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Capture of *Vibrio cholerae* by charged polymers inhibits pathogenicity 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 lifestyle, without creating selective pressure favoring the emergence of antimicrobial resistant strains.

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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 7th 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

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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**). Bacterial clusters were stable for at least 24 hours and had an extended 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

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and cluster size (**Figure 1b**). 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. Capture of bacteria in polymer clusters abolished motility, and full motility was restored upon exposure of clusters to high salt concentration (**Figure 1e**), confirming that clustering was driven by electrostatic interactions.

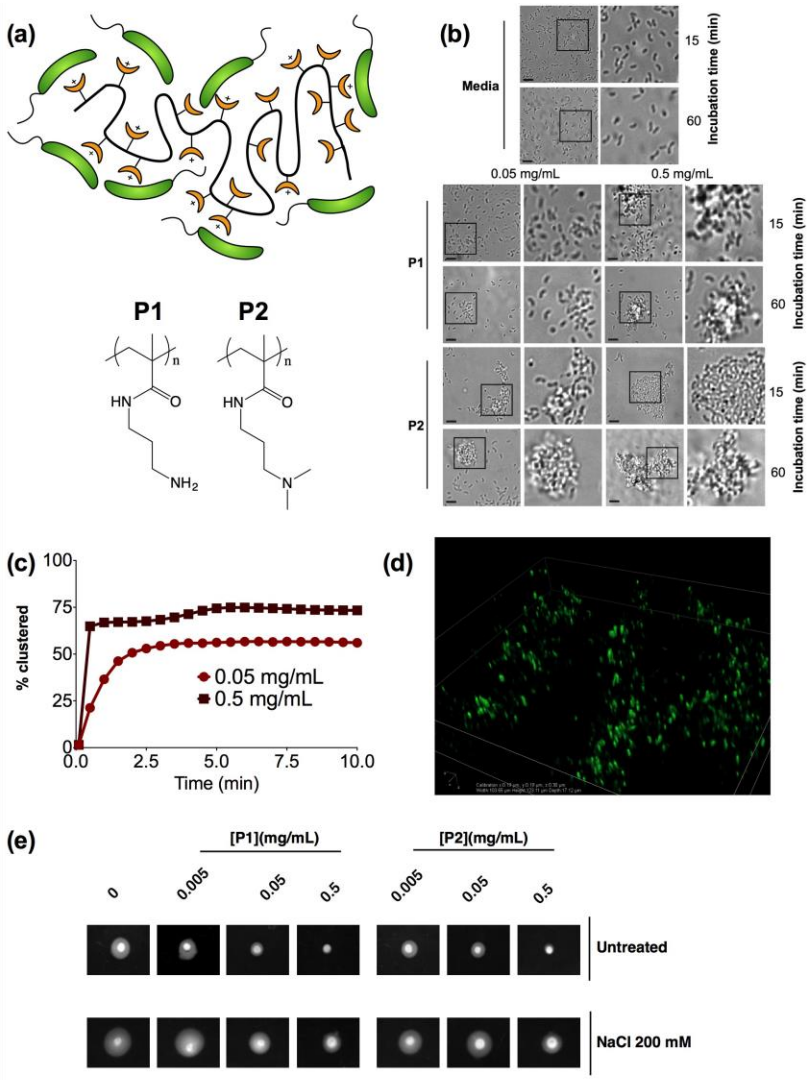

Figure 1 | Cationic polymers rapidly form three-dimensional clusters upon contact with *Vibrio cholerae*. (a) Bacteria are clustered via electrostatic interactions between polymers containing positively charged sidechains (yellow) and *V. cholerae* (green). Chemical structures of polymers P1 and P2 are 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. **(e)** Bacterial clustering by polymers abolishes *V. cholerae*

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swarming motility. Soft agar plates were inoculated with bacterial clusters and bacterial growth imaged following overnight incubation. The addition of 200 mM NaCl released bacteria from the clusters and restored motility.

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 0.005 and 0.0005 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.

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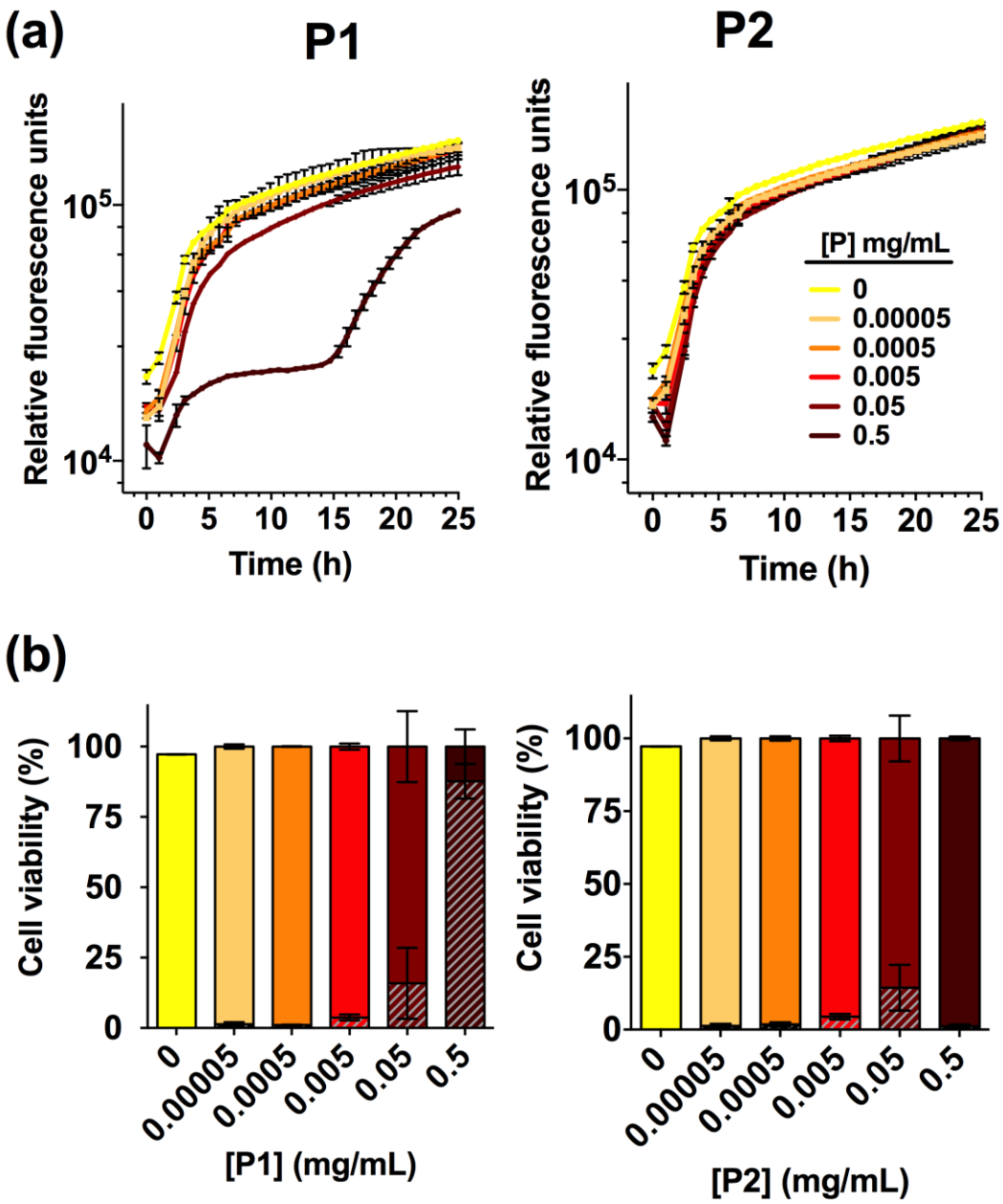

Figure 2 | Effect of polymer-induced clustering on bacterial growth and viability. (a) GFP-*V. cholerae* were adjusted to an initial OD_{600} of 0.02, added to polymers to give final concentrations as indicated in the legend, and grown under shaking at 37 °C for 24 hrs, with GFP fluorescence measured 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.

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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 motility (11, 20). Since we observed during imaging experiments that bacterial clustering abrogated bacterial motility, we investigated whether polymer-induced clustering would also affect *in vitro* biofilm formation. Polymer-induced clustering led to a significant induction of biofilm formation in *V. cholerae*, as measured using crystal violet assays and imaging of GFP-*V. cholerae* (**Figure 3a-d**). Polymer-induced biofilms also released higher levels of extracellular DNA into the biofilm, compared to untreated *V. cholerae*. Treatment of polymer-induced biofilms with DNAse I abolished the additional blue fluorescence, confirming the released substance was indeed extracellular DNA (**Figure 3e,f**).

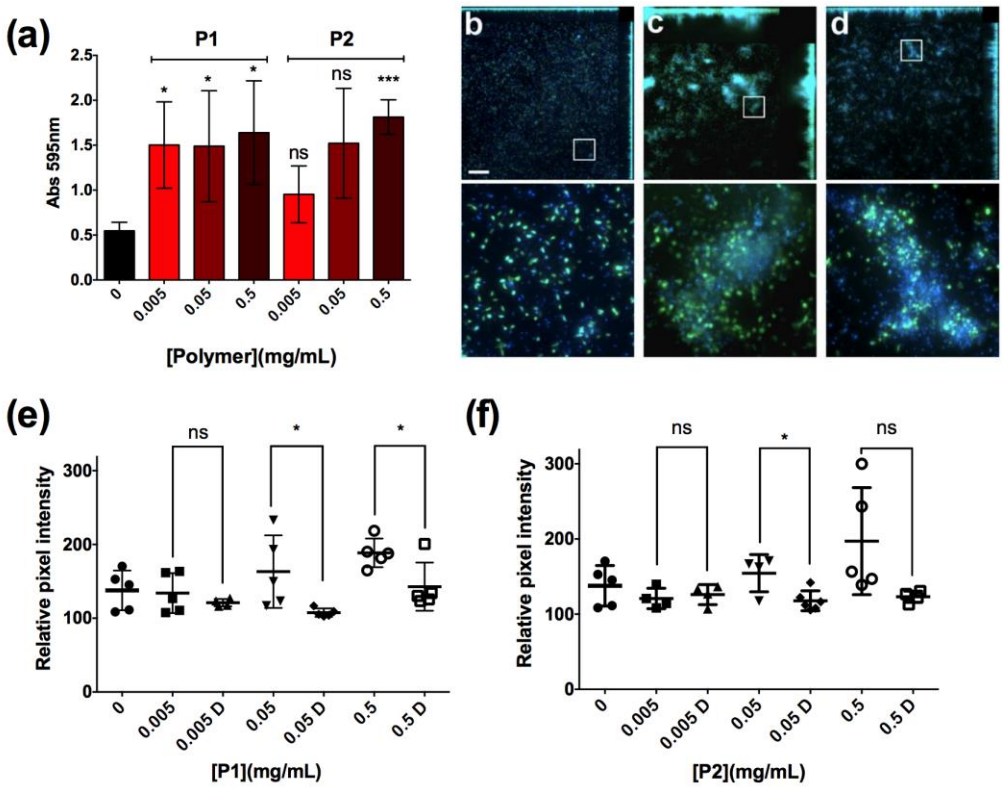

Figure 3 | Polymer-induced clustering leads to increased *V. cholerae* biofilm formation. Biofilm formation of *V. cholerae* after overnight incubation in the absence or presence of polymers **P1** or **P2** at concentrations as indicated, was quantified using crystal violet plate assays **(a)**. Analysis 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 $p<0.001$

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(***). Biofilms of GFP-*V. cholerae* were imaged following 20 hours of growth in glass-bottom plates at 37 °C, in 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 stained with Hoechst (blue). Scale bar, 50 μm. Area within the square has been expanded (bottom row) for clarity. Levels of extracellular DNA in biofilms formed in the presence of P1 (e) or P2 (f) were quantified by image analysis in Image J. Treatment with DNAse I (D) decreased levels of blue fluorescence to those of untreated samples. Data are individual measurements from at least four representative images, means and stdev. Analysis of variance (ANOVA), followed by Tukey's post hoc test, was used to test for significance. Statistical significance was defined as $p<0.05$ (*).

Since sequestration into polymer clusters promotes a sessile state, we investigated what impact this environmental signal would have on virulence regulation. For this purpose, we created a series of transcriptional reporter strains, by introducing, via conjugation, a variant of the pRW50 plasmid containing oriT into *V. cholerae* (**Figure 4a**). Thus, we could follow transcription from *V. cholerae* promoters during infection of Caco-2 intestinal epithelial cells, using β-galactosidase assays. Contact with cultured epithelial cells has been described as a strong activating cue for expression of virulence factors (50). AphA is a master regulator of virulence that is required for the activation of *tcpP* (53). 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 in *V. cholerae* during infection showed a relative activity of ~3 compared to vector control (**Figure 4b**). Sequestration of *V. cholerae* into clusters significantly repressed the *aphA* promoter activity (approx. 50% and 80% suppression by **P1** and **P2**, respectively). The *toxT* promoter showed a relative transcriptional activity of ~17 in the absence of polymer, but transcription was significantly repressed in bacterial clusters (~60% and 88% inhibition by **P1** and **P2**, respectively). Similarly, the promoters of the two key virulence factors, *ctxAB* and *tcpA*, showed relative activities of 17 and 3 during infection, compared to vector control. **P1** and **P2** suppressed *ctxAB* transcription by approximately 65% and 75%, respectively. The effect on *tcpA* differed between P1 and P2, with P1 causing a decrease but P2 causing an increase in transcriptional activity (**Figure 4b**). Despite its low transcriptional activity during host cell infection, TcpA was still required for attachment and cytotoxicity towards host cells, and a *tcpA* mutant (49) showed decreased attachment and cytotoxicity, but caused similar cAMP production than wild type *V. cholerae* (**Figure S6**). We also investigated the effect of physical immobilization within artificial clusters on transcriptional

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regulation of genes encoding for components of the flagellar systems (*flaE* and *flaA*, respectively). Both of these were comparatively weak promoters (relative activities of ~2 to 2.5 compared to vector control). Interestingly, both *flaE* and *flaA* transcription were suppressed by **P2** mediated clustering, while **P1**, which is less hydrophobic than **P2**, had no effect on *flaA* or *flaE* transcription levels (**Figure S5**).

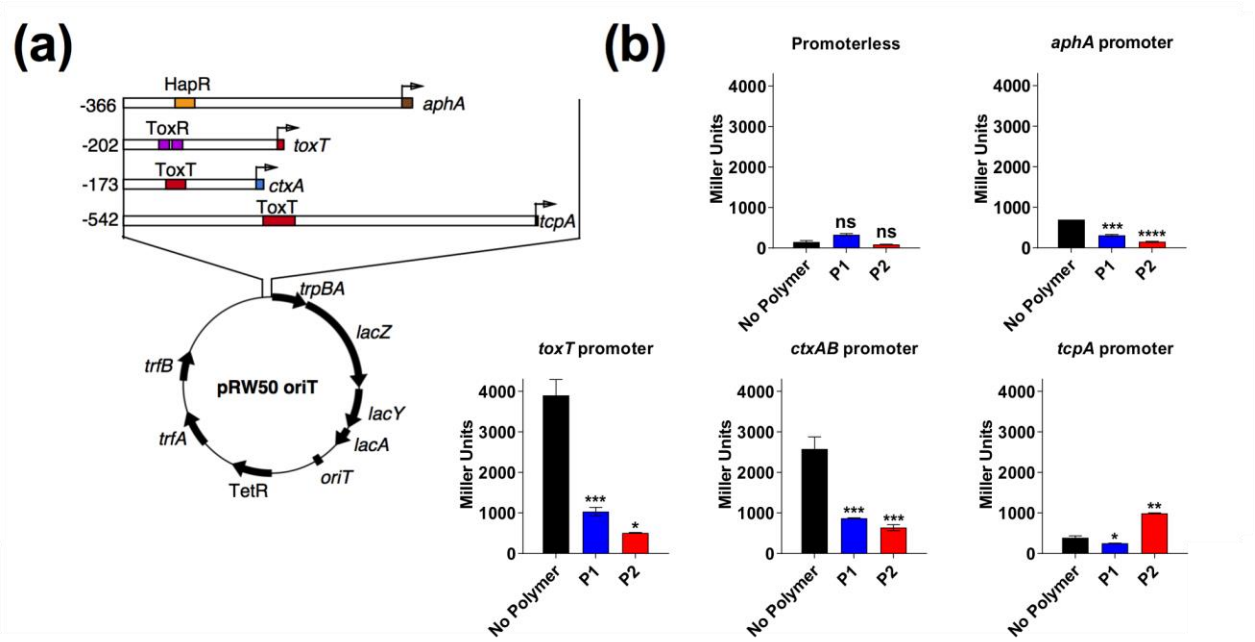

Figure 4 | Sequestration of *V. cholerae* into polymer clusters suppresses bacterial virulence at the transcriptional level. (a) Schematic depiction of the reporter plasmid (pRW50-*oriT*) and promoter regions of *aphA*, *toxT*, *ctxA* and *tcpA* cloned as transcriptional fusions to *lacZ* in pRW50-oriT. Numbering refers to base number relative to the transcriptional start site and promoter binding sites for HapR, ToxR and ToxT are indicated. (b) Promoter activities of *aphA*, *toxT*, *ctxA* and *tcpA* promoter-*lacZ* fusions were measured following infection of Caco-2 cells for 7 hours in the absence (black) or presence of 0.05 mg/mL P1 (blue) or 0.5 mg/mL P2 (red). Student's paired t-test was used to test for significance. Statistical significance was defined as $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), or $p<0.0001$.

Polymer sequestration of bacteria abolishes *V. cholerae* infection *in vitro*. Since polymer-induced clustering repressed the transcription of key virulence factors during infection of intestinal epithelial cells, we asked what impact clustering would have on the *V. cholerae* infection phenotype. Initially, we tested the effect of clustering on colonization and toxicity towards cultured intestinal epithelial cells. Sequestration of *V. cholerae* by both polymers led to a significant reduction in bacterial attachment to host cells, as determined by dilution plating/CFU

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counts, but the effect of **P1** on adhesion was much more pronounced than for **P2** (**Figure 5a**). 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 (**Figure 5d**). Host cells infected with clustered bacteria showed less cell death and more intact cell-cell junctions than cells infected with planktonic bacteria (**Figure 5d**). 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**). Additionally, we tested the specific effect of clustering on Cholera-toxin activity, by measuring cAMP activity in infected cells (**Figure 5c**). Elevated cAMP production due to CTx activity is the main hallmark of cholera infection, and is responsible for diarrhea in humans. While cAMP levels were significantly elevated in *V. cholerae* infected Caco-2 cells, clustering of bacteria with both polymers decreased cAMP levels during infection, in agreement with the reduction in CTx transcription observed before (**Figure 4b**).

We postulate that **P1** and **P2** ameliorate infection by a dual mode of action: the polymers abolish bacterial motility and thus physically interfere with bacterial adhesion to host cells, and they interfere with the transcription of key virulence factors. To assess the relative contribution of these modes of action to the polymers' efficacy in the Caco-2 infection model, we tested their effect on the *V. cholerae* BH1651 strain. BH1651 is an El Tor *luxO*^{D47E} mutant and constitutively produces virulence factors including CTx (52). The absolute attachment, cytotoxicity and cAMP levels following 7 hours of infection with BH1651 was lower than with wild type El Tor, likely due to the slower growth rate of the mutant (**Figure S7**). However, we were still able to assess the relative contribution of polymers to infection phenotypes. Bacterial attachment, cytotoxicity and cAMP levels of the genetically locked strain were all less affected by **P1**-mediated clustering, compared to the wild type strain, and were completely unaffected by **P2**-mediated clustering (**Figure 5e-h**), suggesting that although mechanical and transcriptional effects mediated by clustering synergize to abrogate virulence, the transcriptional effect on virulence factor production caused by bacterial clustering is dominant over the mechanical effect of clustering *in vitro*.

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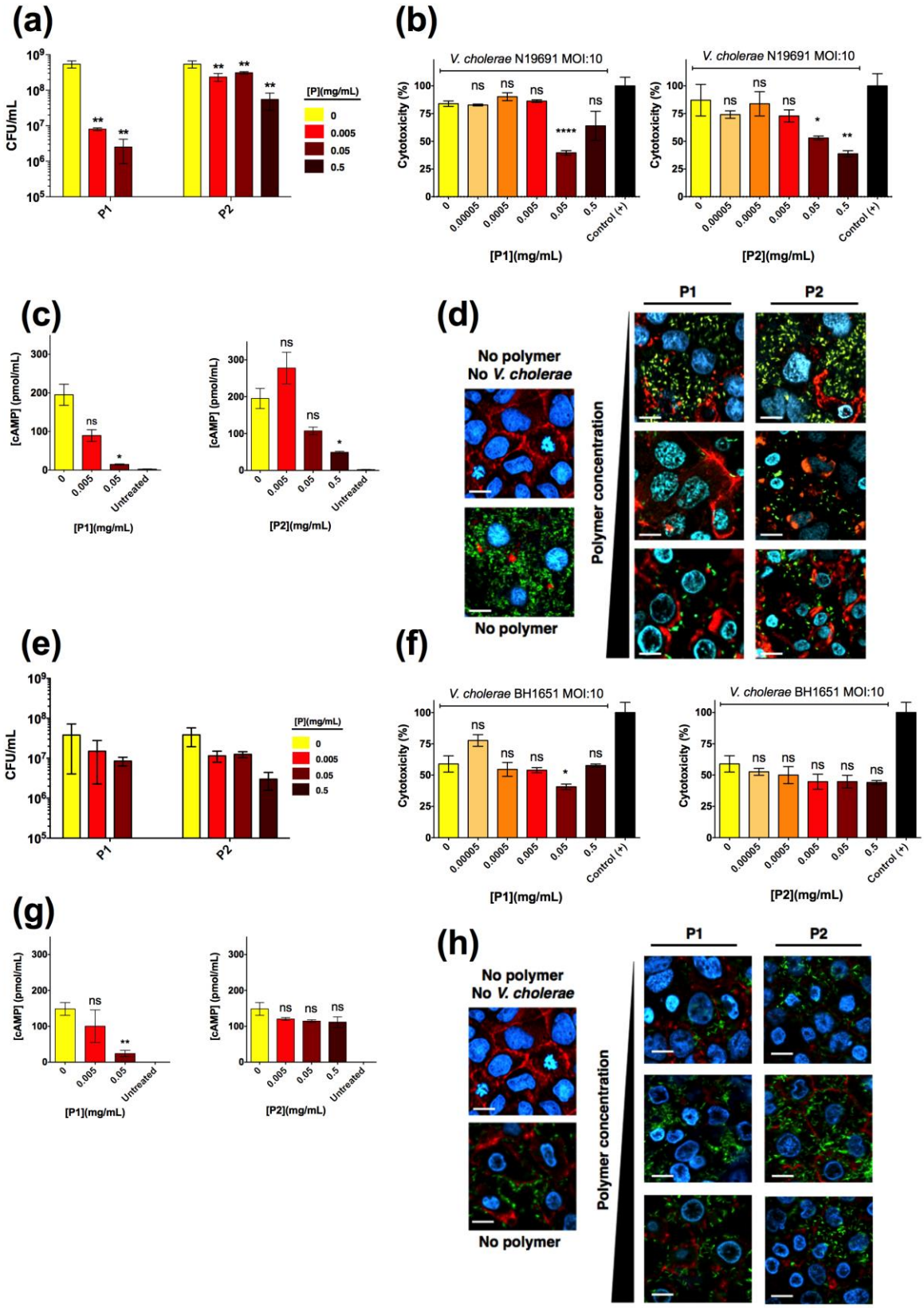

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Figure 5 | Polymer sequestration of bacteria abolishes *V. cholerae* infection of cultured epithelial cells. *V. cholerae* N19691 cultures were adjusted to an MOI of 10, and incubated in the absence or presence of polymers for 1 hr prior to infection 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%). **(c)** Levels of cAMP production in Caco-2 cells were measured by ELISA**. (d)** Following infection, *V. cholerae* (green), DNA (blue), and F-actin (red) were visualized by fluorescence microscopy. Scale bar, 10 μm. **(e-h)** Similar to (a-d), but cells were infected with the genetically locked, hypervirulent *V. cholerae* strain BH1651. 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 $p<0.05$ (*), $p<0.01$ (**) or $p<0.0001$ (****).

Polymer sequestration is an effective way of removing *V. cholerae* from contaminated liquid.

Finally, we tested whether non-bactericidal concentrations of polymers would efficiently decontaminate liquid and prevent colonization with *V. cholerae* upon ingestion. 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). Zebrafish larvae exposed to media containing 10^7 CFU/mL of GFP-expressing *V. cholerae* for 6 hours 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**). 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**). Treatment of contaminated media with polymers significantly reduced colonization levels – ingested clustered bacteria colonized ~1000-fold less than unclustered bacteria (**Figure 6a**) and ingestion of the residual liquid following removal of the clusters reduced bacterial burdens ~100-fold (**Figure 6b**).

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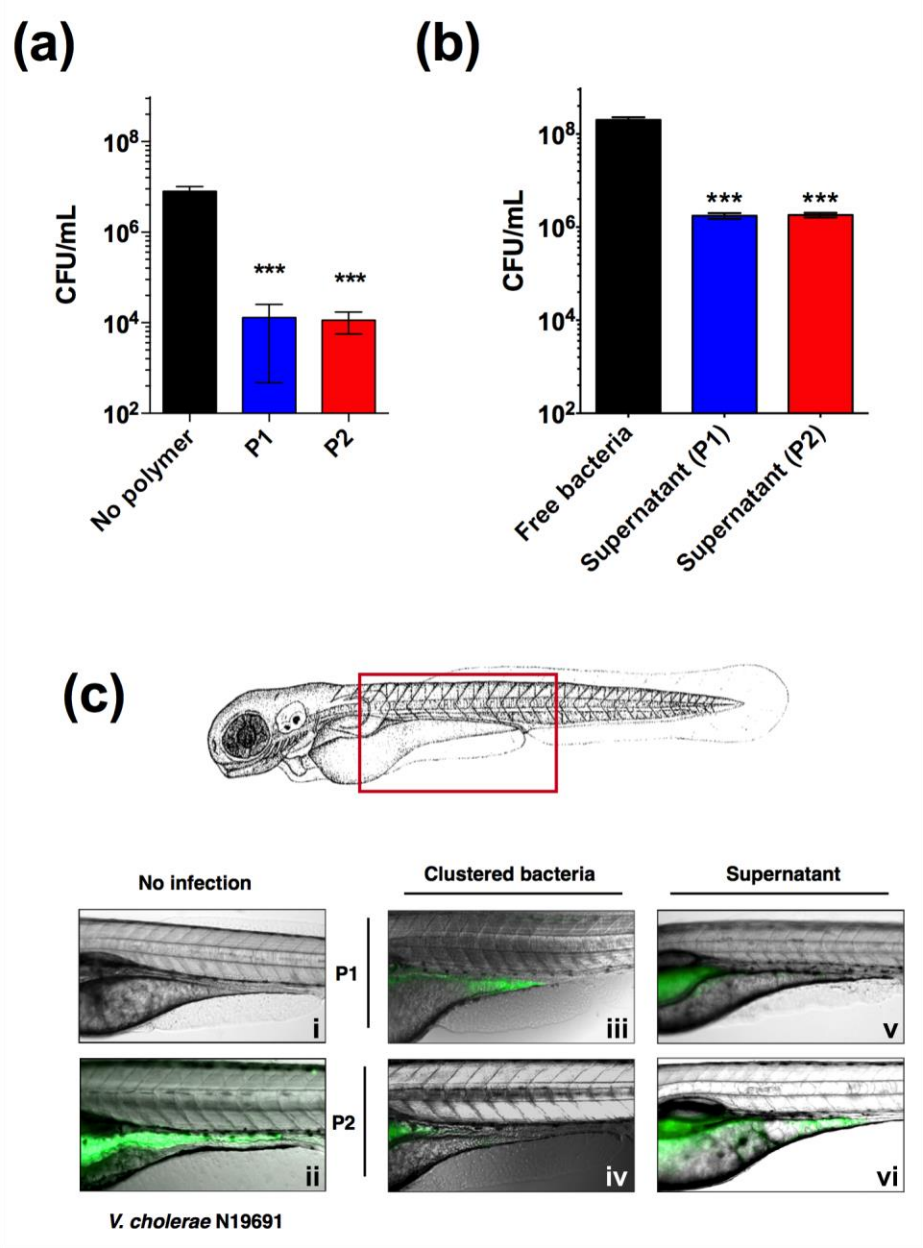

Figure 6 | Polymer sequestration of bacteria abolishes *V. cholerae* colonization in a zebrafish larval infection model. 10^7 CFU/mL of *V. cholerae* N19691 were incubated in the absence (black) or presence of polymer (0.05 mg/mL of **P1**, blue or 0.5 mg/mL of **P2**, red), as indicated, for 1 hr prior to infection experiments. Zebrafish larvae (n=10 per experimental condition) were exposed either to clustered bacteria **(a)** or remaining supernatant following removal of clustered bacteria **(b)** 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 $p<0.001$ (***). **(c)** Imaging of zebrafish left uninfected (panel i), infected with GFP-*V. cholerae* (ii), *V. cholera*e clustered with P1 (iii) or P2 (iv), or remaining decanted supernatant following removal of clustered *V. cholerae* exposed to 0.05 mg/mL of P1 (v) or 0.5 mg/mL of P2 (vi).

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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

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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 such as that piloted in Figure 6. 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 (**Figure 1e,f**). Interestingly, while both polymers cause immobilization to a similar extent, **P2** but not **P1** caused transcriptional repression of flagellar genes (**Figure S5**). At the same time, transcriptional responses to clustering by both polymers show suptle differences: while the *aphA/toxT*-mediated virulence cascade is equally affected by both materials, *tcpA* responses differed. 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

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different effects on gene regulation in response to immobilization, both polymers led to an increase in bacterial deposition on an abiotic surface upon bacterial clustering, which was accompanied by an increased release in extracellular DNA (**Figure 3**). 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* led to a repression of 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. Interestingly, the effect of clustering on a quorum sensing mutant genetically locked in a low-density, hypervirulent state is minimal, suggesting that the effect on transcription is dominant over the polymers' physical effect on motility (**Figure 5**). The net effect of this polymer-induced phenotypical switch towards avirulence is a decrease in attachment, cytotoxicity and cAMP release *in vitro* (**Figure 5**) and a decrease in colonization *in vivo* (**Figure 6**). The effect on cytotoxicity is in part an indirect effect of decreased attachment, as a *tcpA* deficient mutant also showed less cytotoxicity (**Figure S6**), but it likely also due to transcriptional repression of MARTX, an RTX family toxin which is physically linked to the CTx gene cluster and causes damage to epithelial cells (51).

Finally, we evaluated the potential of these dual-action polymers to remove bacteria and decontaminate liquids prior to ingestion, using an *in vivo* colonization 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 following ingestion of polymer-treated media (**Figure 6**) supports the notion that polymer-induced clustering would be an effective way to

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"neutralize" *V. cholerae in vivo*. Overall, our results show that the tested materials act to modulate bacterial behavior in a way that positively impacts on the outcome of infection (**Figure 7**).

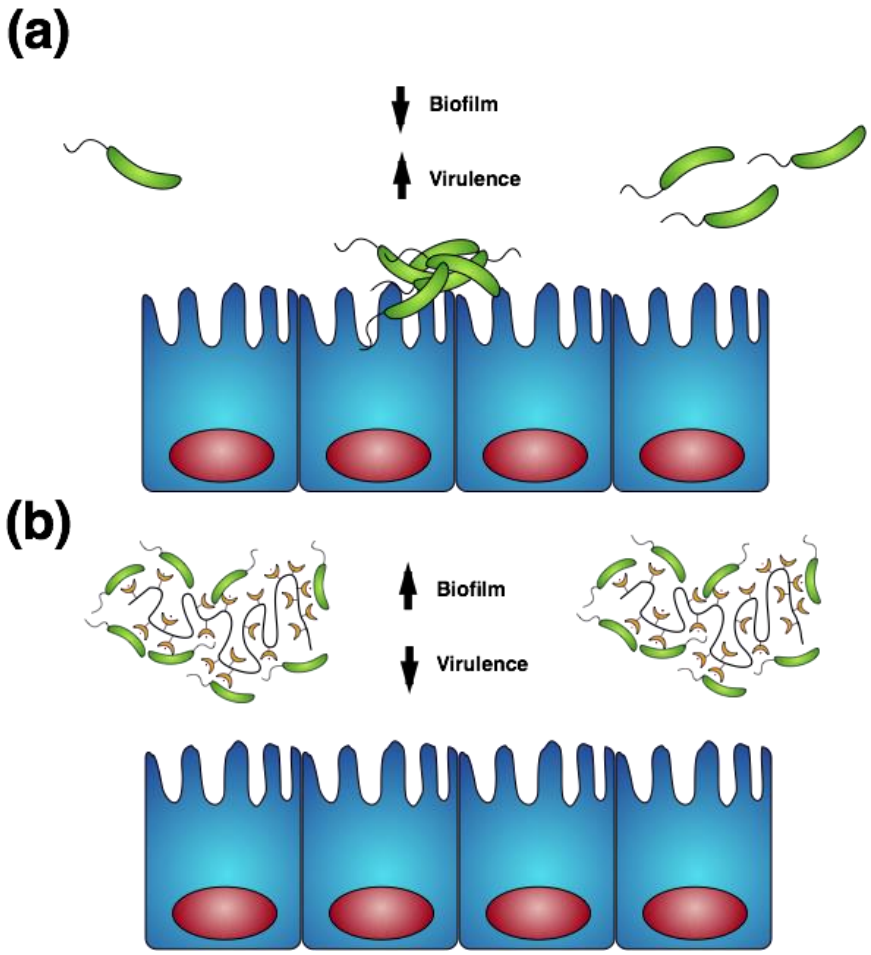

Figure 7 | Dual action polymers abrogate *V. cholerae* infection by physical and transcriptional interference with host colonization and virulence. (a) *V. cholerae* are often ingested as small biofilms, from which they escape to initiate infection of the host intestinal epithelium. Downregulation of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

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Conclusions

Here, we have shown that linear polymers that can sequester the human pathogen *V. cholerae* into clusters, downregulate virulence and mitigate colonization and toxicity in relevant *in vitro* and *in vivo* models. Using cationic polymers and a combination of phenotypic and transcriptional assays, we demonstrate that this reduction in virulence is a result of *V. cholerae* switching to a 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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