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

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## ABSTRACT

*Vibrio cholerae*, the causative agent of cholera, is an abundant environmental bacterium that can efficiently colonize the intestinal tract and trigger severe diarrheal illness. Motility, and the production of colonization factors and cholera toxin, are fundamental for the establishment of disease. In the aquatic environment, *V. cholerae* persists by forming avirulent biofilms on zooplankton, phytoplankton and chitin debris. Here, we describe the formation of artificial, biofilm-like communities, driven by exposure of planktonic bacteria to synthetic polymers. This recruitment is extremely rapid and charge-driven, and leads to the formation of initial "seed clusters" which then recruit additional bacteria to extend in size. Bacteria that become entrapped in these "forced communities" undergo transcriptional changes in motility and virulence genes, and phenotypically mimic features of environmental biofilm communities by forming a matrix that contains polysaccharide and extracellular DNA. As a result of this lifestyle transition, pathogenicity and *in vivo* host colonization decrease. These findings highlight the potential of synthetic polymers to disarm pathogens by modulating their lifestlye, without creating selective pressure favoring the emergence of antimicrobial resistant strains.


## SIGNIFICANCE

*Vibrio cholerae* is an important human pathogen and causes watery diarrhea after consumption of contaminated water. Its reservoir are aquatic environments, where it persists in an avirulent biofilm state. Upon ingestion, it escapes biofilms and expresses virulence factors, leading to colonization and pathogenicity within the human host. Here, we show that capture by charged polymers rapidly immobilizes *V. cholerae* and artificially forces it into a sessile state. This mimics environmental cues for biofilm formation and leads to repression of virulence factors through loss of motility. This work highlights a novel artificial lifestyle and an efficient way to neutralize virulent *V. cholerae* and block disease and transmission.

## INTRODUCTION

*Vibrio cholerae* is a Gram-negative bacterium responsible for several million incidences of enteric disease and up to 142,000 deaths every year (1). Many of these cases are attributable to the *V. cholerae* El Tor biotype, which is the cause of an ongoing global epidemic, the 7$^{th}$ to sweep our planet in recorded history. A natural inhabitant of aquatic environments, El Tor's success has been attributed to genetic systems for quorum sensing, chitin breakdown and virulence. Within the human host, the bacterium initiates a virulence programme including the induction of colonization factors and toxins. The two major virulence factors expressed by El Tor strains are cholera toxin and the toxin-coregulated pilus (TCP). Cholera toxin is an ADP-ribosyltransferase of the $AB_5$ family, which leads to the profuse watery diarrhea and electrolyte loss characteristic of the disease. Its subunits are encoded by *ctxA* and *ctxB* which are organized in an operon(2). TCP, a type IV pilus, is required for formation of bacterial microcolonies in the small intestine and leads to a local enhancement in toxin concentration at the site of infection. The major pilus subunit is encoded *by tcpA*(3, 4). In aquatic environments, *V. cholerae* persists by forming biofilms on the surfaces of phytoplankton, zooplankton and chitin debris (5, 6). Biofilms, which are composed of a matrix of exopolysaccharide and extracellular DNA surrounding the bacteria, offer a protective environment against aquatic predators as well as the host environment. Thus, biofilm formation is an important contributing factor to human disease (7, 8). Efficient colonization of the host intestine, however, requires disassembly of biofilms, and a switch to active motility allowing the bacterium to search out and attach to the host epithelium (8, 9). Indeed, escape from the biofilm is a prerequisite for induction of the virulence programme (8) and failure to escape from the biofilm state results in decreased host colonization fitness *in vivo* (10). Clearly, the ability to switch between motile and sessile lifestyles, along with the carefully controlled induction of virulence factors, is central to the establishment of disease and the emergence of Cholera epidemics (11).

Although efforts to develop a widely effective vaccine against *V. cholerae* are ongoing, the efficacy of existing vaccines is low. Attenuated *V. cholerae*, for example, elicits protective immunity in as few as 16% of patients in developing countries (12). In addition, current vaccines offer protection for only two years, a time period shorter than many epidemics. Currently, and in the absence of a vaccination program, the best way to prevent such outbreaks is by water

treatment and provision of sanitation infrastructure. As such, low-tech measures to facilitate decontamination of drinking water, such as simple means of filtration, are desirable and can have a dramatic impact on disease incidence (13). Moreover, there is a lack of understanding of how *V. cholerae* responds to these strategies, and how this pathogen regulates virulence and motility upon immobilization onto a filtration device. To this end, we demonstrate here that linear, cationic polymers can rapidly capture *V. cholerae* from aqueous environments and, upon sequestration into these artificially precipitated communities, induce an avirulent phenotype in *V. cholerae* that mimics environmental biofilm formation. Overall, the polymers force *V. cholerae* into an artificial sessile lifestyle, which inhibits virulence factor production, colonization and dissemination.

## RESULTS

**Cationic polymers rapidly form three-dimensional clusters upon contact with *V. cholerae*.** Many polycationic polymers have been designed to maximize their antimicrobial effects (14-16) and previously published work demonstrated a trade-off between the charge and hydrophobicity in cationic polymers and their ability to cluster bacteria, and/or affect bacterial viability within clusters (17-19). Based on our previous work with closely related species *Vibrio harveyi* (17, 18), we decided to investigate the potential of (poly(*N*-(3-aminopropyl)methacrylamide), pAPMAm – P1 and (poly(N-[3-(dimethylamino)propyl]methacrylamide), pDMAPMAm - P2, to remove *V. cholerae* from aqueous environments (**Figure 1a**). These polymers are both cationic under neutral aqueous conditions and were synthesized via free radical polymerization with high purity (**Figure S1** and **Figure S2**). Upon contact with *V. cholerae*, both polymers rapidly formed clusters *in situ* in a concentration dependent manner (**Figure 1b**), and cluster formation reached equilibrium within minutes (**Figure 1c**). Cluster formation proceeded via initial nucleation of small layers or sheets of bacteria, which increased in size both by lateral interaction with additional bacteria, as well as stacking of bacteria on existing sheets to form clusters over the first 15 minutes, and then remained stable over the duration of the experiment (**Figure 1b,d**). No significant differences were observed between both polymers, suggesting that clustering was mainly dominated by electrostatic interactions between the positively charged polymers and the negatively charged bacteria. Bacterial clusters were stable for at least 24 hours and had a well-

defined three-dimensional structure (**Figure 1d** and **Figure S3**). Both polymers induced bacterial clustering with high efficiency, and the endpoints were practically indistinguishable in terms of numbers of particles and cluster size (**Figure 1b**).

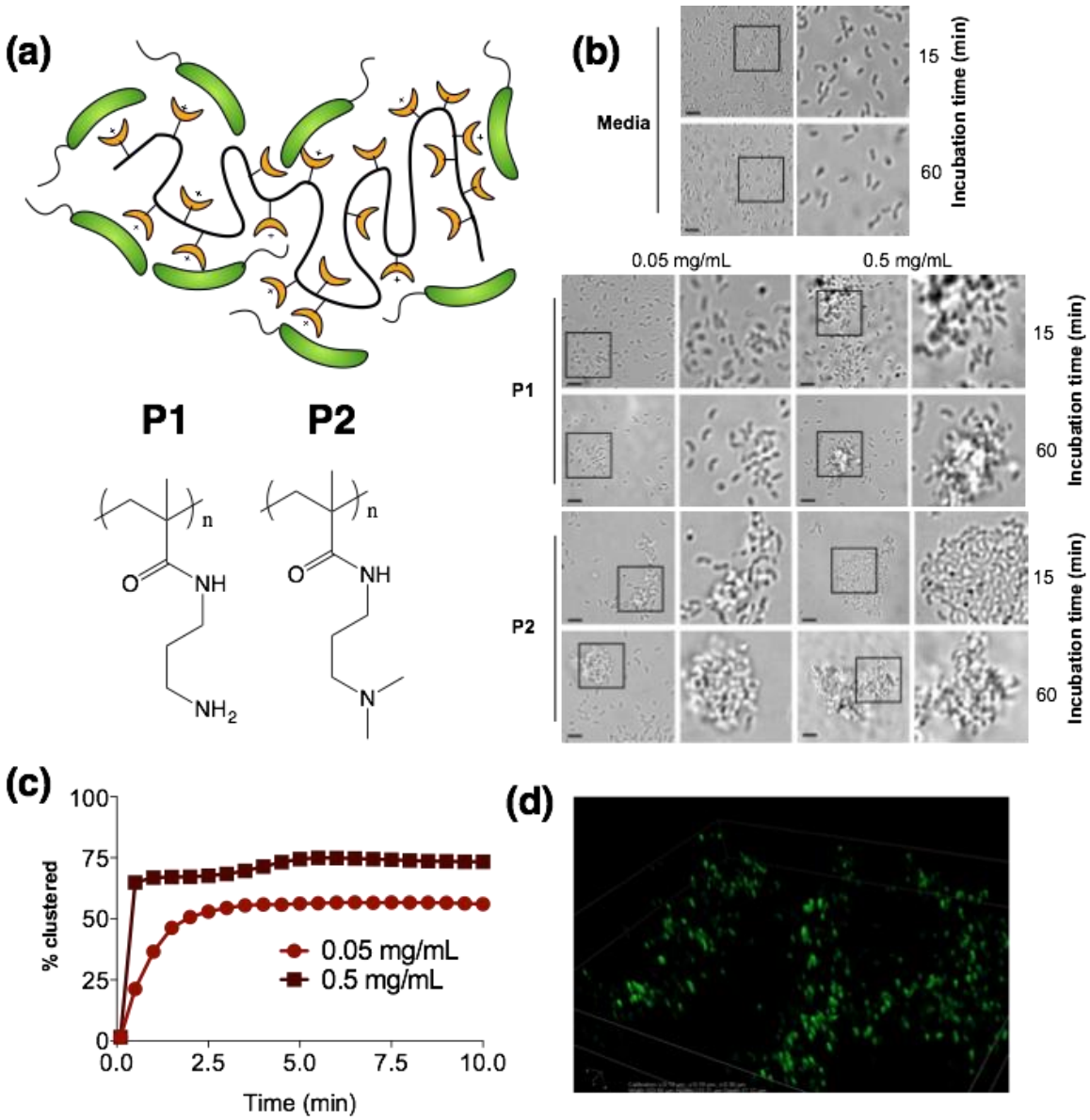


**Figure 1 | Cationic polymers rapidly form three-dimensional clusters upon contact with *Vibrio cholerae*.** (a) Bacteria are clustered via electrostatic interactions between polymers containing positively charged sidechains (yellow) and *V. cholerae* (green). Chemical structures of polymers P1 and P2 are shown. (b) Clusters formed after incubation of *V. cholerae* with 0.05 or 0.5 mg/mL of P1 or P2 for 15 or 60 min were visualized by microscopy using a DIC filter (c) Concentration-dependent kinetic analysis of particle size distribution for P1-induced bacterial clustering. Particle size distribution was analyzed using a Mastersizer. Clusters are defined as particles of a mean diameter larger than that of individual bacteria (2μm). (d) Clusters formed after incubation of GFP-*V. cholerae* with

0.5 mg/mL of P1 for 15 minutes were visualized by spinning disc fluorescence microscopy. Images are representative of at least three independent experiments.

**Effect of polymer-induced clustering on bacterial growth and membrane integrity.** The effect of cationic polymers on bacterial viability varies significantly with charge, hydrophobicity, polymer concentration and the bacterial species tested (17, 18). Thus, we explored if and how clustering of *V. cholerae* affects bacterial proliferation and viability under physiological conditions. Bacterial proliferation during co-incubation of GFP expressing *V. cholerae* with different concentrations of polymers was measured, monitoring GFP fluorescence over 25 hours (**Figure 2a**). Bacterial proliferation was generally unaffected, except at very high polymer concentrations (0.5 mg/ml of P1, **Figure 2a**). However, it was unclear from these experiments whether the slower increase in fluorescence was due to inhibition of bacterial growth, as is observed within subsets of cells within bacterial biofilms (11), or due to cellular damage commonly observed with highly charged cationic polymers (19). Flow cytometry of bacterial samples exposed to polymers and LIVE/DEAD® cell viability stains allowed us to determine bacterial viability via measuring membrane integrity of *V. cholerae* sequestered in clusters at the experimental endpoint (**Figure 2b**). Viability and membrane integrity were largely unaffected by clustering even following overnight incubation, except at very high polymer concentrations (0.5 mg/ml **P1**, **Figure 2b**). We also investigated the effect of both polymers on host cells, in this case cultured Caco-2 intestinal epithelial cells, using lactate dehydrogenase release (LDH) assays to probe cellular membrane integrity (**Figure S4**). **P1** and **P2** both compromised membrane integrity of epithelial cells at $5{\cdot}10^{-3}$ and $5{\cdot}10^{-4}$ mg/mL or above, respectively, compared to untreated control cells. Thus, for functional experiments, we focused on investigating the effects of the highest effective, non-toxic concentration of both polymers.

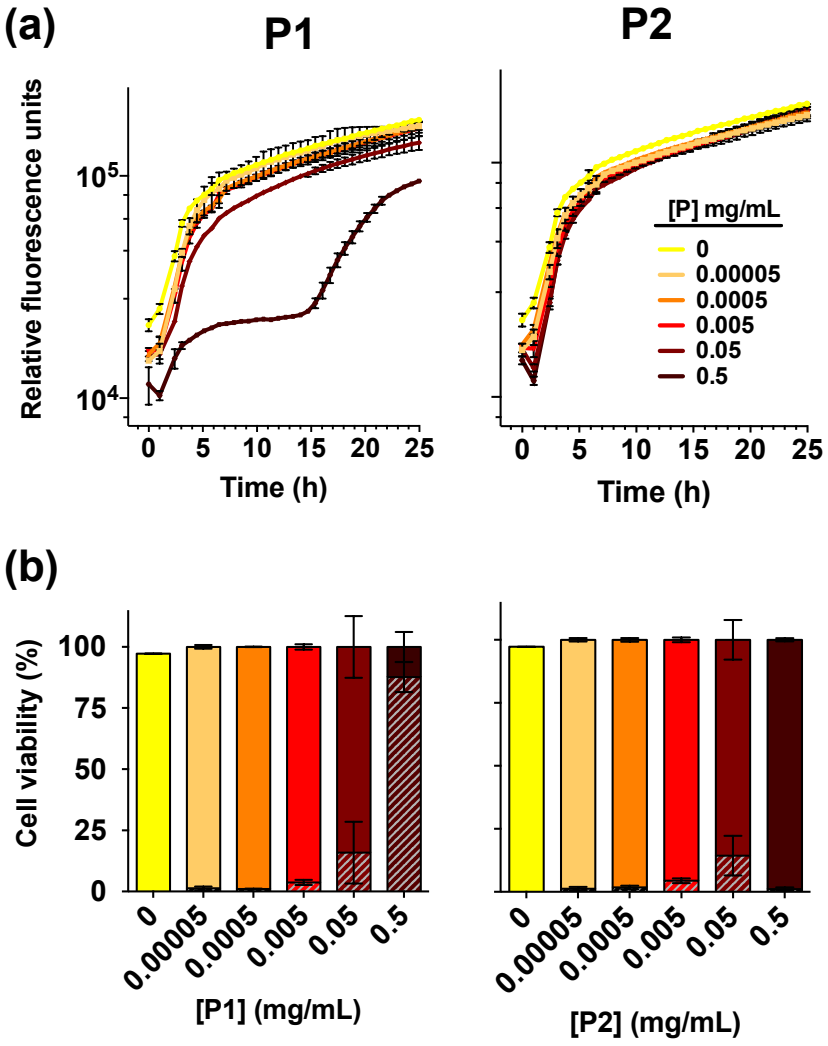


**Figure 2 | Effect of polymer-induced clustering on bacterial growth and viability. (a)** GFP-*V. cholerae* were adjusted to an initial $OD_{600}$ of 0.02, added to polymers to give final concentrations as indicated in the legend, and grown under shaking at 37 °C for 24 hrs, with GFP fluorescence measured every 30 min. Results are means ± s.e.m. of three independent experiments. **(b)** *V. cholerae* were adjusted to an initial $OD_{600}$ of 1, added to polymers to give final concentrations as indicated, and incubated overnight. Samples were stained for membrane integrity using a LIVE/DEAD™ cell viability kit and analyzed by flow cytometry. Experimental samples were gated using untreated (viability 100%) and 2-propanol treated (viability 0%) samples as controls. % bacteria gates as live (filled columns) and dead (hatched columns), respectively, are shown, with results representing means ± s.e.m. from three independent experiments.

**Sequestration of *V. cholerae* into polymer clusters induces a biofilm-like state and suppresses bacterial virulence at the transcriptional level.** The switch to a sessile lifestyle and biofilm formation in *V. cholerae* is initiated by surface sensing and downregulation of bacterial

148 motility (11, 20). Since we observed during imaging experiments that bacterial clustering
149 abrogated bacterial motility, we investigated whether polymer-induced clustering would also
150 affect *in vitro* biofilm formation. Polymer-induced clustering lead to a significant induction of
151 biofilm formation in *V. cholerae*, as measured using crystal violet assays and imaging of GFP-*V.*
152 *cholerae* (**Figure 3a**). Polymer-induced biofilms also released higher levels of extracellular DNA
153 into the biofilm, compared to untreated *V. cholerae* (**Figure 3b-d**).

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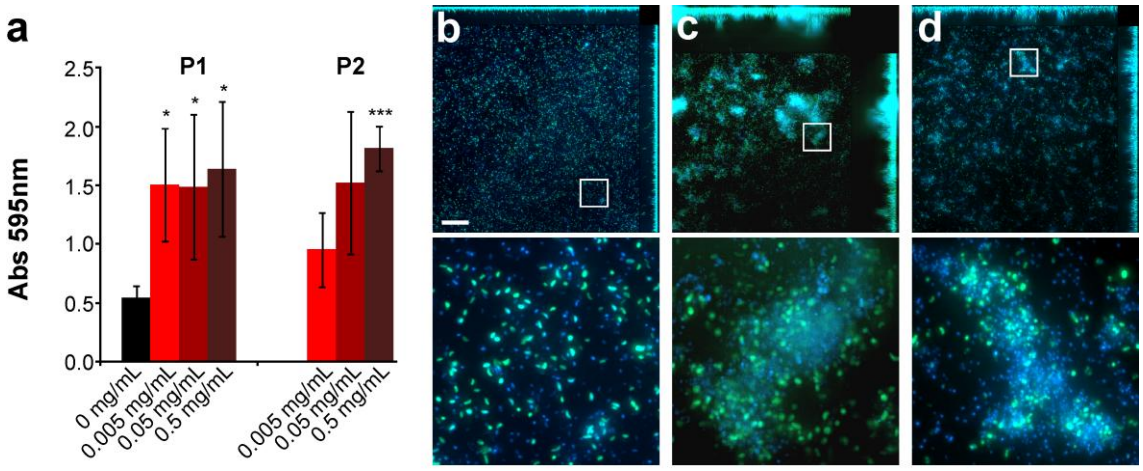


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156 **Figure 3 | Polymer-induced clustering leads to increased *V. cholerae* biofilm formation.** Biofilm formation of *V.*
157 *cholerae* after overnight incubation in the absence or presence of polymers **P1** or **P2** at concentrations as indicated,
158 was quantified using crystal violet plate assays **(a)**. Analysis of variance (ANOVA), followed by Tukey's post hoc
159 test, was used to test for significance. Statistical significance was defined as $p<0.05$ (*), $p<0.01$ (**) or $p<0.001$
160 (***). Biofilms of GFP-*V. cholerae* were imaged following 20 hours of growth in glass-bottom plates at 37 °C, in
161 DMEM only **(b)**, or DMEM containing 0.05 mg/mL P1 **(c)** or 0.5 mg/mL P2 **(d)**. Samples were fixed and DNA was
162 stained with Hoechst (blue). Scale bar, 50 μm. Area within the square has been expanded (bottom row) for clarity.

163

164 Since sequestration into polymer clusters promotes a sessile state, we investigated what impact
165 this environmental signal would have on virulence regulation. For this purpose, we created a
166 series of transcriptional reporter strains, by introducing, via conjugation, a variant of the pRW50
167 plasmid containing oriT into *V. cholerae* (**Figure 4a**). Thus, we could follow the induction of *V.*
168 *cholerae* promoters during growth in medium mimicking inducing conditions within the host
169 environment (DMEM, 37 °C), using β-galactosidase assays. AphA is a master regulator of

virulence that is required for the activation of *tcpP*. TcpP, in turn, activates *toxT*, which activates downstream virulence genes, including those responsible for the production of cholera toxin and the toxin-coregulated pilus (TCP). In the absence of polymer, the *aphA* promoter was strongly induced (~9-fold compared to vector control) in mid-log phase cells (**Figure 4b**). Sequestration of *V. cholerae* into clusters significantly repressed the *aphA* promoter activity (approx. 70% and 55% suppression with **P1** and **P2**, respectively). The *toxT* promoter was also strongly induced (~12-fold over vector control) in the absence of polymer but significantly repressed in bacterial clusters (~30% and 20% inhibition by **P1** and **P2**, respectively). Similarly, the promoters of the two key virulence factors, *ctxAB* and *tcpA*, were strongly induced under conditions mimicking the host environment (12-fold and 4-fold induction over vector control, respectively) with both **P1** and **P2** suppressing *ctxAB* transcription by approximately 60% and 70%, respectively. The effect on *tcpA* was less pronounced, with **P1** showing only mild suppression, and **P2** suppressing transcription by ~50% (**Figure 4b**). We also investigated the effect of physical immobilization within artificial clusters on transcriptional regulation of genes encoding for components of the flagellar systems (*flaE* and *flaA*, respectively). Both of these were comparatively weak promoters (~2 to 2.5-fold induction compared to vector control). Interestingly, both *flaE* and *flaA* were downregulated by P1 mediated clustering, while P2, which is more hydrophobic than **P1**, had no effect on *flaA* or *flaE* transcription levels (**Figure S5**).

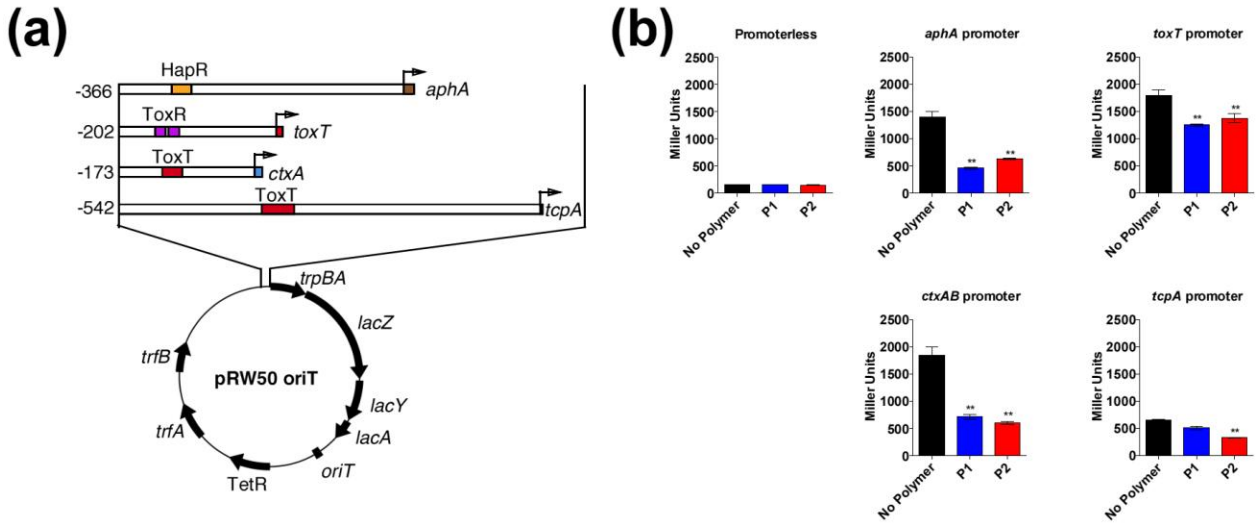



**Figure 4 | Sequestration of *V. cholerae* into polymer clusters suppresses bacterial virulence at the transcriptional level**. (a) Schematic depiction of the reporter plasmid (pRW50-*oriT*) and promoter regions of *aphA*, *toxT*, *ctxA* and *tcpA* cloned as transcriptional fusions to *lacZ* in pRW50-oriT. Numbering refers to base number relative to the transcriptional start site. (b) Promoter activities of *aphA*, *toxT*, *ctxA* and *tcpA* promoter-*lacZ* fusions were measured following 7 hours of growth in the absence (black) or presence of 0.05 mg/mL P1 (blue) or 0.5 mg/mL P2 (red).

**Polymer sequestration of bacteria abolishes *V. cholerae* infection *in vitro* and intestinal colonization *in vivo*.** Since polymer-induced clustering repressed the induction of key virulence factors, we asked what impact clustering would have on *V. cholerae* infection. Initially, we tested the effect of clustering on colonization and toxicity towards cultured intestinal epithelial cells. Sequestration of *V. cholerae* by polymers led to a significant reduction in bacterial attachment to host cells, as determined both by dilution plating/CFU counts and imaging of infected cells (**Figure 5a-c**). Imaging of Caco-2 cells infected with either planktonic or clustered *V. cholerae* also revealed a decrease in *V. cholerae* mediated toxicity as a result of bacterial clustering. Host cells infected with clustered bacteria showed less cell death and more intact cell-cell junctions than cells infected with planktonic bacteria (**Figure 5c**). This protective effect of the polymers was also observed when cytotoxicity was measured by LDH release assays, following infection with planktonic or clustered *V. cholerae* (**Figure 5b**).

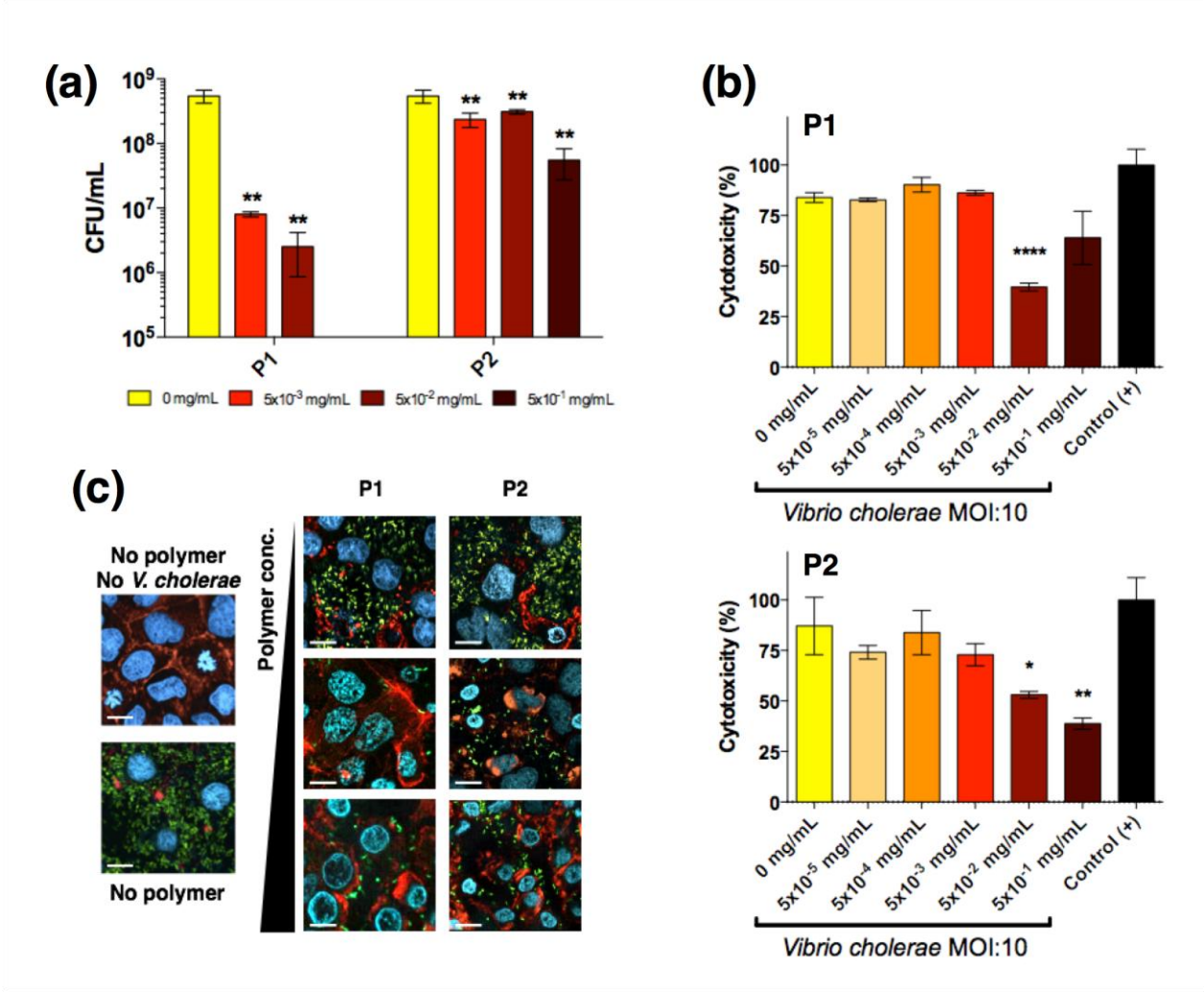



**Figure 5 | Polymer sequestration of bacteria abolishes *V. cholerae* infection of cultured epithelial cells**. *V. cholerae* were adjusted to an MOI of 10, and incubated in the absence or presence of polymers for 1 hr prior to infection of cultured Caco-2 intestinal epithelial cells for 7 hrs. Following 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%). Results in a and b 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 $p<0.0001$ (****). (**c**) Following infection, *V. cholerae* (green), DNA (blue), and F-actin (red) were visualized by fluorescence microscopy. Scale bar, 10 μm.

Finally, we tested whether non-bactericidal concentrations of polymers would protect against intestinal colonization by *V. cholerae in vivo*. Previously, zebrafish (*Danio rerio*) have been established as an aquatic host which can be colonized and infected by *V. cholerae* in a

concentration dependent manner, and infection eventually leads to mortality (21, 22). We tested if bacterial clustering would affect subsequent colonization of zebrafish larvae by *V. cholerae* following a 6 hour exposure. Zebrafish larvae exposed to either $10^7$ or $10^8$ CFU/mL of planktonic or polymer-clustered GFP-expressing *V. cholerae* were first imaged and then sacrificed, and intestinal *V. cholerae* were extracted from the tissue and enumerated by dilution plating on selective TCBS agar (**Figure 6a-b**). Images of infected fish showed that GFP-expressing *V. cholerae* had specifically colonized the gastrointestinal tract, with the majority of bacteria attached to the mid-intestine (**Figure 6c**). The bacterial burden was visibly lower in fish infected with clustered bacteria, compared to fish infected with planktonic bacteria. Bacterial sequestration was more efficient in blocking colonization at an infectious dose of $10^7$ (**Figure 6a**) where bacterial burden was reduced more than 100-fold. At a dose of $10^8$ *V. cholerae*, colonization was still significantly inhibited by polymer-induced clustering, albeit the effect was much smaller (**Figure 6b**).

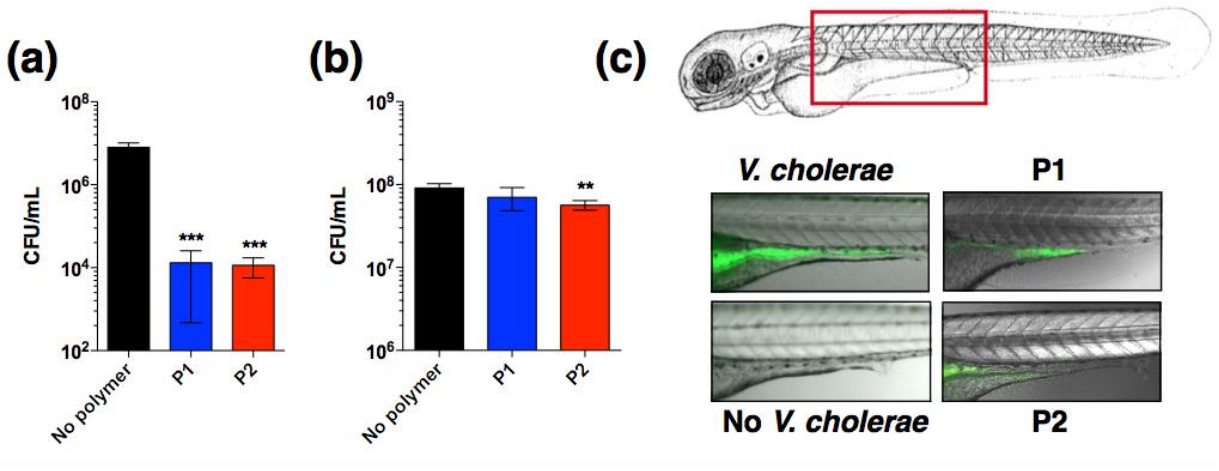



**Figure 6 | Polymer sequestration of bacteria abolishes *V. cholerae* colonization in a zebrafish larval infection model**. (a) $10^7$ or (b) $10^8$ CFU/mL of *V. cholerae* were incubated in the absence or presence of polymer (0.05 mg/mL of **P1** or 0.5 mg/mL of **P2**), as indicated, for 1 hr prior to infection experiments. Zebrafish larvae (n=10 per experimental condition) were transferred into bacterial cluster solutions, and incubated for 6 hrs. Larvae were washed in PBS and homogenized using Triton X-100. Bacterial loads were quantified using dilution plating on selective agar. 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 $p<0.0001$ (****). (c) Imaging of zebrafish infected with GFP-*V. cholerae* exposed to 0.05 mg/mL of P1 or 0.5 mg/mL of P2.

## DISCUSSION

The interaction between bacteria and polycationic molecules plays an important role in nature. Many antimicrobial peptides are polycationic, and their interactions with and impact on bacterial physiology is well characterized (23, 24). Polycationic synthetic polymers have been researched as a cheaper and more stable alternative to mimic the effect of naturally occurring AMPs, and their design and use in previous studies is often targeted at maximizing their antimicrobial effects (25-27). Recent work has however highlighted the potential of such materials to bind bacteria with the aim to manipulate bacterial behaviors, such as quorum sensing, without impacting bacterial viability (17, 18). In the face of increasing problems with the emergence of antibiotic resistant bacterial strains in clinical settings, such strategies, which would potentially provide less selective pressure on the emergence of drug-resistance, become more and more relevant (28). For *V. cholerae*, alternative approaches targeting virulence, rather than bacterial viability, have been the topic of previous research for some time, underpinning the need for novel ways to prevent and treat infections with this globally important human pathogen (29, 30). Anti-adhesion therapies are often proposed as attractive anti-virulence strategies, that compromise the ability of the pathogen to colonise the host and thus establish an infection (31, 32). Similarly, removal of the pathogen through sequestration is often proposed as a cost-effective approach to decontaminate water sources. However, little is known about *V. cholerae* response to these artificial environments and how binding to these materials may affect regulation of virulence in these pathogens.

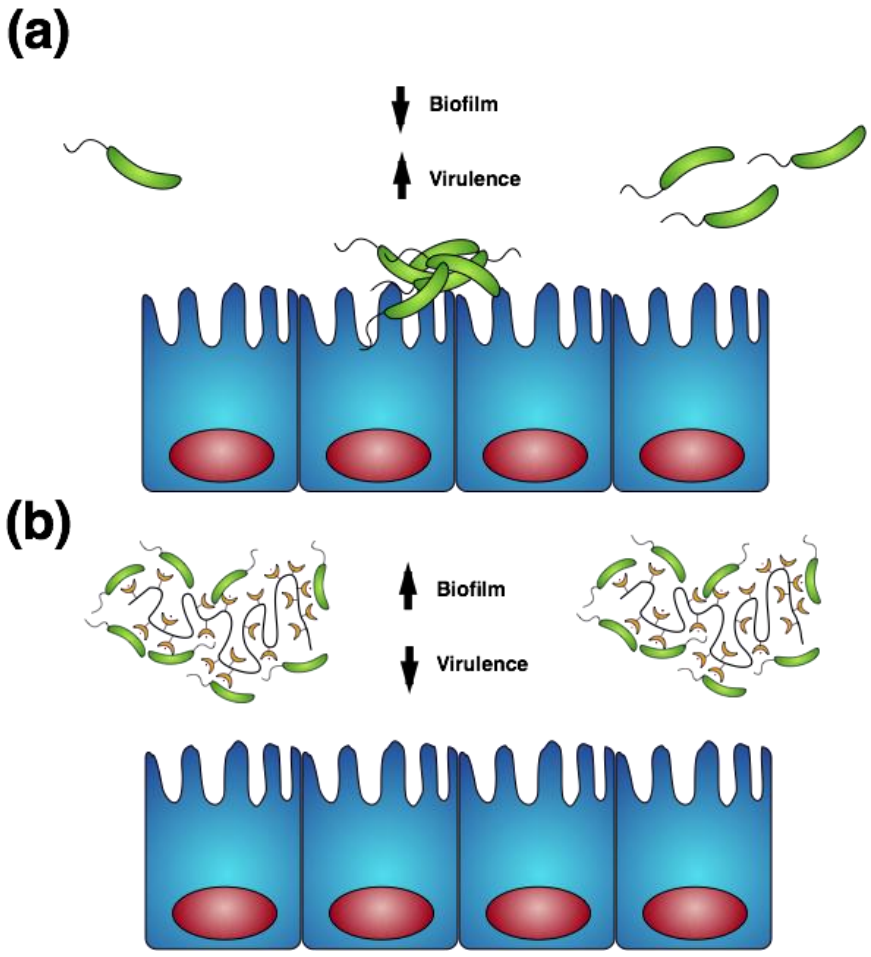
With this in mind, we set out to synthesize a set of two polymers with different cationic groups, but identical backbones, to evaluate their impact on bacterial clustering and behavior in *V. cholerae*. Based on our previous research with *V. harveyi* (17, 18), pAPMAm – P1 and pDMAPMAm - P2, were investigated. We anticipated that P1 would present higher toxicity towards both bacteria and host cells (**Figure 2** and **Figure S4**) (14-16). We found that both polymers were able to induce clustering of *V. cholerae* irrespective of their cationic nature, and the clusters quickly became big enough to precipitate out of solution (**Figure 1**). Both polymers had little impact on bacterial growth, viability and membrane permeability, in particular at concentrations below 0.05 mg/mL (**Figure 2**). Overall, P1 has a bigger effect on bacterial growth and viability than P2, (**Figure 2**), but even P1 showed bactericidal activity only at the highest

concentration tested (0.5 mg/ml). Similarly, charge and buffering impacted on eukaryotic membrane integrity, with both P1 and P2 able to disrupt host cell membranes at high concentrations (**Figure S4**). For both materials, this cytotoxicity would result in a narrow therapeutic window, and thus future applications of polymers inducing bacterial clustering by electrostatic interactions alone would likely lean more towards an *ex vivo* preventative application, for example as part of a low-tech water decontamination/filtration strategy. Although this is in itself a promising approach, our future efforts will also focus on the synthesis of materials with decreased toxicities that exhibit high affinity towards the bacteria by other means (e.g. incorporation of natural ligands for *V. cholerae* such as N-acetyl-glucosamine into the polymer). This approach may open up new avenues to extend future applications of such materials towards a prophylactic or therapeutic use in patients.

Infectious *V. cholerae* are often taken up as small biofilms, from which bacteria escape to colonize the epithelium. Once bound, bacteria initiate microcolony formation, before eventually exiting the host's GI tract, often following re-organization into biofilms (33, 34), to cause environmental dispersal and onward-transmission. The ability to transition between motile and sessile states is thus key to *V. cholerae*'s virulence regulation upon entering the human host and initialization of its colonization programme. Active bacterial motility and induction of virulence factors are both crucially required for *V. cholerae* pathogenesis. In natural environments, the transition of *V. cholerae* to a sessile lifestyle and inhibition of motility is accomplished both by transcriptional repression of flagellar genes, as well as induction of extracellular polysaccharide production, both of which are mediated by c-di-GMP (35). The cues triggering these motile to sessile transition in *V. cholerae* are still subject to investigations, but recent work showed that both lowered temperatures as well as type IV pili-mediated surface sensing can feed into c-di-GMP signaling and thus biofilm formation (36, 37). With our polymers bacterial motility is also largely abolished, albeit by physical deposition into polymer-based clusters. Interestingly, while both polymers cause immobilization to a similar extent, P1 but not P2 caused transcriptional repression of flagellar genes (**Figure S5**). This suggests that physicochemical properties of the adhesive surface, rather than the mechanical process of surface sensing alone, also impact the transition to a sessile lifestyle. Interestingly, despite their different effects on gene regulation in response to immobilization, both polymers lead to an increase in bacterial deposition on an abiotic surface upon bacterial clustering, which was accompanied by an increased release in

extracellular DNA (**Figure 3b-d**). This increase would suggest that these polycationic polymers may act as an alternative cue to promote a transition toward a sessile, community-based lifestyle for *V. cholerae*. At the same time, clustering of *V. cholerae* lead to a decrease in virulence factors at the transcriptional level (**Figure 4b**). Regulation of virulence genes in *V. cholerae* is a complex process and several pathways converge at this point. Crucially, both high cell density, via quorum sensing, and c-di-GMP dependent signaling can act to repress virulence genes (38, 39). Based on the fact that a "biofilm-like state" is induced by clustering in our system, while quorum sensing leads to HapR-dependent suppression of biofilm formation, we conclude that the transcriptional repression of virulence genes we observe here is triggered by a cue that mimics more closely the transition towards a sessile lifestyle in aquatic environments, rather than high cell density dependent signaling. The net effect of this polymer-induced phenotypical switch towards avirulence is a decrease in colonization and a decrease in cytotoxicity towards cultured cells (**Figure 5**).

Finally, we evaluated the potential of these dual-action polymers to inhibit infection in an *in vivo* model. Zebrafish are a suitable natural host model for *V. cholerae* colonization and transmission (21, 22) as their gastrointestinal development and physiology closely mimics that of mammalian organisms (40). Additionally, ease of propagation and live imaging made them a good choice of host for our *in vivo* studies. Due to license restrictions on the experimental duration in the zebrafish infection model, we were unable to characterize the effect of *V. cholerae* on zebrafish survival. However, the observed decrease in initial colonization (**Figure 6**) supports the notion that clustering would be an effective way to "neutralize" *V. cholerae in vivo*. Overall, our results show that the tested materials mainly act to modulate bacterial behavior in a way that positively impacts on the outcome of infection (**Figure 7**).




335

336 **Figure 7 | Dual action polymers abrogate *V. cholerae* infection by physical and transcriptional interference**
337 **with host colonization and virulence**. (a) *V. cholerae* are often ingested as small biofilms, from which they escape
338 to initiate infection of the host intestinal epithelium. Downregulation of biofilm genes and upregulation of virulence
339 genes, such as cholera toxin and toxin-coregulated pilus, are necessary for successful infection. (b) Polycationic
340 polymers form clusters of *V. cholerae*. Clustering abolishes active motility, induces a biofilm-like state, and leads to
341 a downregulation of virulence genes. This dual mode of action inhibits infection of the intestinal epithelium.

342

343 **Conclusions**

344 Here, we have shown that linear polymers that can sequester the human pathogen *V. cholerae*
345 into clusters, downregulate virulence and mitigate colonization and toxicity in relevant *in vitro*
346 and *in vivo* models. Using cationic polymers and a combination of phenotypic and transcriptional
347 assays, we demonstrate that this reduction in virulence is a result of *V. cholerae* switching to a
348 non-pathogenic environmental-like phenotype upon clustering. Our observations suggest that

polymeric materials can underpin the development of novel cost-effective strategies to minimize *V. cholerae* pathogenicity without promoting antimicrobial resistance. As such we anticipate that these materials can act as a blueprint for the development of novel cost-effective prophylactic or therapeutic polymers, but to this end, a clear understanding of how these materials trigger phenotypic responses in these pathogens is essential. Our efforts to optimize affinity toward *V. cholerae* while minimizing toxicity towards the host will be reported in due course.

## MATERIALS AND METHODS

Polymers used in this study were poly-*N*-(3-aminopropyl)methacrylamide p(APMAm), P1 and poly-*N*-[3-(dimethylamino)propyl]methacrylamide p(DMAPMAm), P2. Their synthesis and characterization, as well as their use in biological assays, are described in detail in the Supporting Materials and Methods.

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## CONTRIBUTIONS

All authors contributed to the experimental set-up and discussed the results. A.M.K, F.F.-T. and K.V. secure funding. N.P.-S., L.M., I.I. and D.N.C. synthesised and characterised the polymers. N.P.-S., L.M., K.V., A.M.K. performed the biological assays. N.P.-S., K.V., A.M.K. and F.F.-T. analysed the data, and N.P.-S., K.V., A.M.K. and F.F.-T. wrote the manuscript, with all other authors contributing to its final version.

## Declaration of competing interests

None to declare.

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Supporting Information for

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

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This file contains:

Supporting Figures S1-S5

Supporting Tables S1 and S2

Supporting Materials and Methods

## SUPPORTING FIGURES

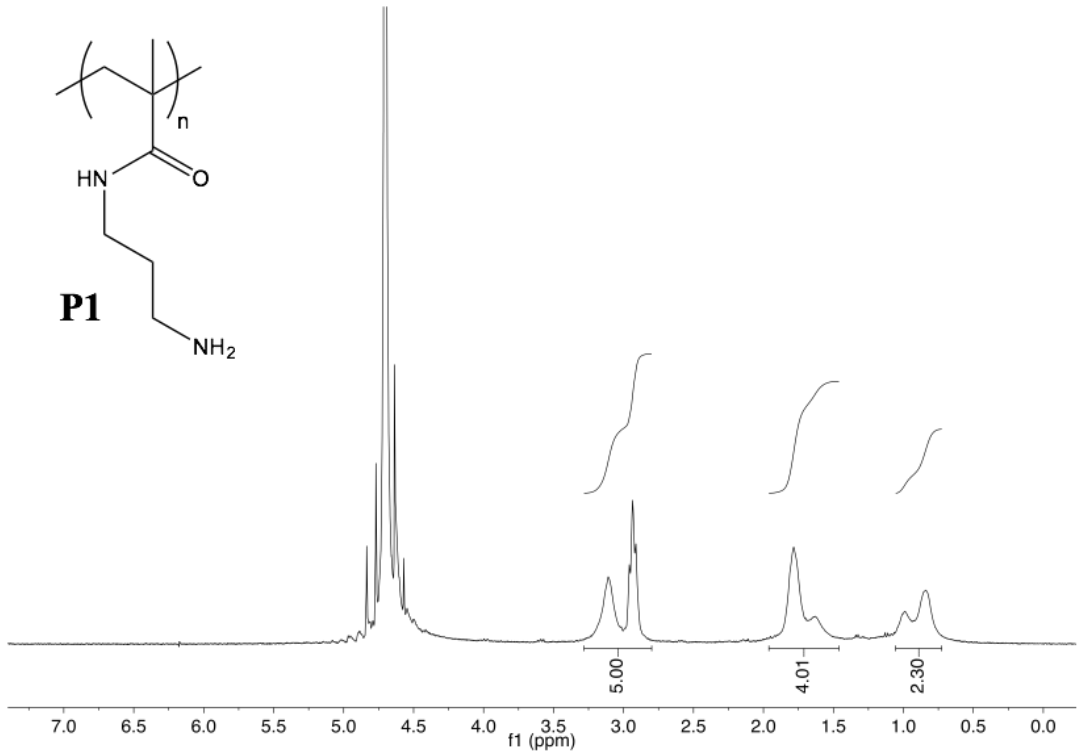
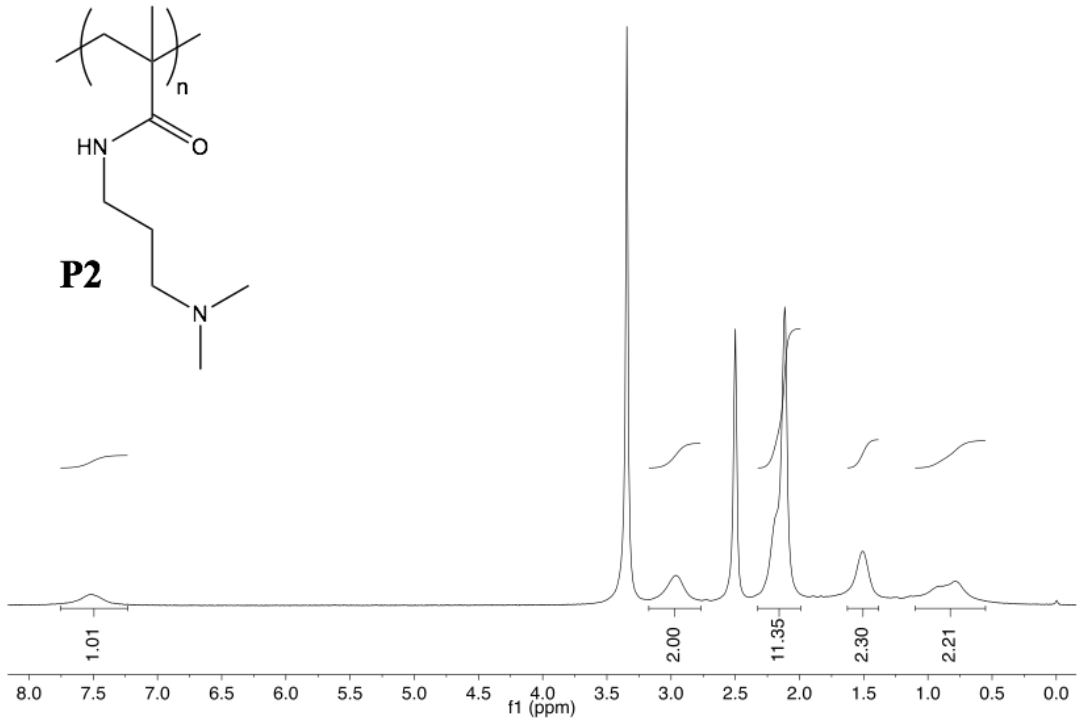



**Figure S1**| $^{1}$H-NMR (300 MHz, $D_2O$) spectrum of **P1**.

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

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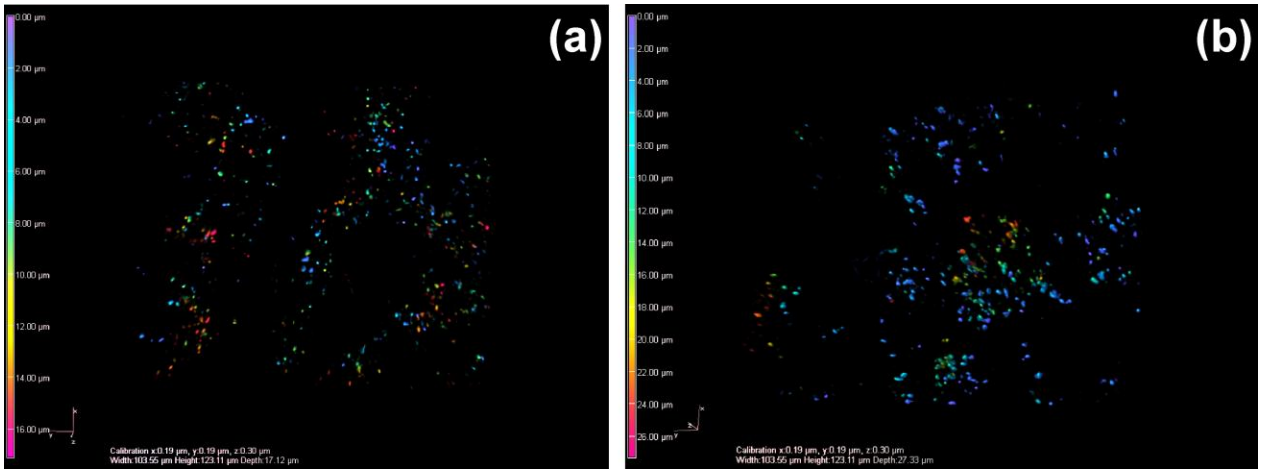


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517 **Figure S3**| **Polymers induce rapid formation of three-dimensional bacterial clusters.** GFP-*V. cholerae* were
518 incubated with 0.5 mg/mL of P1 **(a)** or P2 **(b)** for 15 minutes, and z-stack images were collected using a spinning
519 disc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

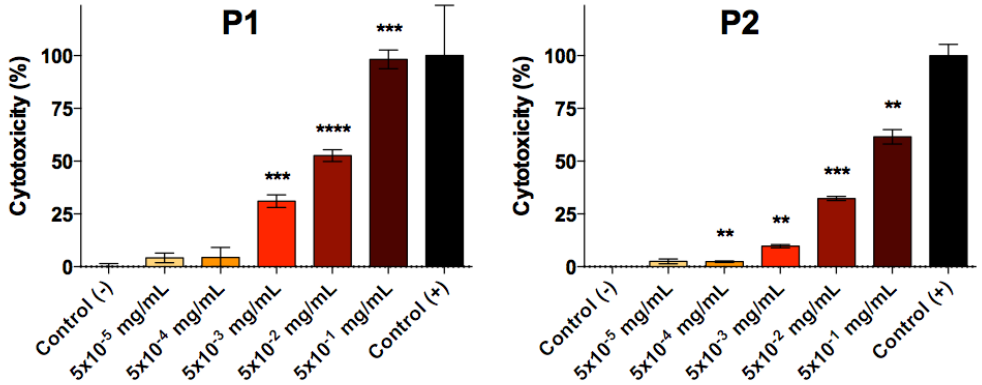


**Figure S4| Effect of polymers on membrane integrity of cultured epithelial cells.** Solutions of polymers in DMEM at concentrations as indicated, were incubated with cultured Caco-2 intestinal epithelial cells for 7 hrs. Cytotoxic effect on host cells was quantified by measuring the amount of lactate 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 $p<0.0001$ (****).

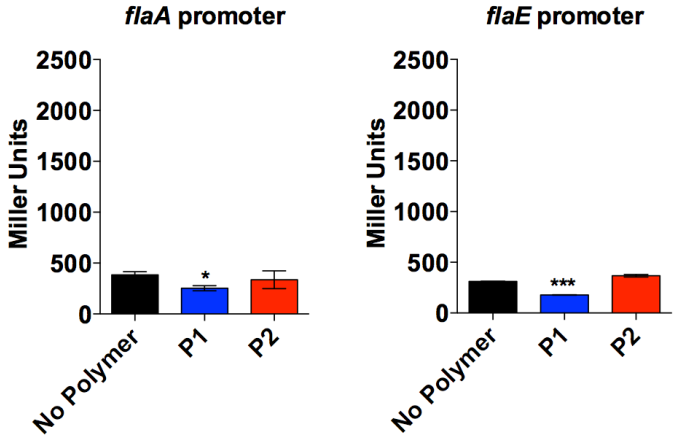


**Figure S5| Effect of bacterial clustering on transcriptional regulation of flagella-driven motility**. Promoter activities of *flaA-lacZ* and *flaE-lacZ* fusions in *V. cholerae* were measured following 7 hours of growth in the absence (black) or in the absence (black) or presence of 0.05 mg/mL P1 (blue) or 0.5 mg/mL P2 (red) in DMEM at 37 °C. Statistical significance was defined as $p<0.05$ (*), or $p<0.001$ (***).

## 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.(42) |
| 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 |
| NPMW1 | N16961 carrying pMW-*gfp* plasmid; $Spect^R$. | Ritchie et al.(48) |

539 **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 |

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## 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 (41). 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 (42). 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 (43), which has been modified by inserting the *oriT* sequence from the vector pRK2

(44). 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).

**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 (45). 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 (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.

**Transciptional assays: β-galactosidase activity.** *V. cholerae* reporter strains were assayed for β-galactosidase activity as previously described (46). 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 (46).

**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 (47).

**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.