Cationic polymer mediated bacterial clustering: cell-adhesive properties of homo- and copolymers.

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ABSTRACT New anti-infective materials are needed urgently as alternatives to conventional biocides. It has recently been established that polymer materials designed to bind to the surface of bacteria can induce the formation of cell clusters which enhance the expression of quorum sensing controlled phenotypes. These materials are relevant for anti-infective strategies as they have the potential to inhibit adhesion while at the same time modulating Quorum Sensing (QS) controlled virulence. Here we carefully evaluate the role that charge and catechol moieties in

these polymers play on the binding. We investigate the ability of the cationic polymers poly(N-[3-(dimethylamino)propyl] methacrylamide) (pDMAPMAm, P1), poly(N-dopamine methacrylamide-co-N-[3-(dimethylamino)propyl] methacrylamide) (pDMAm-co-pDMAPMAm, P2) and p(3,4-dihydroxy-L-phenylalanine methacrylamide), p(L-DMAm, P3) to cluster a range of bacteria, such as *Staphylococcus aureus* (Gram-positive), *Vibrio harveyi*, *Escherichia coli* and *Pseudomonas aeruginosa* (Gram-negative) under conditions of varying pH (5, 7 and 8) and polymer concentration (0.1 and 0.5 mg/mL). We identify that clustering ability is strongly dependent on the balance between charge and hydrophobicity. Moreover, our results suggest that catechol moieties have a positive effect on adhesive properties, but only in the presence of cationic residues such as for P2. Overall, our results highlight the subtle interplay between dynamic natural surfaces and synthetic materials, as well as the need to consider synergistic structure-property relationship when designing antimicrobial polymers.

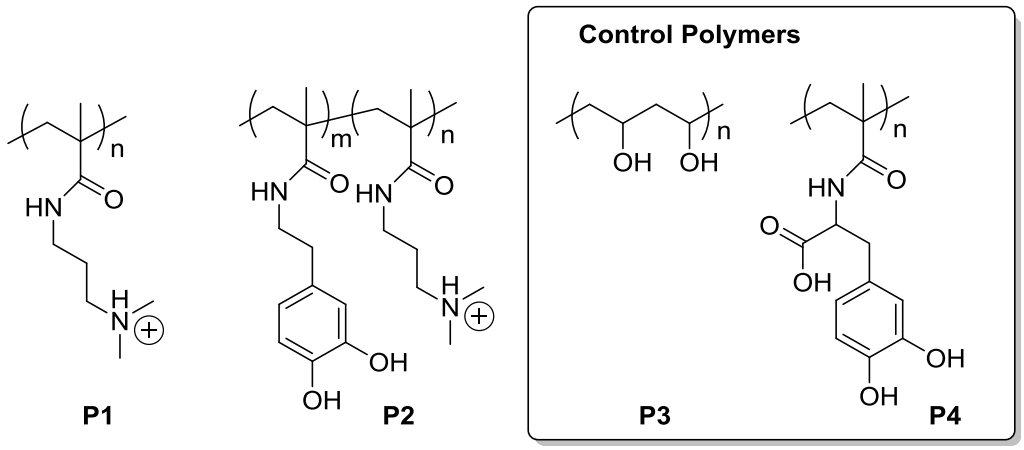
1. Introduction

The rise in bacteria-related diseases worldwide demands new anti-infective materials and improved healthcare strategies. Current materials designed to target microbial infections include a) cytotoxic agents, both small molecule and macromolecular; b) antifouling materials, to prevent bacterial colonization and infection; c) controlled delivery systems, able to release bioactive molecules at the infection site or over a defined timespan to combat pathogenicity.[1] Polymers have become increasingly important as an alternative to existing biocides and antibiotics since, in principle, they can be prepared with any, or combinations, of the properties described above. Synthetic polymers are often easily scalable, their properties can be tuned by pre[2]- or post-polymerization[3, 4] modifications and the accessibility of many different functional monomers allows a wide variety of structures, architectures and properties to be obtained. In addition,

polymers are being considered for therapeutic applications beyond direct anti-infective or anti-microbial fields, but in which bacterial-polymer interactions might be relevant, such as, for example, celiac disease[5, 6] or to reduce consequences related to high cholesterol or iron levels[7]. However, the mechanism of action of many anti-microbial polymers is not fully understood, in part due to their large structural variety [8] and the complexities of association and transport at bacterial cell walls. Microbial cells generally carry a negative net charge at the surface due to specific cell wall and membrane components, which include teichoic acids for Gram-positive bacteria and charged lipopolysaccharides (LPS) and phospholipids in the outer membranes of Gram-negative bacteria[9]. Polycations are therefore electrostatically attracted to bacteria, and if they have a suitable amphiphilic character, are able to disrupt the outer and cytoplasmic membranes, causing lysis of the cell and subsequent cell death. [8, 10] Consequently, when designing cationic polymers which might control infection through binding and sequestering bacteria, there is a trade-off in cation content and charge accessibility for high cell binding, against the type and amphiphilicity of charged side chains which might cause cell death and thus select for resistant strains.

Another strategy to control infection is through interruption of bacterial communication. Of particular interest are the signaling systems known as Quorum Sensing (QS),[11-13] by which bacteria control population behaviour by secreting and sensing small diffusible signal molecules. QS systems act to regulate a diverse range of activities, which for pathogenic species include biofilm formation, host invasion pathways and the production of virulence factors. A number of reports have indicated that polymers capable of binding the signal molecules utilized for QS can lead to changes in the ability of certain bacterial strains to form surface-associated colonies or biofilms.[14, 15] However, it is also known that cell sensing mechanisms are activated when

bacteria are clustered or confined,[16] thus there are possibilities to interfere with QS by inducing cell aggregation in a controlled manner. Recently, we showed that polymer-induced aggregation led to predictable changes in QS for a variety of cell types,[17] and that QS signal activation and cell binding by polymers can lead to feedback in the QS pathways. Nevertheless, there are many complex interactions between bacterial cell signals, host cells, [18] biofilm formation[14, 19-21] and polymeric materials which remain to be clarified in order to design appropriate materials to combat or to prevent bacterial infections. A number of polymeric materials with the ability to bind bacteria and/or QS signals have now been demonstrated. [1, 22, 23] Amongst these materials, poly(N-[3-(dimethylamino)propyl] methacrylamide) (pDMAPMAm, P1 in Scheme 1) and poly(N-dopamine methacrylamide-co-N-[3-(dimethylamino)propyl] methacrylamide) (pDMAm-co- pDMAPMAm, P2 in Scheme 1) have shown their capacity to bind the Gram-negative bacterium *Vibrio harveyi* and form strongly-associated bacteria-polymer clusters. In addition, P2 is able to reduce the concentration of autoinducer-2 (AI-2), a QS signaling molecule used by *V. harveyi.* Accordingly, through this mechanism, P2 can interfere in the cell-cell communication system for this strain.



Scheme 1. Chemical structures of the polymers used in this study.

The link between bacterial clustering and QS in *V. harveyi* with P1 and P2 is of direct relevance for anti-infective strategies since microcolony formation following bacterial attachment is an important stage of biofilm development.[8, 24] While *V. harveyi* is not a human pathogen, other species of the genus *Vibrio* such as *Vibrio cholerae* and *Vibrio parahaemolyticus* are important causative agents in human disease and there are many other bacteria which are of immediate concern regarding infection. In particular, bacteria such as *Escherichia coli, Pseudomonas aeruginosa* and *Staphylococcus aureus* have pathogenic and antibiotic resistant strains and accordingly we were interested in whether polymers P1 and P2 would induce aggregation in these species, especially as QS signaling in some of these microorganisms had been shown to be altered in the presence of polymers P1 and P2.[17]

Here we present data describing the ability of the above polymers to induce aggregation in three Gram-negative and one Gram-positive bacteria species i.e. *V. harveyi*, *E. coli*, *P. aeruginosa* and *S. aureus* respectively. We also report aggregation properties at different pH values, as an additional mechanistic probe of bacteria-polymer interactions. Laser diffraction at different pH, fluorescence and confocal laser scanning microscopies were the main techniques used in these studies.

2. Materials and methods

2.1. Materials

Initiator V-501 was purchased from Fluka® and recrystallized from MeOH. Cascade Blue® ethylenediamine, trisodium salt was purchased from Life Technologies Ltd. Dulbecco's Phosphate Buffer Saline (PBS) 10X without Ca and Mg was purchased fromSigma-Aldrich®. Acetate Buffer (pH 5.5) was prepared according to literature.[25] Citrate Buffer 100 mM (pH 5.0) and Carbonate

Buffer 100 mM (pH 10.0) were prepared by dissolving the appropriate amount of citric acid and sodium bicarbonate respectively to achieve the indicated molarity. Solutions of sodium hydroxide 2M and hydrochloric acid 1M were used to adjust the pH. Poly(vinyl alcohol) (Mw 89,000-98,000, 99+% hydrolyzed) was purchased from Sigma-Aldrich®. All other chemicals were purchased from Sigma-Aldrich® or Acros® and used without further purification. All solvents were HPLC grade, purchased from Sigma-Aldrich® or Fisher Scientific®, and used without further purification.

Dopamine methacrylamide (DMAm)[26], benzyl 2-hydroxyethyl carbonotrithioate (CTA),[23] and p(L-DMAm)[23] (**P4**) [M_n (^{1}H NMR) 3934, DP=15] were synthesized according to protocols described in the literature.

2.2. Instrumentation

Nuclear Magnetic Resonance (NMR) spectra were recorded on a Bruker 400 MHz spectrometer. Chemical shifts are reported in ppm (δ units) downfield from internal tetramethylsilane (dmso-d_6) or the -OD signal (D_2O).

Cationic Gel Permeation Chromatography (CatGPC) was performed on a Polymer Laboratories GPC 50 with RI detector. Separations were performed on series of Eprogen columns [CatSEC 100, 300 and 1000 columns (250 x 4.6 mm), 5 μm bead size, 100, 300 and 1000 Å pore size respectively] fitted with a matching guard column (CatSEC100, 50 x 4.6 mm). The mobile phase was 0.1% TFA solution (pH 2) containing 100 mM NaCl at a flow rate of 0.5 mL/min. Aqueous Gel Permeation Chromatography (AqGPC) was performed on a Polymer Labs GPC50 Plus fitted with differential refractometer (RI), capillary viscometer (DP) and dual angle laser light-scattering (15° and 90°) detectors. The eluent was PBS, at 30 °C and a flow rate of 1 mL/min. The instrument was fitted with a Polymer Labs aquagel-OH guard column (50 × 7.5 mm, 8 μm) followed by a pair

of PL aquagel-OH columns (30 and 40, 300 × 7.5 mm, 8 μm). Molecular weights were calculated based on a standard calibration method using poly(vinylpyridine) (Scientific Polymer Products, CatGPC) or poly(ethylene glycol) (Polymer Laboratories, AqGPC) narrow standards.

Bacterial aggregation was determined by laser diffraction using a Coulter LS230 particle size analyser (Beckman Coulter, High Wycombe, UK). A EVOS™ FL Digital Inverted Fluorescence Microscope and a Zeiss LSM 700 confocal microscope were used for optical and fluorescent microscopy studies. A Beckman Coulter DU 800 UV spectrophotometer was used to confirm the absence of RAFT agents in polymers. The labelling of polymers was confirmed by a Cary Eclipse fluorimeter.

2.3. Bacteria strains and growth conditions

V. harveyi BB170 was a gift from Bonnie Bassler (Department of Molecular Biology, Princeton University). *E. coli* MG1655 mCherry was generated using a plasmid obtained from the Tsien laboratories.[27] The GFP-labelled strains were *P. aeruginosa* PAKR76, a pyocyanin-negative mutant (Δ*phzAG1*) of the Nottingham PA01 strain[28] and *S. aureus* Newman[29] carrying plasmid pSB2030.[30]

All strains were collected from the frozen stock and incubated overnight on LB-agar plates containing appropriate antibiotics (*V. harveyi*: 50 μg/mL of kanamycin; *E. coli*: 100 μg/mL of ampicillin; *P. aeruginosa*: 150 μg/mL of tetracycline; *S. aureus*: 10μg/mL of chloramphenicol). Then single colonies were selected from the LB-agar plates and grown in standard LB medium overnight. All bacterial cultures were incubated at 37 °C, except *V. harveyi* which was incubated at 30 °C.

2.3.1. Bacterial cell counts via colony forming units (CFU)

Bacterial suspensions were prepared from the overnight cultures of the strains growing in LB medium. The obtained suspensions were centrifuged (9,500 rpm, 4 °C, 10 min) and washed with PBS. The cells were finally resuspended with buffer to OD_{600} 1.0, 0.5 and 0.2. Then 10^6 fold dilutions in PBS were prepared. 100 μL of these working dilutions were added and spread over an agar plate in triplicate. The plates were incubated overnight at 37 °C and the number of cells was count and calculated (units x 10^7 cell/mL).

2.4. Polymer Synthesis

RAFT polymerizations were performed as previously described, with removal of RAFT agent prior to microbiological assays. [17, 23]

2.4.1. Polymer characterization data:

Poly(N-[3-(dimethylamino)propyl] methacrylamide) (DMAPMAm, **P1**) ^{1}H-NMR (D_2O, 400 MHz) δ (ppm) 4.0-3.1 (m, 4H, CH_2-N DMAPMAm), 2.90 (s, 6H, CH_3-N DMAPMAm), 2.1-1.9 (m, 3H, CH_3 MAm), 1.9-1.6 (m, 2H, CH_2 DMAPMAm), 1.2-0.8 (m, 2H, CH_2 MAm backbone), DP=46, M_n (CatGPC) 5304, PDI 1.31.

Poly(N-dopamine methacrylamide-co-N-[3-(dimethylamino)propyl] methacrylamide), [p(DMAm-co-DMAPMAm), **P2**] ^{1}H-NMR (D_2O/TFA 5:1, 400 MHz) δ (ppm) 7.70-6.69 (m, 3H, Ar-H), 3.65-3.48 (m, 2H, CH_2-N DMAm), 3.45-3.05 (m, 4H, CH_2-N DMAPMAm), 3.05-2.66 (m, >8H, N-CH_2-CH_2 DMAm + CH_3 DMAP), 2.24-1.55 (m, >5H, CH_3-MAm + HN-CH_2-CH_2-DMAPMAm), 1.34-0.73 (m, 2H, CH_2-MAm backbone) DP(DMAm)=9, DP(DMAPMAm)=91, M_n (AqGPC) 10812, PDI 1.04.

UV spectra of both P1 and P2 before and after RAFT agent removal are depicted in the Supplementary Material (Fig. S1).

2.5. Polymer Labelling

All the solutions containing fluorescent dyes were protected from the light by wrapping the glassware with aluminum foil.

2.5.1. Labelling of p(DMAPMAm) (P1)

Carboxylic acid terminated p(DMAPMAm) (P1) (50.0 mg, 4.00 μmol), N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) (2.31 mg, 12.0 μmol) and N-hydroxysuccinimide (NHS) (1.40 mg, 12.0 μmol) were dissolved in acetate buffer (9.00 mL, 10mM, pH 5.5). The solution was kept in an ice bath under stirring. Cascade Blue (CB) (2.51 mg, 4.00 μmol) was dissolved in acetate buffer (1.00 mL, 10mM, pH 5.5) and added to the mixture. The reaction was carried out overnight. p(DMAPMAm)-CB (P1-CB) was purified by dialysis against water and recovered as a white powder after freeze-drying from water (2 days). The labelling was confirmed by spectrofluorometry (λ_{em} 423 nm).

2.5.2. Labelling of p(DMAm-co-DMAPMAm) (P2)

p(DMAm-co-DMAPMAm) (P2) (50.0 mg, 23.0 μmol), 7-methoxycoumarin-3-carboxylic acid (MCCA) (10.5 mg, 46.0 μmol) and EDC (13.3 mg, 69.0 μmol) were dissolved in propionitrile/triethylamine (10.0 mL, 1:1, v/v). The solution was kept in an ice bath under stirring. 4-(Dimethylamino)pyridine (DMAP) (1.43 mg, 11.5 μmol) was added to the mixture. The reaction was carried out overnight. p(DMAm-co-DMAPMAm)-MCCA (P2-MCCA) was purified by dialysis against water and recovered as a light brown powder after freeze-drying from water (2 days). The labelling was confirmed by monitoring by spectrofluorometry (λ_{em} 402 nm).

2.5.3. Labelling of p(L-DMAm)

p(L-DMAm) (50.0 mg, 17.0 μmol), 7-methoxycoumarin-3-carboxylic acid (MCCA) (7.78 mg, 34.0 μmol) and EDC (9.86 mg, 51.0 μmol) were dissolved in propionitrile/triethylamine (10.0 mL, 1:1, v/v). The solution was kept in an ice bath under stirring. DMAP (1.06 mg, 8.57 μmol) was added to the mixture. The reaction was carried out overnight. p(L-DMAm)-MCCA was purified by dialysis against water and recovered as a light brown powder after freeze-drying from water (2 days). The labelling was confirmed by spectrofluorimetry (λ_{em} 402 nm).

2.6. Preparation of bacterial suspensions

Bacterial suspensions were prepared from overnight cultures of the strains grown in LB medium. The cells were diluted with buffer to an optical density (OD) 1.0 at 600 nm before they were mixed with the polymer solutions.

2.7. Measurement of polymer-bacteria clusters

Mean size and size distributions of bacterial clusters were determined under moderate stirring (default speed 5 setting, Beckman Coulter LS230) at the required concentration as indicated by the in-built display software. Particle size ranges were defined using PSS-Duke standards (Polymer Standard Service, Kromatek Ltd, Dunmow, UK). Particle size distribution was then determined as a function of the particle diffraction using the Coulter software (version 2.11a) and plotted as a function of the percentage of distribution volume.

In a typical experiment, 300-600 μL of a bacterial suspension with an OD_{600} of 1.0 were added to the flow cell filled with water (~14 mL) to obtain an obscuration of 8-12%. At this point the t_0 population distribution was recorded with constant mixing. Then 0.3 mL of 1 mg/mL or 5

mg/mL polymer solution in the corresponding buffer was added to 2.7 mL of the bacterial suspension. The mixture was allowed to equilibrate and the population distributions of bacterial suspensions in the absence and presence of polymers were recorded after 5, 15, 30 and 60 minutes. The experiments were performed in triplicate.

2.7.1. Microscopy

Aliquots (10 μL) of the samples used to measure average cluster size were collected after 60 min, mounted on a glass slide with a cover slip on top and examined with an optical/fluorescent microscope.

2.8. Statistical Analysis

GraphPad Prism 6 was used for statistical analysis by 2-way analysis of variance (ANOVA) using Tukey's multiple comparison test. The degree of significance is represented by intervals of *P* value, defined as: **** when $P \leq 0.0001$; *** when $P \leq 0.001$; ** when $P \leq 0.01$; * when $P \leq 0.05$ and *ns* (no significant) when $P > 0.05$.

2.9. Cell viability assays Bacteria viability in the absence and presence of polymer was evaluated using a LIVE/DEAD® BacLight Bacterial Viability Kit (Life Technologies, L13152). *V. harveyi* BB170, *E. coli* MG1655, *P. aeruginosa* PAKR76, a pyocyanin-negative mutant (Δ*phzAG1*) of the Nottingham PA01 strain[28] and *S. aureus* Newman[29] without incorporating additional plasmids for fluorescent protein synthesis were cultured as described in Section 2.3.

Overnight bacterial cultures in LB medium were centrifuged (9,500 rpm, 4 °C, 10 min) and resuspended in PBS (pH 7.4) to an optical density (OD) 1.0 at 600 nm. Aliquots (0.9 mL) of this bacterial suspension were mixed with 0.1 mL of PBS (positive control), P1 solution (1 mg/mL),

P2 solution (1 mg/mL) or 70% i-PrOH (negative control). The mixture was incubated for 4 h under shaking (200 rpm). After this time, 3 μL of a solution containing equal volumes of green (SYTO 9, 3.34 mM in DMSO) and red (Propidium iodide, 20 mM in DMSO) stains were added and this mixture was incubated at room temperature in the dark for 15 minutes. To remove i-PrOH, the negative control was centrifuged (13,000 rpm, rt, 1min) and resupended in PBS before staining.

For analysis, samples (10 μL) of the stained bacterial suspension were placed between a slide and an 18 mm square coverslip and observed in a fluorescence microscope. Three images of each sample were acquired in transmission, red and green channels. The fluorescent micrographs were then analysed by using ImageJ software. The values obtained (n=3) were then normalized against the corresponding controls in the red and green channels (negative and positive controls respectively) by using GraphPad Prism. In addition, the obtained values were also normalized against the sum of values of both red and green fluorescence together.

3. Results

3.1. Aggregation size analysis: Coulter Counter experiments

Polymers P1 and P2 were synthesized as previously described. [17, 23] Tertiary amine residues in both polymers are protonated at physiological pH providing an overall polycationic charge. Since the cell walls for bacterial strains used in these experiments are normally negatively charged [8, 10] under 'ambient' pH ranges, we anticipated the formation of polyelectrolyte-type clusters when these polymers were added to bacterial suspensions in biological buffer solutions. However, our hypothesis was that changes in solution pH, and also in the relative polymer-bacteria ratios would alter the nature and number of associative interactions. Accordingly, clustering experiments were

performed both at different pH and at different concentrations to observe the differences in clustering properties.

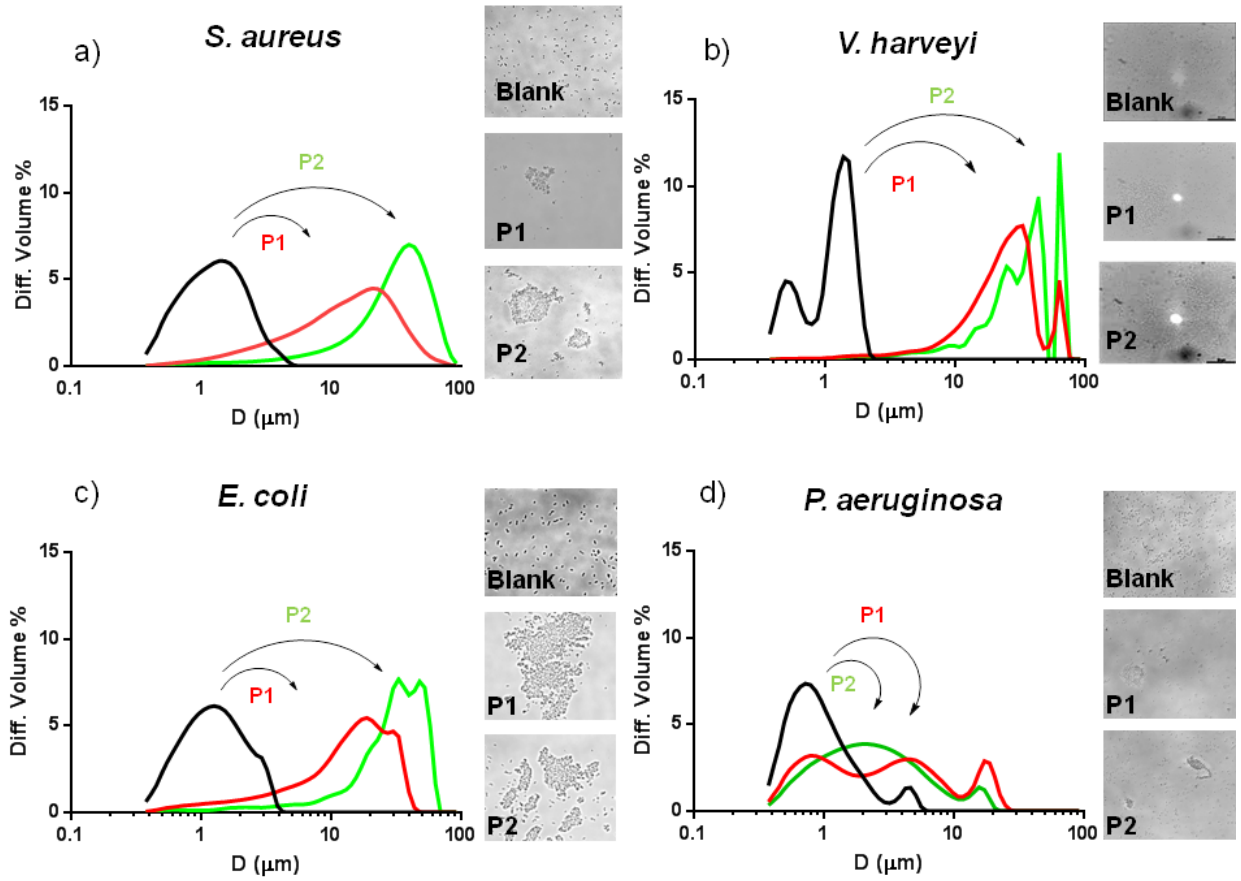


Fig. 1. Size distributions and optical micrographs of a) *S. aureus*, b) *V. harveyi*, c) *E. coli* and d) *P. aeruginosa* suspensions of OD_{600} 1.0 in the absence (black) and in the presence of P1 (red) and P2 (green) at a polymer concentration of 0.1 mg/mL after 60 min of mixing in PBS (pH 7.4). Only mean values are shown for clarity. Allexperiments were performed in triplicate.

Suspensions of four representative bacteria, the Gram-positive *S. aureus* and the Gram-negative species *V. harveyi, E. coli* and *P. aeruginosa,* were used in PBS pH 7.4 for the aggregation assays.

Stock solutions of P1 and P2 in PBS were added to these suspensions and after 60 min of incubation a significant level of cell-clustering was apparent in all cases (Fig. 1) although for *P. aeruginosa* the aggregation was less than for other species and a notable population of dispersed single cells remained (Fig. 1d). Optical micrographs taken in the absence and presence of P1 and P2 (Fig. 1) demonstrated cluster sizes in agreement with those obtained by Coulter counter measurements. Analogous time dependent experiments were performed at two polymer concentrations and are depicted in Fig. 2. Poly(vinyl) alcohol (PVA, P3 in Scheme 1) and poly(3,4-dihydroxy-L-phenylalanine methacrylamide) [P(L-DMAm), P4 in Scheme 1], were used as control polymers in these experiments as they are known QS signal sequestering agents [17, 23]. The greatest increases in cell cluster sizes were observed for mixtures of the Gram-positive *S. aureus* with copolymer P2 at a concentration of 0.1 mg/mL (Fig. 2) with the mean sizes increasing stepwise over time. The effects of P1 with increasing time followed the same pattern as P2, but smaller aggregates were generated.

At higher polymer concentrations, the aggregation induced by the homopolymer P1 was faster and comparable with P2, after 5-15 minutes of mixing, generating aggregates of similar size than those obtained at lower concentration. P2 also increased the size of microbial clusters at both concentrations with increasing time. However, the mean size of aggregates was reduced by 30% at 0.5 mg/mL in comparison with aggregates prepared at 0.1 mg/mL polymer, suggesting that lower sizes were related to higher polymer-bacteria ratios. Most experiments were performed with P1 at a degree of polymerization (DP) corresponding to 49 monomers per polymer chain, but additional experiments performed with an analogous polymer with DP of 99 (similar to that of P2) showed only slight increases in cluster sizes (see Supplementary Material).

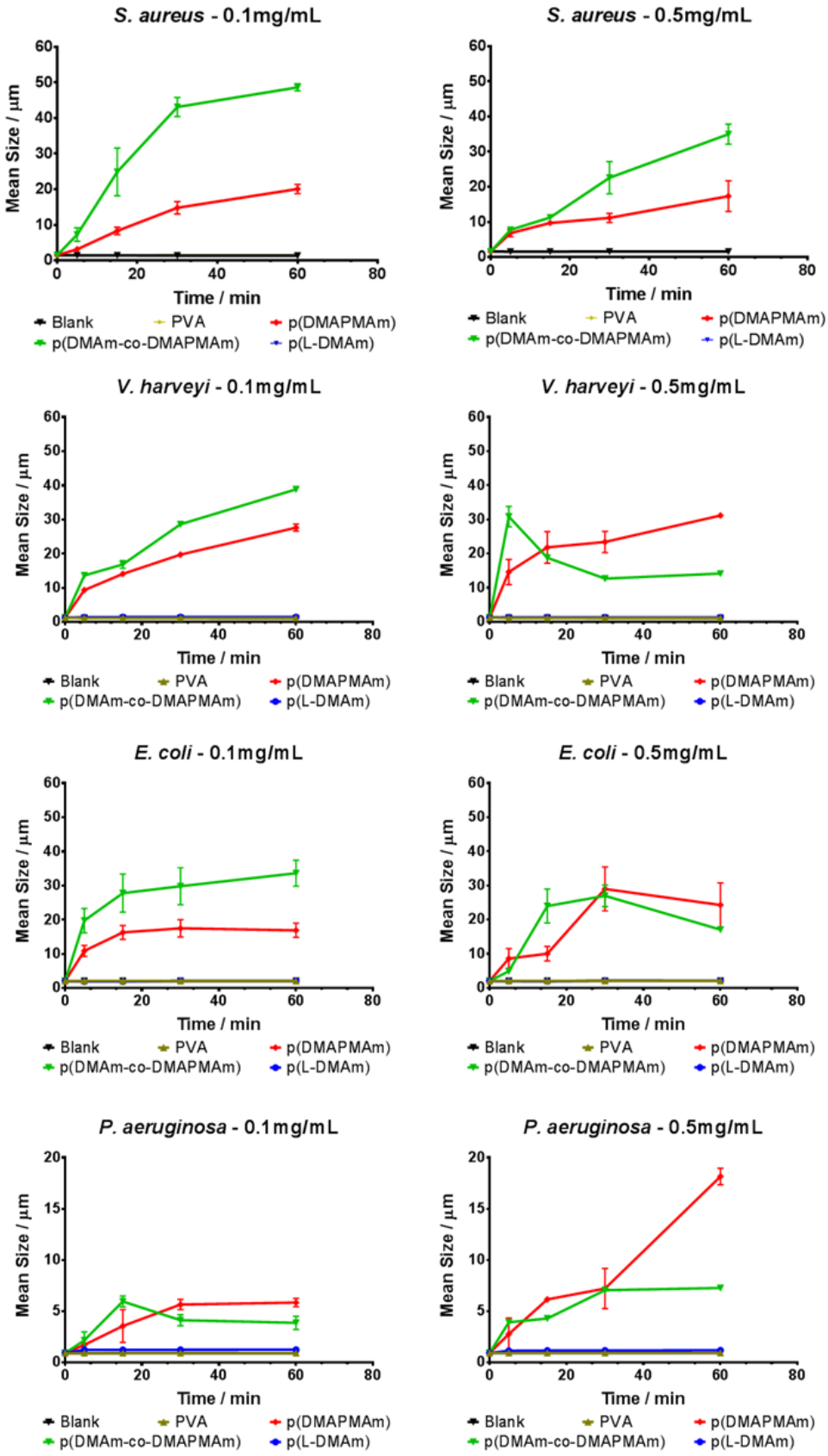

S. aureus - 0.1mg/mL
S. aureus - 0.5mg/mL
V. harveyi - 0.1mg/mL
V. harveyi - 0.5mg/mL
E. coli - 0.1mg/mL
E. coli - 0.5mg/mL
P. aeruginosa - 0.1mg/mL
P. aeruginosa - 0.5mg/mL
Mean Size / µm
Time / min
Blank
PVA
p(DMAPMAm)
p(DMAm-co-DMAPMAm)
p(L-DMAm)

Fig. 2. Mean size of aggregates *vs.* time for bacterial suspensions in the absence (black) and presence of different concentrations of p(DMAPMAm) (P1, red), p(DMAm-co-DMAPMAm) (P2, green), PVA, (P3, yellow) and p(L-DMAm) (P4, blue) (left 0.1 mg/mL, right 0.5 mg/mL). In the case of *P. aeruginosa*, the y-axis has been re-scaled for clarity. Error bars represent standard deviations (n=3).

The Gram-negative *V. harveyi* and *E. coli* behaved similarly regarding aggregation mediated by P1 and P2, and at polymer concentrations of 0.1 mg/mL. For P1 at lower concentrations there were no significant changes in polymer-bacteria aggregate size, but for P2 at 0.5 mg/mL, the cluster sizes increased rapidly over the first 15 min of experiments but subsequently reached a steady state or declined in size at later stages. This suggested that an equilibration related to polymer-bacteria ratios occurred, which may also have accounted for the narrow size deviations obtained. Regarding *P. aeruginosa*, the size of the aggregates was lower than in other cases, although significantly higher than the controls. Surprisingly, at 0.5 mg/mL of P1, the cluster sizes were similar to those induced in the other strains, indicating that for this bacterium, higher concentrations of strongly protonated side-chains on the polymer backbone were necessary to associate with the various charges on the *P. aeruginosa* cell wall (for statistical analysis see Table S1, Supplementary Material).

To investigate further the nature of these interactions, aggregation experiments at different pH were performed. Negative charges on bacterial surface polymers were anticipated to be partially neutralised in acidic media whereas the polymers were expected to have been fully polycationic under such conditions. Conversely, in basic media the polymers were envisaged to display a net reduction in their positive charges, becoming more hydrophobic, whereas bacteria might exhibit increased anionic content at their cell walls to enable substrate binding. This simple model was

based on the premise that although changes in bacterial wall charges are more difficult to predict (since living organisms are inherently dynamic), it was nevertheless expected the net charge in *S. aureus* would be less dependent of the pH than the Gram-negative strains[31, 32], and the negative charge of *S. aureus* and *E. coli* higher than that of *P. aeruginosa* at each pH value tested.[33].

3.2. Aggregation size analysis: pH variable experiments

The aggregation properties of both polymers with *S. aureus* were found to be mostly unaffected by pH changes (Fig. 3), although a faster clustering effect of P1 was observed when increasing the pH from 6 to 8 (Fig. 3a). Nevertheless, P2 clusters were larger at both pH 6 and 8 without an enhanced aggregation rate at the first time points of the experiment. The Gram-negative bacteria *E. coli* (Fig. 3c) and *P. aeruginosa* (Fig. 3e) followed a similar trend in the presence of P1. In both cases the cluster sizes were higher at higher pH, while there was no aggregation of *P. aeruginosa* at pH 6. Regarding P2 however, the difference in clustering of *E. coli* at pH 7 and 8 was less significant, whereas the mean size for aggregates of this polymer with *P. aeruginosa* increased dramatically at pH 8 (see Supplementary Material, Table S2).

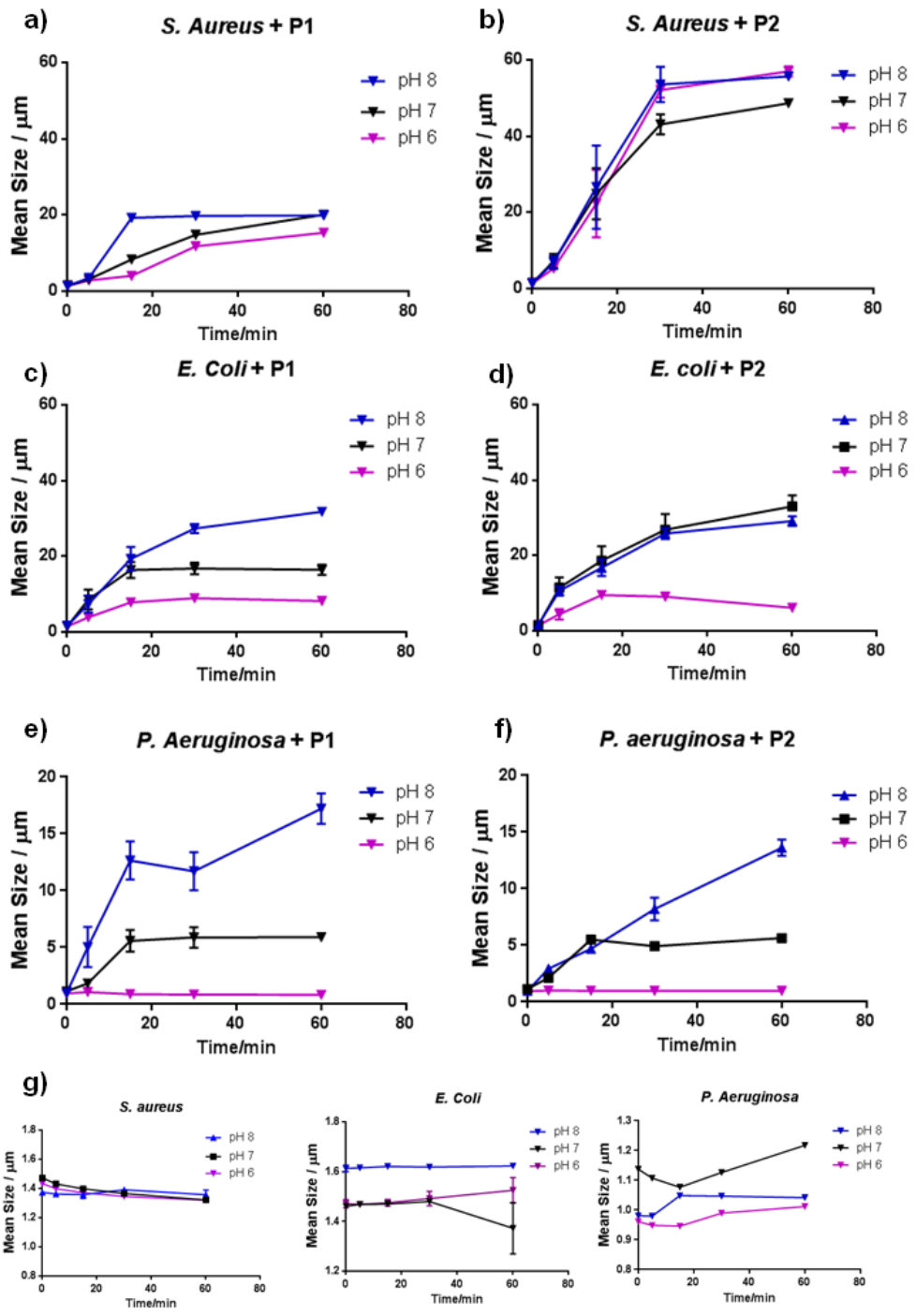

a)
S. Aureus + P1
b)
S. Aureus + P2
c)
E. Coli + P1
d)
E. coli + P2
e)
P. Aeruginosa + P1
f)
P. aeruginosa + P2
g)
S. aureus
E. Coli
P. Aeruginosa
Mean Size / µm
Time/min
pH 8
pH 7
pH 6

Fig. 3. Mean size of aggregates *vs.* time for bacterial suspensions of *S. aureus* (a,b), *E. coli* (c,d), *P. aeruginosa* (e,f) in the presence of P1 (left) and P2 (right) (0.1 mg/mL) at pH 6, 7 and 8 (PBS). Untreated bacteria (g) are shown for comparison. In the case of *P. aeruginosa*, the y-axis has been re-scaled for clarity. Error bars represent standard deviations (n=3).

For full consideration of polymer-cell associative properties it is important to assess the proportion of bacteria that remain unbound rather than just the polymer-cell cluster sizes. We reasoned that the effectiveness of the polymers at certain conditions would be linked to the absence of unbound bacteria independently of the cluster size. In Fig. 4, some selected sizing experiments are depicted. The cell-polymer 'association effectiveness' of P2 was manifestly higher in the case of *S. aureus* at 0.1 mg/mL at every pH, whereas cluster sizes in the presence of P1 revealed a higher population of unbound bacteria (Fig. 4a). For *V. harveyi*, free cells were only observable in the presence of P2 at a concentration of 0.5 mg/mL (Fig. 4b), whereas the non-associated fraction was negligible in the presence of P2 at a concentration of 0.1 mg/mL. For *E. coli* the effects of the polymers were similar to those with *S. aureus*, with P2 being more effective at 0.1 mg/mL at pH 7 (Fig. 4c), but less so at pH 8 (See Supplementary Material, Fig. S3). The relatively low *P. aeruginosa* cluster sizes mediated by both P1 and P2 showed however a low fraction of unbound bacteria at pH 8 and at a concentration of P1 of 0.5 mg/mL. These results reveal the optimal conditions for an efficient binding in every strain.

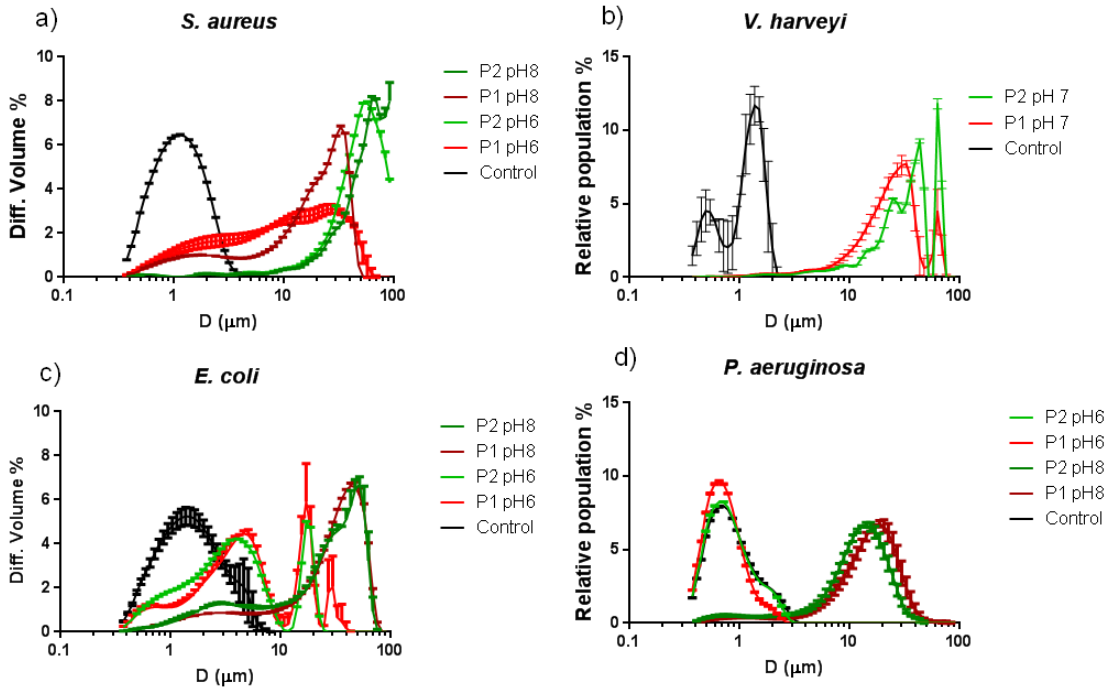


Fig. 4. Size distributions of a) *S. aureus*, b) *V. harveyi*, c) *E. coli* and d) *P. aeruginosa* suspensions of OD_{600} 1.0 in the absence (black) and in the presence of P1 (red) and P2 (green) at a polymer concentration of 0.1 mg/mL after 60 min of mixing at the pH shown.

We also performed variable pH experiments in the presence of P1 of DP 99. As expected from our previous experiments with *V. harveyi*,[17] clustering was essentially independent of the molecular weight (see Supplementary Material, Fig. S2, S3 and Table S3). In the case of *P. aeruginosa*, experiments at different concentrations and at pH 8 were also performed (see Supplementary Material, Fig. S4). However, the most efficient clustering of *P. aeruginosa* was observed, as depicted in Fig. 4d, in the presence of the homopolymer P1.

3.3. Colony forming units (CFU) experiments: Estimation of the bacterial cell number

For a robust analysis, experiments were required to correlate the number of bacteria with the overall optical density (OD) of suspensions containing the bacteria. The OD_{600} value indicates the optical density of a cell suspension at 600 nm and is a standard method to estimate the number of cells in suspension. This technique is based on the light scattered by particulates in suspension, so different cells sizes and shapes result in different intensities of scattered light. It is important to note that the experiments for this study were performed at a known polymer concentration and a constant OD_{600}, meaning that for bacteria of different sizes and shapes, a variation in cell numbers for a given OD_{600} would be observed. Accordingly, experiments to quantify colony forming units (CFU) were performed. The estimated numbers of cells for OD_{600} =1 were $1x10^9$ cell/mL for *E. coli*, $1.5x10^9$ cell/mL for *S. aureus*, and $1.7x10^9$ cell/mL for *P. aeruginosa*. Based on the experimentally-derived molecular weights of the polymers, the average numbers of individual chains in the cell clustering experiments were determined to be around $6x10^{15}$ for lower polymer concentrations (0.1 mg/mL) and $3x10^{16}$ for higher concentrations (0.5 mg/mL). The polymer/bacteria ratios were therefore $6x10^6$ chains/*E. coli* cell, $4x10^6$ chains/*S. aureus* cell and $3.5x10^6$ chains/*P. aeruginosa* cell for 0.1 mg/mL polymer solution with a DP of 99-100. We thus envisioned that individual polymer chains were bound to more than one individual cell, playing a role as crosslinker within a bacterial network.

3.4. Fluorescence and CLSM experiments

In order to probe further the aggregation of cells by the polymers, engineered bacterial strains expressing either the fluorescent proteins mCherry (*E. coli*) or the green fluorescent protein GFP (*S. aureus* and *P. aeruginosa*) were mixed with polymers P1 and P2 which had been labelled

with Cascade Blue (CB) and 7-methoxy coumarin-3-carboxylic acid (MCCA) respectively. Confocal Laser Scanning Micrographs (CLSM) obtained from suspensions of *E. coli* mixed with MCCA labeled P2 clearly indicated, from the analysis of image Z-stacks, that the polymers were located in between individual bacteria. The ortho projections (Fig. 5) from the overlaid red and blue fluorescent Z-stacks (Fig. 4a) showed separated regions of fluorescence deriving from the red-labelled cells and blue-labelled polymers within the cluster.

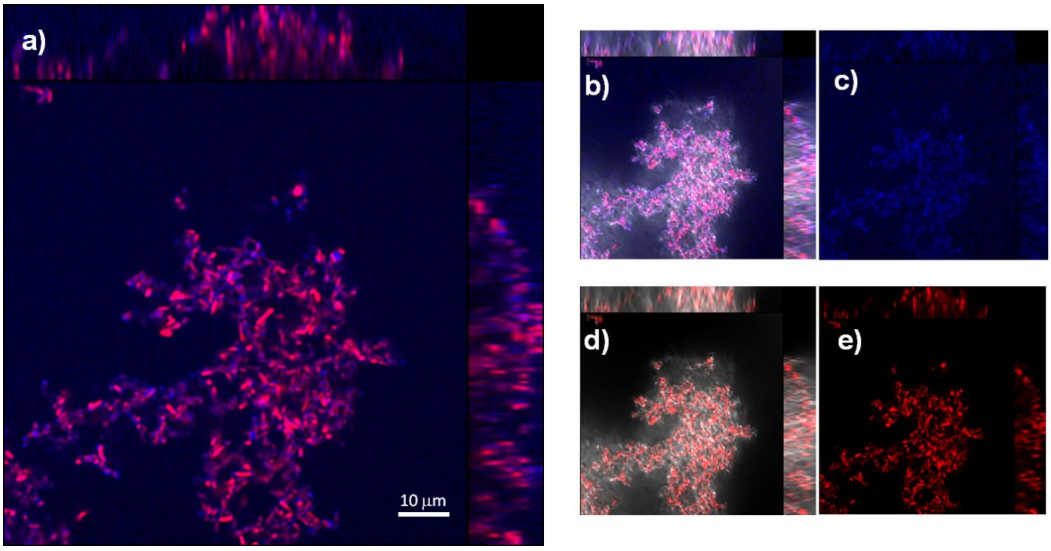


Fig. 5. Confocal Laser Scanning Micrographs (CLSM) of mCherry-*E. coli* in the presence of coumarin labeled P2. Ortho projections from the overlaid Z-stacks in the blue and red channel without (a) and including (b) the transmission micrograph; from the blue fluorescence (c) and red fluorescence including (d) and without including (e) the transmission micrograph. The contrast of the blue channel was increased for clarity.

The results were in agreement with additional fluorescence micrographs of other labeled bacteria in the presence of both P1 and P2 (Fig. 6). These results clearly demonstrate the ability of these

polymers to promote cell aggregates in all of the bacterial species analysed. Additional fluorescence micrographs and controls are shown in Supplementary Material (Fig. S7 and S8).

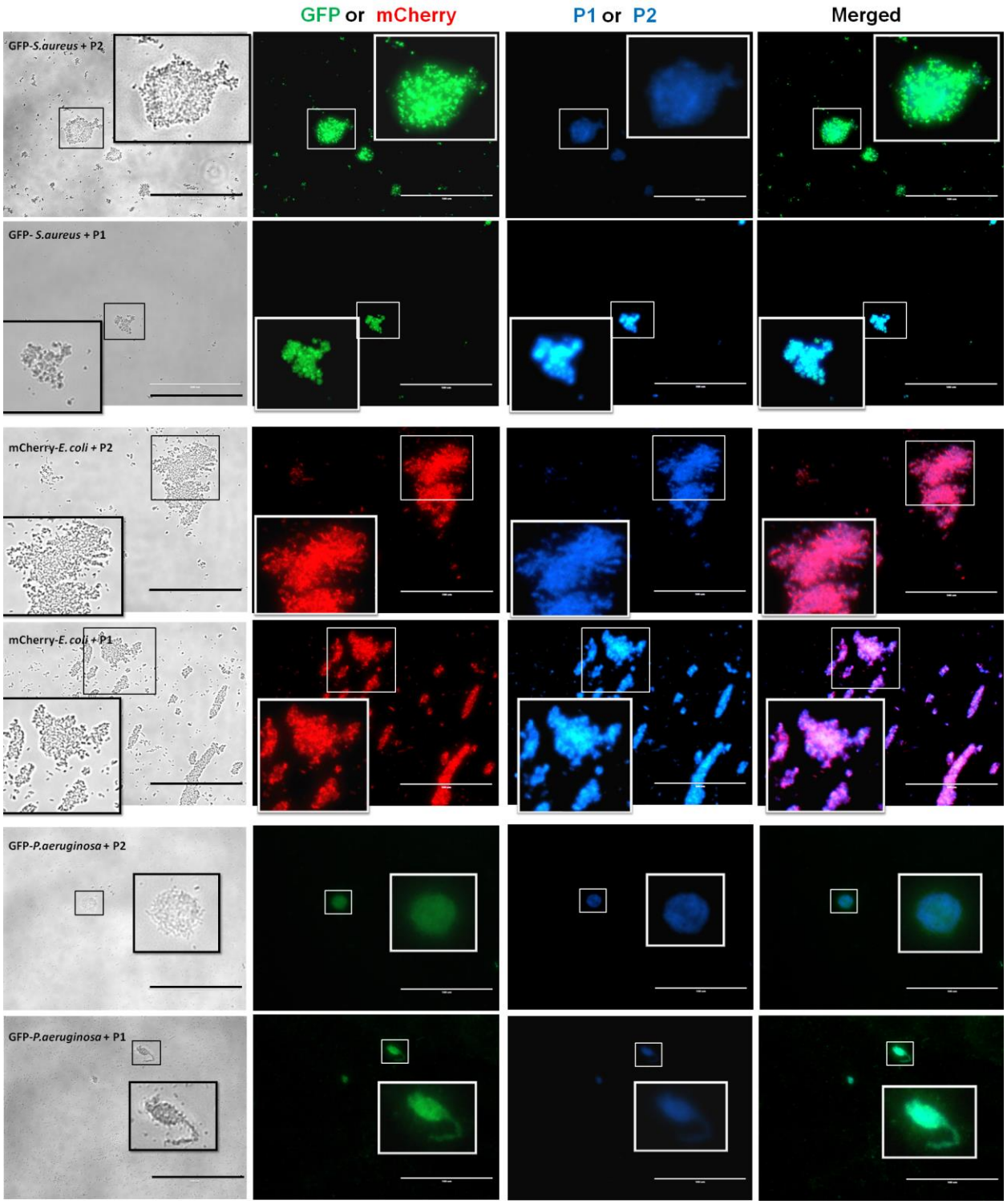


Fig. 6. Transmission and fluorescence micrographs of bacteria expressing the fluorescent proteins GFP (*S. aureus* and *P. aeruginosa*, green) or mCherry (*E. coli*, red) incubated with CB-

P1 or MCCA-P2 polymers (blue fluorescence). From left to right: transmission, red or green channel, blue channel and merged channels. Scale bar: 100 μm. Inset magnifications of the framed areas are shown for clarity.

3.5. Cell viability assays

In order to investigate possible effects of polycations on bacterial integrity and viability, fluorescent labelling assays were performed. SYTO 9 (green fluorescence) was used as a stain for both intact and damaged cell membranes, while propidium iodide was used as a marker for 'leaky' cells as it binds DNA (red fluorescence) only when it can enter a cell through a non-intact membrane. Therefore. any cells with damaged membranes display red fluorescence since the green fluorescence of SYTO 9 is attenuated by propidium iodide.

The results observed by fluorescence microscopy are depicted in Fig. 7. In general, there were both red fluorescent and green fluorescent bacteria in every sample, although the majority of cells fluoresced green. For a more in depth analysis, those red and green fluorescent intensities were normalized against the negative and positive controls respectively. The results are depicted in Fig. 8. However, as the number of bacteria in the different images is not the same, both intensities need to be compared and are shown for this purpose. In addition, the values corresponding to green and red fluorescence in the presence of P1 and P2, normalized against the sum of both fluorescence intensities are also shown. As the red (dead) and green (live) fluorescence intensities are also corresponding to the same images, both percentages of intensity are comparable. Positive and negative controls are depicted in Fig. S9 (Supplementary Material).

Although interpretation of the data in terms of specific cell viability is inexact, the intensity of red fluorescence is an indication of the degree of toxicity. From those experiments and analyses

the following can be inferred: a) The membrane damage produced by P1 ranged from less than 1% of the overall cell numbers (0.67% for *E. coli*) to 12% (*P. aeruginosa*), whereas b) P2 disrupted from between 1.7% of the cells in the assay (*S. aureus*) to 9.4% (*E. coli*). These mean values also include the numbers of cells which may have died during the timeframe of the experiments as a consequence of the inherent conditions of imaging (See Supplementary Information, Fig. S9).

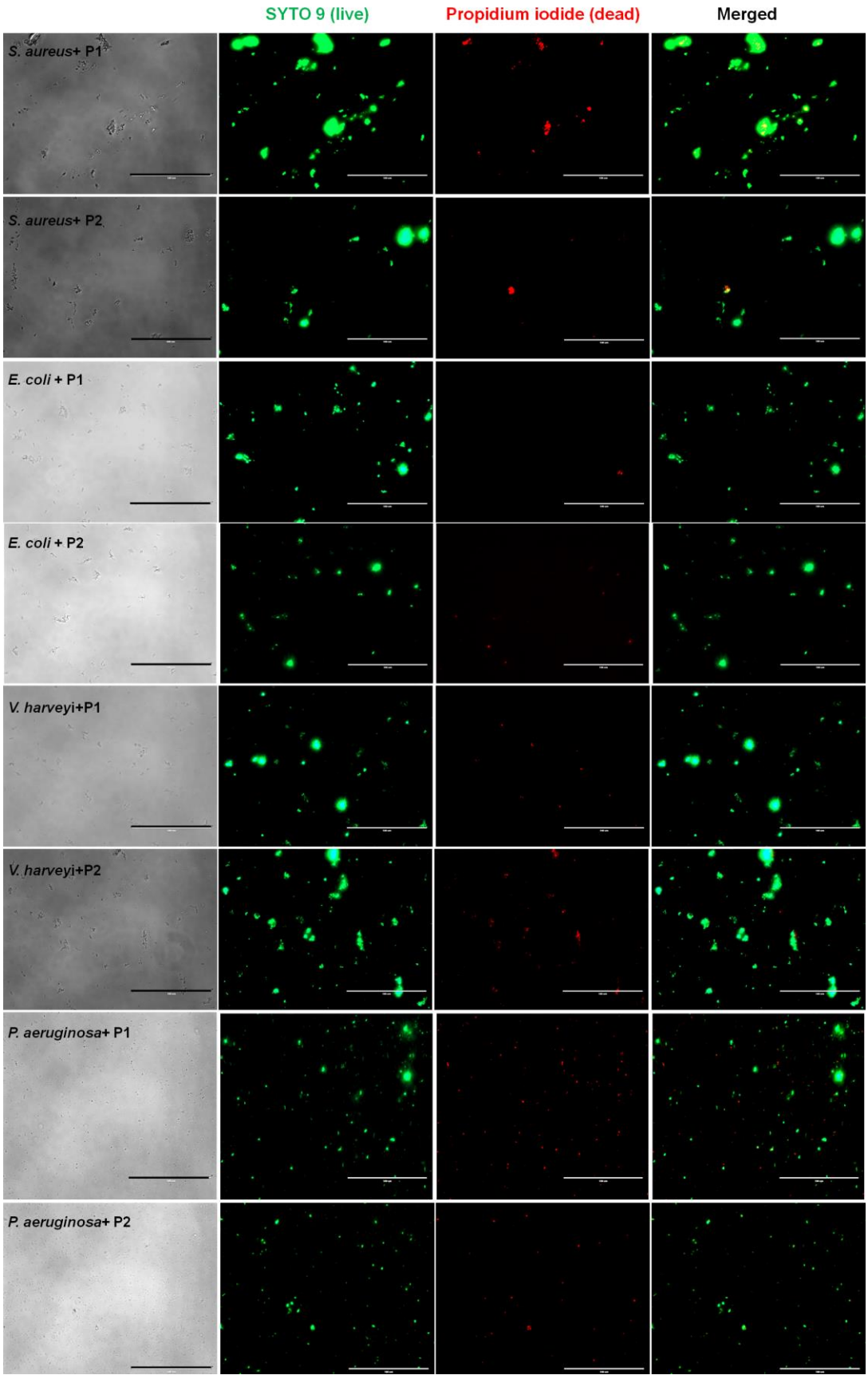

SYTO 9 (live)
Propidium iodide (dead)
Merged
S. aureus+ P1
S. aureus+ P2
E. coli + P1
E. coli + P2
V. harveyi+P1
V. harveyi+P2
P. aeruginosa+ P1
P. aeruginosa+ P2

Fig. 7. Transmission and fluorescence micrographs of bacteria with SYTO 9 (green fluorescence) and propidium iodide (red fluorescence) after incubation with the polymers P1 and P2. From left to right: transmission, green channel, red channel and merged channels. Scale bar: 100 μm.

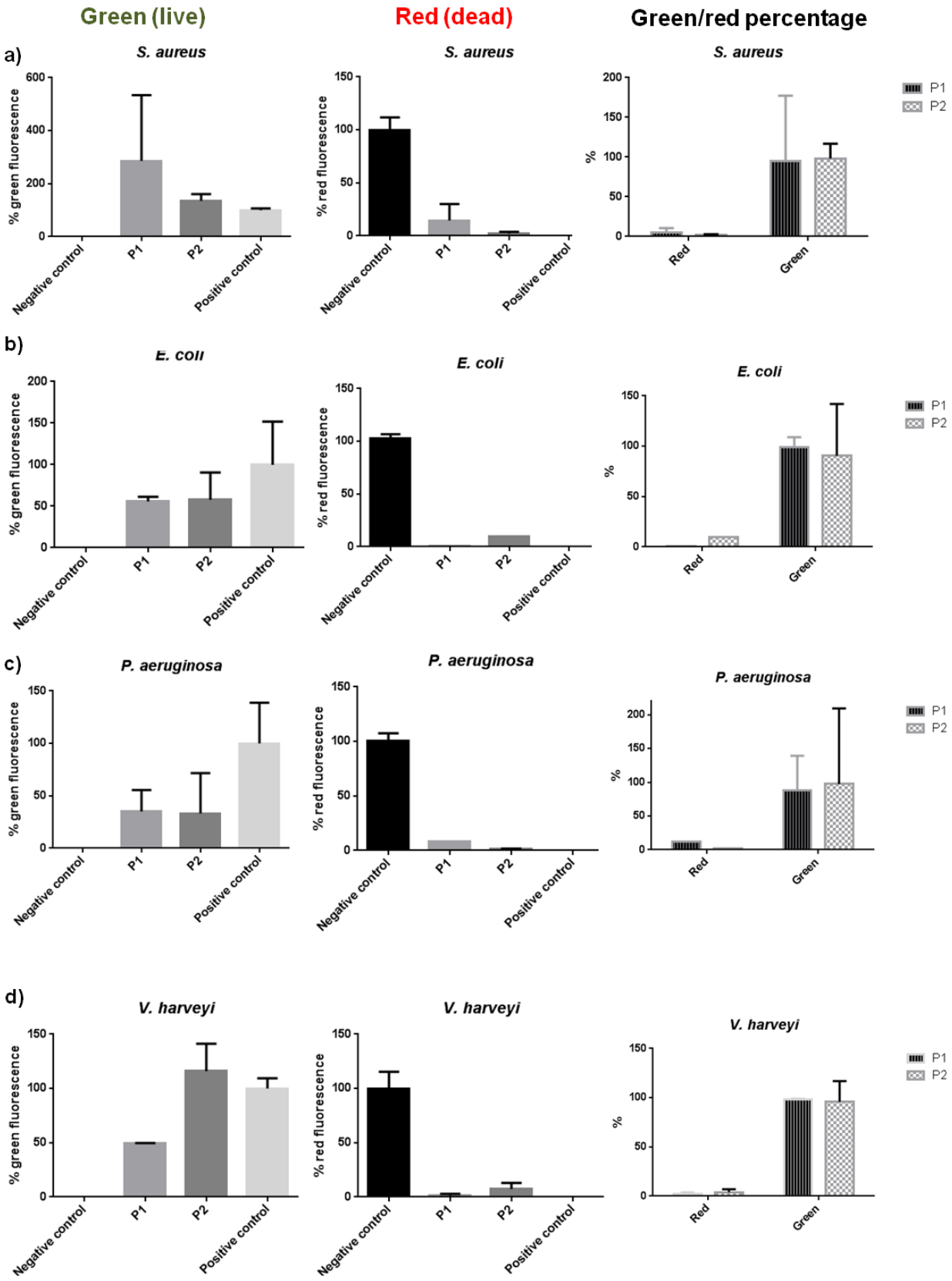

Green (live)
Red (dead)
Green/red percentage
a)
S. aureus
S. aureus
S. aureus
% green fluorescence
% red fluorescence
%
P1
P2
Negative control
P1
P2
Positive control
Red
Green
b)
E. coli
E. coli
E. coli
c)
P. aeruginosa
P. aeruginosa
P. aeruginosa
d)
V. harveyi
V. harveyi
V. harveyi

Fig. 8. Percentages of green (live) and red (dead) fluorescence for a) *S. aureus*, b) *E. coli*, c) *P. aeruginosa* and d) *V. harveyi* in the presence of P1 and P2 normalized against positive and negative controls. Right: Percentage of green and red fluorescence intensities normalized against the sum intensities. The error bars represent the standard deviation (n=3).

4. Discussion

We have recently described the ability of P1 and P2 to bind and cluster a range of Gram-negative bacterial species.[17] Since both P1 and P2 are polycationic and bacterial walls are negatively charged, attractive electrostatic interactions are the main driving force for this aggregation.[1] The marine pathogen *V. harveyi* was used as a model system because of the secretion and sensing of a QS molecule termed auto-inducer 2 (AI-2)[15, 23, 34]. Above a certain threshold concentration, *V. harveyi* responds to AI-2 by expressing the enzyme luciferase and becoming luminescent. As P1 is able to cluster these bacteria, increasing the local population density, the QS process and phenotypic responses were observed by an increased luminescence. However, P2 is able to cluster both bacteria and sequester AI-2 signals, emerging as an interesting case in which 'opposite' effects on cell behavior can occur concurrently [17, 23].

In the present work, we aimed to investigate the binding abilities of those polymers in different bacterial strains. The associative interactions of the polymers to bacteria and the subsequent clustering were expected to occur to varying extents based on a consideration of the biochemical features of the cell walls. Gram-positive and Gram-negative bacteria possess different surface components,[35, 36] although as noted earlier, we expect both to be negatively charged. The membranes of Gram-positive bacteria consist of a thick layer (up to 30 nm thick for *S. aureus*)[37] of cross-linked peptidoglycans, which lie outside the plasma membrane and which

contribute to make the cell wall porous. In these bacteria, the outer layer displays mainly teichuronic acid and lipoteichoic acids, whereas in Gram-negative cells a thinner layer of peptidoglycan occurs just underneath a second membrane, the so-called outer membrane, which consists of phospholipids and lipopolysaccharides (LPS), and contains membrane proteins.

The presence of negative charges in the bacterial cell walls has led to the development of synthetic polymers containing cationic ammonium groups as cell-binding materials with potential applications in detecting and inactivating bacteria. [8, 38] Of particular interest have been quaternary ammonium based polymers[10, 39, 40] as in the first instance, electrostatic attractive forces can be used to establish binding interactions of the polymers with the bacterial cell walls. Our results utilising differential fluorescent labels showed a cell surface binding and partial membrane disruption effect of both P1 and P2 for the bacteria used in this study and in the timeframe of our experiments. It is also possible that the polymers P1 and P2 exhibited other effects on bacteria, such as inhibition of growth and interference in cell communication as a result of their surface activity. However, these effects are downstream of cell surface binding and thus the key parameters to investigate were those controlling the initial polymer-cell clustering activity.

The different binding affinities of certain cationic polymers for disease-causing bacteria has recently been exploited to detect pathogens[41-44] or to discriminate between Gram-positive and Gram-negative bacteria and fungi.[45] It has been suggested that negative charges arising from teichoic acids are responsible for the higher binding of polycations with Gram-positive bacteria. In our studies we found that the P1 polycationic homopolymer formed bacterial clusters, as a

proxy for binding affinity, to a similar extent for both Gram-negative and Gram-positive bacteria in 10 mM PBS. However, in the case of P2, a copolymer containing both amino groups and catechol moieties, cluster formation was greater for three of the tested bacterial species compared to the solely polycationic nature of P1, and the effects were particularly pronounced for the Gram-positive *S. aureus*.

The tertiary amine groups within the main monomer component of both polymers, i.e. DMAPMAm, exist in solution as an equilibrium mixture of protonated and deprotonated (free amine) forms depending on local pH. The backbone and the alkyl chains connected to the side-chain amine provide hydrophobicity, thus any pH changes modify the cationic/hydrophobic ratio in the polymers. According to previous reports, polymers similar to P1 containing tertiary amines were completely protonated at pH values of 6 or below, but were completely deprotonated at pH above 10.[39] Therefore, at pH 6, both P1 and P2 should be fully cationic, maximising the attractive electrostatic interactions with the bacterial cell walls. In typical bacterial binding experiments, the number of polymer chains is higher than the numbers of individual bacteria, and thus following binding interactions with the polymer the bacterial surface charge is expected to change from a net negative value to an overall positive charge.[45] An excess, therefore, of polymer chains at the cell surfaces would disfavor the formation of cell polymer aggregates, as in effect, the bacteria have been coated by a charge-inverting cationic polymer layer. However, the binding properties of both polymers against *S. aureus* is less pH-dependent[31], in agreement with an expected constant cell wall charge,[31, 32] resulting in a lower positive net charge of the clusters and consequently, less repulsive aggregates. The results suggested that an increased bacterial surface charge or the possibility of partial hydrophobic interactions due to the decrease of polymer charge yielded larger clustering in Gram-negative bacteria. At higher pH (both pH 7

and 8), the protonated and deprotonated amines are in equilibrium, so the cationic nature and the electrostatic attractions are preserved, the eventual repulsions are weaker, and the hydrophobic properties increases, allowing additional interactions with hydrophobic components of the bacterial wall.

An increase of the polymer concentration does not increase cluster size or bacteria binding, with the exception of *P. aeruginosa*. In our opinion, increasing the number of polymer chains per bacterium may diminish the size of the clusters because a single chain is bound to a lower number of individuals. However, it is also important to note that a higher content of polycationic polymers within the clusters increases repulsive electrostatic effects.

Data obtained from the Coulter Counter experiments also indicates that the DP was not a significant factor in the overall size of cell-polymer agglomerates at the different conditions tested, although it was noted that the aggregates formed more quickly.

4.1. The Role of the Catechol (dopamine derived monomer in P2)

Catechol motifs are common within biomolecules, e.g the neurotransmitter dopamine or the amino acid L-DOPA (L-3,4-dihydroxyphenylalanine). Within the context of the present paper, catechol moieties may take part in the following interactions: a) with the siderophore receptors and b) with the fimbriae or pili. Although it is reasonable to assume a primary electrostatic interaction, the differences regarding cluster sizes and efficiencies in different conditions suggest a secondary binding effect from the dopamine derived monomers. This effect seems to take place at certain experimental conditions of concentration and pH, as cooperative or synergistic binding.

Catechols are a common motif in siderophores that bacteria use for the uptake of essential metals.[46] Gram-negative bacteria are able to interact with both the holo- and apo-forms of their siderophores[47] by means of Outer Membrane Transporters (OMT)[48]. Enterobactin, the *E. coli* siderophore, (Fig. S10, Supplementary Material) contains three catechol moieties and interacts with the specific OMT by means of the "plug" domain. It would not be surprising if additional interactions between these membrane proteins and P2 may take place, reinforcing therefore the overall bacteria-polymer affinity. Similar reasoning applies for *V. harveyi*, which utilizes the siderophore amphi-enterobactin (Fig. S10) which shares most structural features with enterobactin.[49] *P. aeruginosa* mainly uses pyoverdin and pyochelin as siderophores,[47, 50] peptide based structures that contain one catechol motif and a phenol group respectively (Fig. S10). As the number of catechol (or phenol) moieties in these siderophores is lower than the other ones previously mentioned, it is expected that a weaker additional binding takes place with *P. aeruginosa*. As expected from prior literature,[8, 10] strong electrostatic forces are the main interaction between the polymers and the bacterial walls. The secondary interactions between the catechol moieties and the possible features within the outer layers are expected to be much weaker. Since the control polymer p(L-DMAm) does not produce any bacteria clustering and the negative surface charge for *P. aeruginosa* is lower than the other species included in this study,[33] both P1 and P2 would be expected to behave similarly in binding interactions with *P. aeruginosa*, and indeed this was found experimentally (Fig. 3, 4). Also, a combination of positive charges and hydrophobic interactions seems to be the optimal combination for the polymers to bind *P. aeruginosa* wall together with a possible increase of the net negative charge at pH 8. Gram-positive bacteria also secrete siderophores that are retrieved by cell surface proteins. Specifically, *S. aureus* secretes polyacid based siderophores, staphyloferrin A (SA) and

staphyloferrin B (SB) (Fig. S10). These siderophores have been recently characterized[51-53] and they do not have catechol motifs in their structures. However *S. aureus* strains unable to express siderophores are able to grow in the presence of catechol based siderophores,[53-55] such as enterobactin or human hormones, for example epinephrine and dopamine. The Staphylococcus siderophore transfer protein D (SstD), is the receptor lipoprotein which recognizes catechol-based siderophores[56] and the K_d value for SstD-ferric dopamine is as low as 0.49 μM.[53] To interact with this protein the polymer would need to penetrate the highly crosslinked thick wall of peptidoglycan. The cut-off for free diffusion of hydrophilic proteins through an unstretched murein wall of Gram-positive bacteria has been calculated to be 25 kDa[57] in the absence of interactions within the wall components.[58] The molecular weight dependent ability of some polymethyl acrylate based linear polymers to penetrate bacterial walls has been previously described [59] in studies investigating their antimicrobial mechanisms. The antimicrobial activity of the polymers was found to be optimal between 50 and 120 kDa, and was assumed to be based on the permeability through the bacterial cell wall, until the polymers reached the cytoplasmic membrane. As a consequence, we would expect the polymers in this study to establish strong electrostatic interactions with the bacterial surface, but some polymer chains may penetrate the wall for additional interactions with SstD lipoproteins to promote a synergistic binding effect followed possibly by inter-chain entanglement.

It is known that fimbriae or pili are nonflagellar structures present in both Gram-positive and Gram-negative bacteria, and are involved in bacteria-host interactions, motility, biofilm formation and immunomodulation. [60] Although much is still unknown regarding their function, several strains of *E. coli* possess pili that promote biofilm formation and host-tissue adhesion by specific interactions [61],[18, 62-65] involving specific carbohydrate receptors. Pili

of Gram-positive bacteria are structurally different. [60, 66] The most studied sub-component is the Protein A from *S. aureus*, which is assumed to play a role in cell-substrate adhesion,[60, 67] and which has also been suggested to be important for iron uptake,[68]. We cannot rule out that the catechols may weakly interact with these features. However, the most common pili among *P. aeruginosa* strains [21, 60, 69] are more involved in nonspecific surface adhesion and motility. As a corollary, metal chelators have been found to disrupt biofilms of Gram-negative bacteria and to inhibit their adhesion to surfaces.[18] As catechols are also chelators, and in the case of P2 in our study, are polymer bound, both effects may have occurred in the cell-binding experiments, and may have counteracted each other in terms of clustering. It is not possible from results to date to ascertain whether chelation-induced changes in bacterial behavior or polymer-surface interactions are the most dominant in the induction of clustering. However, it is clear that there are relationships in the chemistry underlying these effects, and these in turn may have arisen from the environments in which bacteria evolved, wherein competition for co-factors in solution would have existed alongside advantages for surface-bound bacteria in obtaining nutrients.

Many of the L-DOPA and dopamine polymers described in recent literature have been synthesized because of their adhesive properties, inspired by the L-DOPA containing peptides that mussels utilize to attach to wet surfaces.[70-72] The mechanisms of catechol-mediated adhesion to surfaces are still not fully established, but metals may be important in some environments. The formation of catechol-metal complexes (e.g. Fe, Ca) and the ability of

catechol residues to polymerise to give cross-linked networks of polymers have been suggested as possible routes to promote adhesion. We cannot rule out similar events happening at the bacterial surface, particularly when electrostatic interactions arising from other residues have attracted the catechol residues close to the bacterial surface. In this respect, it should be noted that L-DOPA homopolymer (P4, Fig. 2) was not able to induce any clustering. This lack of clustering contrasts with the behavior of P1 and P2 and we therefore suggest that the combined action of polycationic polymers containing dopamine monomers produced a synergistic effect due to a primary electrostatic interaction and a secondary interaction based on the complex formation between the dopamine monomers and the bacterial wall in three of the bacterial strains tested.

Overall, the results described herein suggest a binding model schematically represented in Fig. 9. Bacteria cluster formation (Fig. 9a) is induced by the polymers through primary electrostatic forces between the polycationic polymers and the negatively charged cell wall in bacteria (Fig. 9b). As a consequence, polymers act as crosslinkers between individual bacteria (Fig. 9c). In the case of P1, this is the only interaction involved (Fig. 9d). P2 normally generate larger clusters than P1 and a more efficient binding (measured by the high population of cluster-forming bacteria). We believe this is a result of the additional moieties (catechols) in the structure of P2, which can establish weaker (but complementary) secondary interactions with the bacterial wall (Fig. 9e).

The lower net negative charge in *P. aeruginosa* may be responsible for a decreased binding and smaller cluster sizes at physiological pH. However, both the aggregate size and the binding

efficiency are higher at pH 8 or at higher polymer concentrations, with no apparent difference between P1 and P2. We believe that the lower positive charges of the polymers at higher pHs, together with the increase in their hydrophobicity (operating as a second interaction in this case) are responsible for these particular behaviors in *P. aeruginosa* (Fig. 9f).

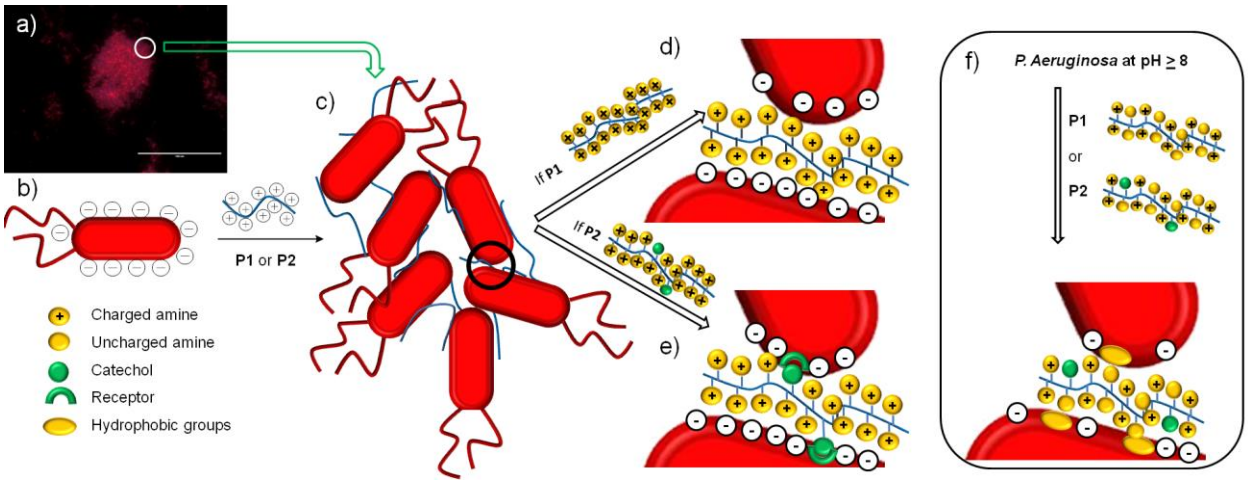


Fig. 9. Scheme of the proposed mechanism of binding: Initial clustering is driven by the electrostatic attraction between positively charged polymer and negatively charged bacteria (a and b). As these polycationic polymers are able to interact with several individuals simultaneously (c), the formation of clusters is favored. In the presence of P1 this is the only interaction (d). In the presence of P2 (e), secondary interactions between catechols and bacterial wall reinforce the binding. The combination of a possible increase in negative charge of *P. aeruginosa* and a lower polycationic nature of both P1 and P2 at pH 8 accordingly results in more efficient binding at this pH. Hydrophobic interactions between the outer membrane and the uncharged alkyl amines result in a secondary binding combined with electrostatic forces (f). Polymers and bacteria are not to scale.

5. Conclusions

In this work we have evaluated the ability of cationic and catechol containing polymers to cluster bacteria. The clustering ability of the polymers was evaluated against a range of bacteria, including Gram-negative *E. coli* and *P. aeruginosa*, and Gram-positive *S. aureus,* known human and antibiotic resistant pathogens, as well as the previously reported *V. harveyi*. Experiments at different pH have shown that clustering of bacteria is not a simple linear function of electrostatic interactions, and that balancing overall positive charge and hydrophobicity (by modulation of pH) has a profound effect on the clustering ability of these polymers. The effect of pH was marked for all gram-negative bacteria, and aggregation could be even inhibited for *P. aeruginosa* at low pH values (i.e. pH 6), where amine residues will be fully protonated, minimizing the hydrophobic character of the polymers. We have also shown that catechol moieties in these polymers have an unexpected synergistic effect, and act to enhance the binding of cationic polymers for most of the bacteria evaluated, especially in the case of gram-positive *S. aureus*.

These polymers may be useful if developed to interfere in processes which lead to human or animal infections such as biofilm formation, attachment of bacterial clusters to surfaces, and host-bacteria interactions. When it is considered that bacterial clustering has an effect in QS regulated gene expression, it may be possible to attenuate virulence of relevant pathogens such as *P. aeruginosa* through inducing 'undesirable' (from the cell's point of view) QS by clustering with these or related polymers. Initial clustering is driven by the electrostatic attraction between positively charged polymer and negatively charge bacteria In the future, incorporation of other binding motifs to achieve specificity in binding and /or cell signal sequestration for different microbial strains may have important benefits for the applications of these materials as non-biocidal antimicrobials .

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