Supporting Information for

# Capture of *Vibrio cholerae* by charged polymers inhibits pathogenicity by inducing a sessile lifestyle

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This file contains:

Supporting Figures S1-S7

Supporting Tables S1 and S2

Supporting Materials and Methods

Supporting References

## SUPPORTING FIGURES

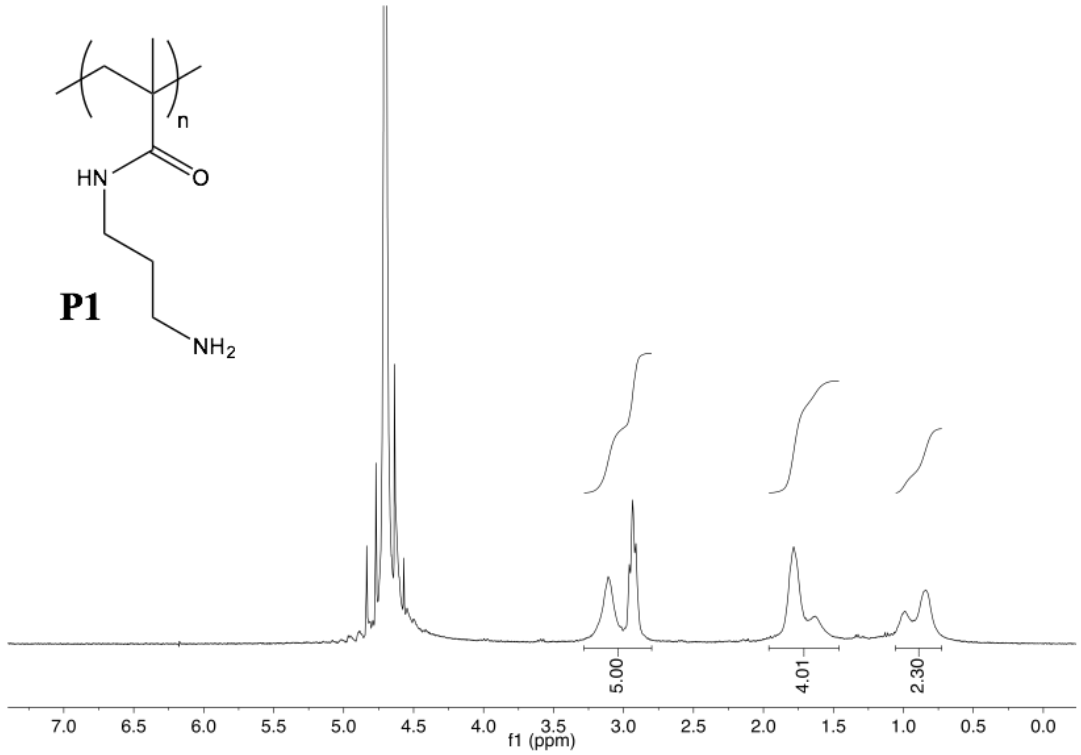


**Figure S1**| $^{1}$H-NMR (300 MHz, $D_2O$) spectrum of **P1**.

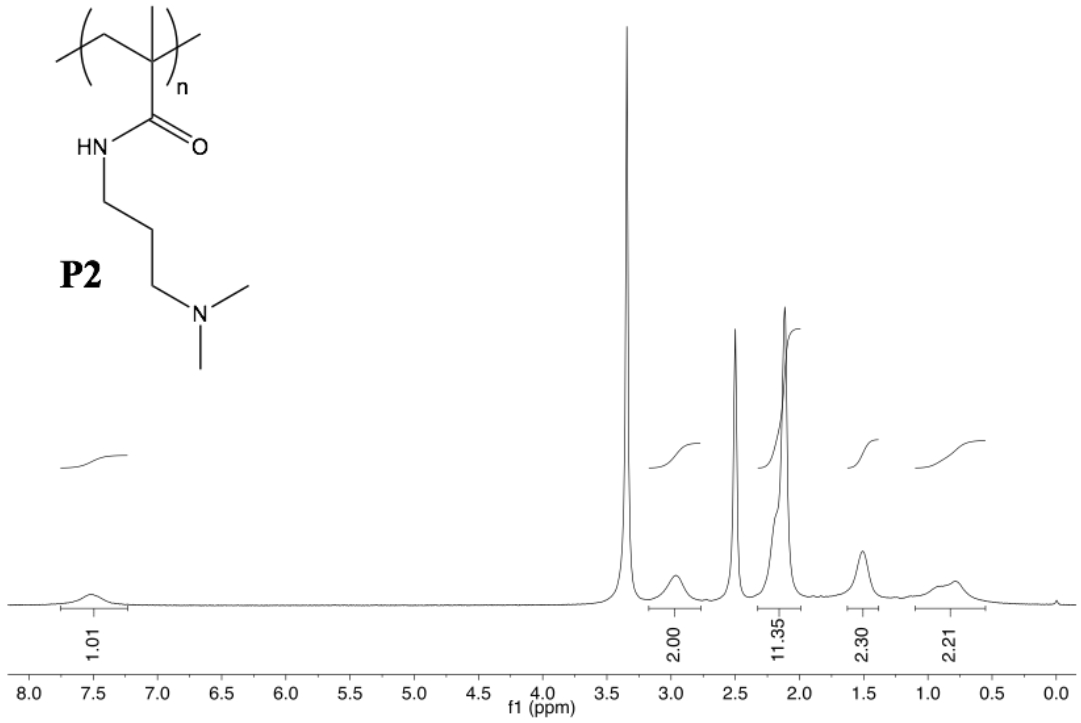


**Figure S2**| $^{1}$H-NMR (300 MHz, DMSO-d6) spectrum of **P2**.

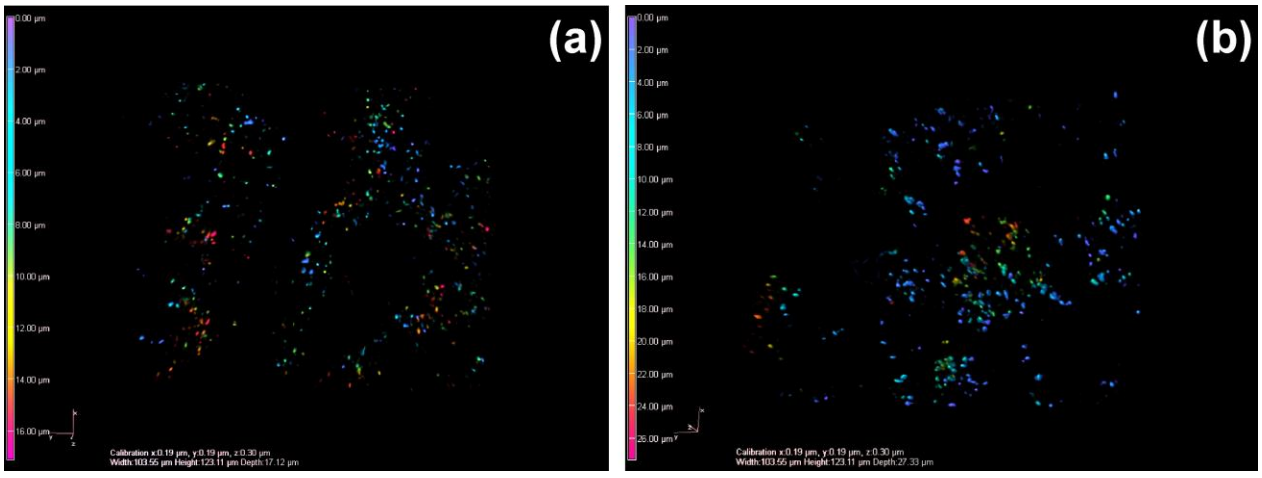


**Figure S3**| **Polymers induce rapid formation of three-dimensional bacterial clusters.** GFP-*V. cholerae* were incubated with 0.5 mg/mL of P1 **(a)** or P2 **(b)** for 15 minutes, and z-stack images were collected using a spinning disc confocal microscope. Maximum intensity projections are shown, with color-coded Z-depth. Total depth of the clusters shown here are 17 and 27 μm, respectively.

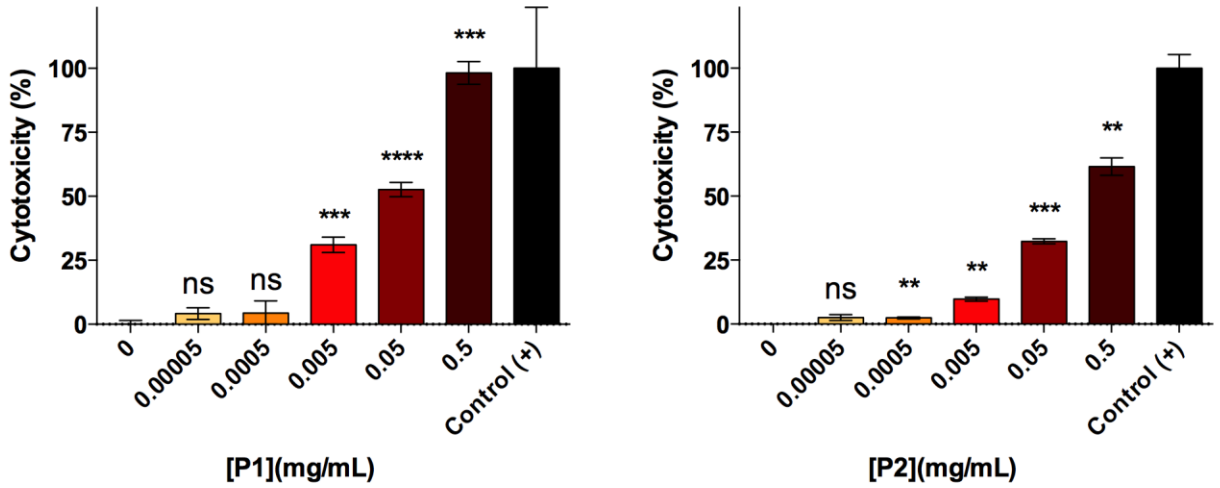


**Figure S4| Effect of polymers on membrane integrity of cultured epithelial cells.** Solutions of polymers in DMEM at concentrations as indicated, were incubated with cultured Caco-2 intestinal epithelial cells for 7 hrs. Cytotoxic effect on host cells was quantified by measuring the amount of lactate dehydrogenase (LDH) released into the culture medium. Results were normalized to untreated Caco-2 cells (0%) and cells lysed with Triton X-100 (100%). Results are means ± s.e.m. of three independent experiments. Analysis of variance (ANOVA), followed by Tukey's post hoc test, was used to test for significance. Statistical significance was defined as $p<0.01$ (**), $p<0.001$ (***), or $p<0.0001$ (****).

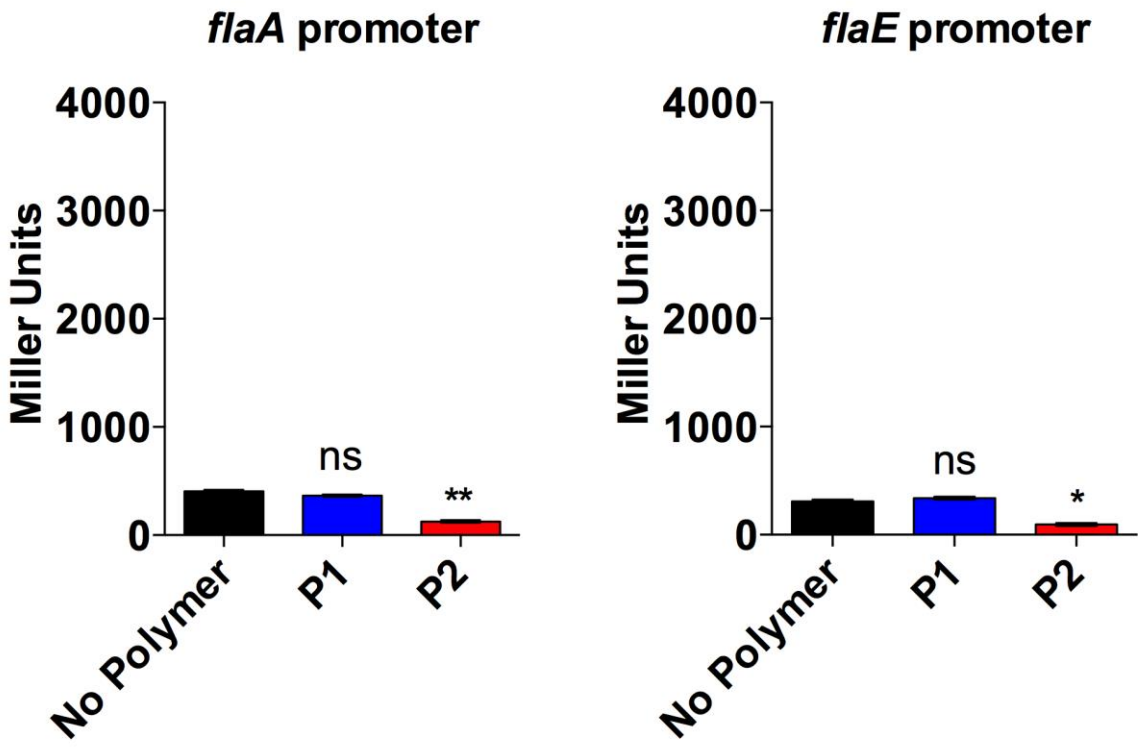


**Figure S5| Effect of bacterial clustering on transcriptional regulation of flagella-driven motility**. Promoter activities of *flaA-lacZ* and *flaE-lacZ* fusions in *V. cholerae* were measured following infection of Caco-2 cells with *V. cholerae* reporter strains at an MOI of 10 for 7 hours at 37 °C in the absence (black) or in the absence (black) or presence of 0.05 mg/mL P1 (blue) or 0.5 mg/mL P2 (red) .

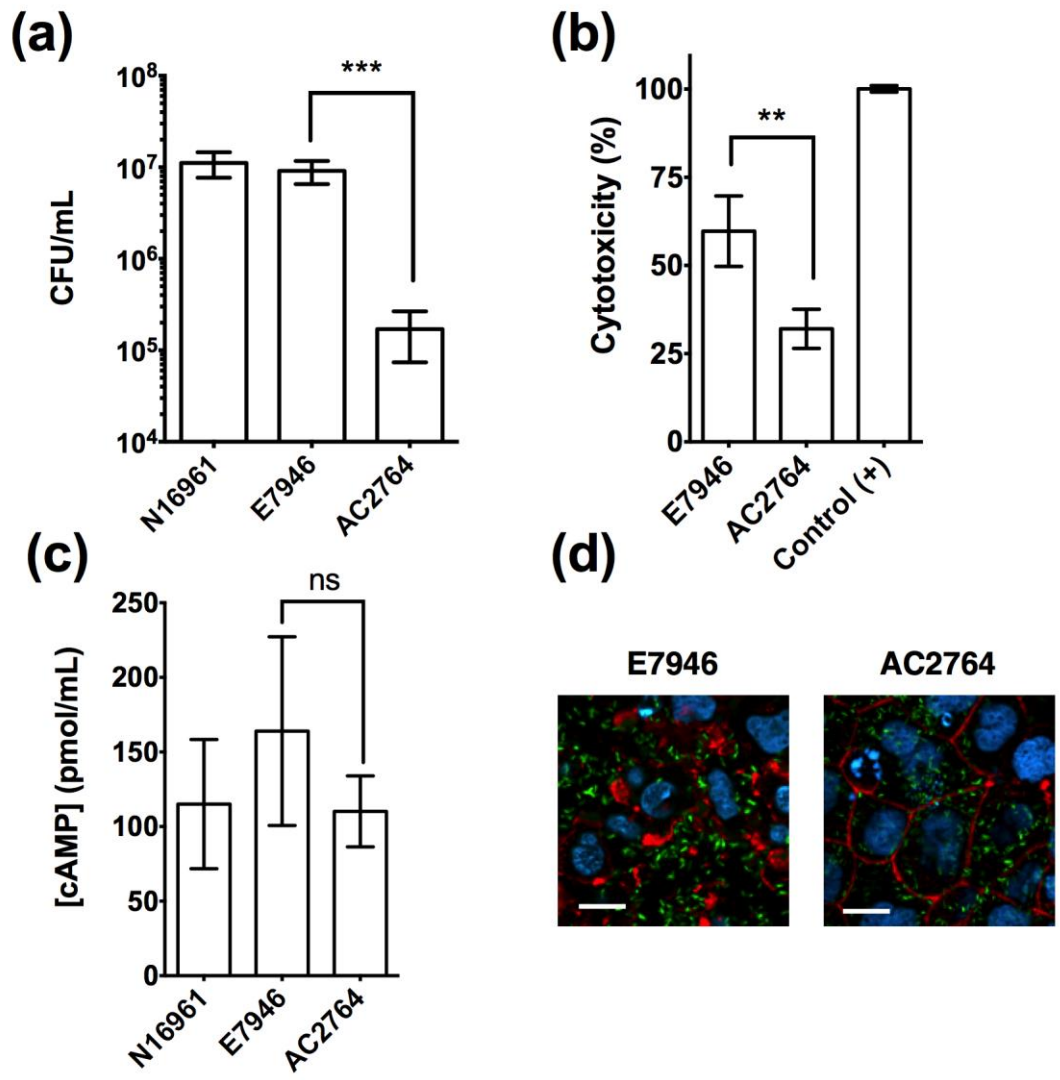


**Figure S6| Effect of *tcpA* deletion on bacterial attachment, cytotoxicity and cAMP release in a Caco-2 infection model**. *V. cholerae* E7946 and E7946 Δ*tcpA* cultures were adjusted to an MOI of 10, and used to infect cultured Caco-2 intestinal epithelial cells for 7 hrs. Following the infection, (**a**) bacteria attached to Caco-2 cells were quantified by dilution plating, following washing and lysis of Caco-2 cells. (**b**) Cytotoxic effect on host cells was quantified by measuring the amount of lactate dehydrogenase (LDH) released into the culture medium. Results were normalized to untreated Caco-2 cells (0%) and cells lysed with Triton X-100 (100%). **(c)** Levels of cAMP production in Caco-2 cells were measured by ELISA**.** Results in a-c are means ± s.e.m. of three independent experiments. Analysis of variance (ANOVA), followed by Tukey's post hoc test, was used to test for significance. Statistical significance was defined as $p<0.05$ (*), $p<0.01$ (**) or $p<0.0001$ (****). (**d**) Following infection, *V. cholerae* (green), DNA (blue), and F-actin (red) were visualized by fluorescence microscopy. Scale bar, 10 μm.

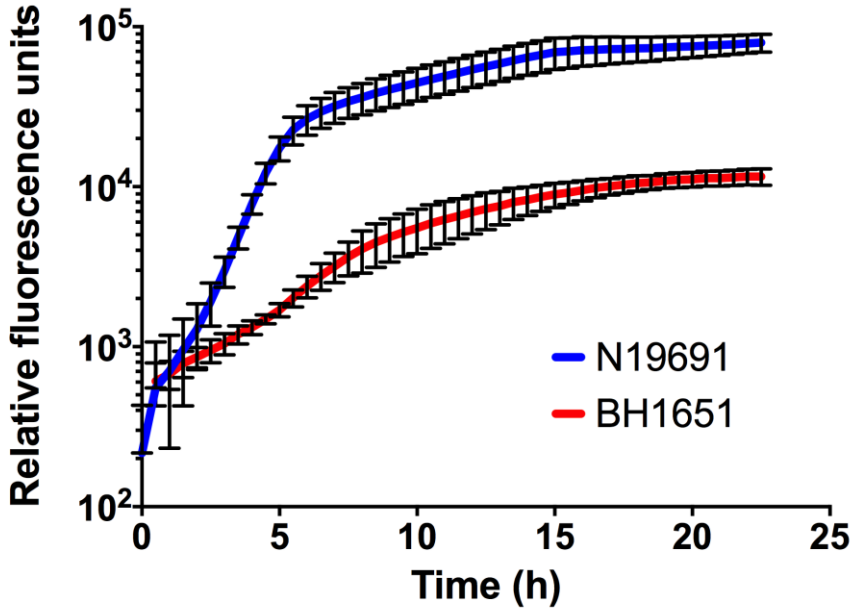


**Figure S7| Growth curves of *V. cholerae* wild type and BH1651 mutant**. Bacterial cultures were grown at 200rpm, 37 °C and GFP fluorescence was measured every 30 minutes for 20 hours. Results are means ± s.e.m. of three independent experiments.

## SUPPORTING TABLES

**Table S1 | *V. cholerae* strains used in this study**.

| Strain | Description | Source or reference |
|---|---|---|
| N16961 | Wild-type; O1 biovar El Tor. | Heidelberg et al.(1) |
| NP5001 | N16961 carrying promoterless pRW50-oriT plasmid; $Tet^R$. | This study |
| NP5002 | N16961 carrying pRW50-oriT plasmid containing the upstream region of *toxT* promoter; $Tet^R$. | This study |
| NP5003 | N16961 carrying pRW50-oriT plasmid containing the upstream region of *ctxAB* promoter; $Tet^R$. | This study |
| NP5004 | N16961 carrying pRW50-oriT plasmid containing the upstream region of *tcpA* promoter; $Tet^R$. | This study |
| NP5005 | N16961 carrying pRW50-oriT plasmid containing the upstream region of *aphA* promoter; $Tet^R$. | This study |
| NP5006 | N16961 carrying pRW50-oriT plasmid containing the upstream region of *flaA* promoter; $Tet^R$. | This study |
| NP5007 | N16961 carrying pRW50-oriT plasmid containing the upstream region of *flaE* promoter; $Tet^R$. | This study |
| NPMW1 | N16961 carrying pMW-*gfp* plasmid; $Spect^R$. | Ritchie et al.(2) |
| E7946 | Wild type *V. cholerae* El Tor O1 Ogawa | A. Camilli lab |
| AC2764 | *tcpA* deletion mutant of E7946 | A. Camilli lab |
| BH1651 | $luxO^{D47E}$ | Ng et al.(30) |

**Table S2**| Primers used in this study, with restriction sites underlined.

| Primer | Sequence (5´-3´) |
|---|---|
| pRW50 F | GTTCTCGCAAGGACGAGAATTTC |
| pRW50 R | AATCTTCACGCTTGAGATAC |
| aphApF1 | TGCAGAATTCCTGGTTAACAAATCGCTAAATGTCAG |
| aphApR1 | ATTCAAGCTTGTGTGGTAATGACATGTCTTCAATC |
| toxTpF1 | TGTAGAATTCGATAAGATAACAGCCATATTCGTGG |
| toxTpR1 | GATCAAGCTTTCCCAATCATTGCGTTCTACTC |
| ctxABpF1 | GCTTGAATTCCTGTGGGTAGAAGTGAAACGG |
| ctxABpR1 | TCATAAGCTTTATCTTTACCATATAATGCTCCCTTTG |
| tcpApF1 | CTTAGAATTCGGTCTTATCATGAGCCGCC |
| tcpApR1 | TGATAAGCTTTGCATATTTATATAACTCCACCATTTGTG |
| flaApF1 | ATCTGAATTCGTCAGCGGCAAAATGGATTG |
| flaApR1 | GTCAAAGCTTCATAGTTTGCTCTCCTATCGAGTTC |
| flaEpF1 | ATCTGAATTCGATCACGGCCTACTGTTTATTG |
| flaEpR1 | GTCAAAGCTTATTTACCGTCATGGCCATGG |

## SUPPORTING MATERIALS AND METHODS

### Polymer synthesis and characterization.

***Materials:*** *N*-(3-aminopropyl)methacrylamide hydrochloride (APMAm), *N*-[3-(dimethylamino)propyl]methacrylamide (DMAPMAm), 2,2'-azobis(2-methylpropionitrile) (AIBN) and Dulbecco's Modified Eagle's Medium (DMEM) were purchased from Sigma-Aldrich. 4,4'-azobis(4-cyanovaleric acid) (ACVA) and 2-mercaptoethanol were bought from Alfa Aesar. Polyethylene glycol standards were purchased from Agilent Technologies. All other chemicals were purchased from Fisher Scientific and VWR and were used without further purification. Polymers were synthesized by radical polymerisation as described below.

***Instrumentation:*** Polymers were characterized by Nuclear Magnetic Resonance (NMR) and Gel Permeation Chromatography (GPC). NMR spectra were recorded on a Bruker Avance III spectrometer operating at 300 MHz and fitted with a 5 mm BBFO probe. Chemical shifts (Figure S1 and Figure S2) are reported in ppm (δ) referenced to the corresponding solvent signals: DMSO-*d6* (δ = 2.50) and $D_2O$ (δ = 4.79). GPC was recorded on a Shimadzu Prominence LC-20A, fitted with Shodex Asaphipak GF-510 HQ (300 × 7.5 mm, 5 μm) and GF-310 HQ (300 × 7.5 mm, 5 μm) columns in series, and equipped with a Thermo Fisher Refractomax 521 detector. GPC analysis was carried out using 100 mM acetate buffer at pH 2.9 as eluent at 40 °C and a flow rate of 0.6 $mL \cdot min^{-1}$. Molecular weights were calculated based on a standard calibration method using polyethylene glycol.

***Synthesis and characterization of p(APMAm) (P1):*** *N*-(3-aminopropyl)methacrylamide (APMAm) hydrochloride (505.0 mg, 2.770 mmol), 4,4'-azobis(4-cyanovaleric acid) (ACVA) (12.4 mg, 0.033 mmol) and 2-mercaptoethanol (1.0 μL, 0.014 mmol) were dissolved in MilliQ water (2.2 mL). This solution was degassed under argon for 10 minutes and then heated at 70 °C under stirring for 17 hours. After this time, the reaction flask was opened to the air and the crude was precipitated three timSes into diethyl ether (50 mL). The precipitate was freeze-dried and a crystalline white solid was obtained (70.0 mg, 14% yield). $^1$H-NMR (300 MHz, $D_2O$) δ (ppm): 3.11 (br, 3H, $CH_{3\ backbone}$), 2.93 (br t, *J*= 7.0 Hz, 2H, CO-NH-$\underline{CH_2}$), 1.78 (br, 2H, -$C\underline{H_2}$-$NH_2$), 1.63 (br, 2H, -$C\underline{H_2}$-$CH_2$-$NH_2$), 0.98 (br, 1H, $CH_{2\ backbone}$), 0.83 (br, 1H, $CH_{2\ backbone}$). Mn (GPC) 46997, $Đ_M$ (GPC) 1.16.

***Synthesis and characterization of* p(DMAPMAm) (P2):** *N*-[3-(dimethylamino)propyl]methacrylamide (DMAPMAm) (2.2 mL, 12.025 mmol), 2,2'-azobis(2-methylpropionitrile) (AIBN) (19.6 mg, 0.117 mmol) and 2-mercaptoethanol (4.0 µL, 0.056 mmol) were dissolved in toluene (9.5 mL). This solution was degassed under argon for 10 minutes and then heated at 70 °C under stirring for 18 hours. After this time, the reaction flask was opened to the air and the crude was precipitated twice: first into diethyl ether (200 mL) and then into a diethyl ether/ hexane mixture (1:1) (100 mL). The precipitate was freeze-dried and a crystalline white solid was obtained (1.66 g, 87% yield). $^{1}$H-NMR (300 MHz, DMSO-*d6*) $\delta$ (ppm): 7.51 (br, 1H, CO-NH), 2.96 (br, 2H, CO-NH-$\underline{CH_2}$), 2.19 (br, 5H, $\underline{CH_2}$-N-$(CH_3)_2$ + $CH_3$ $_{backbone}$), 2.11 (br, 6H, N-$(CH_3)_2$), 1.50 (br, 2H, $\underline{CH_2}$-$CH_2$-N), 0.78 (br, 2H, $CH_2$ $_{backbone}$). Mn (buffer GPC) 46331, $Đ_M$ (buffer GPC) 1.14.

**Bacterial strains and culture conditions.** *Vibrio cholerae* strains used in this study (**Table S1**) were derived from the El Tor strain N16961 used as parental strain. Strains were propagated at 37 ºC in Luria-Bertani (LB) broth supplemented with 50 µg/µL spectinomycin, 30 µg/µL kanamycin or 10 µg/µL tetracycline where selection was required. Plasmids were introduced into *V. cholerae* by conjugation, as previously described (3). Briefly, aliquots of overnight cultures of *V. cholerae* N16961, *E. coli* DH5a carrying the desired plasmid (donor) and an *E. coli* SM10 helper strain carrying pRK2013 were mixed at a volumetric ratio of 1:2:2 and spotted onto brain-heart infusion (BHI) agar. Following overnight incubation, spots of bacterial growth were dislodged and suspended in 3 mL of PBS. 100 µL of bacterial suspension were plated onto M9 media containing 50 µg/µL of spectinomycin. Resulting colonies were checked by PCR and sequencing in the case of pRW50-oriT constructs, and also by screening for green fluorescence in the case of pMW-*gfp* transformants.

**Plasmid construction.** Primers used for cloning and sequencing the constructs used in this study are listed in **Table S2** and were designed based on the sequence of *V. cholerae* N16961 (1). Primers were paired as appropriate to amplify upstream regions of *toxT*, *ctxAB*, *tcpA*, *aphA*, *flaA* and *flaE* (**Figure 4** and **Figure S5**). Amplified DNA was digested using EcoR1 and HindIII, and ligated into digested pRW50-oriT vector (gift from the Grainger lab). The vector is a derivative of pRW50 (4), which has been modified by inserting the *oriT* sequence from the vector pRK2

(5). The resulting reporter constructs were used to transform *V. cholerae* and the resulting strains are listed in **Table S1**.

**Imaging and sizing of bacterial clusters.** An overnight culture of *V. cholerae* was diluted to an $OD_{600}$ of 1.0 in DMEM without phenol red and polymers were added to final concentrations ranging from 0-0.5 mg/mL (**Figure 1** and **Figure S3**). For imaging of clusters, aliquots were taken after 15 and 60 min of incubation and mounted with ProLong® Antifade Gold solution (LifeTechnologies). Cured samples were visualized using a Nikon-Eclipse TE2000-U microscope and Plan Apo 60x/ 1.40 NA oil DIC objective (Nikon) and captured with QICAM Fast1394 camera (Q imaging). Representative images were taken using Nikon NIS-Elements software and prepared with ImageJ and Corel Draw X5 software. The size distribution of bacterial clusters was determined using a Mastersizer 3000 (Malvern) through a period of time of 10 minutes following addition of polymer. P1 was added at $5x10^{-2}$ mg/mL and $5x10^{-1}$ mg/mL with stirring and recorded particle diffraction was plotted as percentage of the particles with a diameter larger than 2 μm. The cutoff size for clusters versus individual bacteria was determined on the median diameter of particles from a sample containing no polymer (2 μm).

**Motility assay**. Motility of *V. cholerae* was assessed as previously described (30). Briefly, overnight cultures of *V. cholerae* were diluted to an $OD_{600}$ of 1.0 and then, clustered with either P1 or P2. An aliquot of 1 uL from clustered bacteria was placed into soft agar LB plates (0.4% agar) and incubated overnight at room temperature. For the high salt control, clustered bacteria were treated with 1 mL of high-salt PBS (200 mM NaCl), when the solution turned cloudy an aliquot of 1 uL was placed into soft agar plates and incubated overnight at room temperature. After incubation, pictures of the plates were taken.

**Determination of bacterial growth and membrane integrity.** Overnight cultures of GFP-expressing *V. cholerae* were diluted into DMEM containing 50 μg/μL of spectinomycin to an $OD_{600}$ of 0.02 as starting density. Polymers aliquots were added to give the desired final concentrations (**Figure 2**) in 200 μL culture using a 96-well plate. The plate was covered with a BEM-1 breathe easy gas permeable membrane to avoid evaporation and incubated at 37 ºC with constant shaking at 200 rpm. GFP fluorescence was recorded every 30 minutes over 24 hours using a FLUOstar Omega plate reader. Membrane integrity of *V. cholerae* cells was assessed by

fluorescent-activated cell sorting (FACS) using the LIVE/DEAD *Bac*Light™ kit (LifeTechnologies). Overnight cultures were diluted to an $OD_{600}$ of 1.0 in 1 mL of DMEM without phenol red, and containing polymers at final concentrations ranging from 0 to 0.5 mg/mL and incubated for 20 hours (**Figure 2**). Following incubation, samples were stained according to the manufacturer's instructions. Readings were taken on an Attune Flow Cytometer, at a flow of 100 µL/min counting up to 10,000 events. Samples containing no polymer were used as "LIVE" controls. In the case of "DEAD" controls, DMEM was replaced by 70% 2-propanol and incubated for 1 h. Prior to staining, 2-propanol was removed and cells were washed once and resuspended in fresh DMEM to carry out the staining procedure.

**Quantification of biofilm formation** (**Figure 3a**)**.** The amount of biofilm was determined by crystal violet staining, as previously described (6). Briefly, *V. cholerae* was exposed to different concentrations of polymers and incubated overnight at 37ºC with shaking in a 96-well plate. Cultures were removed and biofilm was rinsed with PBS and stained with 200 µL of 1% crystal violet solution in water. After 30 minutes, the crystal violet solution was removed and the well was rinsed again with PBS. In order to detach the dye, 200 µL of 95% ethanol was added to each well. The amount of biofilm was determined by measuring at a wavelength 595 nm.

**Imaging of biofilm formation and extracellular DNA quantification. (Figure 3b-d).** Biofilms of GFP-*V. cholerae* were grown for 20 hours at 37°C in 96-well glass-bottom plates containing bacteria at an initial $OD_{600}$ of 0.2 in DMEM only, or DMEM containing 0.05 mg/mL P1, or 0.05 mg/mL P2. Following incubation, plates were rinsed with PBS, samples fixed with 4% formaldehyde in PBS for 15 minutes and then washed with PBS. DNA was stained with 10 µg/mL Hoechst in PBS for 10 minutes. Samples were imaged using a Zeiss Axio Observer.Z1 microscope, Zeiss 40x/1.4 Plan Apochromat,objective, ORCA-Flash4.0 camera (Hamamatsu) and ZEN 2.0.0.10 software, Images were processed using Image J and Corel Draw X5. Extracellular DNA was determined from pictures take under a Nikon TE2000-U microscope using a 100x Plan Apo objective (Nikon) and captured with Digital Sight DS-Qi1MC camera (Nikon). From each picture, the intensity of pixels was measured from the blue channel using ImageJ.

**Transciptional assays: β-galactosidase activity.** *V. cholerae* reporter strains were assayed for β-galactosidase activity as previously described (7). Briefly, *V. cholerae* cultures were diluted to an

$OD_{600}$ of 0.2 and incubated for 7 hours at 37 ºC in DMEM containing polymers. Separate aliquots were taken and used to measure β-galactosidase activity, or washed with PBS and resuspended in high salt solution to disrupt clusters prior to measuring $OD_{600}$. Transcriptional activity (in Miller Units) was calculated as previously described (7).

**Cytotoxicity in Caco-2 cells.** Overnight cultures of the GFP-expressing *V. cholerae* were grown in LB at 37 ºC. Prior to infection, cultures were adjusted to an MOI of 10 and incubated for 1 hour in 1 mL of DMEM without phenol red and polymers as indicated, at 30 ºC. Caco-2 cells were washed with PBS to remove media containing antibiotics and infections were started by transferring the solution of bacteria into the wells. Plates were centrifuged at 1000x g for 5 min at 20 ºC to synchronize the infections. After an incubation of 7 hours at 37 ºC under 5% $CO_2$, supernatants were used to measure lactate dehydrogenase (LDH) activity using LDH Cytotoxicity Detection Kit (Takara Clonetech) according to the manufacturer's instructions. The data was expressed as percentage of cytotoxicity, normalized to untreated cells (0%) and 0.1% Triton X-100 lysed cells (100% lysis) and was calculated according to the formula: % cytotoxicity=100 x ([OD490 for experimental release - OD490 for spontaneous release]/ [OD490 for maximum release– OD490 for spontaneous release]).

**Zebrafish care and maintenance.** Zebrafish (*Danio rerio* wild type strain AB) were kept in a recirculating tank system at the University of Birmingham Aquatic Facility. Zebrafish were kept under a 14h-10h light-dark cycle with water temperature maintained at 28 ºC. Zebrafish care, breeding and experiments were performed in accordance with the Animal Scientific Procedures Act 1986, under Home Office Project License 40/3681. After collection of eggs, larvae were kept in a diurnal incubator under a 14h-10h light-dark cycle with the temperature maintained at 33 ºC. Eggs were maintained at 40 eggs per 50 ml in E3 media plus 0.00003% methylene blue for 8 h and E3 media plus 26.6 µg/ml 1-phenyl-2-thiourea (PTU) to inhibit melanization. The fish line used was wild-type AB zebrafish. All zebrafish care and husbandry procedures were performed as described previously (8).

**Infection of zebrafish embryos with *V. cholerae*.** Prior to infection, $10^6$ and $10^7$ CFU/mL of *V. cholerae* were incubated in 3 mL of E3 medium containing polymers as indicated in the figure legends. After 1 hour of incubation to ensure cluster formation, zebrafish embryos (5 d.p.f) were placed into cluster solutions and incubated with rotation at 25 ºC for 6 hours. Embryos were

euthanised with an overdose of Tricaine-S (1600 μg/mL) and homogenised by washing the embryos with PBS and incubating them in 1% Triton X-100 for 30 minutes. Lysates were passed several times through a needle to homogenize and 100 μL of the resulting solution, as well as serial dilutions, were plated onto TCBS agar and colonies counted following overnight incubation at 37 ºC.

**Imaging of infected Caco-2 cells and zebrafish embryos.** In the case of samples for imaging, Caco-2 cells were seeded onto sterilized glass cover slips inserted into wells of the plate. Imaging of infected Caco-2 cells was done by fixing the samples with 4% formaldehyde in PBS for 15 minutes and then washing with PBS. Cells were permeabilized by adding 0.1% Triton X-100 in PBS and incubation at room temperature for 5 minutes, and washed three times with PBS. Samples were stained with 10 μg/mL Hoechst and 66 ng/mL of rhodamine-phalloidin in the dark for 10 minutes to visualize DNA and F-actin, respectively. Staining was followed by three washing steps with PBS (5 minutes each). Samples were mounted using antifade gold mounting solution (Life-Technologies) and cured overnight at 22 ºC prior to visualization. Visualization of zebrafish embryos was done by directly mounting the embryos in 0.4% low melting point agarose containing 160 μg/mL of Tricaine-S. Samples were viewed under a Zeiss Axio Observer.Z1 microscope with 63x/1.4 Plan Apochromat objective for the Caco-2 infection and 20x/0.8 Plan Apochromat objective in the case of the larvae. Images were processed using ImageJ software.

**Supporting References**


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