Polyacrylamide-based Antimicrobial Copolymers Immune to Typical Resistance Mechanisms to Replace or Rescue Antibiotics

Shoshana C. Williams,[1,2] Madeline B. Chosy,[1] Carolyn K. Jons,[3] Changxin Dong,[3] Alexander N. Prossnitz,[3] Xinyu Liu,[1] Hector Lopez Herandez[3], Lynette Cegelski,[1] Eric A. Appel[2,3,4,5,6*]

[1] Department of Chemistry, Stanford University, Stanford, CA 94305, USA.

[2] Sarafan ChEM-H, Stanford University, Stanford, CA 94305, USA.

[3] Department of Materials Science & Engineering, Stanford University, Stanford, CA 94305, USA.

[4] Department of Bioengineering, Stanford University, Stanford, CA, 94305, USA.

[5] Woods Institute for the Environment, Stanford University, Stanford CA 94305, USA.

[6] Department of Pediatrics (Endocrinology), Stanford University, Stanford CA 94305, USA.

[*]Corresponding author: eappel@stanford.edu

BIGGER PICTURE:

Antibiotics save countless lives each year and have dramatically improved human health outcomes since their introduction in the 20th century. Unfortunately, bacteria are now developing resistance to antibiotics at an alarming rate, with many new strains of "superbugs" showing simultaneous resistance to multiple classes of antibiotics. To combat this growing crisis, we must develop new antibiotics that can overcome typical resistance mechanisms. Copolymer drugs such as the ones presented in this paper can delay or prevent the development of resistance and rehabilitate existing antibiotics by improving their potency and limiting the ability of bacteria to develop resistance. This work represents a significant step towards combatting antimicrobial resistance.

SUMMARY:

To mitigate the global burden of antimicrobial resistance, we must develop antibiotics that are broadly effective, safe, and highly stable for the purpose of global access. In this manuscript, we report the development of polyacrylamide-based copolymers as a novel class of broad-spectrum antibiotics with efficacy against several critical pathogens. We demonstrate that these copolymer drugs are selective for bacteria over mammalian cells, indicating a favorable safety profile. We show that they kill bacteria through a membrane disruption mechanism, which allows them to overcome traditional mechanisms of antimicrobial resistance. Lastly, we demonstrate their ability to rehabilitate an existing small-molecule antibiotic that is highly subject to resistance development by improving its potency and eliminating the development of resistance in a combination treatment.

INTRODUCTION:

Antimicrobial resistance (AMR) is a growing global crisis. It is estimated that by 2050, AMR will cause over 10 million deaths annually, overtaking cancer as a leading cause of death.[1] In the United States alone, AMR already causes over 35,000 deaths each year and costs over $4.6 billion in direct healthcare spending.[2,3] The toll is even higher in low- and middle-income countries, with the highest death rates attributable to AMR occurring in sub-Saharan Africa and South Asia.[4] Unfortunately, progress in the development of new antibiotics has waned in recent decades, while AMR bacteria continue to arise at an alarming rate.[5,6] Even as the identification of multidrug resistant bacterial isolates increases,[7] publications on antibiotics are decreasing (Figure 1A).[8]

The mechanisms of AMR are molecularly defined and include inhibition of drug uptake, modification of the drug target, inactivation of the drug, and enhanced drug efflux (Figure 1B).[9,10] Novel antibiotics that can evade these mechanisms are of immense interest to combat the growing threat of AMR.

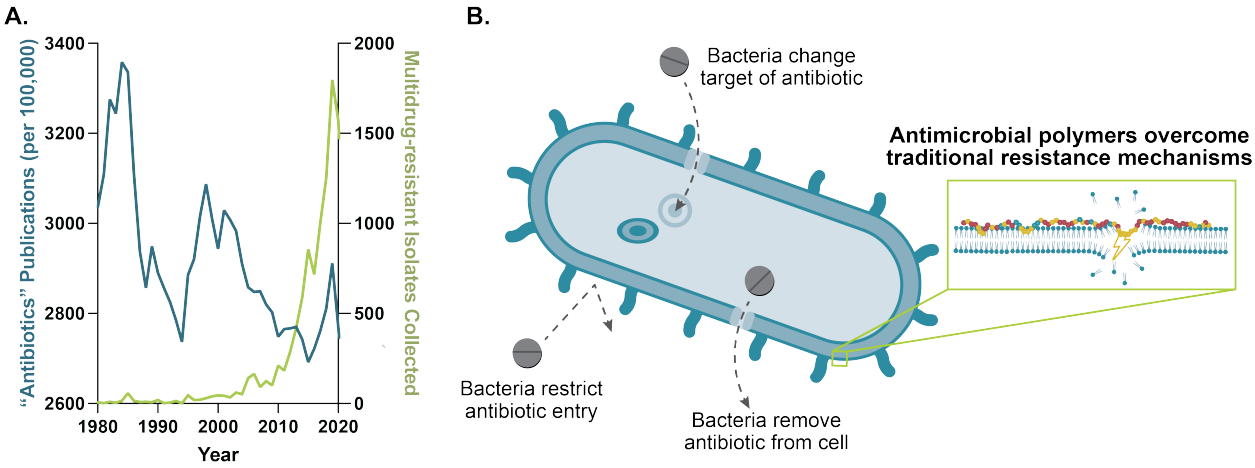

Figure 1. Novel polymers can combat antimicrobial resistance. A. Graph comparing the decreasing research into antibiotics with the increasing incidence of multidrug resistant bacteria. **B.** Schematic showing common mechanisms of AMR and the hypothesized ability of novel polymers to overcome these mechanisms.

Previous work has explored the development of antimicrobial polymers and oligomers, both as coatings for devices or surfaces[11–14] and as treatments.[15–27] These studies have identified positive charge and hydrophobicity as key parameters to enable antibacterial activity, and several of these polymers were shown to work through a physical membrane disruption mechanism.[28–31] This mechanism overcomes traditional resistance mechanisms, since these polymers do not need access to the intracellular space, and they are not specific to a singular molecular target where mutations yield resistance.[32] Despite these exciting developments, the further translation of these polymers has been hampered by challenging synthesis and toxicity by hemolytic activity. Several approaches have relied on multi-step syntheses to prepare monomers and perform post-polymerization modifications, which can be costly and time-intensive.[16–18,22,25,27] Other investigations have utilized sequence-controlled polymers, which similarly require greater synthetic control.[20,21,23,24,26] Even with these approaches, several candidates have shown unfavorable hemolytic activity and toxicity profiles.[17,23–25,27] To address these challenges, we generated a library of polyacrylamide-derived copolymers for use as antimicrobial agents. Polyacrylamides were selected for their impressive chemical stability, commercial availability, and ease of synthesis. We investigated their efficacy, safety, mechanism of action, and ability to rehabilitate the potency of existing small-molecule antibiotics in resistant bacterial strains.

RESULTS AND DISCUSSION:

Development of a library of polyacrylamide derivative copolymers.

We developed a library of unique polyacrylamide derivatives designed to arrest bacterial growth through a physical membrane disruption mechanism, which does not rely on specific protein transporters or pathways that are subject to resistance mechanisms. Each copolymer comprises

various ratios of three monomers, including: (i) a cationic monomer to drive polymer adsorption to the negatively charged surface of bacteria, (ii) a hydrophilic "carrier" monomer to appropriately tune water solubility, and (iii) a hydrophobic "dopant" monomer to permeabilize the bacterial membrane (Figure 2). We designed this library to systematically vary monomer identity, charge density, hydrophobicity, and molecular weight to allow us to explore how these parameters affect activity and safety. To this end, the introduction of the hydrophilic carrier monomer was significant, not only for its ability to impart water solubility, but also for its utility in allowing for the independent variation of charge and hydrophobicity. Previous work in the field has explored these parameters through the development of polymers where the hydrophobicity and charge were linked, either in the same monomers or using two monomers.[11,25–27] Inclusion of a third monomer allowed for greater control in exploring the independent effects of charge and hydrophobicity.

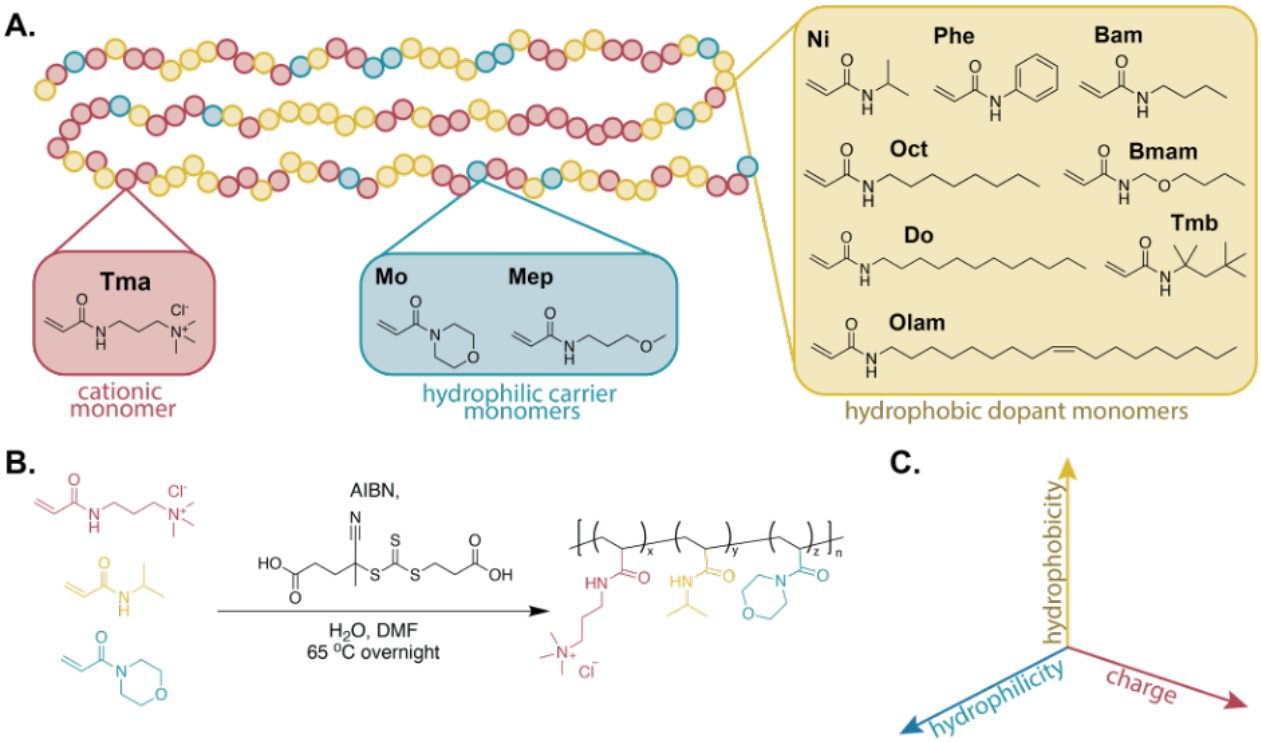

Figure 2. Development of a library of polyacrylamide derivatives. A. Schematic of statistical copolymer showing monomers used and their classes. **B.** RAFT polymerization conditions. **C.** The use of a ternary system allows for independent tuning of charge and hydrophobicity.

Copolymers were synthesized through statistical copolymerization using a variety of commercially available or easily synthesized monomers by reversible addition-fragmentation chain transfer (RAFT) polymerization with a trithiocarbonate chain-transfer agent. Each entry contained the same cationic monomer (Tma). High (H) molecular weight copolymers were designed with a target degree of polymerization (DP) of 115, while low (L) molecular weight copolymers had a target DP of 70. These copolymers were designed with molecular weights below 30 kDa, which is near the molecular weight cutoff for glomerular filtration in the kidneys.[33] The copolymers were named by the following scheme: (DP)-(Hydrophobic monomer)$_{wt\%}$(Hydrophilic monomer)$_{wt\%}$, and the composition of each polymer is detailed (Table 1).

The copolymers were analyzed by NMR (Figure S3) and GPC (Figure S4). Molecular weights were determined by comparison to PEG standards, except for L-Tmb$_5$Mo$_{90}$. Because of this formulation's low cationic density and higher hydrophobicity, it was more appropriately evaluated by comparison to PMMA standards. The dispersities ranged from 1.1 to 1.4, as expected for controlled radical polymerization techniques (Table 1).

Table 1. Composition of polyacrylamide library.

	Target DP	**Target M_w (kDa)**	**M_w (kDa)**	**Đ**	**% Conversion**	**% Yield**
L-Ni$_{31}$Mo$_{10}$	70	11.1	11.1	1.12	n.d.	103
L-Ni$_{31}$Mep$_{10}$	70	11.1	11.9	1.12	n.d.	113
L-Phe$_{31}$Mo$_{10}$	70	12.4	10.3	1.16	n.d.	94.5
L-Phe$_{31}$Mep$_{10}$	70	12.4	10.5	1.14	n.d.	99
L-Do$_{31}$Mo$_{10}$	70	14.4	10.4	1.36	n.d.	92.5
L-Do$_{31}$Mep$_{10}$	70	14.4	12.1	1.15	n.d.	58
L-Ni$_{13}$Mo$_4$	70	12.8	13.6	1.15	n.d.	94
L-Ni$_{13}$Mep$_4$	70	12.8	13.6	1.18	n.d.	65
L-Phe$_{13}$Mo$_4$	70	13.5	12.6	1.16	n.d.	92.5
L-Phe$_{13}$Mep$_4$	70	13.5	11.5	1.25	n.d.	81
L-Do$_{13}$Mo$_4$	70	14.4	11.9	1.32	n.d.	69.5

L-$Do_{13}Mep_4$	70	14.4	12.1	1.37	n.d.	78.5
H-$Ni_{31}Mo_{10}$	115	18.3	21.3	1.21	100	91.5
H-$Ni_{31}Mep_{10}$	115	18.3	22.0	1.15	93.6	85.5
H-$Phe_{31}Mo_{10}$	115	20.3	22.5	1.18	99.3	84.5
H-$Phe_{31}Mep_{10}$	115	20.3	19.1	1.15	100	92.5
H-$Do_{31}Mo_{10}$	115	23.6	21.0	1.27	100	95
H-$Do_{31}Mep_{10}$	115	23.7	19.3	1.24	96.7	81.5
L-$Bam_{31}Mep_{10}$	70	11.7	15.1	1.25	85.6	69
L-$Bmam_{31}Mep_{10}$	70	12.7	15.4	1.27	88	68
L-$Tmb_{31}Mep_{10}$	70	13.3	11.3	1.28	99.3	63
L-$Oct_{31}Mep_{10}$	70	13.3	12.6	1.22	99	51
L-$Olam_{31}Mep_{10}$	70	15.5	10.3	1.27	96.7	71
L-$Do_{30}Mep_5$	70	14.75	14.0	1.23	n.d.	59
L-Tmb_5Mo_{90}	70	10.16	12.8	1.21	n.d.	100
L-Oct_5Mep_5	70	14.07	15.0	1.20	n.d.	100
L-$Phe_{15}Mo_{30}$	70	12.06	14.2	1.18	n.d.	89

Antibiotic efficacy and safety of polyacrylamide copolymers

To evaluate the efficacy of each novel copolymer, its minimum inhibitory concentration (MIC) was determined against a standard Gram-negative strain, *E. coli* ATCC 25922, and a standard Gram-positive strain, *S. aureus* ATCC 29213 (Figure 3A, Table 2). Of the 27 copolymers evaluated, 20 showed activity against at least one of these targets, and 11 showed activity against both, indicating broad-spectrum efficacy. We observed no trade-off between efficacy against *S. aureus* compared to *E. coli*, with several copolymers demonstrating robust activity against both (Figure S5). The observed MIC values were comparable to those of previously reported antimicrobial polymers and licensed antibiotic drug products (Table 2).[17,18,21,22,25–27,30,34–36]

The hemolytic activity of each copolymer was measured as a metric of mammalian non-toxicity. Most of the copolymers showed remarkably low hemolytic activity, even up to concentrations exceeding 2000 μg/mL, indicating favorable safety profiles (Figure 3B, Table 3). Indeed, for most

polymers, HC_{50} (the concentration at which 50% of red blood cells are lysed) values could not be determined because it fell beyond the concentration range tested. Our polymers demonstrated clear selectivity for permeabilizing bacteria, leaving red blood cells intact. Interestingly, the antibacterial efficacy of the copolymer did not directly correlate with the hemolytic activity. In contrast, previous reports have indicated that polymer antimicrobial activity is typically associated with greater hemolysis. The promising safety profile of our copolymers demonstrates the importance of our ternary copolymer design, where the introduction of a hydrophilic monomer enabled access to copolymers with potent antibacterial activity and low toxicity.

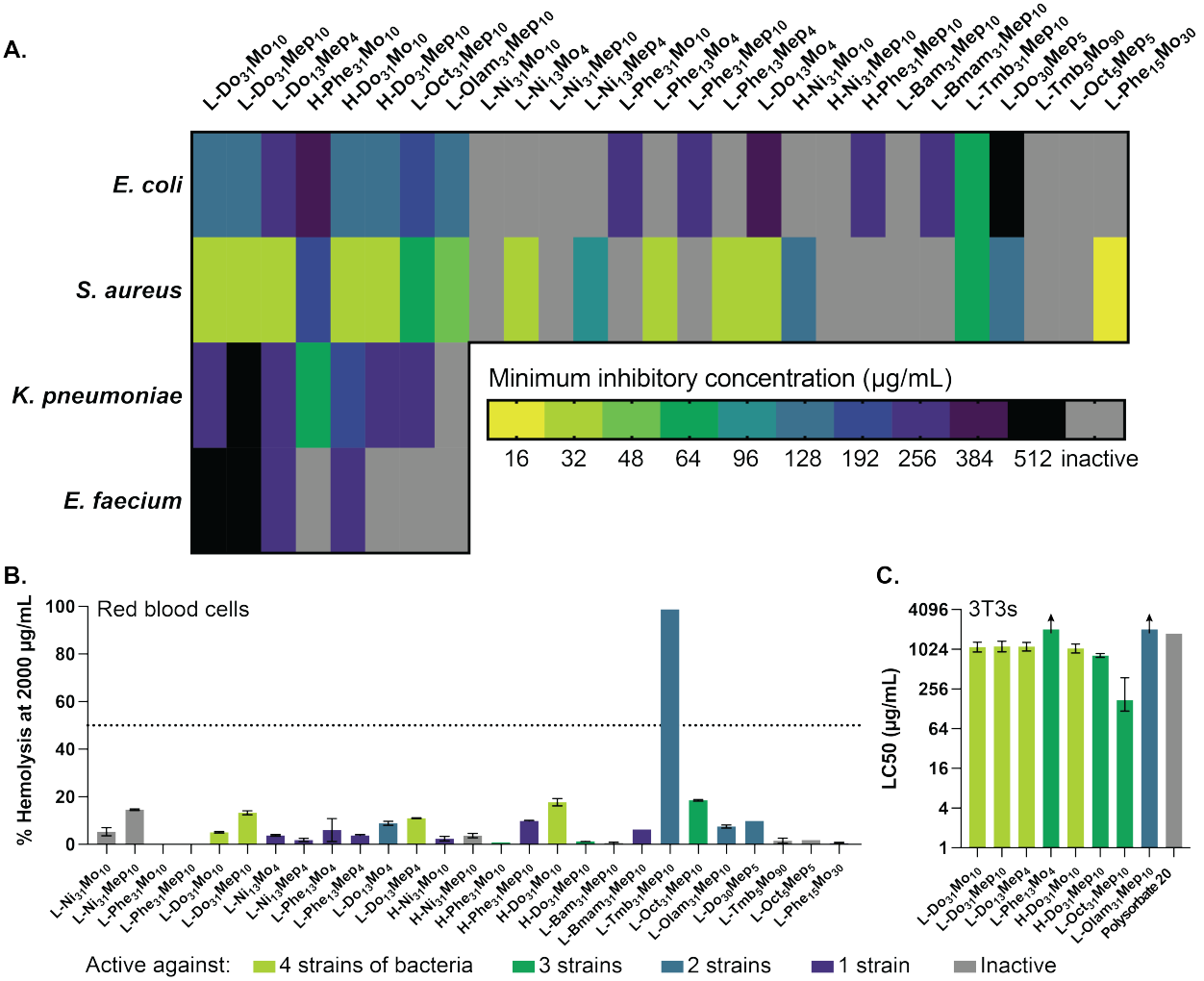

Figure 3. Efficacy and safety of novel polyacrylamides. A. Heat map showing the antibacterial efficacy of each polymer against several bacteria. **B.** Hemolytic activity of each polymer at 2000 µg/mL. **C.** LC50 values of eight copolymers against 3T3 cells.

Table 2. Antibacterial efficacy of novel polyacrylamides and penicillin G.

	Hydrophobic/ cationic molar ratio	Hydrophobicity (ΣLogP)	MIC: *E. coli*, µg/mL (µM)	MIC: *S. aureus*, µg/mL (µM)	MIC: *K. pneumoniae*, µg/mL (µM)	MIC: *E. faecium*, µg/mL (µM)
Penicillin G	N/A	N/A	32 (89.8)	0.5-1 (1.40-2.81)	n.d.	n.d.
L-$Ni_{31}Mo_{10}$	0.95	0.80	>512 (>46.0)	>512 (>46.0)	n.d.	n.d.
L-$Ni_{31}Mep_{10}$	0.95	1.20	>512 (>43.1)	>512 (>43.1)	n.d.	n.d.
L-$Phe_{31}Mo_{10}$	0.73	1.70	256 (24.9)	>512 (>49.7)	n.d.	n.d.
L-$Phe_{31}Mep_{10}$	0.73	2.10	256 (24.3)	>512 (>48.7)	n.d.	n.d.
L-$Do_{31}Mo_{10}$	0.45	5.80	128 (12.3)	32-64 (3.07-6.14)	256 (24.5)	512 (49.1)
L-$Do_{31}Mep_{10}$	0.45	6.20	128 (10.5)	32 (2.64)	512 (42.2)	512 (42.2)
L-$Ni_{13}Mo_{4}$	0.29	0.80	>512 (>37.6)	32 (2.35)	n.d.	n.d.
L-$Ni_{13}Mep_{4}$	0.29	1.20	>512 (>37.5)	64-128 (4.69-9.39)	n.d.	n.d.
L-$Phe_{13}Mo_{4}$	0.22	1.70	>512 (>40.7)	32 (2.54)	n.d.	n.d.
L-$Phe_{13}Mep_{4}$	0.22	2.10	>512 (>44.4)	32 (2.78)	n.d.	n.d.
L-$Do_{13}Mo_{4}$	0.14	5.80	256-512 (21.5-43.1)	32 (2.69)	n.d.	n.d.
L-$Do_{13}Mep_{4}$	0.14	6.20	256 (21.2)	32 (2.64)	256 (21.2)	256 (21.2)
H-$Ni_{31}Mo_{10}$	0.95	0.80	>512 (>24.1)	128 (6.02)	n.d.	n.d.
H-$Ni_{31}Mep_{10}$	0.95	1.20	>512 (>23.3)	>512 (>23.3)	n.d.	n.d.
H-$Phe_{31}Mo_{10}$	0.73	1.70	256-512 (11.4-22.7)	128-256 (5.68-11.36)	64 (2.84)	>512 (>22.7)
H-$Phe_{31}Mep_{10}$	0.73	2.10	256 (13.4)	>512 (>26.8)	n.d.	n.d.
H-$Do_{31}Mo_{10}$	0.45	5.80	128 (6.10)	32 (1.52)	128-256 (6.10-12.2)	256 (12.2)
H-$Do_{31}Mep_{10}$	0.45	6.20	128 (6.65)	32 (1.66)	256 (13.3)	>512 (>26.6)
L-$Bam_{31}Mep_{10}$	0.84	1.50	>512 (>33.8)	>512 (>33.8)	n.d.	n.d.
L-$Bmam_{31}Mep_{10}$	0.68	1.62	256 (16.6)	>512 (>33.3)	n.d.	n.d.
L-$Tmb_{31}Mep_{10}$	0.59	2.84	64 (5.67)	64 (5.67)	n.d.	n.d.
L-$Oct_{31}Mep_{10}$	0.59	3.17	128-256 (10.1-20.3)	64 (5.06)	256 (20.3)	>512 (>40.5)
L-$Olam_{31}Mep_{10}$	0.33	7.03	128 (12.4)	32-64 (3.11-6.22)	>512 (>49.8)	>512 (>49.8)
L-$Do_{30}Mep_{5}$	0.40	6.20	512 (36.6)	128 (9.14)	n.d.	n.d.
L-$Tmb_{5}Mo_{90}$	1.13	2.44	>512 (>39.9)	>512 (>39.9)	n.d.	n.d.
L-$Oct_{5}Mep_{5}$	0.06	3.17	>512 (>34.1)	>512 (>34.1)	n.d.	n.d.

L-Phe$_{15}$Mo$_{30}$	0.38	1.70	>512 (>36.0)	16 (1.13)	n.d.	n.d.

Based on the results of our screen with *E. coli* and *S. aureus*, as well as the hemolysis profiles, eight copolymers were selected as top-performing candidates for further evaluation. These candidates were then tested against two additional bacteria: *K. pneumoniae* ATCC 13884 (Gram-negative) and *E. faecium* ATCC 35662 (Gram-positive). These bacteria, along with *E. coli* and *S. aureus*, are members of a group of virulent pathogens known for their ability to develop resistance to antibiotics. These pathogens are known as the ESKAPEE pathogens, and include *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacter* spp., and *Escherichia coli*.[37,38] Each of the tested pathogens are on the priority list for the World Health Organization on account of their threat to global health.[39] Among these eight copolymers, seven demonstrated activity against at least one of the additional strains, while four exhibited activity against both. The efficacy of our leading copolymers against these species represents a clear step towards combatting antimicrobial resistance.

To further evaluate the safety of our leading copolymers *in vitro*, we examined the cytotoxicity of our eight top-performing candidates. We determined the LC50 following 24 h of exposure with 3T3s, a murine fibroblast cell line (Figure 3C, Table 3), and A549s, a human pulmonary epithelial cell line derived from an alveolar cell carcinoma (Figure S6, Table 3). The LC50 values of the copolymers were compared to those for polysorbate 20, a common excipient in FDA-approved drug products. Notably, polysorbate 20 is approved for intranasal administration at a concentration of 25 mg/mL,[40] well above its LC50 for either of the cell lines tested. The copolymers exhibited lower LC50 values than polysorbate 20 in A549 lung epithelial cells, but generally comparable LC50 values to polysorbate 20 in 3T3 cells. These results corroborate the favorable safety profile

observed previously with hemolysis, and further demonstrate the safety of these potent and broad antibiotic copolymer candidates.

Table 3. *In vitro* safety of polyacrylamide copolymers and polysorbate 20.

	HC_{50}, µg/mL (90% CI)	**LC50, 3T3s, µg/mL (90% CI)**	**LC50, 3T3s, µg/mL (90% CI)**
Polysorbate 20	n.d.	1800	1700 (200-2000)
L-$Ni_{31}Mo_{10}$	>2000	n.d.	n.d.
L-$Ni_{31}Mep_{10}$	>2000	n.d.	n.d.
L-$Phe_{31}Mo_{10}$	>2000	n.d.	n.d.
L-$Phe_{31}Mep_{10}$	>2000	n.d.	n.d.
L-$Do_{31}Mo_{10}$	>2000	1100 (900-1300)	200 (100-300)
L-$Do_{31}Mep_{10}$	>2000	1100 (900-1400)	80 (40-100)
L-$Ni_{13}Mo_{4}$	>2000	n.d.	n.d.
L-$Ni_{13}Mep_{4}$	>2000	n.d.	n.d.
L-$Phe_{13}Mo_{4}$	>2000	n.d.	n.d.
L-$Phe_{13}Mep_{4}$	>2000	n.d.	n.d.
L-$Do_{13}Mo_{4}$	<50	n.d.	n.d.
L-$Do_{13}Mep_{4}$	>2000	1100 (1000-1300)	60 (30-80)
H-$Ni_{31}Mo_{10}$	>8000	n.d.	n.d.
H-$Ni_{31}Mep_{10}$	>8000	n.d.	n.d.
H-$Phe_{31}Mo_{10}$	>8000	>2048	200 (90-300)
H-$Phe_{31}Mep_{10}$	>8000	n.d.	n.d.
H-$Do_{31}Mo_{10}$	>8000	1100 (900-1200)	100 (40-300)
H-$Do_{31}Mep_{10}$	6000 (5000-8000)	820 (770-880)	70 (30-110)
L-$Bam_{31}Mep_{10}$	>8000	n.d.	n.d.
L-$Bmam_{31}Mep_{10}$	6000 (5000-8000)	n.d.	n.d.
L-$Tmb_{31}Mep_{10}$	<62.5	n.d.	n.d.
L-$Oct_{31}Mep_{10}$	5000 (4000-6000)	200 (100-400)	40 (30-100)
L-$Olam_{31}Mep_{10}$	>8000	>2048	90 (70-100)
L-$Do_{30}Mep_{5}$	3000 (3000-4000)	n.d.	n.d.
L-$Tmb_{5}Mo_{90}$	>4000	n.d.	n.d.
L-$Oct_{5}Mep_{5}$	>4000	n.d.	n.d.
L-$Phe_{15}Mo_{30}$	>4000	n.d.	n.d.

Polyacrylamide copolymer antibiotics function through membrane disruption.

Based on the efficacy and safety data, we selected one top candidate copolymer, L-$Do_{31}Mep_{10}$, for further characterization. We sought to verify our hypothesized mechanism of cell-killing behavior through membrane disruption (Figure 3A). In these assays, we incubated *E. coli* with the DNA-intercalating dye propidium iodide in the presence of L-$Do_{31}Mep_{10}$. We observed a dramatic increase in fluorescence over an hour of monitoring (Figure 4A), indicating that both the outer and inner membranes of *E. coli* had been compromised by copolymer exposure, thereby allowing the propidium iodide to access the DNA within the bacteria. Our negative control, penicillin G, kills bacteria through inhibiting cell wall synthesis and did not lead to a similar increase in fluorescence. Further, we visually examined *E. coli* before and after exposure to L-$Do_{31}Mep_{10}$ at to half the lethal level (0.5x MIC) using scanning electron microscopy (SEM) (Figure 4B-C). Clear perturbations in the membrane were observed in the presence of L-$Do_{31}Mep_{10}$. Notably, a pattern of blistering was visible in the treated sample, consistent with disruptions to the physical integrity of the membrane.

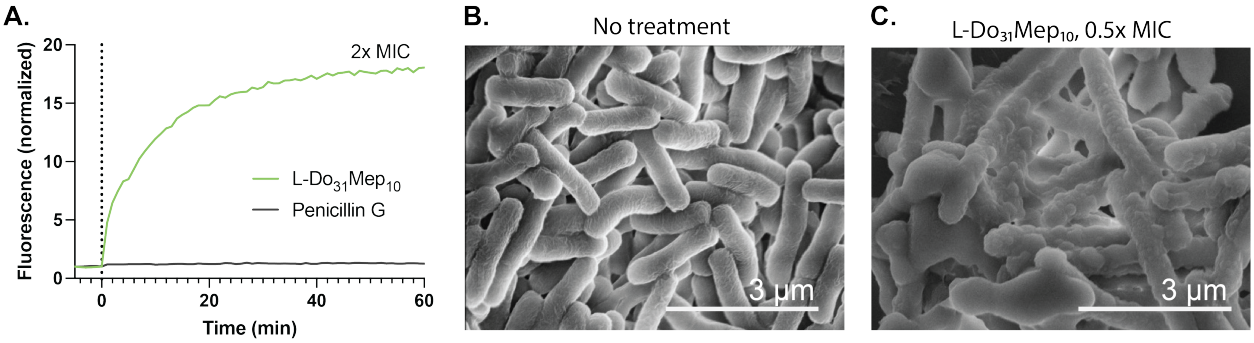

Figure 4. L-$Do_{31}Mep_{10}$ disrupts the membrane of *E. coli*. A. Outer and inner membrane permeabilization assay, using the fluorescent probe propidium iodide. **B.** SEM image of *E. coli* (untreated). **C.** SEM image of *E. coli* treated with L-$Do_{31}Mep_{10}$ (0.5x MIC; 64 µg/mL; 6.27 µM).

Polyacrylamide copolymer antibiotics mitigate onset of resistance.

We hypothesized that a membrane disruption mechanism would allow our treatments to overcome traditional mechanisms of resistance. We tested this hypothesis by exposing *E. coli* ATCC 25922 to half the lethal level of L-$Do_{31}Mep_{10}$ for 7 passages. In each passage, we calculated the MIC of the bacteria (MIC_n) and compared it to the MIC of naïve *E. coli* (MIC_0). In these assays we compared the copolymer efficacy to a small molecule control (penicillin G). Since penicillin G is not reliably effective against clinical *E. coli* isolates on account of well-characterized development of resistance over several decades of penicillin G overuse, newer penicillin derivatives, including ampicillin and amoxicillin, are more commonly used to treat infections in patients.[41] For these assays we selected a strain of *E. coli* with known susceptibility to penicillin G, in accordance with previous reports.[42,43] In these studies, no resistance to the copolymer was observed over the course of the study, whereas resistance to penicillin G was quickly developed over the same timeframe (Figure 5). These results indicate that the physical membrane disruption mechanism of the copolymer described above can mitigate the development of resistance.

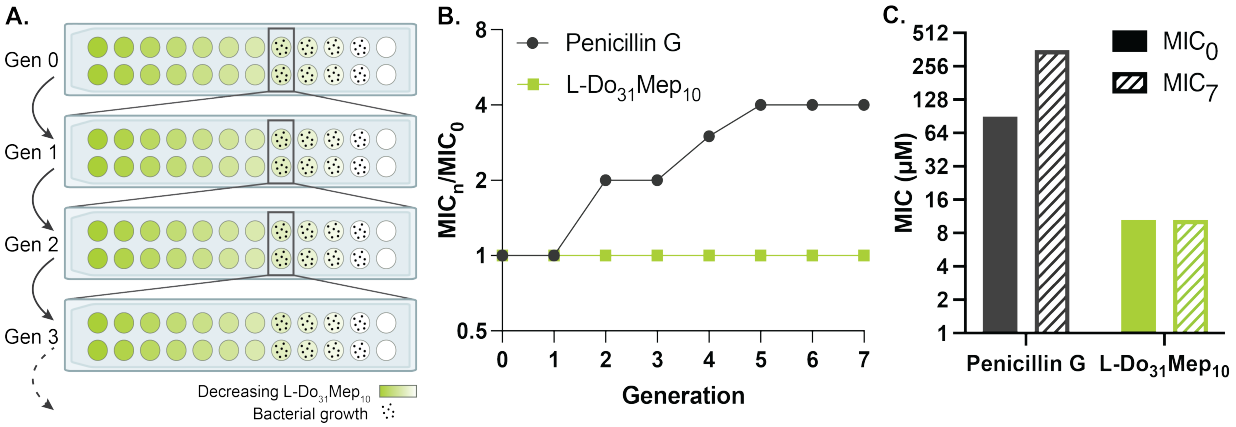

Figure 5. L-$Do_{31}Mep_{10}$ evades resistance mechanisms. A. Schematic indicating the workflow for the resistance assay. **B.** Results of resistance assay, indicating that L-$Do_{31}Mep_{10}$ is immune to resistance over 7 generations. **C.** Comparison of MIC_0 and MIC_7 for penicillin G and L-$Do_{31}Mep_{10}$.

Polyacrylamide copolymer antibiotics can rehabilitate traditional antibiotics.

We then sought to evaluate the potential of our leading polyacrylamide copolymer to improve the efficacy of existing antibiotics. We hypothesized that, since our novel copolymer disrupts the membrane of bacteria, it could facilitate entry of antibiotics into the cell without changing the antibiotic itself (Figure 6A). We evaluated a range of antibiotics, including one that is already effective against *E. coli* (rifampicin), one that is ineffective due to an inability to access to the periplasmic space (vancomycin), and one that shows moderate efficacy due to incomplete access to the periplasmic space (penicillin G). We determined the MIC of each antibiotic alone and in the presence of a sublethal amount (0.5x MIC) of L-$Do_{31}Mep_{10}$ (Figure S7). We observed that the MIC decreased by a factor of four for penicillin G and by a factor of two for vancomycin and rifampicin in the presence of the copolymer, supporting our hypothesis that the copolymer can improve intracellular access for each of these antibiotics. Thus, clinically important antibiotics become more effective in combination treatment with the antibacterial polyacrylamide, likely through enhanced transport of antibiotics across the outer membrane.

Additionally, we were interested in the impact of combination of our copolymer with existing antibiotics, and we sought to determine its ability to affect the development of resistance to penicillin G. We repeated the resistance study described above, but we used L-$Do_{31}Mep_{10}$ as an adjuvant at a constant sublethal concentration (0.25x MIC; 32 μg/mL; 2.64 μM). We evaluated the development of resistance to penicillin G over 7 passages in the presence of L-$Do_{31}Mep_{10}$, and we observed no resistance (Figure 6B-C). This remarkable finding suggests that our leading copolymer antibiotic may serve as a tool to combat the development of resistance against small molecule antibiotics that are subject to traditional resistance mechanisms on their own.

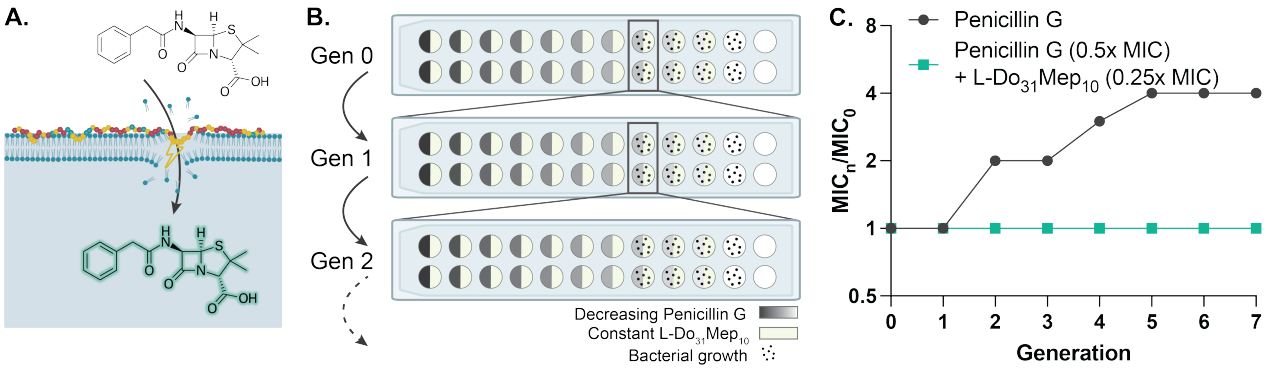

Figure 6. L-Do$_{31}$Mep$_{10}$ improves the efficacy of penicillin G. A. Schematic indicating the hypothesized mechanism through which novel polyacrylamides improve the efficacy of penicillin G by enabling its uptake into the bacteria through the perturbed membrane. **B.** Schematic indicating the experimental workflow to evaluate the ability of a combination treatment to overcome antimicrobial resistance. **C.** Results of resistance assay, indicating that a combination treatment including L-Do$_{31}$Mep$_{10}$ prevents the development of resistance against penicillin G over 7 generations.

Evaluation of the physicochemical properties driving efficacy of polyacrylamide copolymers. In addition to evaluation of the antibacterial efficacy and safety of our polyacrylamide copolymers, we analyzed each copolymer by some of its key physicochemical characteristics to investigate the influence of these properties on antibacterial efficacy. We first featurized each copolymer using the ChemoPy package to compute hundreds of chemical characteristics.[44] We condensed these multidimensional data using a principal component analysis (PCA), which preserved 60% of the variation of the dataset (Figure 7A). In this analysis, we noticed little clustering, indicating that our effective copolymers were spread across the chemical space of our library. This observation likely arose because most of our entries were effective against at least one strain of bacteria. An expansion of the library in future studies might yield more robust patterns in the PCA. Further, we observed a strong relationship between the hydrophobicity of the monomers used and the antibacterial efficacy of the polymer. To explore this, we calculated the sum of the LogP values for the hydrophobic and hydrophilic monomers used for each polymer (Table 2, Figure 7). We also investigated the influence of the molar ratio of the hydrophobic monomer to the hydrophilic

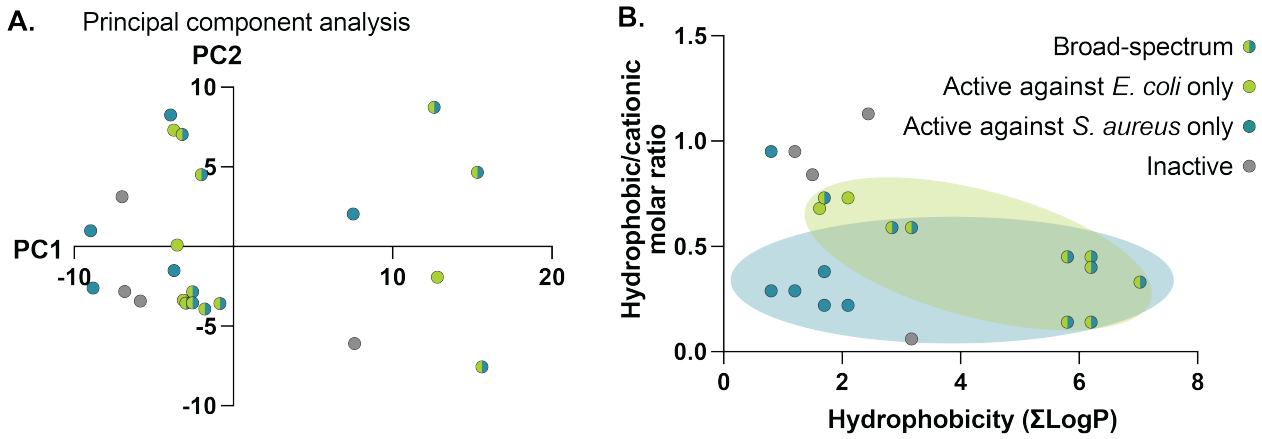

Figure 7. Evaluation of chemical differences in the library of novel polyacrylamides. Each point represents a polymer entry in our library, color-coded by its antimicrobial activity. **A.** Principal component analysis to condense highly dimensional chemical data. **B.** The impact of hydrophobicity and hydrophobic/cationic balance on the efficacy of novel copolymers.

monomer (Table 2, Figure 7). We found that the copolymers with the great breadth of activity were those with the highest LogP values. In contrast, the least effective copolymers had low LogPs and high hydrophobic/cationic molar ratios, suggesting that these copolymers comprised a high concentration of insufficiently hydrophobic monomers. In this analysis, we also noticed that copolymers with a higher charge density (a lower hydrophobic/cationic ratio) showed preferential activity towards *S. aureus*, while those with a slightly higher hydrophobic/cationic ratio were relatively more active towards *E. coli*.

CONCLUSION:

For this study, we synthesized a library of 27 polyacrylamide copolymers at gram-scale with high yield and excellent control over composition and molecular weight using RAFT polymerization. We designed statistical terpolymers to provide for flexibility in independently varying the charge density and hydrophobicity of our library entries. We evaluated their activity as antibacterial agents against *E. coli*, *S. aureus*, *K. pneumoniae*, and *E. faecium* and found several copolymers that showed broad-spectrum activity. Further, we evaluated the safety and tolerability of these

copolymers *in vitro*, and we determined that they were highly selective for bacteria over mammalian cells, leaving the later intact even at high concentrations. Their safety profiles were comparable to used inactive excipients widely used in FDA-approved drug products.

One candidate copolymer, L-$Do_{31}Mep_{10}$, was selected for further characterization on account of its exceptional potency, breadth, and safety profile. Imaging studies confirmed that the copolymer functions through a membrane-disruption mechanism of cell-killing, which was found to both enhance the efficacy of traditional small molecule antibiotics by improving access into the bacteria, as well as to mitigate traditional resistance mechanisms. Furthermore, a combination treatment of this leading copolymer with penicillin G prevented *E. coli* from developing resistance to the penicillin G., despite the copolymer being present well below its own lethal dose. This remarkable result suggests that these polyacrylamide copolymers may be useful as adjuvants to rehabilitate clinically used antibiotics in the fight against antimicrobial resistance.

MATERIALS AND METHODS:

Resource Availability

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Eric A. Appel (eappel@stanford.edu).

Materials Availability

There are restrictions to the availability of the polymers designed for this study due to the scale of synthesis. Furthermore, an MTA is required.

Data and Code Availability

Data generated for this study is available upon request.

ACKNOWLEDGEMENTS:

This work was funded by the Stanford Bio-X Interdisciplinary Initiative Seed Grants Program. Part of this work was performed at the Stanford Nano Shared Facilities (SNSF), supported by the National Science Foundation under award ECCS-2026822. S.C.W. was supported by the Sarafan ChEM-H Chemistry/Biology Interface training program and the National Science Foundation Graduate Research Fellowship. We would like to thank Noah Eckman for his contributions to GPC data processing.

AUTHOR CONTRIBUTIONS:

The study was conceptualized by S.C.W. and E.A.A. with guidance from L.C. Experiments were conducted by S.C.W., with contributions from M.B.C and X.L. (MIC assays), C.K.J. (cell viability assays), C.D. (SEM), and A.N.P (GPC). The original draft was written by S.C.W.

DECLARATION OF INTERESTS:

S.C.W. and E.A.A. are listed as inventors on a patent application describing the materials presented in this manuscript.

REFERENCES:

(1) O'Neill, J. *Antimicrobial Resistance: Tackling a Crisis for the Health and Wealth of Nations*; Review on Antimicrobial Resistance; 2014.

(2) Centers for Disease Control and Prevention (U.S.). *Antibiotic Resistance Threats in the United States, 2019*; Centers for Disease Control and Prevention (U.S.), 2019. https://doi.org/10.15620/cdc:82532.

(3) Nelson, R. E.; Hatfield, K. M.; Wolford, H.; Samore, M. H.; Scott, R. D., II; Reddy, S. C.; Olubajo, B.; Paul, P.; Jernigan, J. A.; Baggs, J. National Estimates of Healthcare Costs Associated With Multidrug-Resistant Bacterial Infections Among Hospitalized Patients in the United States. *Clin. Infect. Dis.* **2021**, *72* (Supplement_1), S17–S26. https://doi.org/10.1093/cid/ciaa1581.

(4) Murray, C. J.; Ikuta, K. S.; Sharara, F.; Swetschinski, L.; Aguilar, G. R.; Gray, A.; Han, C.; Bisignano, C.; Rao, P.; Wool, E.; Johnson, S. C.; Browne, A. J.; Chipeta, M. G.; Fell, F.; Hackett, S.; Haines-Woodhouse, G.; Hamadani, B. H. K.; Kumaran, E. A. P.; McManigal, B.; Agarwal, R.; Akech, S.; Albertson, S.; Amuasi, J.; Andrews, J.; Aravkin, A.; Ashley, E.; Bailey, F.; Baker, S.; Basnyat, B.; Bekker, A.; Bender, R.; Bethou, A.; Bielicki, J.; Boonkasidecha, S.; Bukosia, J.; Carvalheiro, C.; Castañeda-Orjuela, C.; Chansamouth, V.; Chaurasia, S.; Chiurchiù, S.; Chowdhury, F.; Cook, A. J.; Cooper, B.; Cressey, T. R.; Criollo-Mora, E.; Cunningham, M.; Darboe, S.; Day, N. P. J.; Luca, M. D.; Dokova, K.; Dramowski, A.; Dunachie, S. J.; Eckmanns, T.; Eibach, D.; Emami, A.; Feasey, N.; Fisher-Pearson, N.; Forrest, K.; Garrett, D.; Gastmeier, P.; Giref, A. Z.; Greer, R. C.; Gupta, V.; Haller, S.; Haselbeck, A.; Hay, S. I.; Holm, M.; Hopkins, S.; Iregbu, K. C.; Jacobs, J.; Jarovsky, D.; Javanmardi, F.; Khorana, M.; Kissoon, N.; Kobeissi, E.; Kostyanev, T.; Krapp, F.; Krumkamp, R.; Kumar, A.; Kyu, H. H.; Lim, C.; Limmathurotsakul, D.; Loftus, M. J.; Lunn, M.; Ma, J.; Mturi, N.; Munera-Huertas, T.; Musicha, P.; Mussi-Pinhata, M. M.; Nakamura, T.; Nanavati, R.; Nangia, S.; Newton, P.; Ngoun, C.; Novotney, A.; Nwakanma, D.; Obiero, C. W.; Olivas-Martinez, A.; Olliaro, P.; Ooko, E.; Ortiz-Brizuela, E.; Peleg, A. Y.; Perrone, C.; Plakkal, N.; Ponce-de-Leon, A.; Raad, M.; Ramdin, T.; Riddell, A.; Roberts, T.; Robotham, J. V.; Roca, A.; Rudd, K. E.; Russell, N.; Schnall, J.; Scott, J. A. G.; Shivamallappa, M.; Sifuentes-Osornio, J.; Steenkeste, N.; Stewardson, A. J.; Stoeva, T.; Tasak, N.; Thaiprakong, A.; Thwaites, G.; Turner, C.; Turner, P.; Doorn, H. R. van; Velaphi, S.; Vongpradith, A.; Vu, H.; Walsh, T.; Waner, S.; Wangrangsimakul, T.; Wozniak, T.; Zheng, P.; Sartorius, B.; Lopez, A. D.; Stergachis, A.; Moore, C.; Dolecek, C.; Naghavi, M. Global Burden of Bacterial Antimicrobial Resistance in 2019: A Systematic Analysis. *The Lancet* **2022**, *399* (10325), 629–655. https://doi.org/10.1016/S0140-6736(21)02724-0.

(5) Plackett, B. Why Big Pharma Has Abandoned Antibiotics. *Nature* **2020**, *586*, S50–S52.

(6) *Leaving the Lab: Tracking the Decline in AMR R&D Professionals*; AMR Industry Alliance, 2024.

(7) *MicroBIGG-E Map*. https://www.ncbi.nlm.nih.gov/pathogens/microbigge_map/?utm_source=referral+&utm_medium=ncbi_insights&utm_campaign=pathogen-detection-microbigg-e-map-20231206 (accessed 2024-06-10).

(8) *PubMed by Year*. https://esperr.github.io/pubmed-by-year/?q1=Antibiotics&q2=Antimicrobial%20resistance&startyear=1980 (accessed 2024-06-10).

(9) Reygaert, W. An Overview of the Antimicrobial Resistance Mechanisms of Bacteria. *AIMS Microbiol.* **2018**, *4* (3), 482–501. https://doi.org/10.3934/microbiol.2018.3.482.

(10) Munita, J. M.; Arias, C. A. Mechanisms of Antibiotic Resistance. *Microbiol. Spectr.* **2016**, *4* (2), 10.1128/microbiolspec.VMBF-0016–2015. https://doi.org/10.1128/microbiolspec.VMBF-0016-2015.

(11) Yin, L.-H.; Ran, B.; Hu, T.-J.; Yang, C.; Fei, J.-J.; Li, Y.-H. Preparation of Highly Efficient Antibacterial Polymeric Films via the Modulation of Charge Density and Hydrophobicity. *RSC Adv.* **2017**, *7* (10), 6006–6012. https://doi.org/10.1039/C6RA26071C.

(12) Kenawy, E.-R.; Worley, S. D.; Broughton, R. The Chemistry and Applications of Antimicrobial Polymers: A State-of-the-Art Review. *Biomacromolecules* **2007**, *8* (5), 1359–1384. https://doi.org/10.1021/bm061150q.

(13) Qiu, H.; Si, Z.; Luo, Y.; Feng, P.; Wu, X.; Hou, W.; Zhu, Y.; Chan-Park, M. B.; Xu, L.; Huang, D. The Mechanisms and the Applications of Antibacterial Polymers in Surface Modification on Medical Devices. *Front. Bioeng. Biotechnol.* **2020**, *8*. https://doi.org/10.3389/fbioe.2020.00910.

(14) Huang, K.-S.; Yang, C.-H.; Huang, S.-L.; Chen, C.-Y.; Lu, Y.-Y.; Lin, Y.-S. Recent Advances in Antimicrobial Polymers: A Mini-Review. *Int. J. Mol. Sci.* **2016**, *17* (9), 1578. https://doi.org/10.3390/ijms17091578.

(15) Kamaruzzaman, N. F.; Tan, L. P.; Hamdan, R. H.; Choong, S. S.; Wong, W. K.; Gibson, A. J.; Chivu, A.; Pina, M. de F. Antimicrobial Polymers: The Potential Replacement of Existing Antibiotics? *Int. J. Mol. Sci.* **2019**, *20* (11).

(16) Hwang, J.; Barman, S.; Gao, R.; Yang, X.; O'Malley, A.; Nagarkatti, P.; Nagarkatti, M.; Chruszcz, M.; Tang, C. Membrane-Active Metallopolymers: Repurposing and Rehabilitating Antibiotics to Gram-Negative Superbugs. *Adv. Healthc. Mater.* **2023**, *12* (31), 2301764. https://doi.org/10.1002/adhm.202301764.

(17) Hancock, S. N.; Yuntawattana, N.; Diep, E.; Maity, A.; Tran, A.; Schiffman, J. D.; Michaudel, Q. Ring-Opening Metathesis Polymerization of N-Methylpyridinium-Fused Norbornenes to Access Antibacterial Main-Chain Cationic Polymers. *Proc. Natl. Acad. Sci.* **2023**, *120* (51), e2311396120. https://doi.org/10.1073/pnas.2311396120.

(18) Pham, P.; Oliver, S.; Nguyen, D. T.; Boyer, C. Effect of Cationic Groups on the Selectivity of Ternary Antimicrobial Polymers. *Macromol. Rapid Commun.* **2022**, *43* (21), 2200377. https://doi.org/10.1002/marc.202200377.

(19) Pham, P.; Oliver, S.; Boyer, C. Design of Antimicrobial Polymers. *Macromol. Chem. Phys.* **2023**, *224* (3), 2200226. https://doi.org/10.1002/macp.202200226.

(20) Garcia Maset, R.; Hapeshi, A.; Hall, S.; Dalgliesh, R. M.; Harrison, F.; Perrier, S. Evaluation of the Antimicrobial Activity in Host-Mimicking Media and In Vivo Toxicity of Antimicrobial Polymers as Functional Mimics of AMPs. *ACS Appl. Mater. Interfaces* **2022**, *14* (29), 32855–32868. https://doi.org/10.1021/acsami.2c05979.

(21) Kuroki, A.; Sangwan, P.; Qu, Y.; Peltier, R.; Sanchez-Cano, C.; Moat, J.; Dowson, C. G.; Williams, E. G. L.; Locock, K. E. S.; Hartlieb, M.; Perrier, S. Sequence Control as a Powerful Tool for Improving the Selectivity of Antimicrobial Polymers. *ACS Appl. Mater. Interfaces* **2017**, *9* (46), 40117–40126. https://doi.org/10.1021/acsami.7b14996.

(22) Nguyen, T.-K.; Lam, S. J.; Ho, K. K. K.; Kumar, N.; Qiao, G. G.; Egan, S.; Boyer, C.; Wong, E. H. H. Rational Design of Single-Chain Polymeric Nanoparticles That Kill Planktonic and Biofilm Bacteria. *ACS Infect. Dis.* **2017**, *3* (3), 237–248. https://doi.org/10.1021/acsinfecdis.6b00203.

(23) Porel, M.; Thornlow, D. N.; Phan, N. N.; Alabi, C. A. Sequence-Defined Bioactive Macrocycles via an Acid-Catalysed Cascade Reaction. *Nat. Chem.* **2016**, *8* (6), 590–596. https://doi.org/10.1038/nchem.2508.

(24) Porel, M.; Thornlow, D.; Artim, C. M.; Alabi, C. A. Sequence-Defined Backbone Modifications Regulate Antibacterial Activity of OligoTEAs. *ACS Chem. Biol.* **2017**, *12* (3), 715–723. https://doi.org/10.1021/acschembio.6b00837.

(25) AL-Badri, Z. M.; Som, A.; Lyon, S.; Nelson, C. F.; Nüsslein, K.; Tew, G. N. Investigating the Effect of Increasing Charge Density on the Hemolytic Activity of Synthetic Antimicrobial Polymers. *Biomacromolecules* **2008**, *9* (10), 2805–2810. https://doi.org/10.1021/bm800569x.

(26) Engler, A. C.; Tan, J. P. K.; Ong, Z. Y.; Coady, D. J.; Ng, V. W. L.; Yang, Y. Y.; Hedrick, J. L. Antimicrobial Polycarbonates: Investigating the Impact of Balancing Charge and Hydrophobicity Using a Same-Centered Polymer Approach. *Biomacromolecules* **2013**, *14* (12), 4331–4339. https://doi.org/10.1021/bm401248t.

(27) Gabriel, G. J.; Maegerlein, J. A.; Nelson, C. F.; Dabkowski, J. M.; Eren, T.; Nüsslein, K.; Tew, G. N. Comparison of Facially Amphiphilic versus Segregated Monomers in the Design of Antibacterial Copolymers. *Chem. – Eur. J.* **2009**, *15* (2), 433–439. https://doi.org/10.1002/chem.200801233.

(28) Zou, P.; Laird, D.; Riga, E. K.; Deng, Z.; Dorner, F.; Perez-Hernandez, H.-R.; Guevara-Solarte, D. L.; Steinberg, T.; Al-Ahmad, A.; Lienkamp, K. Antimicrobial and Cell-Compatible Surface-Attached Polymer Networks – How the Correlation of Chemical Structure to Physical and Biological Data Leads to a Modified Mechanism of Action. *J. Mater. Chem. B* **2015**, *3* (30), 6224–6238. https://doi.org/10.1039/C5TB00906E.

(29) Leong, J.; Yang, C.; Tan, J.; Tan, B. Q.; Hor, S.; Hedrick, J. L.; Yang, Y. Y. Combination of Guanidinium and Quaternary Ammonium Polymers with Distinctive Antimicrobial Mechanisms Achieving a Synergistic Antimicrobial Effect. *Biomater. Sci.* **2020**, *8* (24), 6920–6929. https://doi.org/10.1039/D0BM00752H.

(30) Concilio, M.; Garcia Maset, R.; Lemonche, L. P.; Kontrimas, V.; Song, J.-I.; Rajendrakumar, S. K.; Harrison, F.; Becer, C. R.; Perrier, S. Mechanism of Action of Oxazoline-Based Antimicrobial Polymers Against Staphylococcus Aureus: In Vivo Antimicrobial Activity Evaluation. *Adv. Healthc. Mater.* **2023**, *12* (29), 2301961. https://doi.org/10.1002/adhm.202301961.

(31) Jung, K.; Corrigan, N.; Wong, E. H. H.; Boyer, C. Bioactive Synthetic Polymers. *Adv. Mater.* **2022**, *34* (2), 2105063. https://doi.org/10.1002/adma.202105063.

(32) Chin, W.; Zhong, G.; Pu, Q.; Yang, C.; Lou, W.; De Sessions, P. F.; Periaswamy, B.; Lee, A.; Liang, Z. C.; Ding, X.; Gao, S.; Chu, C. W.; Bianco, S.; Bao, C.; Tong, Y. W.; Fan, W.; Wu, M.; Hedrick, J. L.; Yang, Y. Y. A Macromolecular Approach to Eradicate Multidrug Resistant Bacterial Infections While Mitigating Drug Resistance Onset. *Nat. Commun.* **2018**, *9* (1), 917. https://doi.org/10.1038/s41467-018-03325-6.

(33) Sigurjonsson, J.; Grubb, D.; Grubb, A.; Christensson, A.; Öberg, C. M.; Ederoth, P.; Koul, S.; Götberg, M.; Yndigegn, T.; Tödt, T.; Viterius, B.; Bjursten, H. A Study of Size-Selective Renal Elimination Using a Novel Human Model. *Scand. J. Clin. Lab. Invest.* **2024**, *84* (2), 115–120. https://doi.org/10.1080/00365513.2024.2338742.

(34) Judzewitsch, P. R.; Nguyen, T.-K.; Shanmugam, S.; Wong, E. H. H.; Boyer, C. Towards Sequence-Controlled Antimicrobial Polymers: Effect of Polymer Block Order on Antimicrobial Activity. *Angew. Chem. Int. Ed.* **2018**, *57* (17), 4559–4564. https://doi.org/10.1002/anie.201713036.

(35) Judzewitsch, P. R.; Zhao, L.; Wong, E. H. H.; Boyer, C. High-Throughput Synthesis of Antimicrobial Copolymers and Rapid Evaluation of Their Bioactivity. *Macromolecules* **2019**, *52* (11), 3975–3986. https://doi.org/10.1021/acs.macromol.9b00290.

(36) Namivandi-Zangeneh, R.; Kwan, R. J.; Nguyen, T.-K.; Yeow, J.; Byrne, F. L.; Oehlers, S. H.; Wong, E. H. H.; Boyer, C. The Effects of Polymer Topology and Chain Length on the Antimicrobial Activity and Hemocompatibility of Amphiphilic Ternary Copolymers. *Polym. Chem.* **2018**, *9* (13), 1735–1744. https://doi.org/10.1039/C7PY01069A.

(37) Huang, D. B.; Brothers, K. M.; Mandell, J. B.; Taguchi, M.; Alexander, P. G.; Parker, D. M.; Shinabarger, D.; Pillar, C.; Morrissey, I.; Hawser, S.; Ghahramani, P.; Dobbins, D.; Pachuda, N.; Montelaro, R.; Steckbeck, J. D.; Urish, K. L. Engineered Peptide PLG0206 Overcomes Limitations of a Challenging Antimicrobial Drug Class. *PLOS ONE* **2022**, *17* (9), e0274815. https://doi.org/10.1371/journal.pone.0274815.

(38) Kurbatfinski, N.; Kramer, C. N.; Goodman, S. D.; Bakaletz, L. O. ESKAPEE Pathogens Newly Released from Biofilm Residence by a Targeted Monoclonal Are Sensitized to Killing by Traditional Antibiotics. *Front. Microbiol.* **2023**, *14*, 1202215. https://doi.org/10.3389/fmicb.2023.1202215.

(39) *WHO Bacterial Priority Pathogens List, 2024: Bacterial Pathogens of Public Health Importance to Guide Research, Development and Strategies to Prevent and Control Antimicrobial Resistance.*; World Health Organization: Geneva, Switzerland, 2024.

(40) *Inactive Ingredient Search for Approved Drug Products*. https://www.accessdata.fda.gov/scripts/cder/iig/index.cfm (accessed 2022-03-09).

(41) *Penicillin, antistaphylococcal penicillins, and broad-spectrum penicillins - UpToDate*. https://www.uptodate.com/contents/penicillin-antistaphylococcal-penicillins-and-broad-spectrum-penicillins#H7 (accessed 2024-08-29).

(42) Fass, R. J.; Barnishan, J. Minimal Inhibitory Concentrations of 34 Antimicrobial Agents for Control Strains Escherichia Coli ATCC 25922 and Pseudomonas Aeruginosa ATCC 27853. *Antimicrob. Agents Chemother.* **1979**, *16* (5), 622–624.

(43) Wiradharma, N.; Khan, M.; Yong, L.-K.; Hauser, C. A. E.; Seow, S. V.; Zhang, S.; Yang, Y.-Y. The Effect of Thiol Functional Group Incorporation into Cationic Helical Peptides on Antimicrobial Activities and Spectra. *Biomaterials* **2011**, *32* (34), 9100–9108. https://doi.org/10.1016/j.biomaterials.2011.08.020.

(44) Cao, D.-S.; Xu, Q.-S.; Hu, Q.-N.; Liang, Y.-Z. ChemoPy: Freely Available Python Package for Computational Biology and Chemoinformatics. *Bioinformatics* **2013**, *29* (8), 1092–1094. https://doi.org/10.1093/bioinformatics/btt105.