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Antimicrobial polymers as therapeutics for treatment of multidrug-resistant *Klebsiella pneumoniae* lung infection

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ABSTRACT

Klebsiella pneumoniae (*K. pneumoniae*) is one of the most common pathogens in hospital-acquired infections. It is often resistant to multiple antibiotics (including carbapenems), and can cause severe pneumonia. In search of effective antimicrobials, we recently developed polyionenes that were demonstrated to be potent against a broad-spectrum of microbes *in vitro*. In this study, polyionenes containing rigid amide bonds were synthesized to treat multidrug-resistant (MDR) *K. pneumoniae* lung infection. The polyionene exhibited broad-spectrum activity against clinically-isolated MDR bacteria with low minimum inhibitory concentrations (MICs). It also demonstrated stronger antimicrobial activity against 20 clinical strains of *K. pneumoniae* and more rapid killing kinetics than imipenem and other commonly used antibiotics. Multiple treatments with imipenem and gentamycin led to drug resistance in *K. pneumoniae*, while repeated use of the polymer did not cause resistance development due to its membrane-disruption antimicrobial mechanism. Additionally, the polymer showed potent anti-biofilm activity. In a MDR *K. pneumoniae* lung infection mouse model, the polymer demonstrated lower effective dose than imipenem with negligible systemic toxicity. The polymer treatment significantly alleviated lung injury, markedly reduced *K. pneumoniae* counts in the blood and major organs, and decreased mortality. Given its potent *in vivo* antimicrobial activity, negligible toxicity and ability of mitigating resistance development, the polyionene may be used to treat MDR *K. pneumoniae* lung infection.

Statement of Significance

Klebsiella pneumoniae (*K. pneumoniae*) is one of the most common pathogens in hospital-acquired infections, is often resistant to multiple antibiotics including carbapenems and can cause severe pneumonia. In this study, we report synthesis of antimicrobial polymers (polyionenes) and their use as antimicrobial agents for treatment of *K. pneumoniae*-caused pneumonia. The polymers have broad spectrum antibacterial activity against clinically isolated MDR bacteria, and eliminate MDR *K. pneumoniae* more effectively and rapidly than clinically used antibiotics. The polymer treatment also provides higher survival rate and faster bacterial removal from the major organs and the blood than the antibiotics. Repeated use of the polymer does not lead to resistance development. More importantly, at the therapeutic dose, the polymer treatment does not cause acute toxicity. Given its *in vivo* efficacy and negligible toxicity, the polymer is a promising candidate for the treatment of MDR *K. pneumoniae*-caused pneumonia.

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1. Introduction

The Gram-negative opportunistic bacterial pathogen *Klebsiella pneumoniae* (*K. pneumoniae*), which exists in the normal flora of the mouth, skin and intestine, is responsible for the majority of hospital-acquired infections [1,2]. *K. pneumoniae*-induced pneumonia has been one of the most urgent global health threats due to its high incidence of complications, and an approximately 50% mortality rate under current antimicrobial therapy [3,4]. Besides, patients infected by *K. pneumoniae* usually have other complications such as asthma, allergic airway inflammation, cystic fibrosis or chronic obstructive pulmonary syndrome, rendering it challenging to treat [5–7]. Moreover, over the past few years, a growing incidence of multidrug-resistant (MDR) *K. pneumoniae* has been frequently reported [8,9]. Carbapenems like imipenem and meropenem are the first recommended antibiotics for treating MDR *K. pneumoniae* infections. Due to a significant increase of extended-spectrum beta-lactamase-producing *K. pneumoniae*, carbapenem has been often used as a common drug of choice, thereby resulting in the emergence of carbapenem-resistant *K. pneumonia* [10,11]. Polymixins are "the last resort" antibiotics for treatment of MDR Gram-negative bacterial infections including carbapenem-resistant *K. pneumoniae* although treatments with polymyxins can cause nephrotoxicity and neurotoxicity [12,13]. With the increased use of polymyxins in recent years, polymyxins resistance has also been reported [14], mainly attributed to chromosomal mutations that lead to altered lipopolysaccharide composition, formation of polysaccharide capsule or efflux pump function [15]. To date, therapeutic options for the treatment of carbapenem-resistant *K. pneumoniae* infections remain strictly scarce. Given the severity of *K. pneumoniae* infections and the lack of effective antibiotics against carbapenem-resistant *K. pneumoniae*, there is an urgent need to develop new antimicrobial agents that are capable of killing MDR *K. pneumonia* and mitigating resistance development.

Several classes of membrane-active cationic polymers have emerged as antimicrobial materials to effectively combat microbes [16–37]. More, importantly, these cationic materials have been shown to work against MDR pathogens [1,21,24,28,38–40]. Recent studies have shown that these membrane-active polymers are effective against numerous MDR species, including *A. baumannii*, *E. coli*, *K. pneumoniae*, methicillin-resistant *S. aureus*, *C. albicans*, to name a few [24,33,41,42]. Compared to conventional antibiotics, these cationic polymers are less likely to develop resistance, rendering them attractive for health care applications [38,39,42,43]. Among numerous classes of antimicrobial compounds, due to ease of synthesis and scale-up, polyionenes have emerged as a promising candidate. Cationic charge resides along the backbone in polyionenes and by careful selection of building blocks, the cationic charge density and hydrophobicity of these polymers can be tailored [44–47]. This additional dimension to tailor the polymer structure-properties along with numerous commercially available building blocks, renders polyionenes attractive materials to combat MDR pathogens.

Recently we have explored the antimicrobial properties of several polyionenes [48]. These polymers were synthesized using commercially available aromatic bis-halides and bis-dimethylamine-containing monomers through addition polymerization. These polymers were found to be potent with broad spectrum of antimicrobial function, excellent killing kinetics and *in vivo* skin biocompatibility. More importantly, these polymers were active against clinically isolated pathogens. These findings demonstrate that polyionenes are an important family of antimicrobial polymers.

Backbone rigidity has been recognized as an important paradigm in designing potent antimicrobials [18,49]. Similarly, the presence of amide bond on cationic polymers has also been demonstrated to improve antimicrobial properties [19,50]. Experimental evidences have shown improvements in antimicrobial activity due to selective interactions with the bacterial membrane in the presence of rigid motifs and hydrogen-bonding groups. Inspired by these studies, herein, we report on polyionenes with rigid amide bonds along the polymer backbone. The polymers were evaluated against standard strains of bacteria and clinically-isolated MDR *K. pneumonia*. The bioactivity of the polyionenes was compared against imipenem and other commonly used antibiotics. Both *in vitro* and *in vivo* studies demonstrated that the polymer had potential to treat MDR *K. pneumoniae* lung infection with negligible toxicity and ability of mitigating resistance development.

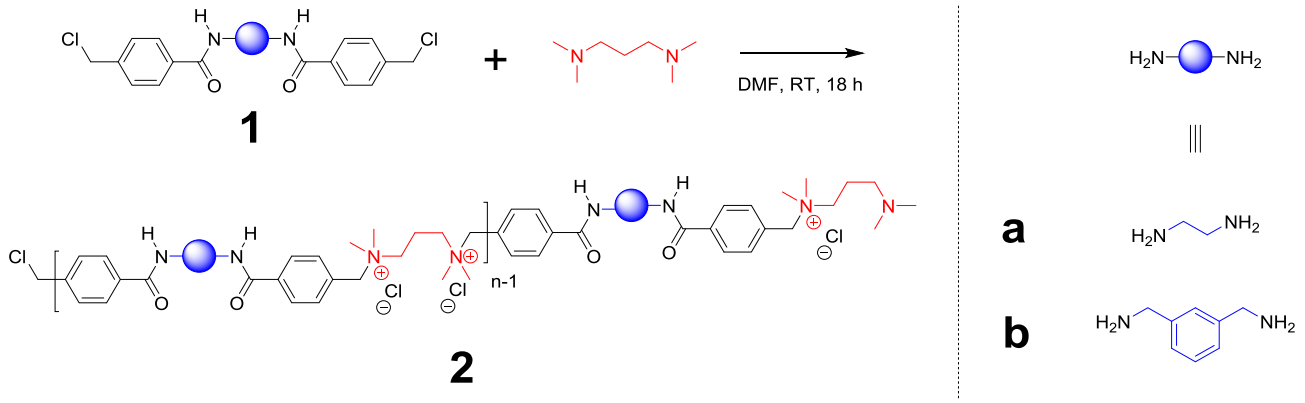
2. Materials and methods

2.1. Synthesis

Unless otherwise stated, all reagents and starting materials were purchased from Sigma Aldrich or Tokyo Chemical Industry (TCI) and were used as received. Solvents were of American Chemical Society (ACS) or high-performance liquid chromatography (HPLC) grade. The monomers and polymers were analyzed for ^{1}H NMR spectra using a Bruker Avance 400 spectrometer: operated at 400 MHz with the solvent proton signal as the internal reference standard (deuterated dimethyl sulfoxide, DMSO d_6 and D_2O for monomer and polymer, respectively). Aqueous size exclusion chromatography (SEC) was conducted in the following solvent mixture [51]: HPLC H_2O: methanol: acetic acid = 54: 23: 23 with 0.5 M sodium acetate (salt concentration with respect to entire solvent mixture) as the eluent at 0.5 mL/min flowrate. SEC was recorded on a Waters 2695 separation module equipped with a Waters 2414 differential refractometer and Waters Ultrahydrogel 120 and 500 columns (7.8 × 300 mm). Polymer solutions were prepared at ~5 mg/mL and injection volume was 100 μL. Empower 3 software (Waters Corporation, U.S.A.) was used for data collection and analysis. The columns were calibrated with a combination of poly(ethylene glycol) and poly(ethylene oxide) standards (PSS Polymer Standard Service, GmbH, Germany).

2.1.1. General procedure for the synthesis of A_2 monomer

*2.1.1.1. N,N'-(ethane-1,2-diyl)bis(4-(chloromethyl)benzamide) (**1a**) (Scheme 1).* In a two-necked round bottom flask (500 mL) equipped with magnetic stir bar and nitrogen inlet adaptor, 4-(chloromethyl)benzoyl chloride (10.06 g, 53.22 mmol, 2.0 equiv.) and tetrahydrofuran (THF, 20 mL) were allowed to equilibrate under ice-cold conditions for about 30 min. To this solution, a mixture of ethylene diamine (1.57 g, 26.12 mmol, 1.0 equiv.) and triethylamine (12.0 mL, 8.7 g, 86.1 mmol, 3.1 equiv.), dissolved in THF (40 mL) were added drop-wise through a dropping funnel over ~30 min. White precipitates were formed immediately. The reaction mixture was allowed to proceed at room temperature for additional 90 min. De-ionized (DI) water (~200 mL) was added to the reaction mixture to dissolve the triethylamine salts and also precipitate the product. This suspension was further chilled in an ice bath for about an hour, followed by isolation of the products as a solid by vacuum filtration. The product was washed with DI water, and was dried under high vacuum to yield white powdery solid. **Isolated yield**: 7.50 g (78.6%); **^{1}H NMR** (400 MHz, DMSO d_6, δ, ppm): 8.66 (t, *J* = 5.6 Hz, 2H, N*H*, **d**), 7.85 (m, 4H, aromatic C*H*,



Scheme 1. Synthesis of polyionenes containing rigid amide motifs from bis-halide monomers and readily commercially available tetramethyl-1,3-diaminopropane.

c), 7.52 (m, 4H, aromatic C*H*, **b**), 4.80 (s, 4H, CH_2Cl, **a**), 3.45 (m, 4H, NHCH_2CH_2NH, **e**).

*2.1.1.2. N,N'-(1,3-phenylenebis(methylene))bis(4-(chloromethyl)benzamide) (**1b**).* General procedure similar to **1a** was used. **Isolated yield:** 9.37 g (80.6%; crop 1 – recrystallized from acetonitrile); **^{1}H NMR** (400 MHz, DMSO d_6, δ, ppm): 9.09 (t, *J* = 6.0 Hz, 2H, N*H*, **d**), 7.85 (m, 4H, aromatic C*H*, **c**), (m, 4H, aromatic C*H*, **c**), 7.50 (m, 4H, aromatic C*H*, **b**), 7.32 – 7.15 (m, 4H, aromatic C*H*, f, g and **h**), 4.81 (s, 4H, CH_2Cl, **a**), 4.46 (d, *J* = 6.0 Hz, 4H, –CONH-CH_2-, **e**).

2.1.2. General procedure for the synthesis of polyionenes

*2.1.2.1. Synthesis of poly(1a-co-tetramethyl-1,3-diaminopropane), **2a**.* In a scintillation vial (20 mL) equipped with magnetic stir bar, monomer **1a** (360.0 mg, 985.6 μmol) and tetramethyl-1,3-diaminopropane (131.0 mg, 1006 μmol) were suspended in DMF (3.0 mL). The reaction mixture gently heated with heat gun to render a clear solution. The clear solution was allowed to stir at room temperature. The reaction mixture turned cloudy and in ~1.5 h, polymer was found to deposit on the walls of the vial. The reaction mixture was allowed to stir overnight (≈18 h), and was then precipitated into diethyl ether (50 mL) to result in a solid white powder. The white power was dissolved/suspended in ~5 mL methanol, and ~ 45 mL diethylether was slowly added to precipitate the polymer and this process was repeated once more. The solids were isolated and dried in vacuuo to result in white solid at near quantitative yields. The polymer was further purified by first dissolving in DI water, followed by extensive dialysis against DI water using dialysis membrane with a molecular weight cut-off (MWCO) of 1 kDa, and lyophilization to result in the target polymer as fluffy solids.

Aqueous size exclusion chromatography indicated that the polymers **2a** and **2b** had M_w of 8.7 and 10.2 kDa, respectively (uncorrected polyethylene glycol equivalent molar mass). Molar-mass dispersity ($Đ_M$) of polymers **2a** and **2b** were found to be 1.61 and 1.73, respectively.

2.2. Bacterial strains

Clinically isolated MDR *K. pneumoniae*, *E. coli*, *A. baumannii*, *P. aeruginosa* and MRSA strains, which were obtained from patients' blood or phlegm, were provided by the First Affiliated Hospital of Medical College, Zhejiang University (Hangzhou, China). All these samples were identified by routine laboratory methods, and susceptibility test of these strains proved resistance towards various antibiotics (Table S1). Commercial strains *S. aureus* (ATCC No. 6538), *E. coli* (ATCC No. 25922) and *P. aeruginosa* (ATCC No. 9027) were obtained from ATCC, U.S.A., and reconstituted according to the suggested protocols.

2.3. Measurement of minimum inhibitory concentration (MIC)

The MICs of the polymers and antibiotics [imipenem was a clinically used drug combination, Tienam (a combination of imipenem and cilastatin = 25/29, wt./wt.; imipenem ≈ 46 wt%), purchased from Tongde Hospital of Zhejiang Province; vancomycin, ceftriaxone, gentamycin, levofloxacin and polymyxin B were purchased from Dalian Meilun Biotechnology Co.] against the MDR bacterial strains and commercial bacterial strains were determined using a broth microdilution method [52]. Briefly, the bacteria were harvested in mid-exponential growth phase after grown overnight in Mueller-Hinton (MH) agar plates at 37 °C. The bacteria suspension was diluted with phosphate-buffer saline (PBS, PH 7.4) to the concentration of 1×10^8 colony-forming unit (CFU)/mL. The bacteria suspension was further diluted by 100-fold with MHB (1×10^6 CFU/mL or 3×10^5CFU/mL for the ATCC bacterial strains). Bacterial suspension and the antimicrobial agent solution were mixed in a 96-well plate, and incubated for 18 h at 37 °C. The MIC was determined as the lowest concentration of the antimicrobial agents, at which no turbidity was seen with unaided eyes or measured using a microplate reader (TECAN, Switzerland) (for the ATCC bacterial strains). Bacterial suspension without any treatment was the negative control. Each MIC was tested in triplicate.

2.4. Hemolysis assay

Rat red blood cells (rRBC) freshly obtained from Animal Handling Unit from Biomedical Research Center, Singapore were used to check on the undesired biological activity of polymers against mammalian cells. The rRBC were diluted to 4% (v/v) using phosphate-buffered saline (PBS) before an equal volume (100 μL) was added to 100 μL of polymeric solution (concentration ranging from 1 to 2000 μg/mL). The mixture was incubated for an hour under 37 °C in an incubator. Following incubation, the mixture was centrifuged at 1000*g* for 10 min and 100 μL of the supernatant was transferred into the 96-well plates. Hemoglobin released was determined using the microplate reader at 576 nm. Untreated rRBC suspended in PBS and rRBC treated with 0.1% (v/v) Triton-X were negative and positive controls, respectively. Percentage hemolysis was calculated as:

$$Hemolysis(\%) = \frac{O.D.\ of\ treated\ sample - O.D.\ of\ negative\ control}{O.D.\ of\ positive\ control - O.D.\ of\ negative\ control} \times 100\%$$

Each assay was performed in 4 replicates and repeated 3 times.

2.5. In vitro time-kill assay

A time-kill test was used to evaluate killing kinetics of the antimicrobial polymer against *K. pneumoniae* 8637. Briefly, *K. pneumoniae* 8637 were grown overnight in an MH agar plate at 37 °C The bacteria suspension (1×10^6 CFU/mL) was prepared as described in the above section. The suspension was exposed to antimicrobial polymer, imipenem and gentamycin at concentrations of 1 × MIC, 2 × MIC, and 4 × MIC at 37 °C. Samples (50 μL) were taken out at 0, 10, 20, 30, 40, 50, 60 and 120 min, and diluted with various dilution factors. Each bacteria suspension (50 μL) was plated on an MH agar plate and then incubated at 37 °C for 24 h before counting the number of viable colonies. An untreated bacteria suspension was employed as the negative control. All experiments were performed in triplicates. The results were presented as mean lg (CFU/mL) ± SD.

2.6. Membrane integrity study

To study the integrity of bacterial membrane after polymer treatment, leakage of cytoplasmic materials (e.g. proteins and genes) was tested with and without polymer treatment. Briefly, *K. pneumoniae* 8637 were grown overnight on an MH agar plate at 37 °C, and then suspended in PBS at 1×10^9 CFU/mL. The bacteria suspension was exposed to polymer **2a** at final concentrations of 1 × MIC, 2 × MIC, 4 × MIC, 8 × MIC and 16 × MIC, and incubated at 37 °C for 2 h. The suspension was filtered with a 0.22 μm filter to obtain the supernatant, and its absorbance at 260 nm was recorded using a UV spectrophotometer (Allsheng, China). Bacteria suspension without any treatment was employed as the negative control. The experiments were performed in triplicates, and the results were shown as mean ± SD.

2.7. Transmission electron microscopy (TEM)

The morphology of *K. pneumoniae* 8637 before and after the treatment with polymer **2a** was examined under a JEM-1230 TEM (JEOL, Japan) with an acceleration voltage of 80 kV [33]. After overnight culture, the bacteria suspension was diluted in PBS to achieve a concentration of 1×10^9 CFU/mL. This suspension was then subjected to 2-h treatment with polymer **2a** at 4 × and 8 × MIC. An untreated bacteria suspension was used as the control. The supernatant was removed, and the bacterial cells were fixed and dehydrated. Ultrathin sections of the bacterial specimens (70–90 nm) were obtained using a Reichert-Jung Ultracut E Ultramicrotome, and stained with uranyl acetate and alkaline lead citrate for 5 and 10 min, respectively. The morphology of the bacterial cells was observed under Hitachi Model H-7650 TEM.

2.8. Confocal microscopic study to observe membrane disruption

K. pneumoniae 8637 suspension with a concentration of 1×10^9 CFU/mL was obtained as described above, and the bacterial suspension (500 μL) was cultured in a 24-well plate at 37 °C with shaking at 150 rpm for 10 h. MHB containing FITC-dextran (100 kD) and polymer **2a** was added to the bacterial suspension at final concentrations of FITC-dextran and polymer **2a** at 250 μg/mL and 4 × MIC, respectively, and incubated for 2 h at 37 °C. Each well was subsequently washed three times with PBS and fixed overnight at 37 °C. The bacteria were observed under a Nikon AIR confocal microscope (100× (oil), Plan Apochromate Lens).

2.9. In vitro resistance evolution study

To study if repeated use of the polymer and antibiotics would lead to resistance development, *K. pneumoniae* 8637 were exposed to the polymer, imipenem or gentamycin at sub-lethal doses for 15 passages, and MICs of these antibacterial agents was monitored and compared with those at passage 0 [53]. Briefly, the bacteria were exposed to these antibacterial agents at various concentrations. Bacterial suspension (20 μL) from wells of 0.5 × MIC determined at passage 0 was taken out and plated on a MH agar plate overnight, and MIC of these antibacterial agents was determined at passage 1. Bacterial suspension from wells of 0.5 × MIC determined at passage 1 was taken out for measurement of MIC at passage 2. Similarly, MIC was determined at passages 3–15. Increase in MIC indicates resistance development.

2.10. Biofilm removal

Biofilm removal ability of the polymer was evaluated according to the protocol reported previously [54]. Briefly, the biofilm of *K. pneumoniae* 8637 was formed after 7 days of culture. The biofilm was then treated with polymer **2a** at MIC, 2 × MIC, 4 × MIC and 8 × MIC for 24 h. The biomass of the biofilm and the viability of cells in the biofilm were measured. The biofilm without polymer treatment was used as a control. Additionally, imipenem was employed as a control antibiotic. The results were presented as a mean ± SD.

2.11. Animals

ICR mice (female, 8 weeks old, 26–28 g) were used for *in vivo* studies. Mice were administered with an intraperitoneal injection of cyclophosphamide (Hengrui Corp, Jiangsu Province, P. R. China) at 200 mg/kg of body weight to induce immunosuppression 4 days prior to infection. Mice were anesthetized by intra-peritoneal injection of 1% pentobarbital (40 mg/kg, Sigma). All animal procedures were performed according to protocols approved by the Animal Studies Committee, P. R. China.

2.12. Pulmonary infection

The *in vivo* efficacy of the polymer was studied in a *K. pneumoniae* 8637-infected pneumonia mouse model. The immunosuppression of mice was induced as described above. Overnight cultures of *K. pneumoniae* 8637 were harvested and suspended in PBS. Before instilled with *K. pneumoniae* 8637, the mice were anesthetized using an intraperitoneal injection with 1% pentobarbital (40 mg/kg). Each of the immunosuppressed mice was infected - intra-nasally with 30 μL of the bacterial suspension at various doses (i.e. 1×10^9, 1.5×10^9, 2.3×10^9, 3.5×10^9, 5.3×10^9 and 8.0×10^9 CFU/ml, four mice per group). The minimum lethal dose was defined as the lowest dose that was sufficient to cause 100% mortality. It was determined from the survival rate of mice at day 5 post-infection using the BLISS method [55].

2.13. In vivo efficacy of the polymer in treating K. pneumoniae-caused pneumonia

The bacteria suspension was given to mice intra-nasally at the minimum lethal dose (30 μL). Polymer **2a** and imipenem were administered intraperitoneally once daily for 3 days starting at 4 h after infection at various doses (i.e. 0.1, 1.0, 2.0, 4.0 and 8.0 mg/kg for polymer **2a**, 0.1, 1.0, 5.0, 10.0, 20.0 and 40.0 mg/kg for imipenem, 200 μL/20 g, four mice per group). The survival of mice in each group was recorded for 5 days to assess ED_{50}, dose of polymer **2a** or imipenem, at which 50% of infected mice are saved, using the BLISS method [55].

To further determine the *in vivo* efficacy of polymer **2a** and imipenem, survival of the infected mice was monitored after treatment with polymer **2a** or imipenem. Briefly, the mice were

randomly divided into PBS-treated group, polymer **2a**-treated group and imipenem-treated group (ten mice per group). After anesthetized, each of the immunosuppressed mice was inoculated intra-nasally with 0.03 mL of bacteria suspension at the minimum lethal dose as determined above. Polymer **2a** or imipenem (1.0 and 2.0 mg/kg of body weight, respectively) was administered intraperitoneally once daily for 3 days starting at 4 h after infection. The mice were monitored for a period of five days and the number of surviving mice in each group was recorded. Survival was determined by using Kaplan-Meier curve.

Besides, bacterial counts in the organs such as lung, blood, liver, spleen and kidney were determined to assess treatment efficacy. Briefly, after anesthetized with an intraperitoneal injection of 1% pentobarbital (40 mg/kg), the immunosuppressed mice were instilled intra-nasally with 2×10^7 CFUs of *K. pneumoniae* 8637 (three mice per group). Polymer **2a** or imipenem at their respective ED_{95} dose (200 μL/20 g of body weight) was administered intraperitoneally once daily for 3 days starting at 4 h after infection. At day 5 post infection, mice were first anesthetized by the method mentioned above. The mice were sacrificed and 1 mL of blood sample and tissue samples including liver, spleen, kidney and lung were obtained. Subsequently, tissue samples were homogenized in 1 mL of PBS. Samples (200 μL) were taken out from the homogenates and diluted with various dilution factors. Each sample (200 μL) was plated on MH agar and grown overnight at 37 °C. The number of viable bacteria in the lungs, blood, liver, spleen and kidneys was counted. The results are presented as lg (CFU/mL of blood or homogenate).

2.14. Histological analysis

In accordance with a routine histological procedure, the lungs were fixed in 10% formalin. The formalin-fixed lungs were used for hematoxylin and eosin (H&E) staining, and the morphological alteration of the lungs was examined using a light microscope (40×, Olympus, Japan) to assess tissue damage.

2.15. In vivo toxicity study

To assess the systemic toxicity of polymer **2a**, its median lethal dose (LD_{50}), which leads to death of 50% mice, was determined. Mice were randomly grouped into six treatment groups (six mice per group). After dissolved in PBS, polymer **2a** was given to mice intraperitoneally at different doses (i.e. 20.0, 30.0, 45.0, 67.5, 101.3 and 151.9 mg/kg of body weight, 200 μL/20 g of body weight). The number of surviving mice in each group was monitored for five days after treatment, and the values of LD_{50} were calculated using the BLISS method [55].

The *in vivo* toxicity of polymer **2a** was further evaluated by analysis of the serum chemistry profile including liver and kidney functions, sodium and potassium ion concentrations. Mice were randomly assigned into the PBS control group and polymer **2a**-treated group (six mice per group). Each mouse received an intraperitoneal injection of polymer **2a** at its ED_{95} dose (200 μL/20 g of mouse body weight) or PBS (200 μL) once daily for 3 consecutive days. The mice were anaesthetized and the blood samples were obtained from periorbital plexus at day 5 after the first treatment with PBS or polymer **2a**, and sent to the First Affiliated Hospital, Zhejiang University (Hangzhou, China) for analysis of alanineaminotransferase (ALT), aspartateaminotransferase (AST), creatinine, urea nitrogen, sodium and potassium ion concentrations. The results were presented as a mean ± SD.

2.16. Statistical analysis

Statistical analysis between two groups was performed using Student's *t*-test. Differences were considered significant with a P value ≤ 0.05. Mouse survival rate was calculated using a Kaplan-Meier curve, with a P value ≤ 0.05 being statistically significant based on a log-rank (Mantel-Cox) test.

3. Results and discussion

3.1. Polymer synthesis

Functional bis-halide monomers (**1a** and **1b**) containing amide groups were synthesized from commercially available diamines (**a** and **b**) and 4-(chloromethyl)benzoyl chloride in the presence of triethylamine with THF as the solvent in high yields. These monomers were reacted with tetramethyl-1,3-diaminopropane at 1:1 M ratio at room temperature in the presence of DMF as the solvent. Post polymerization, the reaction mixture was precipitated thrice into diethylether and the resultant solids were further purified by extensive dialysis against DI water, followed by lyophilization to result in target polymers **2a** and **2b** (Scheme 1.).

3.2. In vitro antimicrobial and hemolytic activities

As shown in Tables 1, S2 and Fig S1, both polymer **2a** and **2b** exhibited broad-spectrum antibacterial activity with low hemolytic activity (HC_5 = 1000 μg/mL, polymer concentration that leads to lysis of 5% rat red blood cells, Table S2). Especially, polymer **2a** was more potent against five MDR clinical bacterial strains with lower MIC values. Polymer **2b** was synthesized from a more rigid bis-halide, and is more hydrophobic than polymer **2a**. Polymer **2b** was slightly difficult to dissolve in water, and a small amount of DMSO (1% DMSO) was used to assist dissolution with negligible impact on growth of bacteria. Its lower antimicrobial activity was probably due to interaction with proteins present in the growth medium. The antimicrobial activity of the polymers was compared with an array of clinically relevant antibiotics, i.e. broad-spectrum levofloxacin and gentamycin, ceftriaxone, imipenem and poly-

Table.1
MIC values of antimicrobial agents against clinically isolated multidrug-resistant bacteria (K.P.: *Klebsiella pneumoniae*; A.B.: *A. baumannii*; P.A.: *P. aeruginosa*; E.C.: *E. coli*).

Agents	MIC (μg/mL)				
	K.P. 8637[a]	A.B. 9861	P.A. 26121	E.C. 58884	MRSA 25312
polymer **2a**	16	32	32	16	8
polymer **2b**	256	128	256	128	128
Ceftriaxone	≥512	≥512	256	≥512	≥512
Levofloxacin	64	4	2	128	32
Gentamycin	64	≥512	16	≥512	256
Imipenem	64	32	128	0.25	64
Polymyxin B	2	2	2	1	≥512
Vancomycin	≥512	≥512	≥512	≥512	1

[a] Defined as resistance when MICs of ceftriaxone, levofloxacin, gentamycin, imipenem and polymyxin B were ≥2, 8, 16, 4 and 8, respectively.

myxin B against Gram-negative bacteria, and vancomycin against Gram-positive MRSA. The MIC values of polymer **2a** were lower than those of ceftriaxone, levofloxacin, gentamycin and imipenem in most of the bacterial strains tested (Table 1). Although polymyxin B and vancomycin have lower MIC values against Gram-negative bacteria and MRSA, respectively, polymer **2a** is superior as it is effective against both Gram-negative and Gram-positive bacteria (Table 1). The polymers were further evaluated in 20 clinical isolates of MDR *K. pneumoniae* in comparison with ceftriaxone, gentamycin and imipenem as these antibiotics especially imipenem are commonly used in clinic to treat *K. pneumoniae* infection. At 32 μg/mL, polymer **2a** completely inhibited the growth of all 20 isolates, while polymer **2b** was only effective against 5 isolates (Tables 2 and S3). Polymer **2a** had MIC of 8.0 μg/mL against 8 isolates, MIC of 16 μg/mL against 10 isolates, and MIC of 32 μg/mL against 2 isolates, while imipenem had MIC of ≤32 μg/mL only against 6 isolates (Tables 2 and S3). The potency of ceftriaxone and gentamycin was even lower (Tables 2 and S3). These results demonstrated broad-spectrum antibacterial activity and higher potency of polymer **2a** as compared to polymer **2b**. Therefore, polymer **2a** was chosen for further studies.

Polymer **2a** was further studied for its bactericidal activity and killing kinetics. Gentamycin and imipenem were employed as control antibiotics because ceftriaxone has very low potency against *K. pneumoniae* (Tables 2 and S3). Both polymer **2a** and antibiotics were bactericidal at MIC after 18-h incubation with MDR *K. pneumoniae* 8637 (more than 99.9% bacterial cells killed). Polymer **2a** eliminated the bacteria more rapidly than the antibiotics gentamycin and imipenem (Fig. 1). Polymer **2a** completely killed all bacterial cells after 2-h incubation at 2 × MIC (Fig. 1B), while a large number of viable bacteria were still observed in gentamycin- and imipenem-treated samples within the same time frame. Different from the antibiotics, the polymer showed a dose-dependent bactericidal effect (Fig. 1D). Two hours were needed for the polymer to remove all bacterial cells at 2 × MIC, while only one hour was required to do so at 4 × MIC (Fig. 1C). The results of statistical analysis between polymer 2**a** and gentamycin, imipenem at time points of 20, 40, 60 and 120 min were presented in Table S4, and the differences observed were significant.

Table 2
Cumulative distribution of MIC values (μg/mL) against clinically isolated MDR *K. pneumoniae* (n = 20).

Agents	Cumulative % of 20 *K. pneumoniae* strains at indicated MICs									
	1	2	4	8	16	32	64	128	256	≥512
polymer **2a**				40	90	100				
polymer **2b**						5	20	70	90	100
Imipenem						30	75	80	100	
Gentamycin						15	25	35	50	100
Ceftriaxone								20	80	100

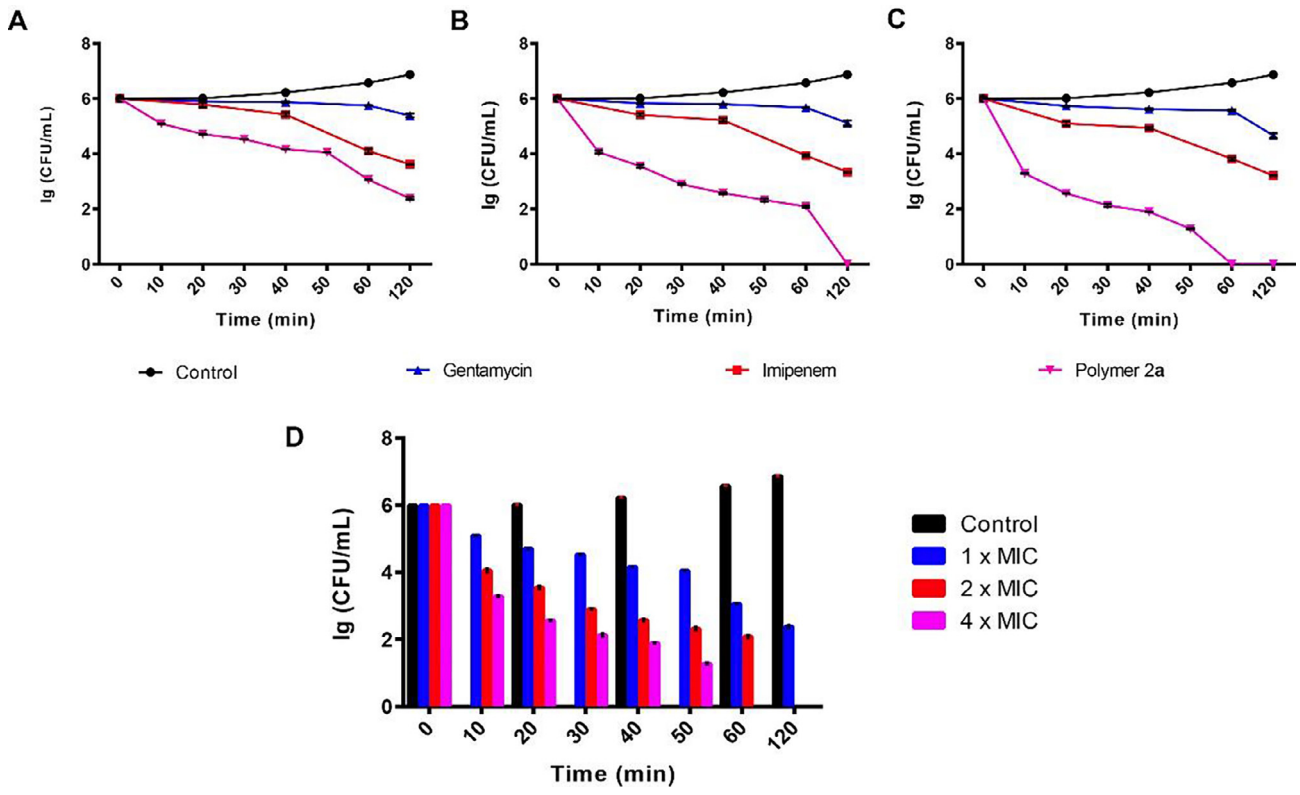


Fig. 1. Killing kinetics of polymer **2a**, gentamycin and imipenem against *K. pneumoniae* 8637. Colony-forming units (CFUs) of *K. pneumoniae* 8637 after treatment at various time points and different polymer concentrations: A) 1 × MIC, B) 2 × MIC and C) 4 × MIC. D) Effect of polymer **2a** concentration on killing kinetics. Error bars indicate respective standard deviations (s.d.); n = 3.

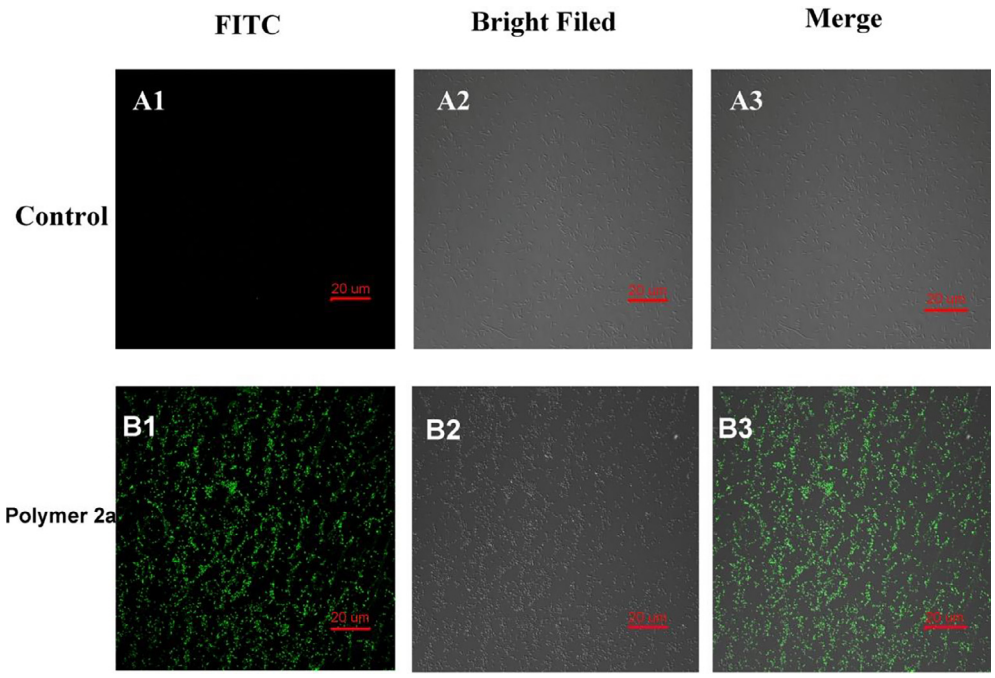


Fig. 2. Confocal microscopic images of *K. pneumoniae* 8637 incubated with FITC-conjugated dextran (100 kDa, 250 μg/mL) in the presence of A) PBS or B) polymer **2a** (4 × MIC) for 2 h. A1, B1) Green region represents uptake of FITC-conjugated dextran. A2, B2) Bright field. A3, B3) Merged images. Scale bar: 20 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.3. Antimicrobial mechanism

Studies that were conducted by our group and other scientists demonstrated that quaternary ammonium-containing antimicrobial polymers function based on membrane-disruption mechanism [1,20,56]. To observe membrane disruption of *K. pneumoniae* 8637, the bacteria were incubated with polymer **2a** at 4 × MIC for 2 h in the presence of fluorescein isothiocyanate (FITC)-labeled dextran (100 kDa, 250 μg/mL), and then observed under a confocal microscope. Without the polymer treatment, FITC-labeled dextran was unable to penetrate through the bacterial membrane (Fig. 2). In contrast, significant uptake of FITC-labeled dextran by the bacteria was observed after the polymer treatment, signifying membrane disruption.

The morphology of the bacteria before and after polymer treatment at 4 × MIC and 8 × MIC was observed under a transmission electron microscope (TEM). Membrane disruption was clearly seen in the polymer-treated bacteria (Fig. 3A1–A3), further supporting membrane-disruption antimicrobial mechanism. Additionally, a decreased density of cytoplasmic contents was observed in the polymer-treated bacteria, which was believed to be attributed to the leakage of intracellular contents. To further study the antimicrobial mechanism of the polymer, leakage of the cytoplasmic materials from the bacteria was quantified after the polymer treatment at different concentrations. The release of the cytoplasmic materials was polymer concentration-dependent, and it increased as a function of polymer concentration (Fig. 3B). Taken together, these results provide convincing evidence that the polymer exerted membrane-disruption antimicrobial mechanism.

3.4. Prevention of resistance development

Antibiotic resistance is a threat to public health [54]. It is caused by many reasons, among which repeated exposure of bacteria to antibiotics at sub-lethal concentrations is one of the most important factors [53]. To evaluate polymer's propensity toward resistance development, *K. pneumoniae* 8637 were serially passaged in the presence of polymer **2a**, gentamycin and imipenem at sublethal doses for 15 passages. Gentamycin and imipenem were employed as control antibiotics as they are the recommended therapy against *K. pneumoniae*-caused infections. As illustrated in the Fig. 3C, there is no change of MIC value of polymer **2a** against *K. pneumoniae* 8637 over 15 passages. However, MIC of imipenem started to increase by the 9th passage, and exponential increase in the MIC of gentamycin was observed after the 4th passage. By the 10th passage, the MICs of imipenem and gentamycin increased by 16 and 256 times, respectively. These results convincingly demonstrated a significantly lower propensity of *K. pneumoniae* to develop resistance toward the polymer when compared with imipenem and gentamycin.

3.5. In vitro anti-biofilm activity

Biofilm, the product of a microbial developmental process, has been reported to be involved in a wide variety of human bacterial infections including *K. pneumoniae* [57]. Biofilm comprises of bacteria embedded in an extracellular matrix (ECM), which is produced by the bacteria. Biofilm is extremely resistant to antibiotic treatment, and the formation of biofilm also prolongs course of infection [58]. The anti-biofilm activity of the polymer was investigated by directly measuring viability of bacterial cells in the biofilm and biomass of the biofilm after polymer treatment. As depicted in Fig. 4, polymer **2a** possessed dose-dependent anti-biofilm activity. For instance, one time treatment with polymer **2a** at 4 × MIC reduced the viability of *K. pneumoniae* 8637 and the biomass to 20% and 30%, respectively. Imipenem was used as a control antibiotic. Polymer 2**a** exhibited a higher reduction of viability of *K. pneumoniae* 8637 in the biofilm and biomass of the biofilm as compared with imipenem as shown in Fig. S2. It is well documented that bacterial biofilms are resistant to most antibiotics due to inherent resistance of bacteria in the biofilm and limited biofilm-penetration ability of antibiotics [56]. Collectively, the

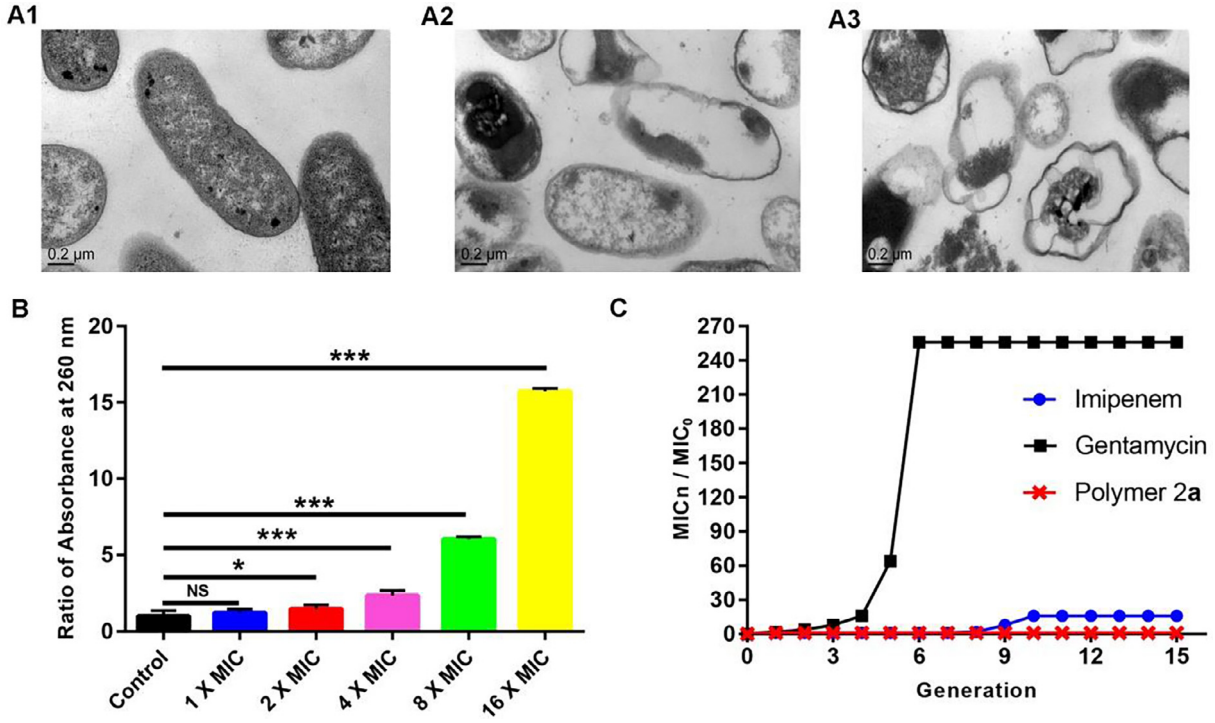


Fig. 3. Membrane-disruption antimicrobial mechanism of the polymer and prevention of resistance development. TEM images of *K. pneumoniae* 8637 before (A1) and after 2-h treatment with polymer **2a** at 4 × MIC (A2) and 8 × MIC (A3) at 37 °C. Size of the scale bar: 0.2 μm. B) Concentration of nucleic acids in the supernatants of *K. pneumoniae* 8637 treated with PBS or polymer **2a** at doses of MIC, 2 × MIC, 4 × MIC, 8 × MIC and 16 × MIC. NS: not significant. $^{*}p < 0.05$; $^{***}P < 0.001$. Error bars represent s.d. for n = 3. C) *In vitro* evolution of antimicrobial resistance in *K. pneumoniae* 8637. The bacteria were serially passaged in the presence of polymer **2a**, gentamycin and imipenem at sub-lethal doses (0.5 × MICs) for 15 passages. MIC was determined at each passage. The data shown is representative of three replicates.

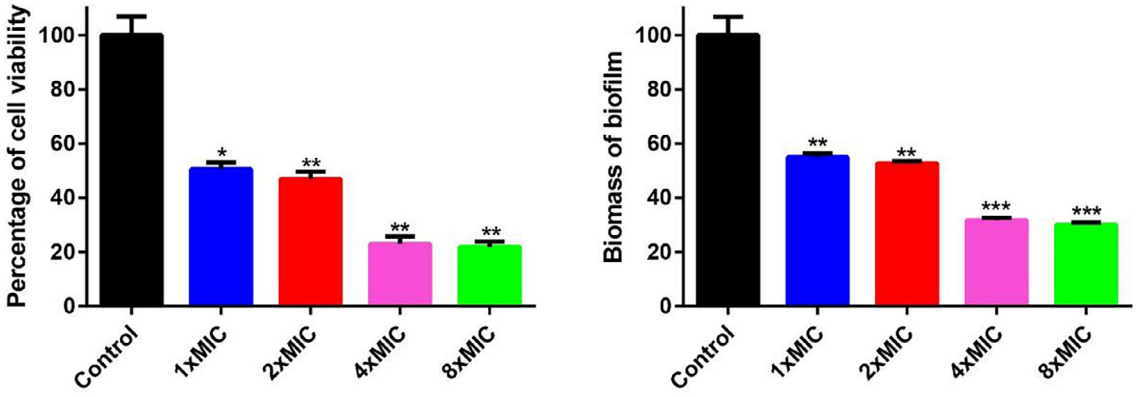


Fig. 4. Anti-biofilm activity of polymer **2a**. Cell viability (Left) and biomass (Right) of *K. pneumoniae* 8637 biofilm after polymer treatment for 24 h at various concentrations (1 × MIC, 2 × MIC, 4 × MIC and 8 × MIC). *K. pneumoniae* 8637 biofilm without polymer treatment was used as a control. $^{*}p < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. Error bars represent s.d. for n = 3.

polymer was capable of removing *K. pneumoniae* biofilm, and more effective than imipenem.

In contrast to small molecular antibiotics, broad spectrum of action, faster killing kinetics, membrane-disruption mechanism, prevention of resistance development and anti-biofilm activity render polymer **2a** an attractive candidate for further development. Moreover, in comparison to numerous other reported polymeric systems [20,25,28,35,41,59,60], this polymer is highly potent and selective, can be obtained in a cost-effective and step-efficient manner. Remarkable antimicrobial properties of this polymer can be partially attributed to rigid arylamide moieties and this is in line with other reports [18,25,49], where the rigidity has been shown to have an impact on antimicrobial properties. As this polymer is of synthetic origin, it eliminates potential immunogenicity concerns [26,28]. Encouraged by these attributes and also promising *in vitro* antimicrobial data of polymer **2a**, further *in vivo* studies were conducted.

3.6. In vivo toxicity and efficacy

Antimicrobial peptides (AMPs) and polymers are often correlated with high systemic toxicity, which is a hurdle that prevents them from being used in clinic [61]. Therefore, the *in vivo* toxicity of polymer **2a** was first evaluated by determining LD_5 and LD_{50}. The LD_{50} and LD_5 values of polymer **2a** were 67.5 mg/kg of body weight and 37.3 mg/kg of body weight, respectively (Table 3). *In vivo* efficacy of polymer **2a** was further evaluated in a MDR *K. pneumoniae* 8637-induced pneumonia mouse model by measuring ED_{50} and ED_{95} (effective doses that lead to survival of 50% and 95% infected mice, respectively). As listed in Table 3, the polymer

Table 3
In vivo efficacy and toxicity (LD_{50}/LD_5) of polymer **2a** against an immunocompromised pneumonia mouse model caused by *K. pneumoniae* 8637.

Minimum lethal dose[a] (CFU/mouse)	Antimicrobial agents	ED_{95}/ED_{50} (95% confidence interval)[b] (mg/kg)	LD_5/LD_{50} (95% confidence interval)[c] (mg/kg)
1.5×10^7	Imipenem	20.0/2.75 (0.28–8.88)	ND[d]
	polymer **2a**	3.08/0.62 (0.14–1.31)	37.3/67.5 (51.1–89.5)

[a] Minimum lethal dose defined as initial inoculum size of a particular bacterial strain required to induce 100% mortality at 5 days post-infection.
[b] Four immunosuppressed mice in each group were injected intraperitoneally with polymer **2a** or imipenem once daily for 3 consecutive days starting at 4 h post-infection.
[c] Polymer **2a** (dissolved in PBS) was administered to mice (n = 6 in each group) intraperitoneally at various doses.
[d] ND, not determined.

showed a superior *in vivo* treatment efficacy than imipenem, with ED_{50} (ED_{95}) of 0.62 (3.08) mg/kg of body weight, as compared to 2.75 (20.0) mg/kg of body weight for imipenem. More importantly, the ED_{95} value of the polymer is far below its LD_5, demonstrating a large therapeutic window (ED_{50}/LD_{50}: 109).

The survival of the infected mice was monitored after treatment with polymer **2a** and imipenem at a dose close to their respective ED_{50} (1.0 and 2.0 mg/kg of body weight, respectively, 3 i.p. injections starting at 4 h post-infection). Only 70% of the infected mice receiving imipenem treatment survived at 3 days post-infection, while all mice treated with polymer **2a** remained alive in the same time frame (Fig. 5A), suggesting that polymer **2a** is more effective than imipenem in improving the survival of the infected mice. Subsequently, the ability of the polymer in removing the bacteria from the major organs (lung, liver, spleen and kidney) and the blood was evaluated in MDR *K. pneumoniae* 8637-induced pneumonia mice in comparison with imipenem. The treatment with the polymer or imipenem at ED_{95} significantly reduced bacterial burden from the organs and the blood (Fig. 5B). Specially, the polymer was more effective than imipenem in reducing the bacterial counts possibly due to its faster killing kinetics (Fig. 1). This is particularly important as the rapid reduction of viable bacteria could potentially decrease the production of bacterial toxins, and prevent septicemia and septic shock [62].

From histological analysis, the infected lungs without any treatment displayed apparent signs of inflammatory response, includ-

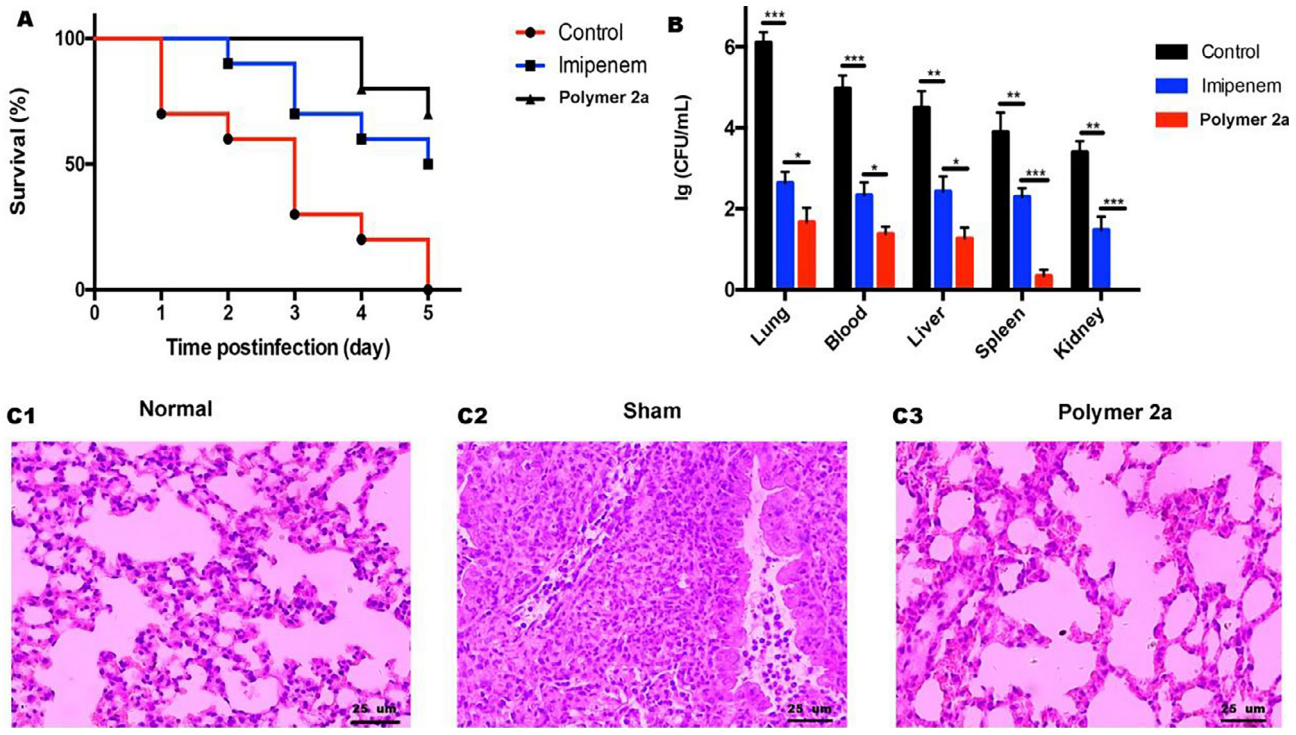


Fig. 5. *In vivo* antibacterial efficacy of polymer **2a** in a MDR *K. pneumoniae* 8637-induced pneumonia mouse model. A) Kaplan-Meier survival curves of the infected mice (ten mice in each group). The mice were monitored for 5 days post-infection. B) Reduction of viable bacterial colonies in the lung, blood, liver, spleen and kidney specimens taken at day 5 post-infection. Error bars represent standard deviations (n = 3). $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$. C1, C2, C3) Histological analysis of lung tissues through H&E staining. Sham: Infected mice without any treatment. Size of the scale bar: 25 μm.

Table 4
Liver and kidney functions as well as blood sodium/potassium ion concentration of mice (n = 6 in each group) after polymer **2a** treatment (3.1 mg/kg of body weight, once daily i.p. injection for 3 consecutive days).

Treatment	ALT (U/L)[a]	AST (U/L)[a]	Creatinine (μmol/L)	Urea nitrogen (mmol/L)	Sodium ion (mmol/L)	Potassium ion (mmol/L)
PBS	27.3 ± 1.2	86.8 ± 3.0	15.8 ± 2.3	8.0 ± 0.7	149.2 ± 4.4	4.4 ± 0.3
polymer **2a**	29.3 ± 2.0	84.4 ± 3.1	16.1 ± 2.0	7.5 ± 1.0	149.6 ± 3.2	4.6 ± 0.5

Polymer **2a** *vs.* PBS: $p > 0.05$.
[a] U/L, international units per liter.

ing inflammatory cell penetration and pulmonary carnification (Fig. 5C). However, polymer **2a** treatment decreased infection-induced tissue injury and showed lower inflammatory characteristics.

The potential acute toxicity of the polymer was further assessed by analyzing liver and kidney functions, sodium and potassium ion concentration in the blood after polymer treatment. As summarized in Table 4, no significant difference between polymer **2a**-treated group and the control group treated with PBS was detected in the serum chemistry, demonstrating negligible acute toxicity of polymer **2a**.

4. Conclusion

Polyionenes were synthesized and used as antimicrobial agents. The polymers have broad spectrum antibacterial activity against clinically isolated MDR bacteria. Polymer **2a** with relatively lower hydrophobicity eliminated MDR bacteria more effectively than polymer **2b**. Additionally, polymer **2a** has stronger activity against MDR *K. pneumoniae* with lower MIC values than gentamycin and imipenem (clinically used antibiotics for treatment of *K. pneumoniae*-caused infections). Particularly, it eliminates the bacteria more rapidly than the antibiotics. In a MDR *K. pneumoniae* 8637-caused pneumonia mouse model, the polymer demonstrates low effective dose with high therapeutic index. The polymer treatment also provides higher survival rate and faster bacterial removal from the major organs and the blood than imipenem. More importantly, at the therapeutic dose, the polymer treatment does not cause acute toxicity towards liver and kidney functions nor interfere electrolyte balance of the blood. Given its *in vivo* efficacy and negligible toxicity, the polymer is a promising candidate for the treatment of MDR *K. pneumoniae*-caused pneumonia.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.actbio.2018.07.038.

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