Dynamic self-assembling supramolecular dendrimer nanosystems as potent antibacterial candidates against drug-resistant bacteria and biofilm

Dinesh Dhumal, Bar Marom, Einav Cohen, Zhenbin Lyu, Ling Ding, Domenico Marson, Erik Laurini, Aura Tintaru, Brigino Ralahy, Suzanne Giorgio, Sabrina Pricl, Zvika Hayouka, Ling Peng

Aix Marseille Univ, CNRS, Centre Interdisciplinaire de Nanoscience de Marseille (CINaM), UMR 7325, Equipe Labelisée Ligue Contre le Cancer, Parc Scientifique et Technologique de Luminy, Marseille, France.

Institute of Biochemistry, Food Science and Nutrition, The Robert H. Smith Faculty of Agriculture, Food and Environment, The Hebrew University of Jerusalem, Rehovot, Israel.

Molecular Biology and Nanotechnology Laboratory (MolBNL@UniTS), DEA, University of Trieste, Trieste, Italy.

Department of General Biophysics, Faculty of Biology and Environmental Protection, University of Lodz, Lodz, Poland.
## Table content

<table>
<thead>
<tr>
<th>Table/Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure S1</td>
<td>3</td>
</tr>
<tr>
<td>Figure S2</td>
<td>4</td>
</tr>
<tr>
<td>Figure S3</td>
<td>5</td>
</tr>
<tr>
<td>Figure S4</td>
<td>6</td>
</tr>
<tr>
<td>Figure S5</td>
<td>7</td>
</tr>
<tr>
<td>Figure S6</td>
<td>8</td>
</tr>
<tr>
<td>General/materials</td>
<td>9</td>
</tr>
<tr>
<td>Synthesis of the amphiphilic dendrimers 1a-d</td>
<td>9</td>
</tr>
<tr>
<td>Critical micelle concentration (CMC)</td>
<td>11</td>
</tr>
<tr>
<td>Transmission electron microscopy (TEM)</td>
<td>12</td>
</tr>
<tr>
<td>Zeta potential measurement</td>
<td>12</td>
</tr>
<tr>
<td>NMR Diffusion Ordered Experiments (DOSY)</td>
<td>12</td>
</tr>
<tr>
<td>Computational methods</td>
<td>13</td>
</tr>
<tr>
<td>Cells</td>
<td>14</td>
</tr>
<tr>
<td>Bacterial cells</td>
<td>14</td>
</tr>
<tr>
<td>PrestoBlue assay</td>
<td>14</td>
</tr>
<tr>
<td>Hemolysis assay</td>
<td>14</td>
</tr>
<tr>
<td>Minimum inhibitory concentration (MIC)</td>
<td>15</td>
</tr>
<tr>
<td>Live/dead cell staining and fluorescence microscopy analysis</td>
<td>15</td>
</tr>
<tr>
<td>Outer membrane permeabilization assay</td>
<td>16</td>
</tr>
<tr>
<td>Inner membrane permeabilization assay</td>
<td>16</td>
</tr>
<tr>
<td>Scanning electron microscopy</td>
<td>16</td>
</tr>
<tr>
<td>References</td>
<td>17</td>
</tr>
</tbody>
</table>
**Figure S1:** Size distribution for the nanomicelles of dendrimers 1a-d measured using TEM. For each sample, at least 300 particles in different TEM images were randomly selected and measured by using ImageJ software to calculate the size distribution of the nanomicelles.
Figure S2: 2D-Diffusion ordered NMR spectrum of 1a in aqueous solution recorded at 300K in D2O.
Figure S3: Cytotoxicity of dendrimers 1a-d in mouse fibroblast (L929) cells and human embryonic kidney 293 (HEK293) cells. Cell viability was measured in triplicate using the PrestoBlue assay.
**Figure S4:** Hemolysis data of dendrimers 1a-d in mouse red blood cells (RBC’s).
Figure S5: Inhibition of biofilm formation of S. aureus JLA513 by dendrimer 1a. The biofilm viability after 24 hr incubation of the different treatments was measured by the tetrazolium dye MTT assay. Results present the mean (± SEM) of 3 independent experiments. Dendrimer 2 was used as negative control.
Figure S6: Scanning electron microscopic (SEM) images of (A) *E. coli* (upper panel) and (B) MRSA (down panel) upon treatment with 1a.
General/materials

The ester-terminating dendrimer 1 was synthesized according to the well-established protocol published in our group [1]. Synthesis of dendrimer 1a and 1b was carried out by amidation of the ester-terminating dendrimer precursor 1 using ethylenediamine and N,N-dimethylethlenediamine respectively according to previously developed protocol [1, 2]. Whereas as synthesis of dendrimer 1c was obtained by conjugation of 1a with the protected arginine followed by subsequent deprotection [3]. The acid-terminated dendrimer 1d was synthesized via hydrolysis of 1 using LiOH [4]. Chemicals were purchased from Sigma Aldrich or Alfa Aesar. Methyl acrylate, ethylenediamine, N,N-Dimethylethlenediamine, dichloromethane and methanol were dried according to the described methods and distilled before use. The other chemicals were used without further purification. For inner/outer membrane assays, N-phenyl-1-naphthylamine (NPN) from Arcos Organics (98%) and 3’-Di-n-propythiadicarbocyanine iodide [DiSC3(5)] from Alfa Aesar (96%) were used. Dialysis tubing was purchased from Sigma Aldrich (St. Quentin Fallavier, France) and Yuanye Biotechnology Co., Ltd. (Shanghai, China). Analytical thin layer chromatography (TLC) was performed using silica gel 60 F254 plates 0.2 mm thick with UV light (254 and 364 nm) as revelator. Chromatography was prepared on silica gel (Merck 200-300 mesh). 1H and 13C NMR spectra were recorded on Bruker Avance III 400 (400 MHz, 1H; 100 MHz, 13C) and Varian Mercury-VX600 (600 MHz, 1H; 150 MHz, 13C). Chemical shifts (δ) are expressed in parts per million (ppm). The HRMS analysis was carried out with a SYNAPT G2 HDMS (Waters) mass spectrometer equipped with a pneumatically assisted atmospheric pressure ionization (API) source. The sample was ionized in positive electrospay mode under the following conditions: electrospray voltage: 2.8 kV; orifice voltage: 20 V; Nebulization gas flow (nitrogen): 100 L/h. The high resolution mass spectrum (MS) was obtained with a flight time analyzer (TOF). The exact mass measurement was done in triplicate with an external calibration. The sample is dissolved in 300 μL of methanol and then diluted 1/10 in a 1.0% solution of methanol formic acid. The solution of the extract is introduced into the infusion ionization source at a flow rate of 10 μL/min.

Synthesis of the amphiphilic dendrimers 1a-d

1a: To a solution of 1 (98 mg, 0.057 mmol) in methanol (2.0 mL) was slowly added ethylenediamine (2.0 mL, 30 mmol) under ice bath. Then the reaction mixture was stirred for 3 days at 30 °C until IR showed the complete disappearance of the ester functions in 1. The reaction solution was evaporated, and the obtained residue was purified by dialysis (change
dialysis water one hour per time for 6 times) and lyophilization. Repeating the operation cycles of dialysis and lyophilization for 3 times, the product was lyophilized to yield the corresponding 1a as a white solid (96 mg, yield: 86 %)

1a: 

1H NMR (400 MHz, CDCl3/CD3OD=3/1): δ 7.54 (s, 1H, CH), 4.17 (t, 2H, J = 7.4 Hz, CH2), 3.62 (s, 2H, CH2), 2.98-3.11 (m, 28H, CH2), 2.51-2.64 (m, 44H, CH2), 2.29-2.42 (m, 12H, CH2), 2.34-2.42 (m, 28H, CH2), 1.64-1.77 (m, 2H, CH2), 0.98-1.21 (m, 30H, CH2), 0.70 (t, 3H, J = 6.8 Hz, CH3);

13C NMR (150 MHz, CD3OD): δ 174.0, 173.6, 173.5, 143.6, 124.0, 53.4, 52.3, 49.9, 49.3, 41.7, 41.5, 40.8, 37.4, 33.6, 31.9, 30.2, 29.6, 29.4, 29.3, 28.9, 26.4, 22.5, 13.3; IR (cm⁻¹): ν 1644.6; HRMS: calculated for C91H186N32O14+ [M+4H]4+ 488.1208, found 488.1294.

1b: To a solution of 1 (100 mg, 0.058 mmol) in methanol (5.0 mL) was added N,N-dimethylethylenediamine (2.5 mL, 28 mmol). The reaction mixture was stirred for 5 days at 50 °C until the IR and NMR analysis showed the complete disappearance of the ester functions in 1. The reaction solution was evaporated, and the obtained residue was purified by precipitation with CH3OH/Et2O for three times and followed by dialysis (change dialysis water one hour per time for 6 times) and lyophilization. Repeating the operation cycles of dialysis and lyophilization for 3 times, the product was lyophilized to yield the corresponding 1b (108 mg, 86%) as a pale viscous oil.

1b: 1H NMR (400 MHz, CDCl3): δ 7.90 (t, 2H, J = 5.3 Hz, NH), 7.73 (t, 4H, J = 5.2 Hz, NH), 7.57 (s, 1H, CH), 7.52 (t, 8H, J = 5.2 Hz, NH), 4.28 (t, 2H, J = 7.4 Hz, CH2), 3.81 (s, 2H, CH2), 3.24-3.32 (m, 28H, CH2), 2.72-2.75 (m, 28H, CH2), 2.51-2.62 (m, 12H, CH2), 2.40-2.43 (m, 28H, CH2), 2.34-2.42 (m, 44H, CH2), 2.30 (br, 48H, CH2), 2.02 (br, 2H, CH2), 1.23 (br, 30H, CH2), 0.84 (t, 3H, J = 6.8 Hz, CH3);

13C NMR (100 MHz, CDCl3): δ 172.7, 172.6, 172.4, 143.4, 122.9, 58.3, 52.6, 50.2, 50.1, 45.3, 45.3, 37.0, 34.5, 31.9, 29.7, 29.7, 29.5, 29.4, 26.6, 22.2, 14.2; IR (cm⁻¹): ν 1648; HRMS: calculated for C107H217N32O143+ [M+3H]3+ at 724.9079, found 724.9038.

1c: To a solution of Fmoc-Arg(pbf)-OH (726 mg, 1.12 mmol), HOBt (171 mg, 1.12 mmol), HBTU (424 mg, 1.12 mmol) and DIPEA (389 μL, 2.24 mmol) in 10 mL DMF stirring at 25 °C under argon, was added a solution of 1a (68 mg, 0.035 mmol) in 5.0 mL DMF. The resulting solution was stirred at 25 °C under argon for 2 days. Then DMF was removed under reduced pressure, and the residue was purified by precipitation with CH3OH/Et2O (1.0 mL/20 mL) at 4 °C for 12 h (three times). The residual precipitate was collected, dried by rotavap and then dissolved in 8.0 mL 30% piperidine in DMF (v/v). The mixture was stirred at 25 °C under argon for 2.0 h, and DMF was then removed under reduced pressure. The residue was purified
by precipitation with CH$_3$OH/Et$_2$O (1.0 mL/12.0 mL) at 4 °C for 12 h (three times). A solution of 95% TFA, 2.5% triisopropylsilane (TIS) and 2.5% H$_2$O (v/v/v, 5.0 mL) was added to the residual precipitate and stirred at 25 °C under argon for 1.0 h. The crude product was dissolved in water (2.0 mL) and filtered, then purified by dialysis (change dialysis water one hour per time for 6 times) and lyophilization. Repeating the operation cycles of dialysis and lyophilization for 3 times, the product was lyophilized to yield the corresponding 1c as a white solid (125 mg, yield: 86%).

1c: $^1$H NMR (600 MHz, D$_2$O): $\delta$ 7.87 (s, 1H, CH), 4.26 (t, 2H, $J = 6.3$ Hz, CH$_2$), 3.76–3.81 (m, 10H, CH$_2$), 3.11–3.28 (m, 28H, CH$_2$), 3.05 (t, 16H, $J = 6.9$ Hz, CH$_2$), 2.91–2.96 (m, 28H, CH$_2$), 2.66–2.80 (m, 28H, CH$_2$), 2.37–2.43 (m, 28H, CH$_2$), 1.71–1.79 (m, 18H, CH$_2$), 1.46–1.48 (m, 16H, CH$_2$), 1.00–1.05 (m, 30H, CH$_2$), 0.65 (t, 3H, $J = 6.6$ Hz, CH$_3$); $^{13}$C NMR (150 MHz, D$_2$O): $\delta$ 173.8, 173.4, 170.2, 157.5, 156.9, 117.4, 115.5, 53.1, 53.0, 51.8, 49.4, 40.4, 39.2, 38.8, 35.6, 32.3, 29.1, 28.2, 23.7; IR (cm$^{-1}$): v 1630.3; HRMS: calculated for C$_{139}$H$_{283}$N$_{64}$O$_{22}$+$^5$ [M + 5]$^+$ 640.4598, found 640.4614.

1d: To a solution of ester dendrimer (300 mg, 0.17 mmol) in MeOH (5.0 mL) was added dropwise LiOH·H$_2$O (117 mg, 2.8 mmol) in H$_2$O (5.0 mL) at 0 °C. The reaction solution was stirred at 0 °C for 10 min and then moved to 25 °C for 24 h. When the reaction was completed indicated by NMR monitoring, MeOH was evaporated and the pH of the aqueous phase was adjusted to 4.0 using 1.0 M HCl solution, then purified by dialysis (change dialysis water one hour per time for 6 times) and lyophilization. Repeating the operation cycles of dialysis and lyophilization for 3 times, the product was lyophilized to yield the corresponding 1d as a white solid (231 mg, yield: 83%).

1d: $^1$H NMR (400 MHz, CD$_3$OD): $\delta$ 8.07 (s, 1H, CH), 4.41 (t, $J = 7.1$ Hz, 2H, CH$_2$), 4.13 (s, 2H, CH$_2$), 3.70-3.60 (m, 12H, CH$_2$), 3.49-3.24 (m, 36H, CH$_2$), 3.01 (t, $J = 6.8$ Hz, 4H, CH$_2$), 2.81 (t, $J = 6.6$ Hz, 8H, CH$_2$), 2.68-2.59 (m, 20H, CH$_2$), 1.95-1.87 (s, 2H, CH$_2$), 1.36-1.24 (m, 30H, CH$_2$), 0.90 (t, $J = 6.7$ Hz, 3H, CH$_3$)$_3$; $^{13}$C NMR (101 MHz, CD$_3$OD): $\delta$ 177.2, 173.9, 173.3, 140.9, 126.8, 53.8, 53.0, 51.8, 51.4, 50.6, 50.1, 47.6, 35.6, 33.1, 32.5, 31.5, 31.3, 31.1, 30.8, 30.8, 30.7, 30.6, 30.5, 30.2, 27.6, 23.7, 14.5; IR (cm$^{-1}$): v 1710.2; HRMS: calculated for C$_{75}$H$_{137}$N$_{16}$O$_{22}$+$^3$ [M+3H]$^+$ 538.0026, found 538.0029.

Critical micelle concentration (CMC)

CMC was determined using pyrene as a fluorescence probe. Dendrimers stock solution was prepared in water and aliquots of the stock solutions were diluted by water to get desired concentration (1.0 µM to 1000 µM) in 1.0 mL solution. 1.0 µL of pyrene was added from its
1.0 mM stock solution prepared in ethanol and solutions were vortexed for 5.0 min and kept at room temperature for 24 h. Fluorescence emission was recorded at 373 nm and 384 nm using an excitation wavelength of 334 nm on CARY Eclipse fluorescence spectrophotometer at room temperature. Excitation and emission bandwidths were 5 nm. The fluorescence intensity ratio of $I_{373}/I_{383}$ was analyzed and a sigmoidal best fit analysis was applied to fluorescence intensity ratio data. The CMC was determined by plotting normalised fluorescence intensity ratio of pyrene $I_1/I_3$ against Log concentration of dendrimers.

**Transmission electron microscopy (TEM)**

TEM was performed using JEOL 3010 transmission electron microscope (Tokyo, Japan) to characterize the size and morphology of the NPs at an accelerating voltage of 300 kV. The dendrimers were dispersed in milliQ water at a concentration of 1.0 mg/mL, and sonicated for 30 s, then the solutions were diluted 100-fold and followed by depositing an aliquot (4.0 μL) onto a carbon-coated copper grid and dried at 37 °C. The grid was then stained with 3.0 μL uranyl acetate (2.0% in aqueous solution) for 4 s, and the excess uranyl acetate was removed by filter paper before measurements. For each sample, at least 300 particles in different TEM images were randomly selected and measured by using ImageJ software to calculate the size and the size distribution of the nanoparticles.

**Zeta potential measurement**

Solutions of dendrimers were prepared in H$_2$O with pH adjusted to 7.4 using 0.10 M NaOH and 0.010 M HCl. After incubated at room temperature for 30 min, zeta potential measurement was performed using Zetasizer Nano-ZS (Malvern, Ltd. Malvern, U. K.) with a He-Ne ion laser of 633 nm. The experiments were done in triplicates.

**NMR Diffusion Ordered Experiments (DOSY)**

NMR sample has been prepared in D$_2$O (99.98% D) provided from EurIsotop (Saint Aubin, France). NMR $^1$H-DOSY experiments were recorded at 300K on Bruker AVANCE spectrometer (Karlsruhe, Germany), operating at $^1$H Larmor frequency of 500 MHz, equipped with a double resonance broadband fluorine observe (BBFO) of 5 mm probe head, and z-gradient coil. Main acquisition parameters have been optimized prior to analysis: relaxation delay (D1) 5s, 90° excitation pulse of 12.9μs, 16 scans. Diffusion parameters have been optimized for 200ms (big delta) and 2.3ms (little delta).

The self-diffusion coefficients D obtained for 1a in D$_2$O solution at 300K, was found D = 7.6 x 10$^{-11}$ m$^2$/s (Figure S2). The average weight of the formed micelles was evaluates using the
calibration method previously described [5]. DOSY results shown the formation of homogeneous micellar species, containing around six molecules of 1a per micelle.

**Computational methods**

A symmetric lipid bilayer containing 244 lipids per leaflet (75% palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE) and 25% 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1’-rac-glycerol) (POPG), was built via the Membrane Builder [6-8] available from the CHARMM GUI web portal, [9] and parametrized using the lipid force fields [10]. The model membrane was solvated in a TIP3P [11] water box extending at least 1.0 nm from the lipid heavy atoms. To achieve electroneutrality, a suitable number of neutralizing counterions (Na\(^+\) and Cl\(^-\)) were added. The solvated bilayer was then energy-minimized with a gradual decrease in the position restraints of the lipid atoms. Finally, the entire simulation box was equilibrated via 20 ns constant volume/constant temperature (NVT) molecular dynamics (MD) simulations, and the equilibrated bilayer model at the end of the MD run was selected for further modeling.

The models for the amphiphilic dendrimers 1a and 1d were built and optimized using the same protocol described in detail in our previous work [12].

To position 1a and 1d on the equilibrated membrane surface, first all water molecules, ions and counterions were deleted. Next, 1a and 1d were manually positioned at the center of the lipid bilayer surface at a distance such that no contacts between the amphiphiles and the membrane could be detected. Each membrane/amphiphile system was then fully solvated in TIP3P water molecules with the required numbers of counterions to reach neutrality. Each hydrated complex system was then gradually heated to 310 K and then 2.0 µs of data collection runs was carried out.

The production MD simulations were performed in the constant pressure/constant temperature (NPT) ensemble at T = 310 K using the Langevin thermostat [13] with a 0.5 ps time constant. The pressure of 1.0 bar was maintained via anisotropic pressure coupling using the Berendsen barostat [14]. All bonds were constrained by applying the SHAKE algorithm [15], thus allowing for the adoption of an integration time step of 2.0 fs. Long-range nonbonded van der Waals interactions were truncated using a cutoff of 9 Å. The particle mesh Ewald method [16] was used to treat the long-range electrostatics with a cutoff of 9Å.

All simulation studies were carried out in AMBER 20 [17] running on our own GPU/CPU hybrid cluster.
Cells
Human embryonic kidney 293 cells (HEK 293 cells) and mouse fibroblast cells (L929 cells) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco, Invitrogen) containing 10% fetal bovine serum (FBS) (Biosera). Cells were maintained at 37 °C with 5.0% CO₂ in a humidified chamber.

Bacterial cells
*Escherichia coli* K12 MG1655 (rp), *Pseudomonas aeruginosa* PAO1, *Staphylococcus aureus* RN4220, JLA513 and Methicillin-resistant *staphylococcus aureus* (MRSA) 1206 were used. The strains were grown in lysogeny broth (LB) or LB agar plates at 37°C. Bacteria were stored at -80 °C in 25% glycerol and at 4.0 °C on LB agar plate.

PrestoBlue assay
L929 and HEK 293 cells were seeded at 1.0 × 10⁴ cells/well and 4.0 × 10³ cells/well in 96-well plates and allowed to grow overnight. Cells were then treated with various concentration (0.10 – 200 μM) of the amphiphilic dendrimers 1a-d for 48 h. Then 10 μL PrestoBlue reagent was added to each well containing 100 μL of blank, control, or treated cells in culture and incubated for another 3.0 h at 37 °C. The changes in cell viability were detected using fluorescence spectroscopy (excitation 570 nm; emission 610 nm). The cell viability was expressed as a percentage relative to the cells untreated with dendrimers. All samples were run in triplicate.

Hemolysis assay
Red blood cells (RBCs) were isolated from the freshly collected whole blood of Swiss nude mice (with 1.0% heparin sodium solution). Blood was centrifuged at 7500 rpm for 10 min. several wash of PBS buffer given to RBCs until no color was seen in the supernatant. 2.0% RBCs concentration was achieved by diluting them in PBS (e.g. 20 μL of RBC suspension added to 0.98 mL PBS). 0.50 mL of 2.0% RBC solution was added into 1.5 mL Eppendorf tubes, and then 0.50 mL of dendrimer at different concentrations (2.0, 10, 20, 100, 200 and 500 μM) were added to make the final concentration 1.0, 5.0, 10, 50, 100 and 250 μM. 0.50 mL RBC suspension incubated with 0.50 mL PBS or 0.50 mL 1.0% TritonX-100 solution was used as the negative control and positive control, respectively. The samples were mixed gently, left at 37 °C for 1.0 h, then centrifuged at 7500 rpm for 10 min. Then the supernatant was analyzed for the absorbance of hemoglobin at 540 nm on fluorescence spectrometer. The percentage of hemolysis was calculated as follows: Hemolysis% = [(sample absorbance – negative control) / (positive control – negative control)] ×100%.
Minimum inhibitory concentration (MIC)
Minimal-inhibition concentration (MIC) assay was performed as described in Hayouka et al [18]. Briefly, stock solutions of the tested compounds were diluted in LB broth and then diluted in 96-well plates (Corning 3650) with a 2-fold dilution, from 200 µg/mL to 3.1 µg/mL. Bacteria were grown overnight (37°C, 200rpm), then diluted 1:100 and grown to OD\_600 of 0.10, subsequently 100 µL were added into the 96-well plate. Bacterial growth was determined after incubation of 24 h at 37 °C by measurement of the optical density (OD\_600nm), using a Tecan infinite Pro Plate reader. MIC value defined as the lowest concentration at which there is complete inhibition of bacterial growth (no increase in OD over the course of the experiment). Each experiment was carried out at least three times, with three replicates per strain/concentration combination in each experiment.

Biofilm Inhibition assay
One colony of *S. aureus* JLA513 was inoculated from agar plate into 5.0 mL TSB media (50 mL tube) and grown overnight. The overnight suspension diluted 1:100 in fresh TSB supplemented with 1.0 % D-glucose (TSBG) and grown until OD\_595 = 0.1. The bacterial suspension was then diluted 1:100 in TSBG and 100 µL were transferred into 96-well plate, which contained a serial concentrations of the dendrimer 1a in TSBG. The plate was then incubated for 48 hours at 37 °C. Then the supernatant liquid was discarded and the wells were washed with DDW 3 times. Dendrimer 2 was used as negative control.

Biofilm quantification by MTT
For quantification of biofilm viability, the tetrazolium dye 3-[4,5-dimethyl-2-thiazolyl]-2, 5-diphenyl-2H-tetrazolium-bromide (MTT) was used as previously described [19] with modifications. Briefly, after the 96-well plate washed from unattached cells and dried, 50 µL of MTT dissolved in PBS (0.50 µg/mL, pH 7.4) added to each well and incubated at 37 °C for 2 hours. Subsequently, 100 µL of DMSO were added to each well and incubated for 15 minutes. Then, the OD\_595 was measured using Tecan plate reader. The results were normalized to the control strain that was grown in the absence of antimicrobial agents.

Live/dead cell staining and fluorescence microscopy analysis
*E. coli* rp/MRSA culture was grown overnight in LB, and was then centrifuged and washed in PBS buffer for three times. The suspension was diluted to OD\_600 of 0.10. Incubation of 1.0 mL bacterial cells suspension was performed with shaking (200 RPM) with 25 µg/mL of tested compound (1a) for 30 min (37 °C). Incubation of the bacteria with PBS was used as control.
After incubation, the bacterial cells were stained using LIVE/DEAD BacLight Bacterial Viability Kit (ThermoFisher). Each sample was incubated with 2.5 μL propidium iodide (red) and 2.5 μL SYTO9 (green) for 15 min in the dark. Afterwards fluorescent images were taken using EVOS M5000 imaging system (ThermoFischer). Images were analyzed using the software imageJ.

**Outer membrane permeabilization assay**
The outer membrane permeability was determined using the fluorescent probe N-phenyl-1-naphthylamine (NPN). *E. coli* cells were grown overnight in LB media, then diluted 100-fold in LB and grown at 37 °C to OD_{600} of 0.50. The cells were then washed with 5.0 mM sodium HEPES buffer (pH 7.2), and resuspended in the same buffer to OD_{600} of 0.20. Subsequently, 100 μL of the suspended bacteria was added into 96-well plate (Nunc, Thermo Scientific, 167008) with 50 μL of NPN solution (10 μM). Fluorescence intensities were measured using Synergy H1 Biotek microplate reader (excitation: 350, emission: 420) for 5.0 min, then 50 μL of the tested dendrimer was added into each well and the fluorescence intensities were measured for additionally 40 min. Dendrimer 2 was used as negative control. The displayed results are representative of 3 independent experiments with three replicates for each treatment.

**Inner membrane permeabilization assay**
Depolarization of the inner membrane was assessed using the lipophilic potentiometric dye 3,3’-Dipropylthiadicarbocyanine Iodide [DiSC\(_3\)(5)]. *E. coli* and MRSA bacterial cells were grown overnight in LB media (37 °C, 200rpm), then diluted 1:100 in LB and grown to OD_{600} of 0.50. Bacteria were then washed with 5.0 mM HEPES, 20 mM glucose and 0.10 M KCl, and resuspended in the same buffer to approximately 10^6 CFU/mL. The bacterial cells were then incubated with 0.40 μM DiSC\(_3\)(5) for 1.0 h in 96-well plates (Nunc, Thermo Scientific, 167008), until a stable reduction of fluorescence was achieved. The tested dendrimer at the indicated concentrations, was then added to the bacterial sample and the changes in the fluorescence intensities were monitored using Synergy H1 Biotek microplate reader (excitation: 647, emission: 677). Triton-X 0.50% was used as positive control, dendrimer 2 (200 μg/mL final concentration) was used as a negative control. Results displayed are representative of 3 independent experiments with three replicates for each treatment.

**Scanning electron microscopy**
*E. coli* rp and MRSA cultures were grown respectively overnight in LB, then centrifuged and washed in PBS buffer for three times. The suspension was diluted to OD_{600} of 0.1, to get ~10^7
CFU/mL. Incubation of 1.0 mL bacterial suspension was incubated with shaking (200 RPM) with 6.3 or 50 μg/mL with the tested compound in PBS buffer at 37 °C for 30 min. Incubation of the bacteria in PBS only, and dendrimer 1a in PBS were used as control. After incubation, the bacteria were centrifuged (4000 RPM, 5.0 min) and resuspended with 100 μL 4.0% glutaraldehyde in PBS added to the samples for 1.0 h at room temperature. The treated bacterial cells were dropped on a glass disk with poly-lysine, 30 μL at each side of the disk. Dehydration of the samples with the treated or non-treated bacteria was done in increasing concentrations of ethanol (25, 50, 75, 95, 100% ethanol in deionized water), 5.0 min twice for each concentration. Drying was performed with K850 critical point dryer (CPD BAL-TEC CPD-030). The samples were dried with ethanol and for 3.0 min in ~10 °C and 20 min in 34 °C. The ethanol was replaced by liquid CO₂ at ~40 °C for 20 min. Then, iridium coating was performed with Q150T ES Spatter Coater. The microphotographs were recorded using imaging Jeol JSM 7800 SEM.

References:


