Published in final edited form as:

J Mater Chem B. 2018 September 7; 6(33): 5353–5361. doi:10.1039/C8TB01610K.

Antibacterial and Potentiation Properties of Charge-Optimized Polyrotaxanes for Combating Opportunistic Bacteria

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Abstract

Bacteria are now becoming more resistant to most conventional antibiotics. Approaches for the treatment of multidrug-resistant bacterial infections are urgently required. Cationic polymers have broad-spectrum antibacterial activity but can also induce non-specific damage to mammalian cells. Herein, we report on the design of cationic polyrotaxanes (cPRs) with variable charge densities. cPRs were prepared by conjugating neutral ethanolamine and cationic ethylenediamine at various ratios onto threaded alpha-cyclodextrins and their antimicrobial and cytocompatible properties were investigated in vitro. In contact with Gram-negative bacteria, cPRs can disrupt the bacterial outer membrane integrity via electrostatic interactions and penetrate into the cytosol. The ability of cPRs to serve as potentiators at sub-MIC concentrations, to enhance the permeability and activity of poorly permeable antibiotics such as vancomycin, erythromycin and rifampicin, was also investigated against Gram-negative *P. aeruginosa* PAO1 and *E. coli* ATCC 25922.

GRAPHICAL ABSTRACT

Introduction

Bacterial infections pose an alarming threat to human health due to widespread use of antibiotics by the healthcare industry.¹ Alarming, multidrug resistance (MDR) is rising,

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Conflicts of interest

There are no conflicts to declare.

with MDR strains accounting for a nearly two-fold increase in mortality compared to susceptible infections.² Conventional antibiotics utilized in the clinics generally inhibit or kill microbes by targeting cell wall synthesis, protein synthesis, or DNA replication.³ Unfortunately, bacteria can evolve internal resistance mechanisms to many of these drugs over time. In the most extreme cases, polymyxins have been used as a last defence against MDR Gram-negative pathogens,⁴ though plasmid-mediated resistance to colistin has been reported.^{5–6} One approach to potentially circumvent internal drug resistance mechanisms is to investigate nanomaterial strategies targeting the bacterial cell wall, with antimicrobial peptides (AMPs)^{7–8} and antimicrobial polymers^{9–10} being the most promising. This group of anti-infective is expected to result in fewer incidences of resistance since such a mechanism would involve an extensive and costly restructuring of the bacterial lipids in the membrane.^{11–13}

AMPs are produced by many multicellular organisms and are generally short peptide molecules comprised of clusters of hydrophobic and cationic amino acids capable of interacting with the negatively charged outer bacterial bilayer.^{14–16} Though highly potent, the utilization of AMPs as antimicrobials *in vivo* has been hampered by cytotoxicity issues in the host, susceptibility to degradation by proteases, and scale up costs;^{17–18} other types of antimicrobial polymers include cationic polymers^{19–21} and biocide-release polymers.²² Among these, cationic polymers have attracted much attention due to their stability *in vivo* and their ability to effectively disrupt the negatively charged bacterial outer wall; unfortunately, this also means that cationic polymers can similarly cause charge-induced damage to mammalian host cell membranes.²³ Although the large surface area characteristic of such nanomaterials is ideal for promoting interactions with the bacterial cell wall and subsequently generating antimicrobial effects, this has to be balanced by a proper optimization of its surface properties to minimize cytotoxicity to host mammalian cells.

In addition, the outer membrane (OM) of Gram-negative bacteria is a formidable barrier consisting of a negatively-charged lipopolysaccharide (LPS) layer stabilized by divalent cations which is known to prevent some large antibiotics from crossing. Design agents aimed at increasing the permeability of the Gram-negative bacterial membrane, or potentiators, could be used therapeutically to widen the spectrum of clinically used antibiotics. Potentiators designed to permeabilize the OM are typically cationic in nature²⁴ and suggest that cationic nanomaterials may also serve as potentiators to allow conventional antibiotics easier access into the bacterial cell.^{25–26} A promising small molecule potentiator currently under Phase I clinical development is SPR741 (Spero Therapeutics), a modified peptide derived from polymyxin B, which displayed favourable OM permeabilizing properties and low renal toxicity.²⁷ Apart from SPR741, there are few other small molecule potentiator molecules in the clinical drug development pipeline. Overall, the activity of poorly effective antibiotics may be improved through strategies such as: (1) inhibition of antibiotic resistance elements (e.g. β -lactamase inhibitors^{28–29} and synthesis of inhibitors of teichoic acid³⁰); (2) increasing the uptake of the antibiotic through the bacterial membrane;²⁴ (3) inhibition of efflux pumps (e.g. reserpine³¹); (4) changing the physiology of resistant cells (targeting biofilm, e.g. nitric oxide,³² superoxide³³). Herein, we investigate whether antimicrobial nanomaterials such as cationic polyrotaxanes can also serve as potentiators to increase the uptake of poorly effective antibiotics against Gram-negative bacteria.

To investigate the design of safe nanomaterials possessing antimicrobial and potentiation properties, a series of cationic polyrotaxanes (cPR) was synthesized. Polyrotaxane (PR) is a type of supramolecular material in which several cyclic molecules are threaded onto a linear polymer chain capped by bulky end groups.³⁴ Due to their unique bead-on-a-necklace structure and rod-like shape, beads on PR can be easily functionalized to serve a variety of applications.^{35–36} To ensure biocompatibility of the nanomaterial, cyclodextrin-based PR assembled from alpha-cyclodextrins (α -CDs) and polyethylene glycol (PEG) were prepared. Herein, we report on the charge-optimized design of cPR possessing potent antibacterial activity and low mammalian cytotoxicity, and antibiotic potentiation properties against *P. aeruginosa* and *E. coli* (Scheme 1). PR was first prepared by threading α -CDs onto PEG-(COOH)₂ (MW 2000 Da) and the cationic properties of final cPR were optimized by conjugating neutral ethanolamine and cationic ethylenediamine at different ratios onto threaded α -CDs. The positive charges present along rod-shaped cPR present a large surface area for disrupting the bacterial surface and permitting antibiotic potentiation, yet the narrower end of this rod-shape nanomaterial (<2 nm) also facilitates its penetration deeper into the bacterial cell to induce additional intracellular damage. Herein, we show that charge-optimized cPR can safely exert antimicrobial activity against Gram-positive and Gram-negative bacteria at concentrations ranging from 90–180 μ g/mL and can display synergism at sub-MIC concentrations (15–30 μ g/mL) with erythromycin (ERY) and rifampicin (RIF) against *P. aeruginosa* PAO1 and *E. coli* ATCC 25922.

2. Experimental section

2.1 Characterization of Cationic Polyrotaxanes

To investigate the morphology of cPRs, transmission electron microscopy (TEM) images were taken on a Philips/FEI Tecnai20 with an acceleration voltage of 120 kV. Sample was prepared by air-drying a drop of 0.01 mg/mL suspension on copper grid. Thereafter, the copper grid was inverted onto a drop of aqueous solution of sodium phosphotungstate (1%, w/v, pH = 7) and rinsed gently with deionized water. Zeta potentials and average particle sizes of cPRs were measured with a Zetasizer Nano ZS (Malvern) and analyzed with Zeta sizer software v7.10. Prior to measurements, cPRs solutions were filtered through Millipore membranes with a 0.22 μ m pore size. All the measurements were performed at 25°C in 10 mM phosphate buffer at pH 7.2. The concentration of cPR was set at 0.01 mg/mL.

2.2 Bacterial Strains and Cell Culture Conditions

P. aeruginosa strain PAO1 as the wild type, *E. coli* (ATCC 25922) and *S. aureus* (ATCC 25923) were obtained from American Type Culture Collection (ATCC). From the frozen stock (–80 °C), bacteria were inoculated into cation-adjusted Mueller–Hinton agar plates under aerobic conditions at 37 °C for 24 h. A colony was picked and sub-cultured in cation-adjusted Mueller–Hinton broth medium and grown to stationary phase at 37 °C in an incubator shaker prior to all experiments. The concentration of bacteria was monitored by measuring the optical density (OD) at a wavelength of 600 nm (OD₆₀₀). All growth media and Milli-Q water used for bacterial cultures were sterilized by autoclave. Sterile polypropylene culture tubes and sterile polystyrene 96-well plates used for culturing were manufactured by VWR and Corning Incorporated, respectively.

2.3 Minimum Inhibitory Concentrations of cPRs

The MIC values of different charged cPRs were evaluated using the broth microdilution method. Briefly, a 135 μL aliquot of the MHB medium containing dilution series of each compound was then mixed with 15 μL aliquot of bacterial solution in a 96-well plate. The bacterial suspension was diluted to an OD_{600} value of 0.01 in MHB medium, and the final OD_{600} value in 96-well is 0.001. The plate was subsequently wrapped in parafilm and incubated at 37 °C for 18 h. Bacterial sample (A_{Sample}) was assayed by measuring OD_{600} using a Spectramax® Plus 384 Microplate Reader. Wells with only medium served as negative controls (A_{Neg}) and bacteria grown in medium without the addition of cPRs served as positive controls (A_{Pos}). Each well condition was prepared in triplicate. The resulting mean OD_{600} is reported, where the error bars are the standard deviation of the mean obtained from three independent replicates. The MIC values were determined as the lowest concentration at which the optical density read zero. Bacteria relative growth was calculated using the following formula:

$$\text{Relative Growth} = \frac{A_{\text{sample}} - A_{\text{Neg}}}{A_{\text{Pos}} - A_{\text{Neg}}}$$

2.4 Antimicrobial Activity

Antibacterial activity of cPR₂ was evaluated using time-kill assay. Inoculate bacterial strains of *P. aeruginosa*, *E. coli* and *S. aureus* were cultured until the OD_{600} reached a value of 0.4–0.6 in MHB medium. At time 0, each tube contained bacteria at an $\text{OD}_{600} = 0.1$ was incubated with 100 μM cPR₂ in PBS solution. Samples were incubated at 37°C with shaking (250 rpm) and 100 μL aliquots from each solution were sampled at 0, 1h, 2h, and 3h, and immediately serially diluted with saline and plated onto MH agar plates using a spreader. The MH agar plates were incubated between 16–18 h at 37 °C, after which single colonies were enumerated and CFU per milliliter (CFU/mL) were determined.

2.5 Mammalian Cell Viability Assay

The cytotoxicity of cPRs on mammalian cells was assessed by using the metabolism-based resazurin assay. J774A.1 mouse macrophage cells were seeded in a 96-well plate at a density of 5,000 cells per well. Cells were incubated in DMEM supplemented with 10% Fetal bovine serum (FBS), and 1% penicillin/ streptomycin, at 37 °C in 5% CO₂ for 24 h. Next, cells were incubated with growth medium containing cPRs at various concentrations. The cPRs concentrations of each formulation were prepared by serial dilution with DMEM medium. After 24 h, the substrate resazurin was dissolved in cell culture medium at a concentration of 44 μM and 100 μL was added to each well for 4 h. Cell viability was determined by measuring fluorescence intensity at 590 nm (excitation at 560 nm) on a Spectra Max microplate reader. Readings from the wells without cells were used as blank (I_{blank}) and the readings from control cells without treatment were used to represent 100% cell viability (I_{control}). All the measurements were conducted in triplicate and cell viability was determined as follows:

$$\text{Cell Viability} \left(\% \right) = 100 \times \frac{I_{\text{sample}} - I_{\text{blank}}}{I_{\text{control}} - I_{\text{blank}}}$$

2.6 Hemolysis Assay

Toxicity to sheep red blood cells (RBCs) was assessed by a hemoglobin release assay using the same cPRs stock solutions used for the MIC measurements. Sheep RBCs were first washed three times in PBS (10 mM pH 7.4) and collected by centrifugation (5,000 rpm, 10 min). The RBCs were diluted to a final concentration of 2×10^7 cells/mL. Aliquots (100 μ L) of the RBC solution were seeded into a 96-well plate containing 100 μ L of test cPRs solutions at various concentrations (40 – 4,000 μ g/mL) and incubated in a humidified atmosphere containing 5% CO₂ at 37 °C for 2 h. Following incubation, the solutions were centrifuged (5,000 rpm, 10 min) and aliquots (100 μ L) of supernatant were transferred to a flat-bottomed 96-well plate. Hemoglobin release upon lysis of the RBCs with cPRs (A_{cPR}) was monitored at 405 nm using a microplate reader. Negative control solution that contained only PBS (A_{Neg}) was used as a reference for 0% hemolysis. Positive control solution (A_{Pos}) that contained 1% Triton X-100 was used as a reference for 100% hemolysis. Each hemolysis assay was independently repeated three times using different stock solutions in triplicate on different days. Hemolytic activity is expressed as the concentration observed to lyse 10% of RBCs (HC_{10}). The percentage of hemolysis was calculated using the formula:

$$\text{Hemolysis} \left(\% \right) = 100 \times \frac{A_{\text{cPR}} - A_{\text{Neg}}}{A_{\text{Pos}} - A_{\text{Neg}}}$$

2.7 Lactate Dehydrogenase Assay

Cytotoxicity of cPR₂ was evaluated by the amount of lactate dehydrogenase (LDH) released from the cytoplasm as measured with the Pierce™ LDH Cytotoxicity Assay Kit (Thermo Scientific). In brief, J774A.1 mouse macrophage cells were seeded in a 96-well plate at a density of 5,000 cells per well. Cells were incubated in DMEM supplemented with 10% Fetal bovine serum (FBS), and 1% penicillin/ streptomycin, at 37 °C in 5% CO₂ for 24 h. Next, cells were incubated with growth medium containing cPR₁ and cPR₂ at various concentrations. The cPRs concentrations of each formulation were prepared by serial dilution with DMEM medium. Next 50 μ L of culture supernatant was transferred to a 96-well flat-bottom plate and reaction mix containing 50 μ L substrate was added to each well and incubated for 30 min at room temperature. After the reaction was stopped, the absorbance was read at 490 nm and 680 nm with a microplate reader.

2.8 CLSM Studies With cPR₂

Bacteria cells were inoculated in 3 mL MHB medium, grown to stationary phase at 37 °C, and harvested by centrifuging (4000 rpm for 5 min) and washing with saline 3x. Then the cells were re-suspended in fresh medium to a predetermined concentration. Next 900 μ L of bacterial solution ($\text{OD}_{600} = 0.1$) was mixed with 100 μ L of cPR₂ solution to a final concentration 2x MIC and incubated at 37°C with shaking (250 rpm) for 2 h. The cell

suspension was then centrifuged (8000 rpm, 10 min) and washed with saline twice. All bacteria were visualized by staining with SYTO 13 (green channel) for 30 min and propidium iodide (PI) (red channel) was added for 20 min to label dead bacterial cells. Bacteria cells were then fixed on a poly-D-lysine coated chambered cover glass and imaged with CLSM.

2.9 Sample Preparation for Imaging with 3D-SIM Super-Resolution Microscopy

E. coli ATCC 25922 cells (OD₆₀₀ 0.1) were incubated with cPR₂-FITC (100 µg/mL) in a tube at 37 °C with shaking (250 rpm) for 90 min. The bacteria suspension was collected and washed with DPBS twice (10,000 rpm, 10 min). Then 0.5 mL of FM 4–64FX dye (from a 5 µg/ml stock solution in DPBS) was added to the bacteria suspension and incubated on ice for 10 min. After incubation, *E. coli* cells were washed with DPBS (10000 rpm, 10 min) and fixed on a poly-D-lysine coated chambered cover glass in 4% *wt/vol* paraformaldehyde solution for 10 min at room temperature. The fixative was washed off with DPBS prior to imaging.

2.10 OM/IM Permeabilization Studies

E. coli ATCC 25922 was the model bacteria used to assess OM/IM permeability after treatment with cPR₂. OM permeability was evaluated by using the nitrocefin method. Briefly *E. coli* cells at stationary state were collected, suspended into 10 mM PBS (OD = 0.05) in a 96-well microplate, and incubated with 0.2 mM nitrocefin and 200 µg/mL cPR₂. Hydrolysis of nitrocefin was monitored by measuring OD₄₈₆ at 37 °C for 3 h. OD₄₈₆ of blank control (0.2 mM nitrocefin) were subtracted from raw data to yield (OD₄₈₆). Studies were carried out in triplicate.

Permeabilization of the IM in the presence of cPR₂ was assessed by measuring ONPG cleavage by β-galactosidase using *E. coli* cells prepared as described above. ONPG was added to wells to a concentration of 2.5 mM and substrate cleavage by β-galactosidase was monitored by noting the change in absorption at 400 nm (OD₄₀₀).

2.11 Potentiation Properties of cPR₂ With Antibiotics.

The MICs of VAN, ERY, and RIF in the absence and presence of 7.5 µg/mL, 15 µg/mL and 30 µg/mL cPR₂ against *P. aeruginosa* PAO1 and *E. coli* ATCC 25922 were evaluated and the fractional inhibitory concentration index (FICI) was calculated for each combination. Briefly, a 135 µL aliquot of MHB medium containing dilution series of antibiotic and cPR₂ was added to each well containing 15 µL aliquot of bacterial solutions in a 96-well plate. The plate was subsequently wrapped in parafilm and incubated at 37 °C for 18 h prior to assessing bacterial growth by measuring OD₆₀₀ using a Spectramax® Plus as previously described. The FICI for each combination was calculated to determine whether there was a synergistic interaction for each combination using the microdilution checkerboard method. A synergistic effect is observed for FICI < 0.5, FICI of 0.5–4 is commonly used to define indeterminate effects, and FICI > 4 indicates antagonistic effects.⁴⁰ The following equation was used, where MIC_A and MIC_B are the MICs of drugs A and B alone and MIC_{CA} and MIC_{CB} are the concentrations of the drugs in combination in all the wells corresponding to an MIC.⁴⁰

$$FICI = \frac{MIC_{CA}}{MIC_{A, alone}} + \frac{MIC_{CB}}{MIC_{B, alone}}$$

3. RESULTS AND DISCUSSION

3.1 Synthesis and Characterization of Cationic Polyrotaxanes (cPRs).

PR was first prepared by threading α -CD onto guest polymer PEG₂₀₀₀-(COOH)₂ and end-capping with adamantanamine. Successful formation of PR was verified by ¹H NMR, which showed a peak at δ 3.5 ppm corresponding to the methylene protons of PEG, a peak at δ 4.48 ppm corresponding to the hydroxyl protons of α -CD, and peaks corresponding to the adamantane end groups (2.25 ppm, 1.9 ppm). Based on NMR, there was an average of 16 α -CD threaded per PR which corresponds to a molecular weight of 18 kDa (Fig. S1, ESI[†]). Assuming each α -CD covers two ethylene glycol units of PEG in the full-coverage state,³⁷ a maximum of 22 α -CD can be threaded onto PEG 2000 Da and final PRs were therefore characterized by a threading efficiency of 72%. Next, all the hydroxyl groups of α -CD on PR were successfully activated for efficient conjugation to tert-Butoxycarbonyl Ethylenediamine (Boc-EDA) as the positive ligand and ethanolamine as the neutral ligand (Fig. S2, ESI[†]). At the end of the conjugation reaction, Boc protecting groups on EDA were removed with concentrated aqueous HCl to yield the final cationic polyrotaxanes possessing varying surface charge properties: cPR₀, cPR₁, cPR₂, and cPR₃.

Before modification of PR with the positive and neutral ligands, the material was insoluble in water due to hydrogen bonding between the hydroxyl groups of μ -CD,³⁸ however after conjugation to the ligands the solubility of cPR increased drastically when these hydrogen bonds were interrupted. The freedom of motion of CDs along the polymer chain allows cPR to manifest as random coils in appropriate solvents and explains their crescent shapes when viewed under transmission electron microscopy (TEM) even though PR is itself a linear supramolecular assembly.³⁹ The morphology of final cPRs was confirmed to be crescent-shaped (Fig. 1A and Fig. S3, ESI[†]) and dynamic light scattering (DLS) revealed an apparent diameter of 15 nm (Fig. 1B). As expected, the zeta potential of cPRs increased as the ratio of positive to neutral ligands conjugated to PR increased; all the physical properties of cPRs prepared are summarized in Table 1.

3.2 Minimum Inhibitory Concentrations of cPRs

Antibacterial activity of cPRs was evaluated by determining their minimum inhibitory concentrations (MIC) against strains of bacteria that are commonly associated with infection: *Pseudomonas aeruginosa* (PAO1) and *E. coli* (ATCC 25922) were chosen as the Gram-negative pathogens and *Staphylococcus aureus* (ATCC 25923) was chosen as the Gram-positive strain. The antimicrobial assays were conducted in cation-adjusted Mueller–Hinton broth (MHB), a nutrient-rich bacterial growth medium. As summarized in Table 1, cPR₁ characterized by a zeta potential of +36 mV exhibited the greatest antimicrobial

[†]Electronic Supplementary Information (ESI) available: Details of syntheses and NMR spectra, additional TEM and confocal images, cytotoxicity and hemolysis data, and antibacterial dose response curves.

activity against all three bacterial strains and was characterized by an MIC of 62 $\mu\text{g/mL}$ for *P. aeruginosa*, 90 $\mu\text{g/mL}$ against *E. coli*, and 62 $\mu\text{g/mL}$ against *S. aureus*. cPR₂ at +21 mV was characterized by an MIC of 90 $\mu\text{g/mL}$ for *P. aeruginosa*, 180 $\mu\text{g/mL}$ for *E. coli* and 90 $\mu\text{g/mL}$ against *S. aureus*. Finally, cPR₃ at +15 mV was characterized by an MIC of 250 $\mu\text{g/mL}$ for *P. aeruginosa*, 500 $\mu\text{g/mL}$ against *E. coli*, and 125 $\mu\text{g/mL}$ against *S. aureus*. cPR₀, which had been conjugated only with ethanolamine as the neutral ligand, possessed a zeta potential of +2.5 mV and displayed no antimicrobial activity against the 3 bacterial strains, with MIC >4000 $\mu\text{g/mL}$ (Fig. S4, ESI[†]). It is worth noting that *P. aeruginosa* and *S. aureus* appeared more sensitive to the effects of the cPRs since *E. coli* required 1.5–2 times more nanomaterial before its MIC was reached. Although *P. aeruginosa* and *E. coli* are both Gram-negative bacteria characterized by an outer membrane composed of a bilayer of LPS and phospholipids, their LPS structures and permeability properties differ markedly.^{40–41} Previous studies have shown that the LPS plays a critical role in mediating such cationic nanoparticle–cellular interactions.⁴² The LPS is made up of three different components which contributes to the net negative charge of the bacterial cell: an outer presenting strain-specific O-antigen (also called O-polysaccharide) segment, a core polysaccharide domain, and a lipid A segment responsible for anchoring the LPS to the bacterial membrane. Charged molecules are likely to interact with the core domains and the backbone of lipid A. *E. coli* lipid A has a longer fatty acid chain and is frequently described as being hexa-acylated compared to the penta-acylated lipid A of wild-type *P. aeruginosa* therefore the higher acylated lipid A of *E. coli* may make it less susceptible to positively charged nanomaterials.

3.3 Cytocompatibility of cPRs.

To evaluate the cytotoxicity of cPRs on mammalian cells, the metabolism-based resazurin assay was used. As expected, neutral cPR₀ showed little cytotoxicity against J774A.1 mouse macrophage cells and was characterized by an IC₅₀ >4000 $\mu\text{g/mL}$. Cells incubated with cPR₁ (+36 mV) were characterized by an IC₅₀ of 200 $\mu\text{g/mL}$ whereas cPR₂ (+21 mV) and cPR₃ (+15 mV) were characterized by IC₅₀ of 2000 and >4000 $\mu\text{g/mL}$ respectively (Fig. S5A, ESI[†]). Hemolytic activities of cPRs were investigated by incubating them with sheep red blood cells (RBCs) for 2 h and monitoring the release of hemoglobin from RBCs by absorbance at 405 nm (Fig. S5B, ESI[†]).⁴³ The HC₁₀ (concentration required to lyse 10% of the RBCs) for cPR₁ was 30 $\mu\text{g/mL}$ compared to >4000 $\mu\text{g/mL}$ for both cPR₂ and cPR₃. To evaluate the relative cytocompatible properties of cPRs with respect to their antimicrobial properties, the HC₁₀/MIC was calculated and found to be highest for cPR₂ at >22 compared to cPR₁ (0.3–0.5) and cPR₃ (>8). To further assess plasma membrane integrity of mammalian cells in the presence of cPRs, the colorimetric lactate dehydrogenase (LDH) assay was used. Based on the nanomaterial's ability to disrupt the mammalian plasma membrane, degree of LDH released is a direct indicator of membrane-induced cytotoxicity. LDH release was significantly greater for cPR₁ (+36 mV) compared to cPR₂ (+21 mV) at all concentrations evaluated (Fig. 2). This means that although cPR₁ exerts the best antimicrobial activity, it also causes more damage to mammalian cell membranes. Based on the cytocompatibility data, cPR₂ is therefore the safest nanomaterial to investigate further due to its potent antimicrobial activity, favorable HC₁₀/MIC, and low cytotoxic properties against mammalian cells (Table 1).

3.4 Effect of cPR₂ on Planktonic Bacteria

We monitored the effects of treating *E. coli*, *P. aeruginosa*, and *S. aureus* with cPR₂ at 2x MIC for each strain by counting colony forming units (CFU) at 0, 1, 2, and 3 h time points as previously conducted for other types of nanomaterials.²¹ It is important to note that 2x MIC was chosen because the initial concentration of bacteria used for this assay was 2 orders of magnitude more concentrated (OD₆₀₀ 0.1) than that used in the MIC study described earlier (OD₆₀₀ 0.001). The number of viable bacteria decreased by 99.9 % after 2 h incubation with cPR₂ for *P. aeruginosa* and *S. aureus* compared to 0 h, whereas *E. coli* growth decreased by more than 99.9% after 3 h (Fig. 3A); representative MH agar plates showing the decrease in CFU for all bacterial strains after 3h treatment with cPR₂ is shown in Fig. 3B. To further visualize the antimicrobial properties of cPR₂, live/dead bacterial planktonic cells were imaged using confocal laser scanning microscopy (CLSM). All bacteria were stained with the cell permeable SYTO 13 dye (a green fluorescent nucleic acid stain) and dead bacteria was identified with red propidium iodide (PI). As can be seen from the merged images in Fig. 3C, viable bacterial cells were readily visible in untreated samples whereas samples treated for 2 h with cPR₂ were characterized by substantially fewer planktonic cells that also stained red or yellow. These results visually support the data presented in Fig. 3A–B and confirms that cPR₂ can effectively kill planktonic bacterial cells.

3.5 Mechanism of cPR₂ and its Effect on Bacterial Membrane Permeability

To probe the mechanism by which cPR₂ kills bacteria, interactions between the nanomaterial and *E. coli* ATCC 25922 was visually monitored with super-resolution fluorescence imaging (Zeiss ELYRA S1 SR-SIM Super Resolution Microscope). *E. coli* cell membrane was labelled red with a lipid membrane dye (FM4–64FX) and cPR₂ was conjugated to green fluorescent FITC (FITC-cPR₂). Images of untreated *E. coli* bacterium reveal an intact membrane clearly outlined by the red lipid membrane dye (Fig. 4A and Fig. S6A, ESI†). After treatment with FITC-cPR₂ at 200 µg/mL, the bacterial membrane is no longer intact and penetration of the nanomaterial into the bacterium can be readily observed. In Fig. 4B, the nanomaterial can be seen adhering to the outer membrane of a red-labelled bacterium followed by its internalization and distribution in the cytosol (additional representative images can be found in Fig. S6B–D, ESI†).

To obtain more visual evidence to support the antibacterial mechanism of cPR₂, scanning electron microscopy (SEM) was employed to assess bacterial morphological changes before and after treatment with the nanomaterial. Before treatment, the morphology of *E. coli* bacterium exhibits clear edges and intact bodies (Fig. 4C). After treatment with cPR₂, evidence of bacterium deformation in the form of total collapse, membrane damage, and large holes were observed (Fig. 4D). These studies demonstrate that the antimicrobial properties of cPR₂ result from its ability to electrostatically 1) disrupt the bacterial cell membrane and 2) penetrate into the cytosol to affect critical intracellular processes.

To further elucidate the outer/inner membrane (OM/IM) permeabilizing capability of the nanomaterial against *E. coli* ATCC 25922, poorly permeable substrates of β-lactamase/β-galactosidase in the absence and presence of 200 µg/mL cPR₂ was investigated.⁴⁴ One of

these substrates, nitrocefin (a chromogenic cephalosporin), cannot effectively cross the OM of bacterial cells in the absence of a permeabilizer and is a useful reporter for demonstrating enhanced OM permeability.⁴⁵ Once nitrocefin crosses into the periplasm, β -lactamase enzymes present readily cleave it and this induces a change in nitrocefin's absorbance properties that can be monitored at 486 nm. As shown in Fig. 4E, (OD_{486}) significantly increased only upon co-incubation with cPR₂ in contrast to the small changes observed for untreated cells incubated with nitrocefin. Untreated controls exhibit some (OD_{486}) due to possible hydrolysis of nitrocefin (a relatively slow process) and/or limited diffusion of the drug on its own across the semipermeable OM. Similarly, IM permeability can be monitored at (OD_{400}) for the substrate o-nitrophenyl- β -D-galactoside (ONPG) which can be cleaved by β -galactosidase. This enzyme is present in bacterial cytosols and is released only when the IM integrity has been compromised. The (OD_{400}) monitors cleavage of the substrate in the external buffer solution following β -galactosidase release from cells as well as cleavage of ONPG due to limited diffusion of the substrate into the bacterium cytosol. The (OD_{400}) significantly increased only upon co-incubation with cPR₂ (Fig. 4F) compared to untreated bacteria. Overall, the data confirms that the antibacterial properties of cPR₂ can be ascribed to polycation-induced penetration of OM/IM bacterial cell membranes.

3.6 Potentiation Properties of cPR₂ with Antibiotics

The potentiation properties of cPR₂ in the presence of three poorly permeable antibiotics (vancomycin, erythromycin and rifampicin) was investigated on Gram-negative *P. aeruginosa* PAO1 and *E. coli* ATCC 25922 (Fig. S7–S8, ESI[†]). The MIC for each antibiotic alone as well as in the presence of 7.5 μ g/mL, 15 μ g/mL or 30 μ g/mL cPR₂ was determined and the fractional inhibitory concentration indices (FICI) were calculated to determine whether there was a synergistic interaction. A synergistic effect is observed for FICI < 0.5 , FICI of 0.5–4 is commonly used to define indeterminate effects, and FICI > 4 indicates antagonistic effects.⁴⁶ Based on the indicator range for cPR₂ investigated (Table 2), the vancomycin (VAN) combinations all displayed indeterminate effects against *P. aeruginosa* PAO1 and *E. coli* ATCC 25922. This may be because polycationic-induced disruptions in the OM created by cPR₂ was not pronounced enough to allow for the relatively larger glycopeptide VAN (1449 g/mol) to diffuse through compared to the smaller macrolide ERY (734 g/mol) and ansamycin RIF (823 g/mol) antibiotics. ERY and RIF need to cross a second IM to get into the cytosol to exert their antimicrobial effects but the FICI of ERY and RIF combinations with cPR₂ did display synergism. For *P. aeruginosa*, 15 μ g/mL cPR₂ in combination with 64 μ g/mL ERY or 4 μ g/mL RIF yielded synergistic effects, with the MIC of ERY and RIF each decreasing 4-fold respectively in the presence of cPR₂. Since *E. coli* ATCC 25922 is more resistant than *P. aeruginosa* PAO1 to the cationic effects of cPR₂, it is not surprising that slightly greater concentrations of 15–30 μ g/mL cPR₂ with 64 μ g/mL ERY or 4 μ g/mL RIF were required to display synergism, reducing the respective MIC of ERY and RIF each 4-fold again for combinations compared to the antibiotic alone. Although these reductions in MIC for all three antibiotics tested are encouraging, none of the MICs decreased below the clinical breakpoint for susceptibility against *Staphylococcus* spp. based on the 2017 Clinical and Laboratory Standards Institute (CLSI) guidelines of 2 μ g/mL for VAN, 0.5 μ g/mL for ERY, and 1 μ g/mL for RIF.⁴⁷ Taken together, these data show that the mechanism of potentiation by cPR₂ is complex and unpredictable for different

combinations. Furthermore, intrinsic resistance mechanisms unaccounted for in *E. coli* ATCC 25922 and *P. aeruginosa* PAO1 may also have been present and could have further limited the effectiveness of the combinations observed. Nevertheless, the data presented does demonstrate that cPR₂ can indeed disrupt the bacterial membranes of *E. coli* and *P. aeruginosa* at sub-MIC concentrations in vitro to synergistically potentiate the activity of ERY and RIF.

4. Conclusions

In summary, we report on the preparation of cationic polyrotaxanes (cPRs) with charge-optimized broad antimicrobial properties. cPR₂ characterized by a hydrodynamic size of 15 nm and a zeta potential of +21 mV demonstrated the best balance between antimicrobial activity and lowest cytotoxicity to mammalian cells. The cell kill mechanism of cPR₂ relies on polycationic interactions to disrupt the bacterial cell membrane followed by penetration of the nanomaterial into the bacteria. We have also demonstrated that the addition of cPR₂ at sub-MIC concentrations can increase *P. aeruginosa* PAO1 and *E. coli* ATCC 25922 bacterial membrane permeability to antimicrobial agents and synergy was observed for ERY and RIF combinations but not VAN.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This work was supported in part by NIH grant R01DK099596 awarded to M.P. Xiong.

Notes and references

1. Jones KE, Patel NG, Levy MA, Storeygard A, Balk D, Gittleman JL and Daszak P, Nature, 2008, 451, 990–994. [PubMed: 18288193]
2. Nathwani D, Raman G, Sulham K, Gavaghan M and Menon V, Antimicrob. Resist. In, 2014, 3, 32.
3. Kohanski MA, Dwyer DJ and Collins JJ, Nat. Rev. Microbiol, 2010, 8, 423–435. [PubMed: 20440275]
4. Boucher HW, Talbot GH, Bradley JS, Edwards JE, Gilbert D, Rice LB, Scheld M, Spellberg B and Bartlett J, Clin. Infect. Dis, 2009, 48, 1–12. [PubMed: 19035777]
5. Srinivas P and Rivard K, Curr. Infect. Dis. Rep, 2017, 19,38. [PubMed: 28895051]
6. Liu YY, Wang Y, Walsh TR, Yi LX, Zhang R, Spencer J, Doi Y, Tian GB, Dong BL, Huang XH, Yu LF, Gu DX, Ren HW, Chen XJ, Lv LC, He DD, Zhou HW, Liang ZS, Liu JH and Shen JZ, Lancet Infect. Dis, 2016, 16, 161–168. [PubMed: 26603172]
7. Seo MD, Won HS, Kim JH, Mishig-Ochir T and Lee BJ, Molecules, 2012, 17, 12276–12286. [PubMed: 23079498]
8. Gordon YJ, Romanowski EG and McDermott AM, Curr. Eye Res, 2005, 30, 505–515. [PubMed: 16020284]
9. Li P, Poon YF, Li WF, Zhu HY, Yeap SH, Cao Y, Qi XB, Zhou CC, Lamrani M, Beuerman RW, Kang ET, Mu YG, Li CM, Chang MW, Leong SSJ and Chan-Park MB, Nat. Mater, 2011, 10, 149–156. [PubMed: 21151166]
10. Yang YC, Cai ZG, Huang ZH, Tang XY and Zhang X, Polym. J, 2018, 50, 33–44.
11. Bechinger B and Gorr SU, J. Dent. Res, 2017, 96, 254–260. [PubMed: 27872334]
12. Yeaman MR and Yount NY, Pharmacol. Rev, 2003, 55, 27–55. [PubMed: 12615953]

13. Wimley WC, *Acs Chem. Biol.*, 2010, 5, 905–917. [PubMed: 20698568]
14. Brogden KA, *Nat. Rev. Microbiol.*, 2005, 3, 238–250. [PubMed: 15703760]
15. Zasloff M, *Nature*, 2002, 415, 389–395. [PubMed: 11807545]
16. Takahashi H, Caputo GA, Vemparala S and Kuroda K, *Bioconjugate Chem.*, 2017, 28, 1340–1350.
17. Marr AK, Gooderham WJ and Hancock REW, *Curr. Opin. Pharmacol.*, 2006, 6, 468–472. [PubMed: 16890021]
18. Forde E, Schutte A, Reeves E, Greene C, Humphreys H, Mall M, Fitzgerald-Hughes D and Devocelle M, *Antimicrob. Agents Ch.*, 2016, 60, 2813–2821.
19. Kuroda K and DeGrado WF, *J. Am. Chem. Soc.*, 2005, 127, 4128–4129. [PubMed: 15783168]
20. Engler AC, Shukla A, Puranam S, Buss HG, Jreige N and Hammond PT, *Biomacromolecules*, 2011, 12, 1666–1674. [PubMed: 21443181]
21. Takahashi H, Nadres ET and Kuroda K, *Biomacromolecules*, 2017, 18, 257–265. [PubMed: 27992189]
22. Martins AF, Facchi SP, Follmann HDM, Pereira AGB, Rubira AF and Muniz EC, *Int. J. Mol. Sci.*, 2014, 15, 20800–20832. [PubMed: 25402643]
23. Huang KS, Yang CH, Huang SL, Chen CY, Lu YY and Lin YS, *Int. J. Mol. Sci.*, 2016, 17, 1578.
24. Zabawa TP, Pucci MJ, Parr TR and Lister T, *Curr. Opin. Microbiol.*, 2016, 33, 7–12. [PubMed: 27232956]
25. Ng VWL, Ke XY, Lee ALZ, Hedrick JL and Yang YY, *Adv. Mater.*, 2013, 25, 6730–6736. [PubMed: 24018824]
26. Khalil H, Chen T, Riffon R, Wang R and Wang Z, *Antimicrob. Agents. Ch.*, 2008, 52, 1635–1641.
27. Corbett D, Wise A, Langley T, Skinner K, Trimby E, Birchall S, Dorali A, Sandiford S, Williams J, Warn P, Vaara M and Lister T, *Antimicrob. Agents Ch.*, 2017, 61, e00200–17
28. Drawz SM, and Bonomo RA, *Clin. Microbiol. Rev.*, 2010, 23, 160–201. [PubMed: 20065329]
29. Ehmann DE, Jahi H, Ross PL, Gu RF, Hu J, Kern G, Walkup GK, and Fisher SL, *Proc. Natl. Acad. Sci. U.S.A.*, 2012, 109, 11663–11668. [PubMed: 22753474]
30. Campbell J, Singh AK, Santa Maria JP, Kim Y, Brown S, Swoboda JG, Mylonakis E, Wilkinson BJ, and Walker S, *ACS Chem. Biol.*, 2011, 6, 106–116. [PubMed: 20961110]
31. Farha MA, Koteva K, Gale RT, Sewell EW, Wright GD, Gand Brown ED, *Bioorg. Med. Chem. Lett.*, 2014, 24, 905–910. [PubMed: 24393581]
32. Kakan L, and Wright GD, *Expert Rev. Mol. Med.*, 2011, 13, e511–e517.
33. Courtney CM, Goodman SM, Nagy TA, Levy M, Bhusal P, Madinger NE, Detweiler CS, Nagpal P and Chatterjee A, *Sci. Adv.*, 2017, 3, e1701776. [PubMed: 28983513]
34. Wenz G, Han BH and Muller A, *Chem. Rev.*, 2006, 106, 782–817. [PubMed: 16522009]
35. Garcia-Rio L, Otero-Espinar FJ, Luzardo-Alvarez A and Blanco-Mendez J, *Curr. Top Med. Chem.*, 2014, 14, 478–493. [PubMed: 24354671]
36. Nakashima N, Kawabuchi A and Murakami H, *J. Inclus Phenom. Mol.*, 1998, 32, 363–373.
37. Harada A, Li J and Kamachi M, *Macromolecules*, 1993, 26, 5698–5703.
38. Araki J and Ito K, *Soft Matter*, 2007, 3, 1456–1473.
39. Tardy BL, Tan S, Dam HH, Ejima H, Blencowe A, Qiao GG and Caruso F, *Nanoscale*, 2016, 8, 15589–15596. [PubMed: 27509868]
40. Caroff M and Karibian D, *Carbohydr. Res.*, 2003, 338, 2431–2447.
41. Zgurskaya HI, Lopez CA and Gnanakaran S, *ACS Infect. Dis.*, 2015, 1, 512–522. [PubMed: 26925460]
42. Jacobson KH, Gunsolus IL, Kuech TR, Troiano JM, Melby ES, Lohse SE, Hu D, Chrisler WB, Murphy CJ, Orr G, Geiger FM, Haynes CL and Pedersen JA, *Environ. Sci. Technol.*, 2015, 49, 10642–10650. [PubMed: 26207769]
43. Saha K, Moyano DF and Rotello VM, *Mater. Horizons*, 2014, 1, 102–105.
44. Eriksson M, Nielsen PE and Good L, *J. Biol. Chem.*, 2002, 277, 7144–7147. [PubMed: 11739379]
45. Epand RF, Pollard JE, Wright JO, Savage PB and Epand RM, *Antimicrob. Agents Ch.*, 2010, 54, 3708–3713.

46. Odds FC, J. Antimicrob. Chemoth, 2003, 52, 1-1.
47. Wayne PA, Clinical and Laboratory Standards Institute (CLSI): Performance Standards for Antimicrobial Susceptibility Testing. 2017, 27th ed. CLSI supplement M100, Wayne, PA, 27th ed, 2017, 60–63.

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Fig. 1. TEM of cPR2 reveals ca. 20 nm crescent-shaped morphological structures (A), and hydrodynamic size of all cPRs prepared averaged ca. 15 nm (B).

Fig. 2. LDH release comparing cPR₁ and cPR₂ in J77A4.1 mouse macrophage cells. cPR₂ at +21 mV affects the mammalian cell plasma membrane significantly less than cPR₁ characterized by a zeta potential of +36 mV at all concentrations investigated.

Fig.3.

Bactericidal time-kill of cPR2 at 2x MIC against *E. coli* ATCC 25922, *P. aeruginosa* PAO1 and *S. aureus* ATCC 25923 reveal >99.9% bacteria are dead after 2–3 h incubation (A); error bars represent mean $\pm$ standard deviation for $n = 3$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$. Representative images of MH agar plates showing colony forming units (CFU) for *E. coli*, *P. aeruginosa* and *S. aureus* before (top) and after (bottom) treatment with cPR2 at 2x MIC after 3 h (B). CLSM images of Live/Dead bacteria before (top) and after (bottom) cPR2 treatment for 2 h. SYTO 13 (green) was used to label both live and dead bacteria and propidium iodide (red) was the stain used to identify dead bacteria (C); scale bar: 10 μ m.

Fig. 4.

3D-SIM images of *E. coli* ATCC 25922 bacteria before (A) and after treatment with 200 µg/mL FITC-cPR2 in MHB for 90 min (B); scale bar: 2 µm. SEM of *E. coli* ATCC 25922 bacteria before (C) and after treatment with cPR2 for 90 min with arrows pointing to large holes in the bacterial membrane (D); scale bar: 2 µm. Nitrocefin assay was utilized to monitor OM permeability in the presence (200 µg/mL) and absence of cPR2 (E); IM permeability was evaluated by measuring β-galactosidase activity in the presence (200 µg/mL) and absence of cPR2 (F).

Scheme 1.

Graphical illustration for preparing cationic polyrotaxanes (cPRs) with variable charge densities. All the hydroxyl groups of α -CD on PR were successfully activated with CDI for efficient conjugation to Boc-EDA as the positive ligand ($R = a$) and ethanolamine as the neutral ligand ($R = b$). At the end of the conjugation reaction, Boc protecting groups were removed with concentrated aqueous HCl to yield the final four cPRs possessing varying surface charge properties: cPR₀, cPR₁, cPR₂, and cPR₃

Table 1.

Antimicrobial activity and mammalian cytocompatibility of cPRs.

PRs	D (nm)	Z (mV)	MIC ($\mu\text{g/mL}$)			IC ₅₀ ($\mu\text{g/mL}$) J774A.1	HC ₁₀ ($\mu\text{g/mL}$) RBC	Selectivity HC ₁₀ /MIC
			<i>P. aeruginosa</i> (PAO1)	<i>E. coli</i> (ATCC 25922)	<i>S. aureus</i> (ATCC 25923)			
cPR ₀	14.7±1.5	2.5±1.3	> 4000	> 4000	> 4000	> 4000	>4000	
cPR ₁	15.8±0.7	36±5.4	62	90	62	200	30	0.3–0.5
cPR ₂	15.2±1.2	20.8±4.2	90	180	90	2000	>4000	>22
cRR ₃	16.2±2.5	15±5.6	250	500	125	>4000	>4000	>8

HC₁₀: concentration causing 10% lysis (hemolysis) of red blood cells (RBCs) relative to 1% Triton-X (positive control, 100%) and DPBS (negative control).

Table 2.

Antibiotic potentiation properties for cPR₂ at sub-MIC concentrations (7.5–30 µg/mL) against *P. aeruginosa* PAO1 and *E. coli* ATCC 25922.

Antibiotic	MIC (µg/mL)					
	<i>P. aeruginosa</i> (PAO1)			<i>E. coli</i> (ATCC 25922)		
	Antibiotic alone	Combination (antibiotic, cPR ₂)	FICI (effect)	Antibiotic alone	Combination (antibiotic, cPR ₂)	FICI (effect)
Vancomycin	>1024	256, 30	<0.58(I)	512	256, 30	0.58(I)
		1024, 15	<1.16(I)		512, 15	1.08(I)
		1024, 7.5	<1.08(I)		512, 7.5	1.04(I)
Erythromycin	256	64, 30	0.58(I)	256	64, 30	0.41(S)
		64, 15	0.41(S)		64, 15	0.33(S)
		256, 7.5	0.58(I)		256, 7.5	1.04(I)
Rifampicin	16	4, 30	0.58(I)	16	4, 30	0.41(S)
		4, 15	0.41(S)		8, 15	0.58(I)
		16, 7.5	1.08(I)		8, 7.5	0.54(I)

I = indeterminate, S = synergistic