Dendrimer mediated clustering of bacteria

Leire, Emma; Amaral, Sandra P; Louzao, Iria; Winzer, Klaus; Alexander, Cameron; Fernandez-Megia, Eduardo; Fernandez-Trillo, Francisco

DOI:
10.1039/c6bm00079g

Journal Name

ARTICLE

Dendrimer mediated clustering of bacteria: Improved aggregation and evaluation of bacterial response and viability.

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

Emma Leire,[a,b] Sandra P. Amaral,[a] Iria Louzao,[b,c] Klaus Winzer,[d] Cameron Alexander,[b,*] Eduardo Fernandez-Megia[a,*] and Francisco Fernandez-Trillo[e,*]

Here, we evaluate how cationic gallic acid-triethylene glycol (GATG) dendrimers interact with bacteria and their potential to develop new antimicrobials. We demonstrate that GATG dendrimers functionalised with primary amines in their periphery can induce the formation of clusters in *Vibrio harveyi*, an opportunistic marine pathogen, in a generation dependent manner. Moreover, these cationic GATG dendrimers demonstrate an improved ability to induce cluster formation when compared to poly(N-[3-(dimethylamino)propyl]methacrylamide) [p(DMAPMAm)], a cationic linear polymer previously shown to cluster bacteria. Viability of the bacteria within the formed clusters and evaluation of quorum sense controlled phenotypes (i.e. light production in *Vibrio harveyi*) suggests that GATG dendrimers may be activating microbial responses by maintaining a high concentration of quorum sensing signals inside the clusters while increasing permeability of the microbial outer membranes. Thus, the reported GATG dendrimers constitute a valuable platform for the development of novel antimicrobial materials that can target microbial viability and/or virulence.

Introduction

In recent years there has been an increase in the prevalence of microbial strains that can resist the action of current antibiotics.[1, 2] To overcome this resistance a significant effort has been placed both in the development of new classes of antibiotics, but also in exploring novel antimicrobial strategies[3, 4] which minimise the impact of infection without targeting microbial viability.[5] Anti-virulence methods target key stages in infection such as the adhesion of the pathogen to the host[6, 7] or the signalling networks that coordinate the expression of virulence factors.[8, 9] Because these strategies do not compromise microbial viability and survival, the evolutionary pressure imposed on microbes is much smaller than with conventional antibiotics and hence, resistance is less likely to develop.[10] Moreover, anti-virulence strategies can be combined with other antimicrobial treatments to obtain synergistic benefits.[11]

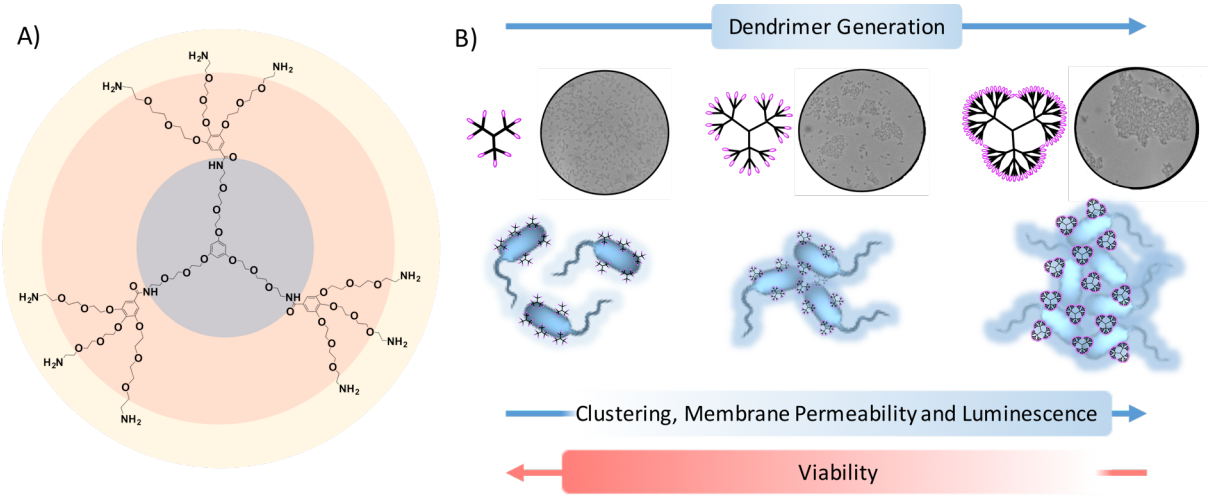
We, and others, have been exploring the use of polymeric materials to target these aspects of infections, reporting the bacterial clustering effect of cationic polymers with Gram-positive and Gram-negative bacteria.[12] Polymers are inherently multivalent and thus can be designed to outcompete the ligand-binding interactions observed during the adhesion of pathogens and toxins to hosts.[13] Since this adhesion is heavily affected by the spatial arrangement of the ligands, it is not surprising that a major effort has been placed in using dendrimers for preventing microbial attachment.[14, 15] Indeed, the monodispersity of dendrimers is a key advantage when interpreting affinity data in term of size and multivalency.[16] However, adhesion of polymers to bacteria surfaces can result in the clustering of these bacteria and the activation of signalling within those clusters.[17, 18] Polymeric materials are now starting to be reported as relevant alternatives to modulate microbial signalling where they can passively and actively target these signalling networks.[19-25]

Alternatively, polymeric materials have been investigated as readily accessible mimics of antimicrobial peptides.[26-28] These antimicrobial polymers exert a bactericidal effect by targeting bacterial membranes. The combination of cationic charge and hydrophobic character in antimicrobial polymers gives them interesting membrane properties, including increasing cell membrane permeability, that can result in bactericidal activity through lysis.

Here, we investigate the use of gallic acid-triethylene glycol (GATG) dendrimers[29] as a platform for the development of novel antimicrobial agents. We have focussed our attention in 3 areas – adhesion/clustering, signalling and membrane disruption – because of the relevance of polymeric materials. The presence of terminal azides in GATG and their derivatives in-

[a] Department of Organic Chemistry and Center for Research in Biological Chemistry and Molecular Materials (CIQUS), University of Santiago de Compostela, 15782 Santiago de Compostela, Spain. E-mail: ef.megia@usc.es
[b] School of Pharmacy, The University of Nottingham, Nottingham NG7 2RD, UK. E-mail: cameron.alexander@nottingham.ac.uk
[c] Additive Manufacturing and 3D Printing Research Group, School of Engineering, University of Nottingham, Nottingham NG7 2RD, UK
[d] School of Molecular Medical Sciences, Centre for Biomolecular Sciences, The University of Nottingham, Nottingham NG7 2RD, UK
[e] School of Chemistry, University of Birmingham. B15 2TT, UK. E-mail: f.fernandez-trillo@bham.ac.uk

† Electronic Supplementary Information (ESI) available: Dendrimer characterisation, additional microscopy images, full details of luminescence experiments and additional membrane permeability and viability data. See DOI: 10.1039/x0xx00000x



Scheme 1: A) Chemical structure of cationic GATG dendrimer (**G1-NH$_2$** is depicted) and B) interaction of GATG dendrimers with bacteria. Increasing generation (and multivalency) increases the ability of **Gn-NH$_2$** dendrimers to cluster bacteria. Similarly, increasing generation increases membrane permeability and enhances quorum sense controlled production of light in *V. harveyi*. Dendrimers and bacteria are not to scale.

troduces great flexibility in their peripheral decoration. These azides can be easily functionalized using highly efficient click-chemistries, and not surprisingly, the resulting materials have found application in the study of receptor-ligand interactions[30, 31] and cell internalization,[32] to interfere with protein-protein interactions[33] and amyloid formation,[34] or as imaging agents.[35] Moreover, these azides can be easily reduced to give primary amines and afford cationic dendrimers that have been applied in gene delivery.[36, 37] Bacteria are negatively charged, so cationic materials are also an obvious starting point in the development of antimicrobial agents that cluster bacteria,[12, 17, 18] potentially inhibiting their adhesion to the host. In addition, adhesion of cationic materials to bacterial membranes can result in membrane damage and bactericidal activity.[26-28]
Using *Vibrio harveyi* as a model organism, here we demonstrate that three generations of GATG dendrimers carrying 9, 27 and 81 primary amines in their periphery (Scheme 1) induce clustering of bacteria in a generation dependent fashion and that their ability to cluster bacteria is higher than p(DMAPMAm), a cationic linear polymer previously used in our laboratories. Also, we demonstrate that quorum sense controlled expression of luminescence by *V. harveyi* is activated in a generation dependent fashion, in agreement with the clustering observed. Finally, we evaluate if these GATG can induce membrane damage and elicit a potential bactericidal effect.

Experimental

Materials

Triphenyl phosphine was purchased from Sigma-Aldrich® and recrystallized from ethanol. Phosphate Buffer Saline (PBS, Dulbecco A) was purchased from Oxoid. 4,5-Dihydroxy-2,3-pentandione (DPD) was purchased from Ommcientific®. p(DMAPMAm), degree of polymerisation 46 and 99, was synthesised according to protocols described in the literature[18]. All other chemicals were purchased from Sigma-Aldrich or Acros and were used without further purification. All solvents were of HPLC grade and purchased from Sigma-Aldrich or Fisher Scientific and used without further purification. Baclight™ Bacterial Viability kit (L32856) was purchased from Life technologies™ and used for fluorescent live/dead staining for flow cytometry and Confocal Laser Scanning Microscopy (CLSM). When possible, experiments have been done in triplicate. Mean values and standard deviations are reported unless otherwise stated.

Instrumentation

Nuclear Magnetic Resonance (NMR) spectra were recorded on a Varian NMR Inova 500MHz spectrometer. Chemical shifts are reported in ppm (δ units) downfield from solvent peak (CD_3OD or $CDCl_3$). FT-IR spectra were recorded on a Bruker IFS-66v using KBr pellets or neat samples (CsI window). MALDI-TOF MS of the dendrimers at the azide stage were performed on a 4800 MALDI-TOF/TOF analyser (Applied Biosystems) with samples dissolved in MeOH supplemented with NaCl as cationizing agent, and 2-(4-hydroxyphenylazo)benzoic acid (HABA) or 2,5-dihydroxybenzoic acid (DHB) as matrixes. Dendrimer hydrodynamic diameters and ζ-potentials were measured by dynamic light scattering (DLS) and laser Doppler microelectrophoresis on a Nano-S Zetasizer (Malvern Instrument Ltd) at 25 °C. Molecular weight distributions were measured by size exclusion chromatography (SEC) on an Agilent 1100 series separation module using a PSS SDV pre-column (5 μm, 8 x 50 mm), a PSS SDV Linear S column (5 μm, 8 x 300 mm) and a PSS SDV Lux Linear M column (5 μm, 8 x 300 mm) with an Agilent 1100 series refractive index (RI) detector with THF as the eluent at 1mL/min flow rate and filtered through 0.45 μm before injection. Size of bacterial clusters was measured on a Coulter LS230 particle size analyser (Beckman Coulter, High Wycombe, USA) using laser diffraction. ζ-potential for bacteria clusters was measured on a NanoZS ZEN3600 Malvern Zetasizer. An L3001 Epifluorescent Microscope was used for optical imaging. A Zeiss LSM 880 confocal laser microscope was used

for fluorescent microscopy imaging. Labelling of the dendrimer was confirmed by a Cary Eclipse fluorimeter. Flow cytometry was performed on a BD LSRII Flow Cytometer. Luminescence and OD_{600} were measured in a Tecan Infinite Pro 200 Reader.

Dendrimer synthesis

Three generations of cationic GATG dendrimers (3[Gn]-NH_2·HCl (n = 1,2,3), abbreviated as **Gn-NH_2**) incorporating an aromatic trivalent core were synthesized through modification of a previously published route.[38-40] In brief, terminal azides in GATG dendrimers were reduced through a Staudinger reaction with PPh_3 in a MeOH – $CHCl_3$ – H_2O mixture and then protonated with HCl to obtain Gn-NH_2·HCl dendrimers. Reactions were monitored by ^{1}H-NMR (disappearance of the CH_2N_3 signal at 3.35-3.39.ppm) and FT-IR (disappearance of the characteristic azide peak at 2100 cm^{-1}) that confirmed a complete reduction of the azide groups (see ESI for further details). After solvent evaporation the crude was dissolved in H_2O and filtered through cotton, then it was washed with DCM and lyophilized to give **Gn-NH_2·HCl** dendrimers in excellent yields (96 - 99%).

G1-NH_2·HCl – ^{1}H NMR (500 MHz, MeOD-d_3) δ_H: 7.3 (s, 6H), 6.16 (s, 3H), 4.32 - 4.26 (m, 18H), 4.10 - 4.09 (m, 6H), 3.95 – 3.72 (m, 96H), 3.64 – 3.62 (m, 6H), 3.21 – 3.17 (m, 18H). IR (KBr) ν_{max}: 2929, 1584, 1119 cm^{-1}. MALDI-TOF MS of **G1-N_3** (HABA, reflected mode): *m/z* calcd for $C_{99}H_{156}N_{30}O_{39}Na$: 2412.1. Found $[M+Na]^+$: 2412.3.

G2-NH_2·HCl – ^{1}H NMR (500 MHz, MeOD-d_3) δ_H: 7.31 – 7.26 (m, 24H), 6.17 (s, 3H), 4.29 - 4.20 (m, 72H), 4.11 - 4.09 (m, 6H), 3.93 – 3.72 (m, 312H), 3.62 – 3.60 (m, 24H), 3.19 – 3.16 (m, 54H). IR (KBr) ν_{max}: 2929, 1638, 1118 cm^{-1}. MALDI-TOF MS of **G2-N_3** (HABA, reflected mode): *m/z* calcd for $C_{324}H_{507}N_{93}O_{129}Na$: 7792.0. Found $[M+Na]^+$: 7791.9.

G3-NH_2 ·HCl – ^{1}H NMR (500 MHz, MeOD-d_3) δ_H: 7.32 – 7.27 (m, 78H), 6.18 (s, 3H), 4.29 - 4.19 (m, 234H), 4.11 (bs, 6H), 3.92 – 3.60 (m, 1038H), 3.20 – 3.16 (m, 162H). IR (KBr) ν_{max}: 2878, 1638, 1117 cm^{-1}.

Fluorescent labelling of G3-NH_2

Stock solutions of 7-methoxycoumarin-3-carboxylic acid (MCCA) (7.9 mg/mL in dry DCM), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC·HCl) (6.9 mg/mL in dry DCM) and N-hydroxysuccinimide (NHS) (8.3 mg/mL in dry MeOH) were prepared. 20 μL of those stock solutions (2.5 equiv.) were placed in a round-bottomed flask. 100 equiv. of Et_3N (3.6 μL) were added. 7.1 mg of G3-NH_2 were dissolved in 600 μL of dry MeOH. This dendrimer solution was then added slowly to the previous mixture under stirring and stirred overnight. The solvent was removed under a nitrogen flow, the crude re-dissolved in H_2O and dialysed against H_2O for 48 h (14 kDa MWCO). The labelling was confirmed by fluorimetry (λ_{ex} 330 nm, λ_{em} 402 nm).

Bacteria strains and growth conditions

V. harveyi BB170 was a gifts from Bonnie Bassler (Department of Molecular Biology, Princeton University). *V. harveyi* BB170 was grown from a frozen stock on Luria Bertani (LB) agar plates containing 50 μg/mL kanamycin. Bacteria were incubated overnight at 30 °C with 50 μg/mL kanamycin. LB medium: 10 g/L of tryptone, 5 g/L of yeast extract and 10 g/L of NaCl. This medium was autoclaved at 120 °C for 20 min before use.

Aggregation Assay

A single colony of *V. harveyi* from LB agar plates was used to inoculate 2 mL of LB medium containing 50 μg/mL kanamycin and incubated overnight at 30 °C. Bacteria were centrifuged at 9,000 rpm for 5 min at 4 °C, the supernatant was discarded and bacteria re-suspended in PBS. This washing step was repeated two times and bacteria were finally re-suspended at an OD_{600} = 1.0. Aliquots of the bacteria culture were mixed with known volumes of stock solutions of Gn-NH_2 in PBS. The values of the concentrations reported correspond to the final concentration (mg/mL) of dendrimer in the bacteria-suspension.

Size: Mean size and size distributions of bacterial clusters were determined under moderate stirring (default speed 5-6 setting, Beckman Coulter LS230) at the required concentration as indicated by the in-built display software. Aliquots of the dendrimer-bacteria suspension in PBS were taken at different times (5-60 min range) and added to a Coulter flow cell filled with H_2O (<14 mL) to reach the required optical obstruction indicated by the built-in software (8-12%, between 500-1,500 μL). Particle size ranges were defined using PSS-Duke standards (Polymer Standard Service), Kromatek Ltd., Dunmow, UK). Particle size or cluster size distribution was determined as a function of the particle diffraction using the Coulter software (version 2.11) and plotted as a function of the percentage of distribution volume. Cluster size was measured in triplicate at each time point. Experiment was repeated three times.

Microscopy: 10 μL of the Gn-NH_2–bacteria suspension in PBS used for measuring cluster size were taken after 60 min and mounted on a glass slide with a cover slip for optical and confocal microscopy imaging. For confocal microscopy, 10 μL of a solution of the MCCA labelled G3-NH_2 was added to 90 μL bacteria in PBS and incubated for 60 min. 1 μL of SYTO-9 stain (0.033 mM in DMSO) was added to this dendrimer-bacteria suspension and incubated in the dark for 10 min at room temperature prior to imaging. For SYTO-9; λ_{ex} = 488 and λ_{em} = 522, for MCCA; λ_{ex} = 405 and λ_{em} = 460. In all cases, confocal micrographs have the following colour representation: green (green channel) and magenta (blue channel).

Membrane Permeability using Flow Cytometry

A single colony of *V. harveyi* from LB agar plates was used to inoculate 2 mL of LB medium containing 50 μg/mL kanamycin and incubated overnight at 30 °C. Bacteria were then centrifuged at 9,000 rpm for 5 min, supernatant was discarded and bacteria were re-suspended in AB medium or PBS at an OD_{600} = 1.0. For membrane permeability during aggregation assays in PBS; 90 μL of the bacteria culture was added to 10 μL of stock solutions of Gn-NH_2 in H_2O or H_2O alone (control) and incubated for 1 h at room temperature. 1 μL of a mixture of SYTO-9 stain (0.033 mM in DMSO) and propidium iodide (20 mM in DMSO) (PI) 1:1.5 was added to each sample and incubated at room temperature in the dark for 10 min prior to experiment.

For membrane permeability during luminescence experiment; bacteria were diluted 5,000-fold in AB medium. 90 μL bacteria in AB medium was mixed with 10 μL of stock solutions of Gn-NH_2 in H_2O or H_2O alone (control). Bacteria-dendrimer suspensions were incubated at 30 °C for 10 h, thereafter, centrifuged for 5 min at 9,000 rpm and re-suspended in 500 μL of 0.85% NaCl. 1 μL of a mixture of SYTO-9 stain (0.033 mM in DMSO) and PI (200 mM in DMSO) 1:1 was added to each sample and incubated at room temperature in the dark for 10 min prior to experiment. Negative controls were incubated with 70% *i*-PrOH for 1 h followed by 2 washes with media (centrifugation 10,000 rpm, 5 min, rt). Bacteria were counted in a Flow Cytometer. 100,000 events per sample were counted. The values of the concentrations reported correspond to the final concentration (mg/mL) of dendrimer in the bacteria-suspension. Each condition was done in triplicate and the experiment was repeated three times. Data were analysed using Kaluza® Flow Analysis Software (Beckman Coulter Inc.).

Luminescence Assay

A single colony of *V. harveyi* from LB agar plates was used to inoculate 2 mL LB medium containing 50 μg/mL kanamycin and incubated overnight at 30 °C. Bacteria were then diluted to OD_{600} = 1.0 in boron-depleted Assay Broth (AB) medium and then inoculated in the same medium (5,000:1). Boric acid was added to a final concentration of 22 μM. 180 μL of the inoculated AB medium was added to each well of a 96-well plate and mixed with 20 μL of the sample to be analysed. Light production and OD_{600} were recorded at 30 °C every 30 min for at least 10 h in a 96-well plate. The normalized luminescence was calculated by dividing the light output by the optical density at each time point. Luminescence recovery time was defined as the time for normalised luminescence to reach the initial value for each individual sample. Each compound was tested over at least 3 different concentrations and each concentration was run in triplicate. Experiment was repeated three times. AB Medium: 0.3 M NaCl, 0.05 M $MgSO_4$ x $7H_2O$, 0.2% vitamin-free casamino acids, 1 mM L-arginine, 1% glycerol, 0.01 M K_2HPO_4, pH 7.0. This medium was autoclaved at 120 °C for 20 min before use.

Results and discussion

Dendrimers are attractive materials to interface with biology.[41-43]. They are monodisperse polymers, with controlled sizes and numbers of terminal groups that can present biological information (e.g. multiple copies of ligands or drugs) in a precise and unique spatial arrangement. Not surprisingly, dendrimers have been investigated as mimics of natural ligands (e.g. glycans) and exploited to interfere with host-pathogen interactions.[13-15] In this work, we decided to investigate the potential of GATG dendrimers to develop novel antimicrobial agents. GATG dendrimers are composed of a repeating unit incorporating a gallic acid core and hydrophilic triethylene glycol arms.[29] They are biocompatible, and easy to manufacture and scale up. Generations in GATG dendrimers are grown following a two-step reaction sequence involving an initial amide coupling between the terminal amines at a certain generation and the carboxylic acid at the GATG repeating unit, followed by reduction of the azides to afford a new layer of primary amines (Scheme 1). This strategy can be easily scaled-up to multigram quantities of these materials.[39] Here, we evaluate the antimicrobial activity of a new family of GATG dendrimers prepared using a 1,3,5-tri(2-(2-(2-aminoethoxy)ethoxy)ethoxy)benzene core. As opposed to traditional GATG dendrimers that carry a gallic acid core, this new family is expected to display a more globular architecture. Dendrimers with 9, 27 and 81 primary amines, corresponding to generations G1-NH_2 – G3-NH_2, were obtained by quantitative reduction of the peripheral azides, easily monitored using FT-IR spectroscopy (Figure S1) (complete disappearance of the azide signal at 2100 cm^{-1}) and determined by ^{1}H-NMR (disappearance of the CH_2N_3 peak at 3.35-3.39 ppm) (Figure S2).[40]

Clustering of *V. harveyi* by Gn-NH_2

The first potential antimicrobial strategy that we evaluated was adhesion and clustering of bacteria. Previous work in our group has shown that linear cationic polymers readily form polymer-bacteria clusters by electrostatic interactions with the negatively charged bacterial surface.[12, 17, 18] Herein, by using aminodendrimers where the size and number of terminal groups can be accurately controlled by design, we aimed to modulate systematically the antimicrobial properties of these polycations. We anticipated that the globular and branched architecture of GATG dendrimers would have a beneficial generation dependent effect on their interaction with bacterial membranes and thus in clustering. As in our previous reports, we used *V. harveyi*, an opportunistic marine pathogen, as a model organism. It is closely related to human pathogens *Vibrio cholerae* and *Vibrio parahaemolyticus* and, like these microbes, uses a complex signalling network to regulate its virulence.[44] Importantly, *V. harveyi* is luminescent and this luminescence provides an easy readout to evaluate *V. harveyi* viability and behaviour.

When *V. harveyi* BB170 was incubated with GATG dendrimers a clear generation dependent increase in cluster formation could be observed (Figure 2). The ability of these dendrimers to cluster bacteria was compared to linear p(DMAPMAm), previously used in our laboratories for this purpose. The high clustering ability of GATG dendrimers allowed for these experiments to be performed in PBS which was significant because under these conditions none of the p(DMAPMAm) polymers tested (DP46 and DP99) were able to induce any clustering of *V. harveyi* (Figure 2A). While no apparent increase in cluster size was observed for G1-NH_2, which only has 9 primary amines in its periphery (Figure 2A), a clear increase in cluster size was immediately apparent when *V. harveyi* BB170 was incubated with the dendrimers of higher generations, with clusters of over 4.5 μm forming for G3-NH_2. Inspection of the size distributions (Figure 2B) suggested that as the multivalency of the dendrimer increased, the number of free bacteria (population centred at 1 μm) was reduced. A similar effect could be observed when these clusters were inspected under the mi-

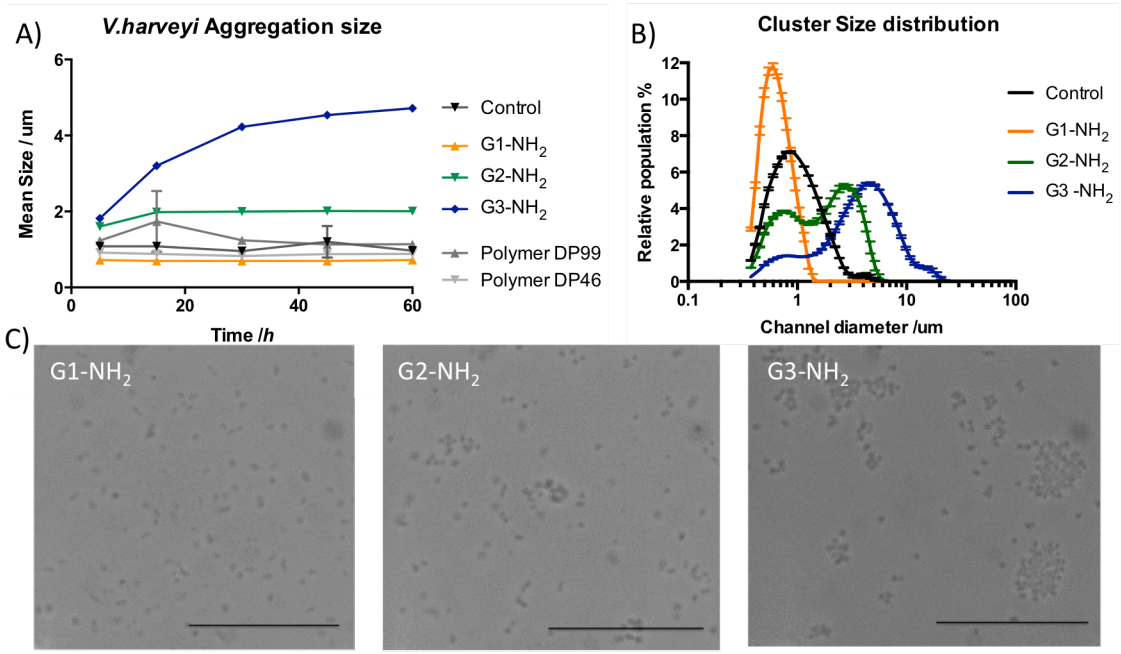


Figure 2: A) Mean cluster size of *V. harveyi* BB170 in the absence (black) and presence of G1-NH_2 (orange), G2-NH_2 (green), G3-NH_2 (blue), and p(DMAPMAm) polymer DP46 (light grey) and DP 99 (dark grey). B) Size distribution of *V. harveyi*'s clusters in the absence (black) and presence of G1-NH_2 (orange), G2-NH_2 (green) and G3-NH_2 (blue). C) Aggregation as observed by optical microscopy (100X magnification). The black bar represents 30 μm. Initial OD_{600} = 1, [NH_2] = 1.64 mM in PBS at pH 7.4.

croscope (Figure 2C), with aggregates of increasing size forming with increasing dendrimer generation.

It is worth noting that the average size obtained for the bacteria in the presence of G1-NH_2 was slightly smaller (orange triangles, Figure 1B, ~0.6 μm) than that of *V. harveyi* in the absence of the dendrimers (black triangles, Figure 1B, ~0.9 μm). However, since reported bacteria sizes are measured relative to calibrants (i.e. polystyrene beads), they do not represent the actual size expected for *V. harveyi* (~1.5 μm), but do inform of relative changes in size. Taking into account the size of these dendrimers (less than 3 nm for G1-NH_2, 3.6 nm for G2-NH_2, and 5.7 nm for G3-NH_2; Table S1), it is quite reasonable that G1-NH_2 does not have the minimum required size and multivalency to efficiently bridge between multiple bacteria and none or few small clusters are formed. Size distributions in the presence of G1-NH_2 were narrower (0.25-1.05 μm) than in its absence (0.30-3.00 μm), suggesting that G1-NH_2 still binds to the surface of *V. harveyi* and modifies its surface properties, potentially preventing the natural tendency of microbes to aggregate. A similar reduction in size could be observed as well for the population of smaller size when *V. harveyi* was incubated with G2-NH_2 (~0.75 μm) and G3-NH_2 (~0.65 μm), suggesting that these cationic dendrimers were able to bind to *V. harveyi* and, depending on their size and multivalency, also induce the formation of bacterial clusters.

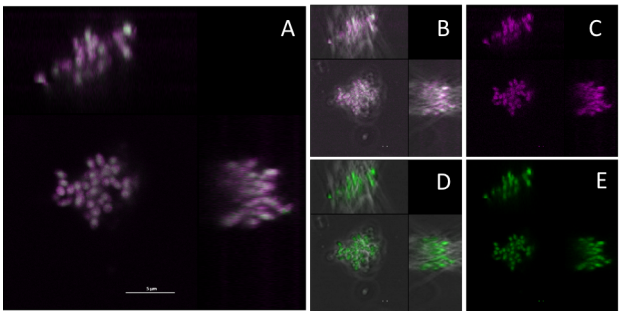


Figure 1: Confocal Laser Scanning Micrographs of *V. harveyi* BB170 (green) in the presence of MCCA labelled G3-NH_2 (magenta). Ortho projections from the overlaid magenta and green channels (white) including Z-stacks without (A) and with (B) transmission micrograph. Ortho projections for the blue (C) and green channel with (D) and without (E) transmission micrograph. In all cases, blue channel has been depicted magenta for clarity.

Further evidence of the interaction between Gn-NH_2 dendrimers and *V. harveyi* was obtained from ζ-potential measurements. As expected, the bacteria presented an overall negative potential (-64.4 ± 5.9 mV, Figure S6), representative of their negatively charged membranes. In the presence of the dendrimers however this negative potential was reduced. Once again, this effect was generation dependent, with G3-NH_2 showing the biggest effect.

In order to verify that the dendrimers were co-located with the bacteria in the clusters, G3-NH_2 was labeled with MCCA, a fluorescent blue dye, and incubated with *V. harveyi* BB170 stained with SYTO-9, a fluorescent green dye permeable to microbial membranes (Figure 1 and Figure S5). Co-localization was apparent by comparing the green (SYTO-9, bacteria) and magenta (MCCA labelled G3-NH_2) channels in confocal laser scanning microscopy (Figure 1). Z-stack analysis showed that the MCCA labelled G3-NH_2 is present throughout the cluster. The high density of white fluorescent clusters and the absence of green fluorescence within the clusters indicates colocalization of both dyes suggesting that this cationic GATG dendrimer was able to bind to the bacterial membrane.[12]

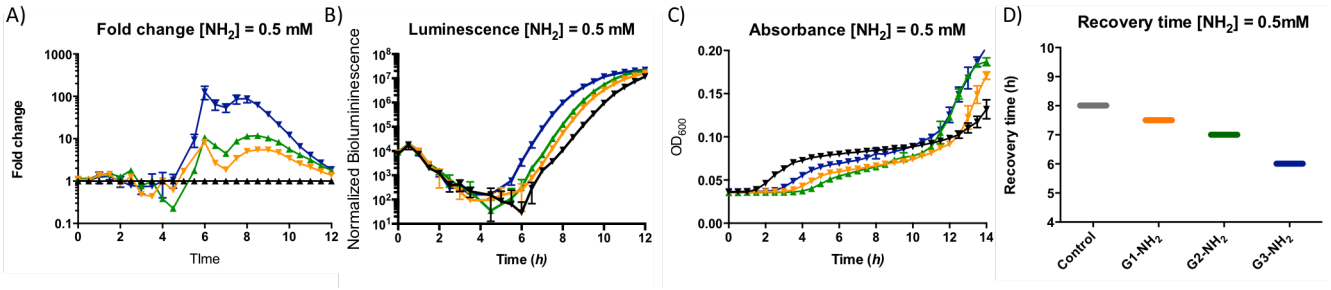


Figure 3 Luminescence of *V. harveyi* BB170 in the absence (black) and presence of G1-NH_2 (orange), G2-NH_2 (green) and G3-NH_2 (blue). [NH_2] = 0.5 mM. A) Fold increase of luminescence, B) normalised luminescence, C) OD_{600} and D) recovery time.

Luminescence *of V. harveyi* in the presence of Gn-NH_2

Based on our previous work with linear cationic polymers,[17, 18] we decided to investigate the ability of these dendrimers to modulate the expression of quorum sense controlled luminescence in *V. harveyi*. We anticipated that a generation dependent effect on the production of light should be observed, in agreement to that identified during the aggregation experiments (Figure 2-Figure 1). When *V. harveyi* BB170 was incubated with these dendrimers, a significant increase in the production of light was observed that was proportional to the amount of dendrimer added (Figure S9-Figure S11). When the same concentration of amines (e.g. 0.5 mM) was evaluated across the different dendrimers used, a clear generation effect could be observed (Figure 3). Light production in *V. harveyi* was increased more than ten times when it was incubated with G1-NH_2 at this concentration, and over two orders of magnitude when treated with the highest generations of dendrimers.

This increase was also apparent when we evaluated the time taken by bacteria to recover the initial levels of luminescence (~3 x 10^3, Figure 3B). Under normal conditions, *V. harveyi* switches off light production following dilution into the assay broth and it takes almost 8 h for *V. harveyi* to reach a population density that can produce similar levels of luminescence than the levels observed at the beginning of the experiment. In the presence of these GATG dendrimers however, this level of luminescence could be reached in as little as 6 h for the dendrimers of higher generation (Figure 3C). This generation effect in recovery time was also observed at the other concentrations of amino groups tested (0.25 and 1 mM, Figure S12).

Such a significant increase in luminescence was noticeable, in particular considering the poor ability of G1-NH_2 to cluster bacteria (Figure 2). This enhancement of light production was even more remarkable when we evaluated the effect these GATG dendrimers had on the growth *of V. harveyi*. Monitoring of optical density at 600 nm (OD_{600}) during the luminescence experiments showed that all three Gn-NH_2 were able to delay growth in *V. harveyi* (Figure S13-Figure S14), suggesting that *V. harveyi* were achieving similar levels of light production at even lower densities of bacteria. This effect was dose-dependent, but not generation dependent, with higher concentrations of dendrimers showing the highest effect in microbial growth. Remarkably, even at the lowest concentration of amino groups evaluated (0.25 mM, Figure S9-Figure S14), where a negligible effect on growth could be observed for all 3 generations of GATG dendrimers (Figure S13A), enhancement of light production could be observed.

Membrane permeability *of V. harveyi* in the presence of Gn-NH_2

Cationic polymers have been extensively investigated as potential mimics of antimicrobial peptides.[26-28] Due to the cationic nature of our GATG dendrimers, we decided to investigate if they were able to induce membrane permeability and as such have an alternative mode of antimicrobial activity. In this regard, delayed growth of *V. harveyi* in the presence of these GATG dendrimers was observed at the highest concentrations tested during luminescence (Figure S14), suggesting a potential bacteriostatic effect.

Incubation of *V. harveyi* (under the aggregation assay conditions) with a mixture of SYTO-9 and propidium iodide (green and red stains for cells with intact and damaged membranes) revealed that as the generation of dendrimers increased, a growing number of bacteria were stained red (Figure 4 and Figure S7). This increase in red staining is indicative of higher permeability of the membrane with larger generations of dendrimer. While membrane permeability for G1-NH_2 was not higher than control, over 50% of the bacteria were stained red in the presence of G2-NH_2 and up to 85% in the case of G3-NH_2, suggesting that these dendrimers could have a complementary bactericidal effect.

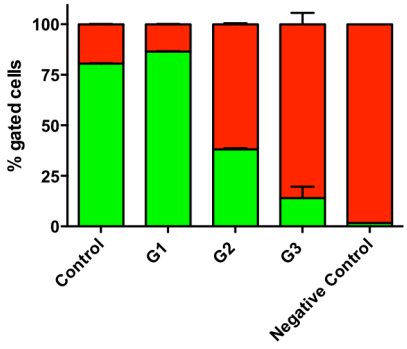


Figure 4: Normalized population of *V. harveyi* BB170 presented as the percentage of green (non-permeable/viable) and red (high permeability/dead) cells as measured by flow cytometry. *V. harveyi* was incubated in the absence (control) and presence of Gn-NH_2 for 1h at room temperature. Bacteria were treated with *i*-PrOH as a negative control. Initial OD_{600} = 1, [NH_2] = 1.64 mM in PBS at pH 7.4.

From the luminescence assays, all GATG dendrimers showed a remarkable effect on the production of light. However, at early time points Gn-NH_2 showed a concentration-dependent bacteriostatic effect (Figure S14), which was consistent across the 3 generations of dendrimers tested (Figure S13). Accordingly, any reduction of the amount of light produced as a result of the slower growth of *V. harveyi* should affect equally all generations. At the same time, these dendrimers were inducing a generation dependent clustering and thereby were likely activating quorum sense controlled light production in the order G1<G2<G3 (Figure 2-Figure 3).[18] Moreover, membrane permeability assays showed that G2 and G3 dendrimers led to increasing membrane permeability under the conditions employed to monitor aggregation (Figure 4), an effect that was also observed when this permeability assay was performed under the luminescence assay conditions (Figure S8). This is not unexpected, as membrane disruption has been observed for primary amines in multivalent scaffolds, but can be alleviated if wanted with alternative cationic GATG dendrimers carrying secondary/tertiary amines or quaternary ammonium salts.[26-28] This increased permeability caused by the higher generation dendrimers could facilitate the diffusion of quorum sensing autoinducers inside the periplasm and thus enhance light production from the bacteria. Experiments to elucidate the relative contributions of aggregation and permeability on light production have commenced in our laboratories using super-resolution microscopy.

Conclusions

In summary, in this manuscript we have evaluated how cationic GATG dendrimers interact with bacteria, interactions that could result in antimicrobial applications for this family of dendrimers. We have focused our evaluation in 3 areas – adhesion/clustering, signalling and membrane disruption – where we anticipate these dendrimers will have a higher impact. Thus, we have shown that cationic GATG dendrimers induce clustering in *V. harveyi* in a generation dependent manner and that this clustering ability is greater than that of a cationic linear polymer, p(DMAPMAm), previously shown to be a potent sequestrant of bacteria. Also, cationic GATG dendrimers induced the expression of quorum sense controlled luminescence in a generation dependent fashion, while inhibiting the growth of *V. harveyi* at the highest concentrations of amines tested. Moreover, these cationic dendrimers were able to increase the membrane permeability of the bacteria, again in a generation dependent manner. These results together highlight the potential of GATG dendritic materials as a platform to develop new antimicrobials that can target microbial viability and/or virulence (e.g. adhesion): Inhibition of growth and increased membrane permeability in combination with cell clustering may be promising antibacterial features of these cationic dendrimers.

Acknowledgements

This work was financially supported by the Spanish Government (CTQ2012-34790) and the Xunta de Galicia (GRC2014/040). E.L. thanks the European Commission, Education, Audiovisual and Cultural Executive Agency (EACEA) for an Erasmus Mundus grant under the NanoFar Joint Doctoral Program. F. F-T. thanks the University of Birmingham for the John Evans Fellowship. C. A. thanks the Engineering and Physical Sciences Research Council for a Leadership Fellowship (EP/H005625/1) and the research grant EP/G042462/1. I. L. has been supported by the EPSRC grant EP/K005138/11. The authors thank Cheng Sui (University of Nottingham) for the gift of p(DMAPMAm), Bonnie Bassler (Department of Molecular Biology, Princeton University) for the gift of *V. harveyi* BB170 and Tim Overton (University of Birmingham) and David Onion (University of Nottingham) for their guidance with regards to flow cytometry.

Notes and references

1. E. A. R. S. Network, *Antimicrobial resistance surveillande in Europe*, European Centre for DIsease Prevention and Control, 2013.
2. W. H. Organization, *Antimicrobial resistrance:global report on surveillance*, World Health Organization, 2014.
3. C. Nathan, *Science translational medicine*, 2012, **4**, 140sr142.
4. J. O'Neill, *Antimicrobial Resistance: Tackling a crisis for the health and wealth of nations*, The Welcome Trust HM Government, 2014.
5. D. A. Rasko and V. Sperandio, *Nature reviews. Drug discovery*, 2010, **9**, 117-128.
6. P. Klemm, R. M. Vejborg and V. Hancock, *Appl. Microbiol. Biotechnol.*, 2010, **88**, 451-459.
7. A. M. Krachler and K. Orth, *Virulence*, 2013, **4**, 284-294.
8. T. Praneenararat, A. G. Palmer and H. E. Blackwell, *Organic & biomolecular chemistry*, 2012, **10**, 8189-8199.
9. R. P. Singh, *MedChemComm*, 2015, **6**, 259-272.
10. R. C. Allen, R. Popat, S. P. Diggle and S. P. Brown, *Nature reviews. Microbiology*, 2014, **12**, 300-308.
11. E. E. Gill, O. L. Franco and R. E. Hancock, *Chem. Biol. Drug Des.*, 2015, **85**, 56-78.
12. I. Louzao, C. Sui, K. Winzer, F. Fernandez-Trillo and C. Alexander, *Eur J Pharm Biopharm*, 2015, **95**, 47-62.
13. A. Bernardi, J. Jimenez-Barbero, A. Casnati, C. De Castro, T. Darbre, F. Fieschi, J. Finne, H. Funken, K. E. Jaeger, M. Lahmann, T. K. Lindhorst, M. Marradi, P. Messner, A. Molinaro, P. V. Murphy, C. Nativi, S. Oscarson, S. Penades, F. Peri, R. J. Pieters, O. Renaudet, J. L. Reymond, B. Richichi, J. Rojo, F. Sansone, C. Schaffer, W. B. Turnbull, T. Velasco-Torrijos, S. Vidal, S. Vincent, T. Wennekes, H. Zuilhof and A. Imberty, *Chem. Soc. Rev.*, 2013, **42**, 4709-4727.
14. A. Imberty, Y. M. Chabre and R. Roy, *Chemistry (Weinheim an der Bergstrasse, Germany)*, 2008, **14**, 7490-7499.
15. M. A. Mintzer, E. L. Dane, G. A. O'Toole and M. W. Grinstaff, *Molecular pharmaceutics*, 2012, **9**, 342-354.

16. Y. M. Chabre and R. Roy, in *Advances in Carbohydrate Chemistry and Biochemistry*, ed. H. Derek, Academic Press, 2010, vol. Volume 63, pp. 165-393.
17. X. Xue, G. Pasparakis, N. Halliday, K. Winzer, S. M. Howdle, C. J. Cramphorn, N. R. Cameron, P. M. Gardner, B. G. Davis, F. Fernandez-Trillo and C. Alexander, *Angewandte Chemie (International ed. in English)*, 2011, **50**, 9852-9856.
18. L. T. Lui, X. Xue, C. Sui, A. Brown, D. I. Pritchard, N. Halliday, K. Winzer, S. M. Howdle, F. Fernandez-Trillo, N. Krasnogor and C. Alexander, *Nat Chem*, 2013, **5**, 1058-1065.
19. A. S. Breitbach, A. H. Broderick, C. M. Jewell, S. Gunasekaran, Q. Lin, D. M. Lynn and H. E. Blackwell, *Chem. Commun.*, 2010, **47**, 370-372.
20. A. H. Broderick, D. M. Stacy, Y. Tal-Gan, M. J. Kratochvil, H. E. Blackwell and D. M. Lynn, *Adv. Healthcare Mater.*, 2014, **3**, 97-105.
21. M. J. Kratochvil, Y. Tal-Gan, T. Yang, H. E. Blackwell and D. M. Lynn, *ACS biomaterials science & engineering*, 2015, **1**, 1039-1049.
22. E. Cavaleiro, A. S. Duarte, A. C. Esteves, A. Correia, M. J. Whitcombe, E. V. Piletska, S. A. Piletsky and I. Chianella, *Macromolecular bioscience*, 2015, **15**, 647-656.
23. E. V. Piletska, G. Stavroulakis, K. Karim, M. J. Whitcombe, I. Chianella, A. Sharma, K. E. Eboigbodin, G. K. Robinson and S. A. Piletsky, *Biomacromolecules*, 2010, **11**, 975-980.
24. E. V. Piletska, G. Stavroulakis, L. D. Larcombe, M. J. Whitcombe, A. Sharma, S. Primrose, G. K. Robinson and S. A. Piletsky, *Biomacromolecules*, 2011, **12**, 1067-1071.
25. H. D. Lu, A. C. Spiegel, A. Hurley, L. J. Perez, K. Maisel, L. M. Ensign, J. Hanes, B. L. Bassler, M. F. Semmelhack and R. K. Prud'homme, *Nano Letters*, 2015, **15**, 2235-2241.
26. A. Som, S. Vemparala, I. Ivanov and G. N. Tew, *Biopolymers*, 2008, **90**, 83-93.
27. R. W. Scott, W. F. DeGrado and G. N. Tew, *Current opinion in biotechnology*, 2008, **19**, 620-627.
28. K. Kuroda and G. A. Caputo, *Wiley Interdisciplinary Reviews: Nanomedicine and Nanobiotechnology*, 2013, **5**, 49-66.
29. A. Sousa-Herves, R. Novoa-Carballal, R. Riguera and E. Fernandez-Megia, *The AAPS journal*, 2014, **16**, 948-961.
30. E. M. Munoz, J. Correa, R. Riguera and E. Fernandez-Megia, *Journal of the American Chemical Society*, 2013, **135**, 5966-5969.
31. E. M. Munoz, J. Correa, E. Fernandez-Megia and R. Riguera, *Journal of the American Chemical Society*, 2009, **131**, 17765-17767.
32. L. Albertazzi, M. Fernandez-Villamarin, R. Riguera and E. Fernandez-Megia, *Bioconjugate Chem.*, 2012, **23**, 1059-1068.
33. R. Domenech, O. Abian, R. Bocanegra, J. Correa, A. Sousa-Herves, R. Riguera, M. G. Mateu, E. Fernandez-Megia, A. Velazquez-Campoy and J. L. Neira, *Biomacromolecules*, 2010, **11**, 2069-2078.
34. B. Klajnert, T. Wasiak, M. Ionov, M. Fernandez-Villamarin, A. Sousa-Herves, J. Correa, R. Riguera and E. Fernandez-Megia, *Nanomedicine : nanotechnology, biology, and medicine*, 2012, **8**, 1372-1378.
35. F. Fernandez-Trillo, J. Pacheco-Torres, J. Correa, P. Ballesteros, P. Lopez-Larrubia, S. Cerdan, R. Riguera and E. Fernandez-Megia, *Biomacromolecules*, 2011, **12**, 2902-2907.
36. M. Raviña, M. de la Fuente, J. Correa, A. Sousa-Herves, J. Pinto, E. Fernandez-Megia, R. Riguera, A. Sanchez and M. J. Alonso, *Macromolecules*, 2010, **43**, 6953-6961.
37. M. de la Fuente, M. Ravina, A. Sousa-Herves, J. Correa, R. Riguera, E. Fernandez-Megia, A. Sanchez and M. J. Alonso, *Nanomedicine (London, England)*, 2012, **7**, 1667-1681.
38. E. Fernandez-Megia, J. Correa, I. Rodríguez-Meizoso and R. Riguera, *Macromolecules*, 2006, **39**, 2113-2120.
39. S. P. Amaral, M. Fernandez-Villamarin, J. Correa, R. Riguera and E. Fernandez-Megia, *Organic letters*, 2011, **13**, 4522-4525.
40. S. P. Amaral, J. Correa and E. Fernandez-Megia, unpublished work.
41. S. Mignani, S. El Kazzouli, M. M. Bousmina and J. P. Majoral, *Chemical reviews*, 2014, **114**, 1327-1342.
42. M. A. Mintzer and M. W. Grinstaff, *Chem. Soc. Rev.*, 2011, **40**, 173-190.
43. R. M. Kannan, E. Nance, S. Kannan and D. A. Tomalia, *Journal of internal medicine*, 2014, **276**, 579-617.
44. A. Nguyen and A. Jacq, *Wiley Interdisciplinary Reviews: RNA*, 2014, **5**, 381-392.