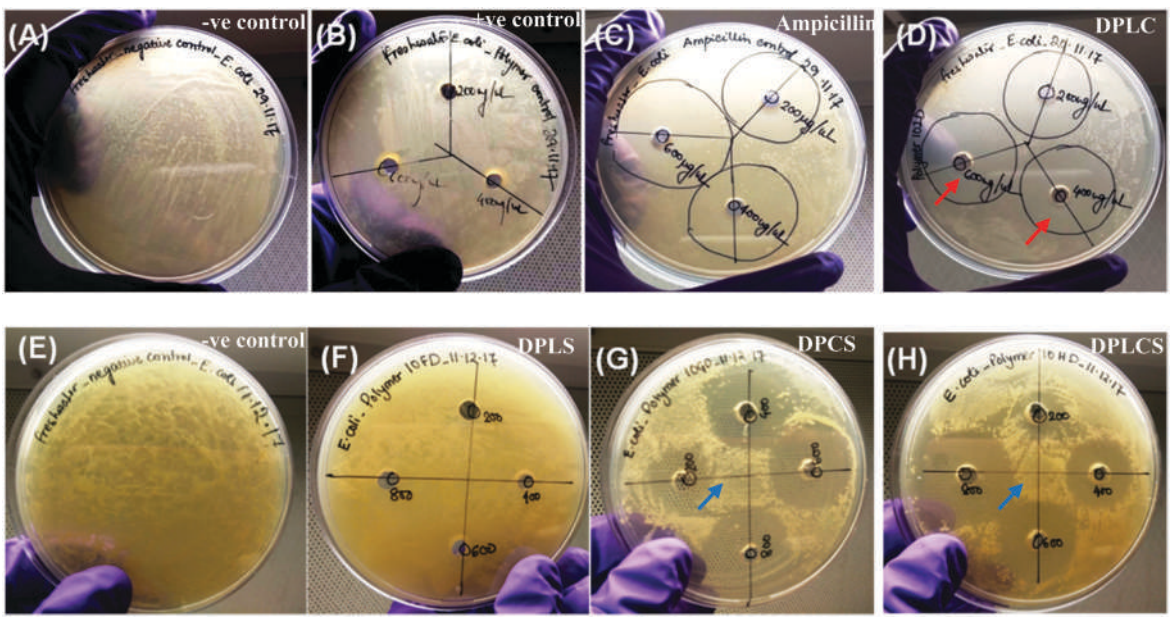


Fig. 2 Zone of inhibition against *E. coli*: (A and E) control (without polymers); treatment with (B) only leucine based cationic polymers; $P(H_3N^+$-Leu-HEMA) (polymer control), (C) ampicillin (well-known antibiotic control), (D) DPLC at 200, 400 and 600 μg mL^{-1}, (F) DPLS, (G) DPCS and (H) DPLCS at 200, 400, 600 and 800 μg mL^{-1}. The red arrows indicate the zone of clearing and the blue arrows indicate cell growth.

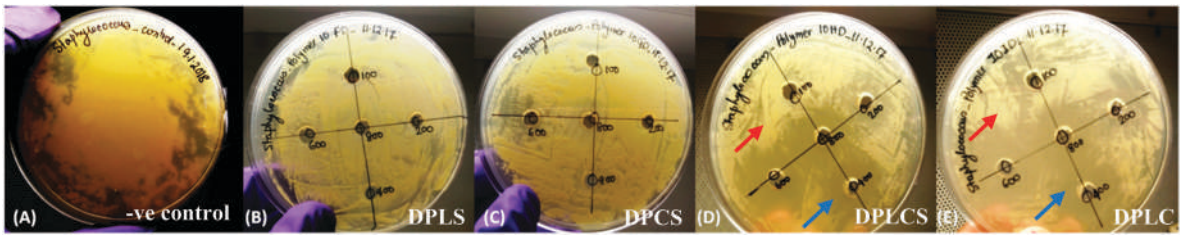


Fig. 3 Zone of inhibition against *S. aureus*: (A) control (without polymers); treatment with (B) DPLS, (C) DPCS, (D) DPLCS and (E) DPLC at 100, 200, 400, 600 and 800 μg mL^{-1}. The red arrows indicate the zone of clearing and the blue arrows indicate cell growth.

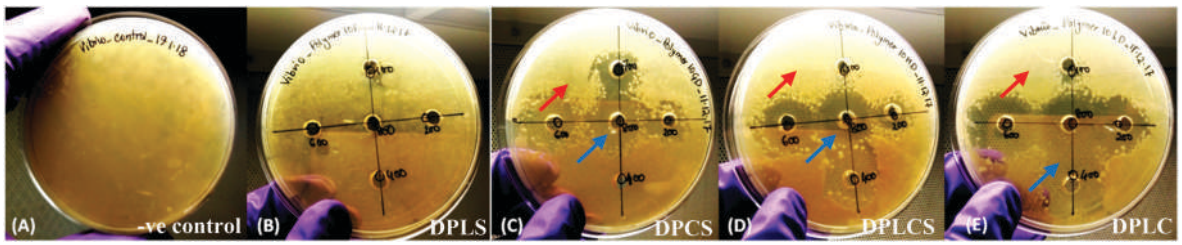


Fig. 4 Zone of inhibition against pathogenic *V. chemaguriensis*: (A) control (without polymers); treatment with (B) DPLS, (C) DPCS, (D) DPLCS and (E) DPLC at 100, 200, 400, 600 and 800 μg mL^{-1}. The blue arrows indicate the ZOI as a result of inhibition of bacterial cell growth by the polymer and the red arrows indicate cell growth in regions without any effect of the polymer.

forming Gram negative *E. coli* (Fig. 2). Quantitative ZOI values are shown in Table 2. For DPLC, DPCS and DPLCS, the increase in concentration of the polymer resulted in a wider ZOI, which clearly indicates the antibacterial properties of the synthesized polymers. The ZOI recorded for all three polymers at 200 μg mL^{-1} clearly shows DPLC to be more efficient than DPCS and DPLCS in killing *E. coli.* However, a two-fold and three-fold increase in concentration only increased the ZOI marginally. The only appreciable increase in the ZOI was recorded for DPCS with a two-fold increase in concentration. This indicates that a low concentration of these polymers may be sufficient to act as a potent antibacterial agent against *E. coli*, which could be highly pathogenic under opportunistic conditions.

Table 2 Quantitative values of the ZOI against *E. coli*, *S. aureus* and pathogenic *V. chemaguriensis*

Polymer	Concentration (μg mL^{-1})	Diameter of the zone of inhibition (cm)		
		E. coli	*S. aureus*	*V. chemaguriensis*
DPLS	200	0.00	0.00	0.00
	400	0.00	0.00	0.00
	600	0.00	0.00	0.00
	800	0.00	0.00	0.00
DPCS	200	2.60	0.00	1.60
	400	3.20	2.00	2.20
	600	3.40	2.80	2.60
	800	3.40	2.00	3.60
DPLCS	200	2.10	0.00	2.20
	400	2.50	2.00	2.80
	600	2.70	2.40	3.20
	800	3.50	2.60	3.60
DPLC	200	3.00	0.00	2.00
	400	3.30	1.40	3.00
	600	3.70	1.60	3.40
	800	—	1.80	3.80

Complete inhibition was observed on treatment with cationic antibiotic based DPLC as compared to a strong antibiotic, ampicillin control (Fig. S10, ESI†). This phenomenon leads to the explanation of the less dominant effect of cationic charge on negatively charged *E. coli* cells. The greater extent of cationic charge on DPLC compared to DPLCS and DPCS, with no cationic component, could result in the highest inhibitory effect. Hence, this result displayed that coupling of cationic charge and antibiotics exhibited a greater extent of bactericidal effect on *E. coli*, whereas only cationic charge was unable to kill the bacterial cells. The expected mechanism might include the cellular disruption by the cationic leucine based polymeric part followed by the incorporation of active antibiotic fragments into the cell. The combined bioactive components might interfere with bacterial DNA replication and transcription through inhibition of DNA gyrase/topoisomerase II and DNA topoisomerase IV leading to the formation of quinolone–enzyme–DNA complexes to produce reactive oxygen species (ROS) with subsequent cellular death.[34] Another possible mechanism might be that the conjugate did not affect the DNA replication but instead inhibited the enzymes involved in septum formation during cell division, which was most likely influenced, as reported elsewhere.[45]

As can be seen from the ZOI values, antibacterial properties were similar when tested against Gram negative *V. chemaguriensis*. All the three polymers showed distinct antibacterial properties at a concentration of 200 μg mL^{-1}. DPLCS and DPLC (the diameter of the ZOI is 2.20 cm and 2.00 cm, respectively) had a stronger inhibitory effect compared to DPCS (a ZOI of 1.60 cm) (Table 2). Unlike as observed against *E. coli*, an increase in concentration of the polymers showed a significant inhibitory effect on *V. chemaguriensis*. This also indicates that the synthesized

polymers may be capable of combating diseases that could be caused by unknown but potentially pathogenic bacteria such as *V. chemaguriensis*. This particular bacterium is a natural isolate but possesses pathogenic genes that show significant homology to its closest relative, *V. alginolyticus*, a bacterium often associated with widespread infections in living organisms. There was a significant increase in the recorded ZOI with an increase in the concentration of the polymer. Against the Gram positive bacterium *S. aureus*, no inhibition was seen at 200 μg mL^{-1}, which is a first hint toward the better antibacterial properties of the synthesized polymers against Gram negative bacteria. Similar to the observations against Gram negative *E. coli*, only a marginal increase in the recorded ZOI is seen with a two-fold and three-fold increase in concentration of the polymers. The only exception was in the case of DPCS where the recorded ZOI (2.80 cm) was increased significantly at a concentration of 600 μg mL^{-1} compared to a concentration of 400 μg mL^{-1} (the ZOI is of 2.00 cm) (Table 2). Thus, the antibiotic based zwitterionic DPCS has a lower inhibition effect on Gram positive bacteria compared to Gram negative bacteria. The differential cell wall structure of Gram positive and Gram negative bacteria might regulate the penetration of active antibiotic fragments into the bacterial cell causing cell death.[46] Interestingly, since common nosocomial infections are often caused by Gram negative bacteria, these synthesized polymers may be developed further into potent antibacterial agents in combating infections.

The coupling effect of antibiotics and cationic charge in bioactive killing was further explored against non-biofilm forming Gram positive *S. aureus* (Fig. 3) and biofilm forming pathogenic *V. chemaguriensis* strains (Fig. 4). This indicates that the synthesized polymers may also be able to inhibit bacteria that form a biofilm. Biofilm is a thick sheath of 'sticky' material around the bacterial cells that inhibits the entry of antibacterial agents into the cell. Designing new antibacterial agents that could inhibit biofilm formation can essentially hinder bacterial cell growth along surfaces of implants, catheters and pace-makers.

Antibacterial activity in a liquid matrix

LB broth medium was chosen as an efficient liquid matrix to explore the *in vitro* antibacterial effect of DPLCS against *E. coli*, *V. alginolyticus*, *V. chemaguriensis* and *B. subtilis* (Fig. 5). Previously, we reported the matrix dependent active ciprofloxacin fragment released from the antibiotic based gel against *V. chemaguriensis*.[45] The designed polymer reported in this paper appeared to be trapped by the EPS layer around the cells of *V. chemaguriensis* under the effect of the polymer selectively in the liquid matrix only.[46] Here, DPLCS was chosen to study the liquid matrix antibacterial effect based on the antibacterial effect observed from the ZOI results. A combined bioactive killing effect of cationic charge and ciprofloxacin, and anti biofilm effect of the zwitterionic part leading to biopassive bactericidal efficacy are expected from DPLCS in the liquid matrix. Interestingly, the effect was prominently observed only in the case of the non-biofilm strains, *E. coli* and *B. subtilis*, which resulted in complete clearance of bacterial growth in the medium (Fig. 5A and B). The minimum inhibitory concentration (MIC)[47] was observed at 200 μg mL^{-1} (11.4 μM). Complete bactericidal effect against biofilm forming *Vibrio*, namely *V. alginolyticus* and *V. chemaguriensis*, was not observed as turbidity of the LB medium indicated cell growth (Fig. 5C and D). This phenomenon can be explained by the formation of biofilm under treated conditions,[48] which usually inhibits the influx of 'active' ciprofloxacin into the cell and thereby stops interaction of cationic charge with the negatively charged cell wall of the bacterium. Though some recent work has been reported on the disruption of biofilm *via* cationic charge,[49] the proposed mechanisms may not be applicable against the studied *Vibrio* species.

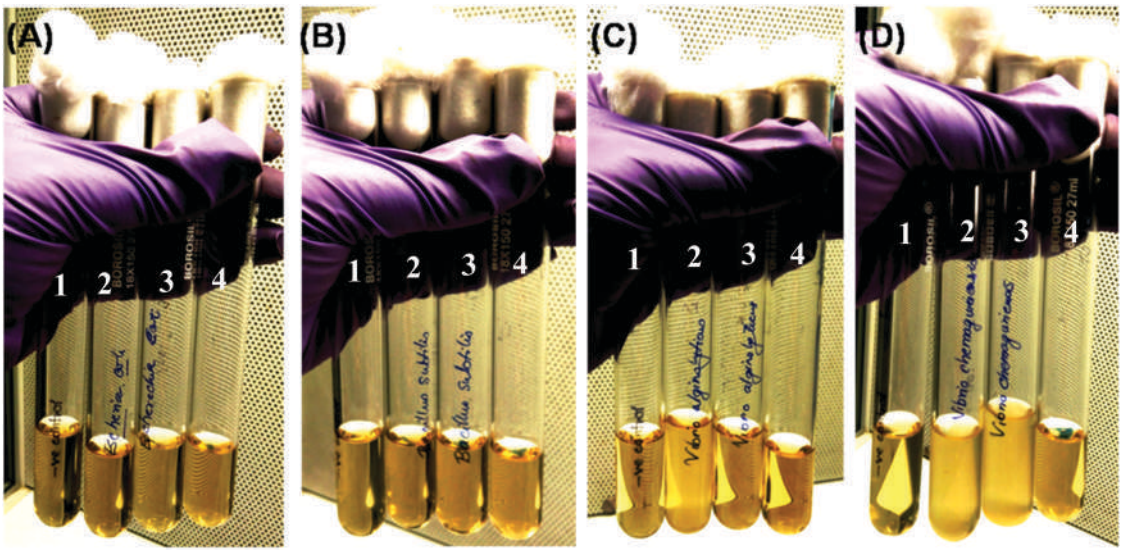


Fig. 5 Growth experiment of (A) *E. coli*, (B) *B. subtilis*, (C) *V. alginolyticus*, and (D) *V. chemaguriensis* in LB broth medium on treatment with 200 μg mL^{-1} DPLCS (test tubes 2 & 3 in each set); only LB medium was taken as negative control (test tube 1 in each set), other blank set included polymer containing LB medium at pH 8.0 (test tube 4 in each set).

Although ciprofloxacin is a widely used broad spectrum antibiotic, it is soluble only at acidic pH and precipitates out from aqueous medium under physiological conditions (pH 7.4).[50] This phenomenon poses a challenge to modern medical science in terms of miscibility of the drug at physiological pH. Some bacterial genera like *Vibrio* selectively prefer slightly alkaline conditions to grow (pH 8.0), which highlights the urgency to develop a new set of bactericidal agents, which could be active at both physiological and higher basic pH through modification of existing antibiotics. We have previously reported the antibacterial efficacy of polymers by the release of active fragments from a ciprofloxacin based gel under physiological conditions.[45] In the present work, the synthesized polymers DPCS, DPLCS and DPLC appear to be water soluble in a pH range including physiological to basic pH and may be antibacterial in nature due to the release of the active fragment from the polymer. The pH dependent antibacterial effect of DPLCS was demonstrated against pathogenic *V. chemaguriensis* in LB medium at a salinity of 11.5. Lower bacterial growth was observed in the basic pH range (pH 8.0 to 9.5) compared to a slightly acidic environment (pH 6.0–6.5) (Fig. 6 and Fig. S11, ESI†). The same result was also observed when cells were grown in liquid trypticase soy broth (TSB) instead of LB broth (Fig. S12, ESI†). Bacterial cell growth was comparable at pH 6.0 and 7.0 in the treatment as well as the control set ups. Interestingly, cell growth observed qualitatively at pH 8.0 and 9.0 was negligible compared to the control set up at these pH values. The biofilm assay clearly showed that biofilm formed at pH 6.0 (OD_{570} = 2.40) and 7.0 (OD_{570} = 2.40) was similar to biofilm formed in the control set up (OD_{570} = 2.40) grown in the absence of polymer (Fig. 7). But, the OD values obtained for biofilm at pH 8.0 (OD_{570} = 0.98) and 9.0 (OD_{570} = 0.72) under polymer treated conditions were lower compared to the control (OD_{570} = 1.99 at pH 8.0 and 9.0). This indicates that the polymer might be capable of inhibiting bacterial biofilm formation under alkaline pH conditions. Absence of biofilm could be a primary reason for the higher antibacterial activity of the polymer observed under alkaline pH conditions. The pH dependent bactericidal effect of DPLCS under basic conditions in the liquid matrix may be due to hydrolytic degradation of the active antibiotic fragment attached through a covalent ester linkage to the water soluble DPLCS polymeric structure. In addition, the cationic charges from leucine moieties in DPLCS can regulate the bactericidal effect in the studied pH range through bacterial cellular disintegration *via* electrostatic interaction. In an acidic environment, reduced bacterial growth may be caused only by the cationic charges of leucine fragments (Fig. 6 and Fig. S11, ESI†), but the effect of cationic charge does not appear to be significant for the potentially pathogenic *V. chemaguriensis*.

Further, the growth curve obtained by treating cells with polymer at pH 6.5, 8.0 and 9.0 (Fig. 8) clearly demonstrated the effect of the polymer on the actively growing cells. The initial lag phase is observed under control and treated conditions at pH 6.5, 8.0 and 9.0. No prominent difference in cell growth was observed for almost 2 hours when the growth appears comparable between control and treated conditions. The growth then reduces marginally at pH 8.0 and 9.0 compared to pH 6.5. The effect of the polymer on cell growth might become significant after prolonged exposure of the cells to treatment. This could be due to an increased release of the active fragment under basic conditions compared to that of acidic conditions. It would be interesting to further explore the reasons driving the time lapse (almost 2 hours) before which the polymer does not seem to affect cell growth considerably.

Morphological variation at different pH

Treatment of *V. chemaguriensis* with the DCLSP polymer induced a multifold effect on the bacterial cells. At pH 6.5, optical density at 600 nm (OD_{600}) indicated a lower number of cells as compared to the control. The cells that may be termed as 'resistant' to bactericidal activity of the polymer indicated a distinct increase in cell length. Field emission scanning electron microscopy (FESEM) and transmission electron microscopy (TEM) images shown in Fig. 9 and 10, respectively, indicate an increase in cell size. This may have been induced

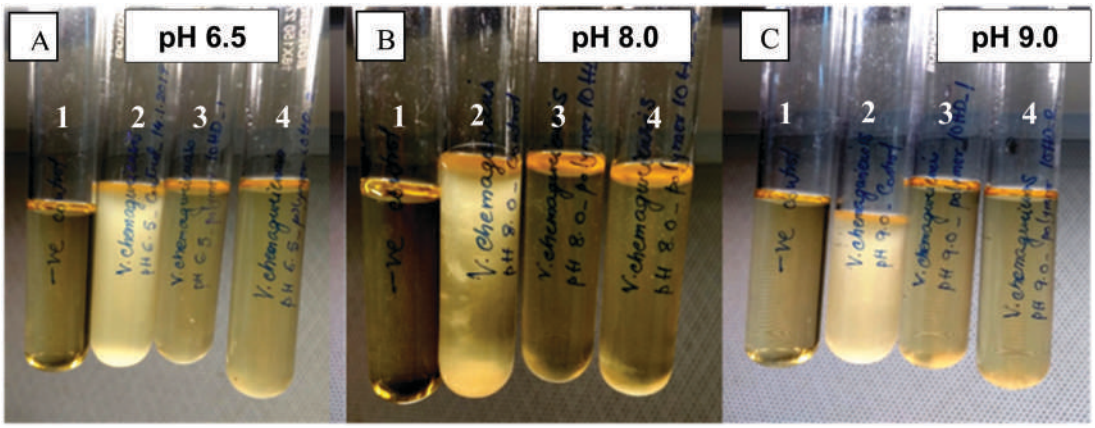


Fig. 6 pH dependent growth experiment in LB broth medium; only LB medium was taken as a negative control (test tube 1 in each set), *V. chemaguriensis* culture in LB medium at a salinity of 11.5 was taken as a positive control (test tube 2 in each set), treatment with DPLCS with the duplicate experimental set at (A) pH 6.5, (B) pH 8.0 and (C) pH 9.0, with a salinity of 11.5 (test tubes 3 & 4 in each set). Turbidity of the medium indicates cell growth. Less turbidity in polymer treated tubes indicates lower cell growth as seen from the equivalent test.

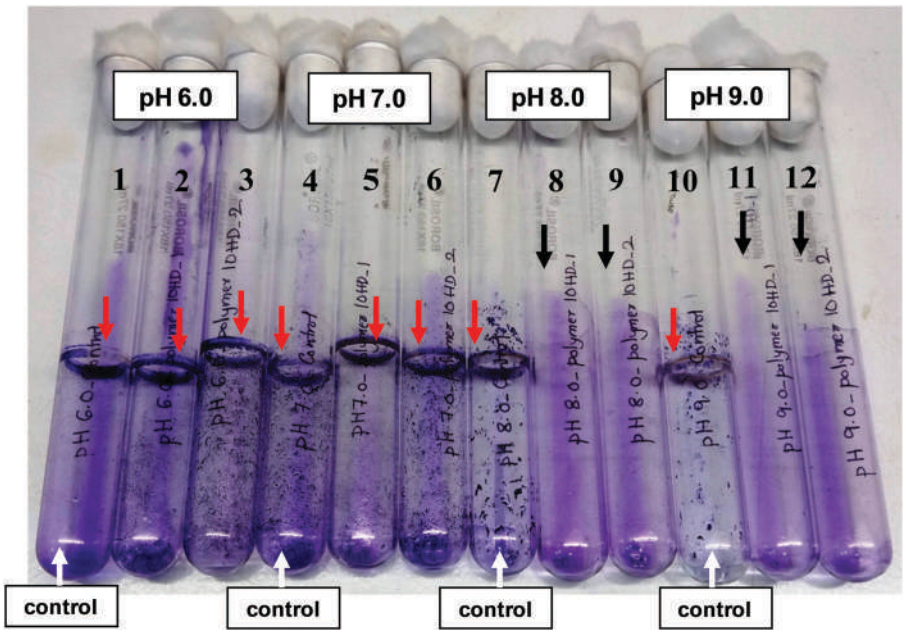


Fig. 7 Biofilm assay of *V. chemaguriensis* at pH 6.0 (test tubes 1–3), 7.0 (test tubes 4–6), 8.0 (test tubes 7–9) and 9.0 (test tubes 10–12) after crystal violet staining treatment with DPLCS (duplicate experimental set). Red arrow indicates the prominent biofilm (test tubes 1–7 and 10) and black arrows indicate no biofilm observed on the test tube wall (test tubes 8, 9, 11, and 12).

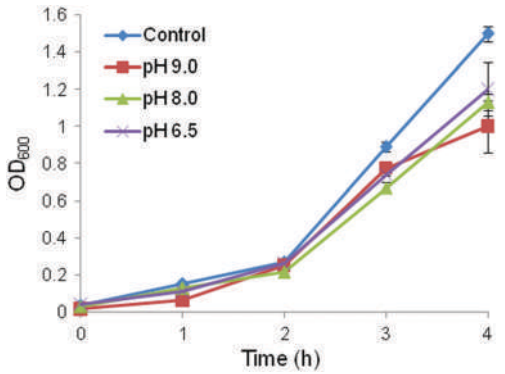


Fig. 8 Growth curve showing the change in cell growth under treated conditions at medium pH of 6.5, 8.0 and 9.0. Cell growth is lower under treated conditions compared to in the absence of the polymer. Further, under basic conditions of pH 8.0 and 9.0, cell growth appears lower than that recorded at pH 6.5. This indicated the greater antibacterial efficiency of the polymer under basic conditions.

by an increase in length of individual cells in order to enhance nutrient uptake under stressed conditions (*i.e.* stress caused by the presence of polymer). It may also be a result of inhibition of septum formation during the final stages of cell division. Some cells also appear to have a curved shape, which is considered as the 'typical comma-shaped cells' characteristic of *Vibrio*. Such comma-shaped cells are not observed in the control at pH 6.5. Elucidation of the exact mechanism of the polymer in cell physiology is a subject of further investigation using extensive molecular techniques.

Both FESEM and TEM imaging at pH 8.0 showed cell debris indicative of cell death. Compared to the control, the lower OD_{600} values observed upon treatment with the polymer at pH 8.0 are further reinstated by the cell debris observed using the imaging techniques. Similar to observations in the case of pH 6.5, some cells show an increase in cell length. It is interesting to note that cells appear to be less elongated compared to the treatment at pH 6.5 (Fig. 9D). Cell lengthening and cell debris are the critical observations upon treatment at pH 9.0 (Fig. 9F). Typical comma-shaped cells were also observed upon treatment at pH 8.0 and 9.0.

Further investigation *via* selective staining [membrane selective fluorescein-5-isothiocyanate (F143) and nucleic acid specific 4′,6-diamidino-2-phenylindole (DAPI)] under epifluorescence microscopy indicated the cellular lengthening (Fig. S13, ESI†). Persistency of the F143 green colour under treated conditions established that the polymer does not kill the bacteria cells through disintegration of the cell membrane. Interestingly, we could not observe the DAPI blue colour binding to DNA prominently after polymeric treatment as compared to the control, which indicates the probability of binding of DPLCS through the bacterial DNA. OD_{600} values along with the morphological features observed by FESEM and TEM imagining indicate the polymer to be more potent under basic pH conditions compared to acidic pH conditions.

Comparative antibacterial activity and cell morphology in liquid and solid matrices in a single set

To study the matrix dependent bactericidal effect of DCLSP against the pathogenic *V. chemaguriensis* strain, a dual matrix growth experiment was performed by coupling LB medium in both solid and liquid matrices in equal proportions. Considerable growth inhibitory effect in both matrices was observed on

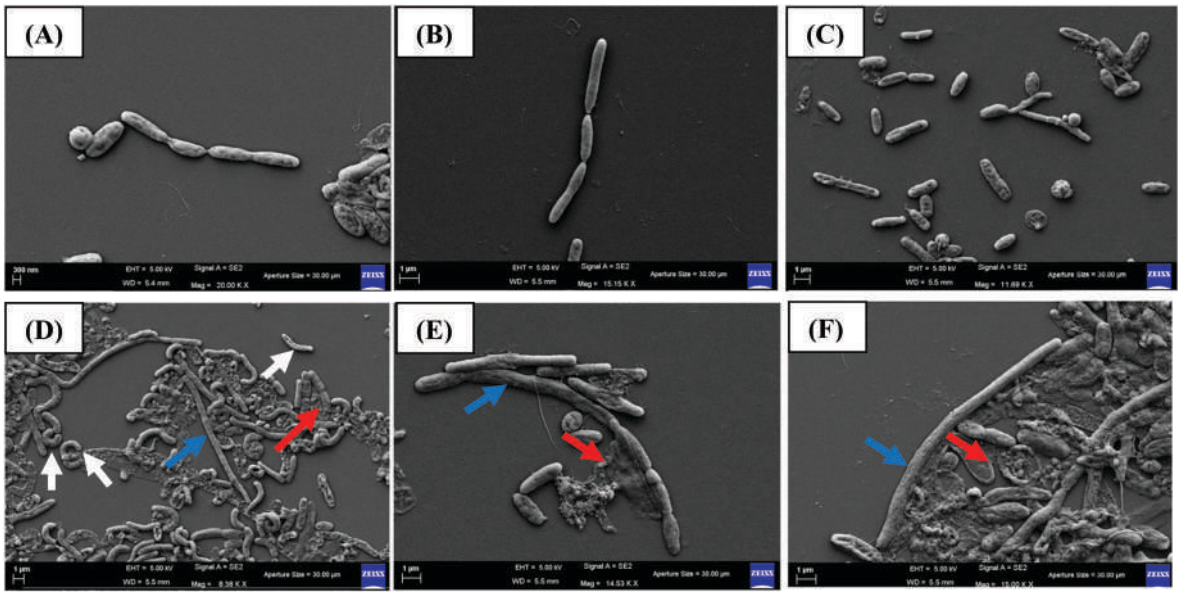


Fig. 9 FESEM image of cells from control at pH (A) 6.5, (B) 8.0 and (C) 9.0 and DPLCS treated cells at pH (D) 6.5, (E) 8.0 and (F) 9.0. The blue arrows indicate cell elongation and the red arrows indicate cell debris (dead cells). The white arrows indicate the comma-shaped cells observed only upon treatment with the polymer at pH 6.5.

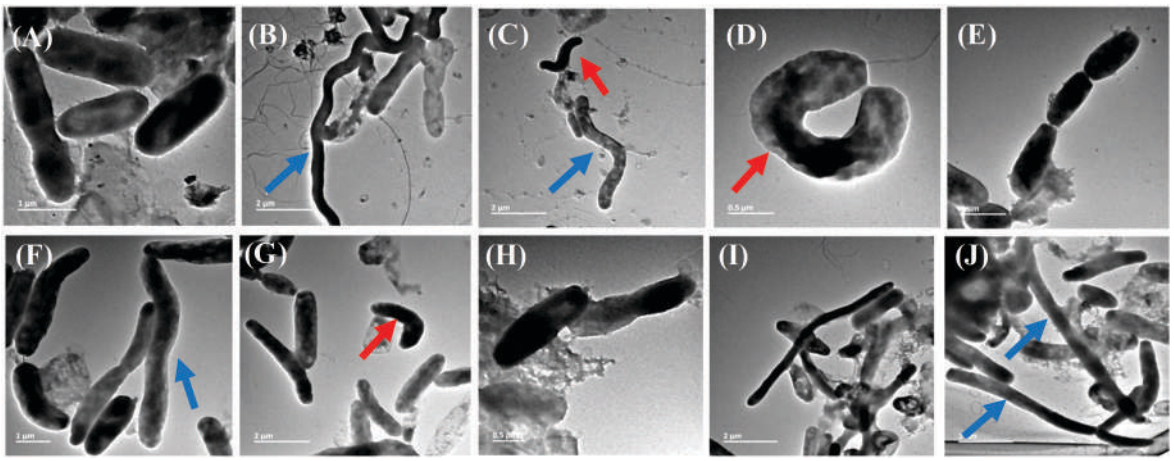


Fig. 10 TEM image of cells at pH 6.5: (A) control, (B–D) the variation in cellular morphology upon treatment with DPLCS; at pH 8.0: (E) control, (F and G) treated with the polymer; at pH 9.0: (H) control, (I and J) treated with the polymer. The blue arrows indicate cell elongation and red arrows indicate the comma-shaped cells.

treatment with DCLSP at 200 μg mL^{-1} (Fig. 11). Cellular morphology under treated conditions was observed by Gram staining under 100×, 400× and 1000× magnification (Fig. 12 and Fig. S14, ESI†). Observable results include cell lengthening and cell curvature under the treated conditions in both liquid and solid matrices. Therefore, the cells exhibit the ability to enhance suface area causing cell lengthing and deformation in the cell wall leading to cell curvature.[51] In addition, fragmentation of the cells leading to chain formation was also observed (Fig. 12B and D). This could indicate that the cellular repair attempts to increase the surface area, which could enhance additional uptake of nutrients for survival during the stress phase.

In vitro cytotoxicity *via* hemolytic assay

To check the bioapplicability of the synthesized PAC, next, we studied the cytotoxicity of DPLCS by performing a hemolytic assay in chicken red blood cells (RBCs) (Fig. 13). The effect of hydrophobicity and molecular weight of antibacterial polymeric agents on mammalian blood erythrocytes is already reported in the literature.[52] An enhanced hydrophobicity always decreases the selectivity between the disruption of the blood cell wall and the bacterial cell membrane by diminishing the HC_{50} value, which was defined by the minimum concentration causing 50% hemolysis, and overall amphiphilicity was affected.[53] Generally, the selectivity is quantitatively judged for any antibacterial

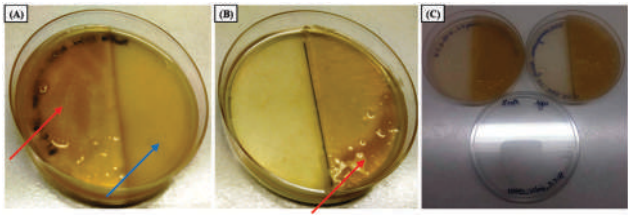


Fig. 11 Dual growth inhibition experiment against *V. chemaguriensis* on both LB agar and LB broth liquid media in a single plate: (A) control, (B) treated with DPLCS and (C) a duplicate of the experiment. The red arrow indicates dense cell growth as observed from colony formation on the solid agar medium. The blue arrow indicates turbidity of the liquid broth upon cell growth.

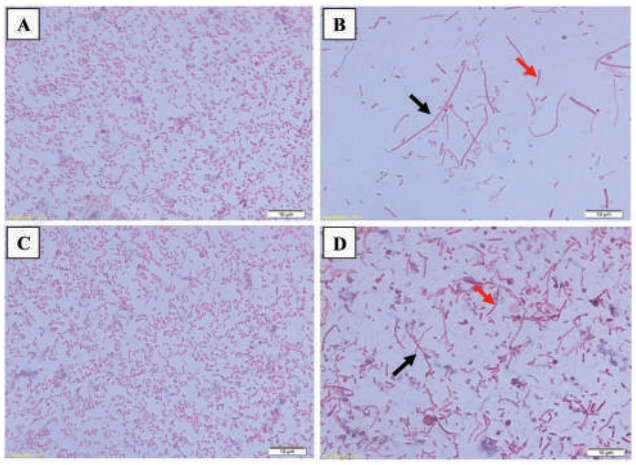


Fig. 12 Optical microscopic images of *V. chemaguriensis* cells following Gram staining. Cells were collected from LB agar: (A) control and (B) after treatment with DPLCS. Cells were collected from the LB broth portion: (C) control and (D) after treatment with DPLCS at 1000× magnification. The control set-up shows cells to be uniformly dispersed throughout the field of view. No irregular cellular morphology was observed. The cells appear rod shaped and are plenty in number. The treated conditions show a much reduced cell number, which is particularly prominent in the solid matrix. The red arrows show cells becoming curved with an increase in cell size. A number of cells in the field of view appear to have elongated significantly (black arrows). Cell lengthening can be understood by comparing with the scale bar shown in the images. Most cells also appear to be much longer under treated conditions than the average cell observed in the control.

agents by measuring the ratio between the HC_{50} value and MIC (selectivity = HC_{50}/MIC). A higher ratio indicates more potential biomedical applications of antibacterial materials with lower cytotoxicity. The DPLCS consists of cationic leucine, antibiotic ciprofloxacin and zwitterionic sulphabetaine units. Non-cytotoxicity of leucine based polymeric units was already explored by our group[54] and others,[55] though there are several reports on ciprofloxacin induced acute hemolytic anemia.[56] But the hemolytic assay indicated the superiority of antibacterial ciprofloxacin based DPLCS in terms of biomedical applications as hemolysis did not reach 50% even at sufficiently high polymer concentration (5 mg mL^{-1}). Hence, HC_{50} must be greater than 5 mg mL^{-1} regulated by proper amphiphilicity with a high selectivity of >25 towards

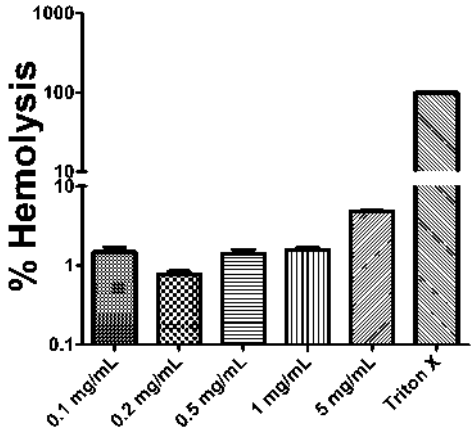


Fig. 13 *In vitro* toxicity of DPLCS by determining the % hemolysis after treating RBC with the polymer up to a concentration of 5 mg mL^{-1} in PBS buffer and measuring the % lysed RBCs at 540 nm with respect to Triton X.

bacterial cells and MIC at 200 μg mL^{-1} against several bacterial strains.

Experimental

Materials, instrumentation, synthesis and characterization of SBMA (Fig. S1, ESI†), Boc-Cipro-HEMA (Fig. S2 and S3, ESI†), copolymers (Scheme S1, ESI†), P($^{+}H_3$N-Leu-HEMA), determination of molecular weight *via* UV-visible spectroscopy ($M_{n,UV}$), deprotection of Boc groups, the specific experimental procedure for the study of antibacterial activity in a solid agar plate and liquid LB broth medium with corresponding morphology study *via* FESEM, TEM and staining process, and hemolytic assay are discussed in the ESI.†

Conclusions

In summary, a zwitterionic non-cytotoxic PAC with the ciprofloxacin moiety and cationic leucine units was developed by potential coupling of bioactive and biopassive activity against both Gram negative and Gram positive bacteria including potentially pathogenic species. Only cationic charge or the antibiotic based active component was less effective as antibacterial agents but the coupling of the antibiotic fragment with cationic charge enhanced the bactericidal efficiency of the polymer. This was proved by comparison with a series of synthesized polymers containing cationic–antibiofilm, antibiotic–antibiofilm and cationic–antibiotic fragments. The combined capacity was more pronounced on non-biofilm forming *E. coli* and *B. subtilis* in both liquid and solid matrices than on biofilm forming *V. alginolyticus* and *V. chemaguriensis.* Enhanced surface area in the treated environment was observed under both circumstances by Gram staining. DPLCS appeared to be bacteriostatic against the studied *Vibrio* species in the liquid matrix though a decrease in growth on altering pH conditions was clearly observed. Stronger antibacterial action of the designed polymer against *Vibrio* species was further supported

by the clear changes in morphology observed by FESEM and TEM imaging. Greater activity of the synthesized antibacterial polymer under physiological to basic conditions (pH 7.0 to 9.0) added a new potential in biomedical applications. But, at the same time, despite the occurrence of all bioactive cationic, antibiotic and zwitterionic antibiofilm components, the lower bactericidal capacity of the system against biofilm forming *Vibrio* strains in the liquid matrix at acidic pH hints toward possible challenges for the modern medical sciences to further optimize the known antibiotics with complete killing efficacy of biofilm forming resistant pathogens in all matrices.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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