

UCLA

UCLA Electronic Theses and Dissertations

Title

Antimicrobial Polymer Delivery via Bacteriophages for Improved Biocompatibility and Reduced Resistance Potential

Permalink

<https://escholarship.org/uc/item/9hq8c8qh>

ISBN

9798247947349

Author

Vexler, Shelby

Publication Date

2026-06-01

Peer reviewed|Thesis/dissertation

UNIVERSITY OF CALIFORNIA

Los Angeles

Antimicrobial Polymer Delivery via Bacteriophages
for Improved Biocompatibility and Reduced Resistance Potential

A dissertation submitted in partial satisfaction of the
requirements for the degree Doctor of Philosophy
in Chemistry

by

Shelby Danielle Vexler

2026

© Copyright by
Shelby Danielle Vexler
2026

ABSTRACT OF THE DISSERTATION

Antimicrobial Polymer Delivery via Bacteriophages for Improved Biocompatibility and Reduced Resistance Potential

by

Shelby Danielle Vexler

Doctor of Philosophy in Chemistry

University of California, Los Angeles, 2026

Professor Irene A. Chen, Chair

The rise of antibiotic resistance necessitates developing alternative treatments with minimal potential for bacterial adaptation. Two promising solutions include membrane-disruptive polymers and phage therapy, which utilizes viruses specific to bacteria, but they face opposite shortcomings; many polymers lack specificity for bacterial cells, resulting in poor biocompatibility, while phages are restricted to a very narrow host range. In this dissertation, we aim to circumvent each agent's individual shortcomings through the synergistic approach of engineering the non-lytic M13 bacteriophage into a polymer delivery platform.

Our first goal was to modify M13 bacteriophage, which normally infects *Escherichia coli*, to target the pathogenic bacteria *Staphylococcus aureus*. We expressed an antibody fragment specific to a conserved surface polysaccharide, poly-*N*-acetylglucosamine, on the receptor-binding protein. Successful binding of the anti-PNAG phage was quantified through

enzyme-linked immunosorbent assays (ELISAs) and visualized by transmission electron microscopy (TEM).

Next, we assessed the antimicrobial activity of three separate libraries of bio-inspired polymers with either methacrylate, hydroxybenzoate-co-lactide, or peptide backbones. We explored the differing trends in activity for each library with respect to degrees of polymerization, hydrophobicity, and charge. Our results highlight the diversity of antimicrobial polymers while demonstrating the combined utility of synthetic biology and chemistry for designing them.

Lastly, antimicrobial polylysines varying by degrees of polymerization (48, 90, or 170) were loaded onto the anti-PNAG phage through electrostatic interactions or EDC crosslinking. Characterization determined higher drug-to-phage ratios for electrostatic loading compared to crosslinking, yet both attachment strategies improved potency, reducing the minimum inhibitory concentration (MIC) five-fold towards *S. aureus*. Critically, all bioconjugates demonstrated improved biocompatibility compared to polylysine alone; mammalian cells exposed to covalent polylysine-phages with $DP \leq 90$ exhibited the highest viability over a five-day period. Furthermore, no resistance was induced in *S. aureus* after thirteen rounds of selection with covalent polylysine-phages, confirming bioconjugates retain the “resistance-resistant” nature of antimicrobial polymers.

In summary, the findings of this dissertation present phage-based delivery of antimicrobial polymers as an effective strategy to target pathogenic bacteria while minimizing harm to human cells and potential for resistance.

The dissertation of Shelby Danielle Vexler is approved.

Junyoung O Park

Jose A Rodriguez

Jorge Z Torres

Irene A Chen, Committee Chair

University of California, Los Angeles

2026

Dedication

I am incredibly grateful for the support of my family, friends, and colleagues on this academic journey. This achievement would not be possible without them.

First and foremost, I want to thank my advisor Irene Chen, for providing the opportunity to pursue the research I've dreamed of since high school, and endless guidance. The support of my lab mates was critical to the completion of this research. I'm grateful for not only their assistance with experiments, but friendship. I especially want to thank Dr. Yanxi "Steven" Yang, my first mentor in the lab, and my fellow phage graduate researchers: Nicholas Zhang, Katherine McCarthy, and Jeremy Pietropaolo. I'd also like to acknowledge the contributions of my mentees to not only my research, but my journey as a scientific mentor. Training undergraduate researchers has been one of my highlights of my doctoral studies, and I want to express my gratitude to my students Sandy He, Beatrice Mihalache, and Marlys Kutach.

Science is a collaborative endeavor, and I received many opportunities to work with talented scientists outside my own group. I am fortunate to call Dr. Casey Morrison both my collaborator and close friend. I am incredibly grateful for all the polymers and emotional support you've provided; half of this dissertation would not be possible without you! To Dr. Saumya Jain, thank you for your friendship and help with the biocompatibility studies. I'd also like to acknowledge Dr. Rajalakshmi "Raj" Sivasankaran and Dr. Pedro Salas-Ambrosio from Professor Heather Maynard's group, as our collaborations on antimicrobial polymers were instrumental to my doctoral studies. Additionally, I'd like to thank my committee, Professors Junyoung "Jun" Park, Jose Rodriguez, and Jorge Torres, for their ongoing support and advice.

Lastly, I want to acknowledge my friends and family. Thank you to my parents, Allen and Cindy Vexler, for their endless support and encouragement. I am incredibly grateful for my partner, Steven Fields, who in addition to being my biggest cheerleader, always picks me up

from lab when I miss the last bus home. Thank you for your unconditional love and believing in my dreams. To all my other friends and family, thank you for supporting me on this journey. In the words of Elle Woods, “We did it!”.

Table of Contents

ABSTRACT OF THE DISSERTATION	ii
Dedication	v
List of Figures.....	ix
List of Tables	xi
List of Schemes	xviii
Acknowledgments	xxi
Vita	xxiv
Chapter 1. Introduction	1
1.1 Antibiotic resistance and pathogenic <i>Staphylococcus aureus</i>	1
1.2 Phage therapy	4
1.3 Antimicrobial polymers	8
1.4 M13 phage as a polymer delivery platform	11
Chapter 2. A poly-<i>N</i>-acetylglucosamine binding phage targets <i>Staphylococcus aureus</i>	15
2.1 Methodology.....	16
2.2 Results.....	21
2.3 Discussion	27
2.4 Supplementary figures and tables.....	29
Chapter 3. Antimicrobial properties of bio-inspired polymers	32
3.1 Methodology.....	37
3.1.1 Preparation of methacrylate copolymers with caffeine and cationic or zwitterionic side chains	37
3.1.2 Preparation of hydroxybenzoate-co-lactide polymers	48
3.1.3 Preparation of polylysine.....	62
3.1.4 Antimicrobial assays	64
3.2 Results.....	67
3.2.1 Methacrylate copolymers with caffeine and cationic or zwitterionic side chains.....	67
3.2.2 Hydroxybenzoate-co-lactide polymers.....	75

3.2.3 Polylysine	85
3.3 Discussion	87
3.4 Supplementary figures	91
3.4.1 Methacrylate copolymers with caffeine and cationic or zwitterionic side chains.....	91
3.4.2. Hydroxybenzoate-co-lactide polymers.....	113
3.4.3. Polylysine	143
3.5 Supplementary tables.....	146
Chapter 4. Characterization of polylysine-phage bioconjugates and complexes.....	149
4.1 Methodology.....	150
4.2 Results.....	157
4.3 Discussion	175
4.4 Supplementary figures	179
4.5 Supplementary tables.....	193
Chapter 5. Conclusions.....	203
References.....	206

List of Figures

Chapter 1

Figure 1.1. Predicted mortality from AMR compared with current common causes of death.....	2
Figure 1.2. Phage morphology and life cycles.....	5
Figure 1.3. Flowchart depicting outcome of all bacteriophage therapy requests at the Center for Innovative Phage Applications and Therapeutics (IPATH)	7
Figure 1.4. General working principles of antimicrobial polymers	9
Figure 1.5. Structure of M13 bacteriophage	12

Chapter 2

Figure 2.1. Engineered M13 binding to <i>S. aureus</i> biofilm polysaccharides.....	23
Figure 2.2. Engineered M13 binding to <i>E. coli</i> , <i>S. aureus</i> , <i>S. epidermidis</i> , and <i>P. aeruginosa</i> cells	25
Figure 2.3. Binding of SYBR Gold-labeled M13 and M13 ^{aPNAG} phages to <i>S. aureus</i> Newman and <i>E. coli</i> ER 2738	26
Figure 2.4. Engineered M13 binding to eukaryotic cells.....	27

Chapter 3

Figure 3.1. Compounds tested for antimicrobial evaluation with different R ₁ and R ₂ groups on the aromatic ring	76
Figure 3.2. Preparation and characterization of polymers derived from 4-(methyl/allyl/benzyl)-oxy-6-alkyl-2-oxybenzoate lactide monomers by ring-opening polymerization.....	79

Figure 3.3. Biofilm inhibition and disruption of small molecules and functional hydroxybenzoate-co-lactide polymers on <i>S. aureus</i>	84
Figure 3.4. Key compounds with antimicrobial activity against <i>S. aureus</i>	89
 Chapter 4	
Figure 4.1. Time-kill kinetics of polyK ₉₀ , PKAP _{90E} and PKAP _{90C}	164
Figure 4.2. Fluorescence and phase contrast microscopy of <i>S. aureus</i> treated with polyK ₉₀ and PKAP ₉₀ phages	165
Figure 4.3. Transmission electron microscopy (TEM) visualizes interactions between M13 ^α PNAG and <i>S. aureus</i>	166
Figure 4.4. Relative metabolic activity of polyK and PKAP treated HEK 293 cells.	167
Figure 4.5. Metabolic activity of polyK and PKAP treated HEK 293 cells over a five-day period	169
Figure 4.6. Representative images of live-dead staining on day 5	170
Figure 4.7. Hemolysis in the presence of polyK and PKAP phages.....	171
Figure 4.8. Release of electrostatically loaded polyK ₉₀ from PKAP _{90E}	172
Figure 4.9. PolyK ₉₀ and PKAP _{90C} do not induce resistance in adaptive laboratory evolution ..	173

List of Supplementary Figures

Chapter 2

Figure S.2.1. Sequences of VH-L-VL and VL-L-VL	29
Figure S.2.2. Binding of M13 ^{aPNAG} to glycan microarrays from the National Center for Functional Glycomics	30

Chapter 3

Figure S3.1. ¹ H-NMR spectrum of CafMA in DMSO- <i>d</i> ₆	92
Figure S3.2. ¹³ C-NMR spectrum of CafMA in DMSO- <i>d</i> ₆	92
Figure S3.3. ¹ H-NMR spectrum of P(CafMA) in CDCl ₃	93
Figure S3.4. SEC chromatogram of P(CafMA) in trifluoroethanol.....	93
Figure S3.5. Solubility of monomer CafMA, oligomers and homopolymer P(CafMA)	94
Figure S3.6. ¹ H-NMR spectrum of Boc-ab-MA in DMSO- <i>d</i> ₆	95
Figure S3.7. ¹³ C-NMR spectrum of Boc-ab-MA in DMSO- <i>d</i> ₆	95
Figure S3.8. ¹ H NMR of a representative copolymer bearing caffeine and cationic side chains	96
Figure S3.9. ¹ H-NMR spectrum of P(Boc-ab-Caf50%)MA in DMSO- <i>d</i> ₆	97
Figure S3.10. ¹ H-NMR spectrum of P(Boc-ab-Caf30%)MA in DMSO- <i>d</i> ₆	97
Figure S3.11. ¹ H-NMR spectrum of P(Boc-ab-Caf10%)MA in DMSO- <i>d</i> ₆	98
Figure S3.12. ¹ H-NMR spectrum of P(Boc-ab)MA in DMSO- <i>d</i> ₆	98
Figure S3.13. Representative chromatograms from refractive index (RI) trace of copolymer bearing caffeine and cationic side chains from SEC in DMF.....	99
Figure S3.14. Light scattering signal (LS) from the series of copolymers P(Boc-ab-Caf)MA by SEC in DMF	99

Figure S3.15. ^1H -NMR spectrum of P(ab-Caf50%)MA in DMSO- d_6	100
Figure S3.16. ^1H -NMR spectrum of P(ab-Caf30%)MA in DMSO- d_6	100
Figure S3.17. ^1H -NMR spectrum of P(ab-Caf10%)MA in DMSO- d_6	101
Figure S3.18. ^1H -NMR spectrum of P(ab)MA in DMSO- d_6	101
Figure S3.19. ^1H -NMR spectrum of <i>t</i> -Bu-DMEA-MA in DMSO- d_6	102
Figure S3.20. ^1H -NMR spectrum of P(<i>t</i> -Bu-DMEA-Caf30%)MA in DMSO- d_6	102
Figure S3.21. ^1H -NMR spectrum of P(<i>t</i> -Bu-DMEA-Caf10%)MA in DMSO- d_6	103
Figure S3.22. ^1H -NMR spectrum of P(<i>t</i> -Bu-DMEA)MA in DMSO- d_6	103
Figure S3.23. Chromatograms from aqueous SEC from RI detector for P(<i>t</i> -Bu-DMEA-Caf)MA series of copolymers	104
Figure S3.24. ^1H -NMR spectrum of P(DMEA-Caf30%)MA in DMSO- d_6	104
Figure S3.25. ^1H -NMR spectrum of P(DMEA-Caf10%)MA in DMSO- d_6	105
Figure S3.26. ^1H -NMR spectrum of P(DMEA)MA in DMSO- d_6	105
Figure S3.27. Chromatograms from aqueous SEC from RI detector for P(DMEA-Caf)MA series of copolymers upon Boc-deprotection	106
Figure S3.28. ^1H -NMR spectrum of Lys-MA in DMSO- d_6	106
Figure S3.29. ^1H -NMR spectrum of P(Lys-Caf50%)MA in DMSO- d_6	107
Figure S3.30. ^1H -NMR spectrum of P(Lys-Caf30%)MA in DMSO- d_6	107
Figure S3.31. ^1H -NMR spectrum of P(Lys-Caf10%)MA in DMSO- d_6	108
Figure S3.32. ^1H -NMR spectrum of P(Lys)MA in DMSO- d_6	108
Figure S3.33. Chromatograms from aqueous SEC from RI detector for P(Lys-Caf)MA series of copolymers	109
Figure S3.34. ^1H -NMR spectrum of P(<i>t</i> -Bu-DMEA-Caf30%)MA DP30 in DMSO- d_6	109

Figure S3.35. ^1H -NMR spectrum of P(<i>t</i> -Bu-DMEA-Caf10%)MA DP30 in DMSO- d_6	110
Figure S3.36. ^1H -NMR spectrum of P(<i>t</i> -Bu-DMEA)MA DP30 in DMSO- d_6	110
Figure S3.37. Chromatograms from aqueous SEC from RI detector for P(<i>t</i> -Bu-DMEA-Caf)MA DP30 series of copolymers	111
Figure S3.38. ^1H -NMR spectrum of P(DMEA-Caf30%)MA DP30 in DMSO- d_6	111
Figure S3.39. ^1H -NMR spectrum of P(DMEA-Caf10%)MA DP30 in DMSO- d_6	112
Figure S3.40. ^1H -NMR spectrum of P(DMEA)MA DP30 in DMSO- d_6	112
Figure S3.41. Chromatograms from aqueous SEC from RI detector for P(DMEA-Caf)MA DP30 series of copolymers upon Boc-deprotection.....	113
Figure S3.42. ^1H -NMR of 3 in acetone- d_6	113
Figure S3.43. ^1H -NMR of 7-hydroxy-2,2-dimethyl-4 <i>H</i> -benzo[<i>d</i>][1,3]dioxin-4-one in acetone- d_6	114
Figure S3.44. ^1H -NMR and ^{13}C -NMR of 7-(allyloxy)-2,2-dimethyl-4 <i>H</i> -benzo[<i>d</i>][1,3]dioxin-4-one in acetone- d_6	115
Figure S3.45. ^1H -NMR and ^{13}C -NMR of 4 in acetone- d_6	116
Figure S3.46. ^1H -NMR of 7-(benzyloxy)-2,2-dimethyl-4 <i>H</i> -benzo[<i>d</i>][1,3]dioxin-4-one in acetone- d_6	116
Figure S3.47. ^1H -NMR of 5 in acetone- d_6	117
Figure S3.48. ^1H -NMR of 7 in acetone- d_6	117
Figure S3.49. ^1H -NMR of 8 in acetone- d_6	118
Figure S3.50. ^1H -NMR and ^{13}C -NMR of 9 in CDCl ₃	119
Figure S3.51. ^1H -NMR of 5-heptyl-7-hydroxy-2,2-dimethyl-4 <i>H</i> -benzo[<i>d</i>][1,3]dioxin-4-one in acetone- d_6	119

Figure S3.52. ^1H -NMR and ^{13}C -NMR of 7-(benzyloxy)-5-heptyl-2,2-dimethyl-4 <i>H</i> -benzo[<i>d</i>][1,3]dioxin-4-one in acetone- d_6	120
Figure S3.53. ^1H -NMR and ^{13}C -NMR of 10 in acetone- d_6	121
Figure S3.54. ^1H -NMR and ^{13}C -NMR of 13 in acetone- d_6	122
Figure S3.55. ^1H -NMR and ^{13}C -NMR of 11 in acetone- d_6	123
Figure S3.56. ^1H -NMR and ^{13}C -NMR of 12 in acetone- d_6	124
Figure S3.57. ^1H -NMR and ^{13}C -NMR of 14 in acetone- d_6	125
Figure S3.58. ^1H -NMR and ^{13}C -NMR of 15 in CDCl_3	126
Figure S3.59. Ring-opening polymerization of monomer 13	127
Figure S3.60. COSY 2D-NMR of the polymer from monomer 13 using the system monomer/3-O urea/MTBD/initiator (50:1:1:1)	128
Figure S3.61. ^{13}C -NMR of the polymer from monomer 13 using the system monomer/3-O urea/MTBD/initiator (50:1:1:1) in CDCl_3	128
Figure S3.62. Refractive index trace from SEC in THF of the resultant polymers at molar ratio 30:1:1 (polymers 18, 21, 24, 27).....	129
Figure S3.63. ^1H -NMR and ^{13}C -NMR of 16 in CDCl_3	130
Figure S3.64. ^1H -NMR and ^{13}C -NMR of 17 in CDCl_3	131
Figure S3.65. ^1H -NMR and ^{13}C -NMR of 18 in CDCl_3	132
Figure S3.66. ^1H -NMR and ^{13}C -NMR of 19 in CDCl_3	133
Figure S3.67. ^1H -NMR and ^{13}C -NMR of 20 in CDCl_3	134
Figure S3.68. ^1H -NMR and ^{13}C -NMR of 21 in CDCl_3	135
Figure S3.69. ^1H -NMR and ^{13}C -NMR of 22 in CDCl_3	136
Figure S3.70. ^1H -NMR and ^{13}C -NMR of 23 in CDCl_3	137

Figure S3.71. ^1H -NMR and ^{13}C -NMR of 24 in CDCl_3	138
Figure S3.72. ^1H -NMR and ^{13}C -NMR of 25 in CDCl_3	139
Figure S3.73. ^1H -NMR and ^{13}C -NMR of 26 in CDCl_3	140
Figure S3.74. ^1H -NMR and ^{13}C -NMR of 27 in CDCl_3	141
Figure S3.75. ^1H -NMR and ^{13}C -NMR of 28 in CDCl_3	142
Figure S3.76. ^1H NMR of polyK ₄₈ and polyZK ₄₈ after reaction with mPEG-NCO for end-group analysis and molecular weight determination.....	143
Figure S3.77. ^1H NMR of polyK ₉₀ and polyZK ₉₀ after reaction with mPEG-NCO for end-group analysis and molecular weight determination.....	144
Figure S3.78. ^1H NMR of polyK ₁₇₀ and polyZK ₁₇₀ after reaction with mPEG-NCO for end-group analysis and molecular weight determination	145

Chapter 4

Figure S4.1. UV-Vis absorbance spectrum of M13 ^{αPNAG} , polyK90, PKAP _{90C} , and PKAP _{90E} ...	179
Figure S4.2. Bradford assay standard curves.....	180
Figure S4.3. OPA standard curves	181
Figure S4.4. Standard curve for virion quantification by Qubit Fluorometer.....	181
Figure S4.5. Chromatograms of amino acid analysis	189
Figure S4.6. HEK293 cell metabolic activity at additional polyK and PKAP ₉₀ concentrations	190
Figure S4.7. Fluorescence microscopy visualizes mammalian cell viability after twenty-four hours of treatment	191
Figure S4.8. Mammalian cell percent viability on days 1 and 5.....	191
Figure S4.9. Dextran sulfate concentration vs rate constant of polyK ₉₀ release from PKAP _{90E}	192

Figure S4.10. MIC of control group after serial passaging.....	192
---	-----

List of Tables

Chapter 3

Table 3.1. Characterization of copolymers bearing caffeine and cationic or zwitterionic side chains by ¹ H-NMR, SEC, and yields	70
Table 3.2. Antimicrobial evaluation of the caffeine copolymers	72
Table 3.3. Antimicrobial screening of 4-(methyl/allyl/benzyl)oxy, 6-(H/alkyl)-2-hydroxybenzoates	77
Table 3.4. Characterization of functional hydroxybenzoate-co-lactide polymers with different M/I.....	81
Table 3.5. Antimicrobial activity on <i>S. aureus</i> of polymers M/I = 5 – 30	82
Table 3.6. Minimum Inhibitory Concentration (MIC) of synthetic polyK with various degrees of polymerization (DP).....	86

Chapter 4

Table 4.1. Synthetic PKAP phage conjugates and complexes prepared from M13 ^α PNAG and three polyK polymers with zeta potentials (ζ)	158
Table 4.2. PKAP phage composition	161
Table 4.3. Minimum inhibitory concentration (MIC) of polyK and PKAP phages.....	163

List of Supplementary Tables

Chapter 2

Table S.2.1. Fitting parameters of binding curves	31
--	----

Chapter 3

Table S3.1. Characterization of the copolymers bearing caffeine and cationic or zwitterionic side chains	146
Table S3.2. Evaluation of the conditions during ring opening polymerization of monomer 13	147
Table S3.3. Characterization of polymers from ROP of monomer 13.....	147
Table S3.4. Dynamic light scattering (DLS) results of the polymers	148
Table S3.5. Minimum Bactericidal Concentration (MBC) of polyK of various degrees of polymerization (DP).....	148

Chapter 4

Table S4.1. Reaction of phage with Bradford reagent	193
Table S4.2. PolyK concentration on PKAP phages measured by Bradford reagent.....	195
Table S4.3. Reaction of PKAP phages with OPA reagent.....	196
Table S4.4. Phage amino acid analysis	197
Table S4.5. Minimum inhibitory concentration (MIC) of polyK and PKAP phages for <i>E. coli</i> and <i>P. aeruginosa</i> determined by broth microdilution.	198
Table S4.6. Minimum bactericidal concentration (MBC) of polyK and PKAP phages	198
Table S4.7. Time-kill curves of polyK ₉₀ , PKAP _{90C} , and PKAP _{90E} fit to an exponential death model.....	199

Table S4.8. Release curves of polyK ₉₀ from PKAP _{90E} with varying concentrations of dextran sulfate fit to a pseudo-first order exponential decay model.....	199
Table S4.9. Cross-evaluation of MIC after thirteen rounds of selection.....	200
Table S4.10. Mutations identified by snippy	202

List of Schemes

Chapter 1

Scheme 1.1. Engineering M13 bacteriophage as an antimicrobial polymer delivery platform ... 14

Chapter 3

Scheme 3.1. General strategy to prepare methacrylate copolymers with caffeine and cationic or zwitterionic side chains..... 34

Scheme 3.2. Polymeric antimicrobial agents against *S. aureus* inspired by depsides..... 36

Scheme 3.3. Synthesis of α -poly(L-lysine)-NH₂ by NCA polymerization 37

Scheme 3.4. General scheme of semi-aromatic lactide monomers 49

Scheme 3.5. Preparation of 2-hydroxy-4-methoxybenzoic acid, “Compound 3” 50

Scheme 3.6. Ring-opening polymerization of the monomers using 3-O urea catalyst, MTBD and 3-MeBuOH as initiator. 56

Scheme 3.7. Preparation of lactide monomers (compounds 11–15) from 4-(methyl/allyl/benzyl)oxy-6-(H/alkyl)-2-hydroxybenzoates..... 78

Acknowledgments

The work presented in this dissertation was made possible by the contributions of my lab mates, collaborators, facilities, and funding agencies. BioRender was used to design figures in Chapter 1. Chapters 2 through 4 are adapted from the work of Vexler, S.; Morrison, C.; Jain, S.; Yang, Y.; Tsunemoto, H.; Zhang, N.; McCarthy, K.; Mihalache, B.; Kutach, M.; Yang, S.; Pogliano, J.; Annabi, N.; Deming, T.J.; Chen, I.A. “Phage-Polymer conjugates inhibit *S. aureus* with high cytocompatibility and low bacterial resistance.”, which is currently under review. The clinical isolates used in Chapters 2 through 4 were collected and characterized by Shangxin (Shaun) Yang.

In Chapter 2, I prepared the anti-PNAG phage, with guidance from Yanxi (Steven) Yang on plasmid design, and carried out the polysaccharide ELISA. The whole-cell ELISA protocol described here was designed by me and originally published in the work by Yang, Y.; Vexler, S.; Jordan, M.C.; Abbondante, S.; Kang, D.; Peng, H.; Marshall, M.; Naini, B.V.; Jain, S.; Lai, Y.; Annabi, N.; Roos, K.P.; Chen, I.A. “Phage-peptide conjugates as potent, broad-range antibacterial agents for gram-negative infections.” *ACS Central Science* 2025.

<https://doi.org/10.1021/acscentsci.5c00562>. Beatrice Mihalache assisted with the whole-cell ELISAs. Nick Zhang ran the whole-cell ELISA for eukaryotic cells. Katherine McCarthy conducted the SYBR-Gold binding assay. Glycan microarrays were provided by the Protein-Glycan Interaction Resource of the CFG and the National Center for Functional Glycomics (NCFG) at Beth Israel Deaconess Medical Center, Harvard Medical School (supporting grant R24GM137763).

Chapter 3 contains the work of Salas-Ambrosio, P.; Vexler, S.; P S, R.; Chen, I. A.; Maynard, H. D. “Caffeine and Cationic Copolymers with Antimicrobial Properties.” *ACS Bio Med Chem Au* 2023. <https://doi.org/10.1021/acsbiochemau.2c00077> and Salas-Ambrosio, P.;

Vexler, S.; P Sivasankaran, R.; Vlahakis, N.; Lai, R.; Johnson, C.; Baas-Maynard, S.; Min, D.; Lower, H.; Doyle, A.; Tang, Y.; Rodriguez, J.; Chen, I.; Read de Alaniz, J.; Maynard, H.

“Biosourced Functional Hydroxybenzoate-co-Lactide Polymers with Antimicrobial Activity.”

Journal of the American Chemical Society 2025. <https://doi.org/10.1021/jacs.5c04624>. I

performed all the MIC and MBC assays. Pedro Ambrosio Salas synthesized the methacrylate and hydroxybenzoate-co-lactide polymers. Colin Johnson prepared the bio-sourced monomers. Casey Morrison synthesized polyK and Jeremy Pietropaolo assisted with acquiring the NMR spectra. I assisted Rajalakshmi P. Sivasankaran with the biofilm assays.

In Chapter 4, polyK was synthesized by Casey Morrison. Marllys Kutach assisted with PKAP phage quantification. The assay for virion quantification by Qubit Fluorometer was designed by me and originally described in the work by Peng, H.; Vexler, S.; Xu, S.; Chen, I. A. “Real-time in vivo bacterial imaging by CT and fluorescence using phage-gold nanorod bioconjugates as contrast agents.” *ACS Biomaterials Science & Engineering* 2025.

<https://doi.org/10.1021/acsbiomaterials.4c02190>. Amino acid analysis was carried out by John M. Schulze at the UC Davis Molecular Structure Facility. Fluorescence microscopy of *S. aureus* was performed by Hannah Tsunemoto. Yanxi (Steven) Yang provided guidance on electron microscopy. Transmission Electron Microscopy data was acquired at the Electron Imaging Center for Nanosystems (EICN) at the University of California, Los Angeles’s California NanoSystems Institute (CNSI) (RRID:SCR_022900). Saumya Jain performed mammalian cell assays with my assistance. All other experiments were performed by me.

This work was funded by National Institute of General Medical Sciences of the National Institutes of Health under Award Numbers R35GM148249 and T32GM145388, and the National Science Foundation BioPACIFIC Materials Innovation Platform (DMR-1933487). I would

especially like to thank the Camille Dreyfus Teacher-Scholar Program for a Supplemental Grant, which enabled the completion of my dissertation research.

Vita

Shelby received her Bachelor of Science in Biochemistry-Molecular Biology from the University of California, Santa Barbara. At UCSB, she conducted research under Professor Deborah Fygenon in the Physics Department, using DNA origami nanostructures to study the bending dynamics of DNA. She also spent a summer at the University of California, Los Angeles as an Amgen Scholar in the lab of Professor Douglas Black researching pre-mRNA splicing. After graduating in 2020, she continued her work in Dr. Fygenon's lab, using M13 bacteriophage to produce DNA for nanostructures.

In 2021, she returned to UCLA to pursue her PhD in Chemistry under Professor Irene Chen's, where she continued working with M13 phage. Shelby's accomplishments during her doctoral studies are as follows:

Publications

- **Vexler, S.**; Morrison, C.; Jain, S.; Yang, Y.; Tsunemoto, H.; Zhang, N.; McCarthy, K.; Mihalache, B.; Kutach, M.; Yang, S.; Pogliano, J.; Annabi, N.; Deming, T.J.; Chen, I.A. Phage-Polymer conjugates inhibit *S. aureus* with high cytocompatibility and low bacterial resistance. (under review)
- Zhang, N.; Kutach, M.; Hsieh, L.; **Vexler, S.**; Chen, I.A. Halictine-II as a potential broad-spectrum antifungal and antibacterial agent. (submitted)
- Yang, Y.; Kang, D.; Mihalache, B.; **Vexler, S.**; Jain, S.; Peng, H.; Annabi, N.; Yang, S.; Chen, I.A. Metagenome-inspired libraries to engineer phage M13 for targeting killing of gram-negative bacterial species. *Nucleic Acids Research* **2025**. <https://doi.org/10.1093/nar/gkaf984>
- Yang, Y.; **Vexler, S.**; Jordan, M.C.; Abbondante, S.; Kang, D.; Peng, H.; Marshall, M.; Naini, B.V.; Jain, S.; Lai, Y.; Annabi, N.; Roos, K.P.; Chen, I.A. Phage-peptide conjugates as potent, broad-range antibacterial agents for gram-negative infections. *ACS Central Science* **2025**. <https://doi.org/10.1021/acscentsci.5c00562>
- Salas-Ambrosio, P.; **Vexler, S.**; P Sivasankaran, R.; Vlahakis, N.; Lai, R.; Johnson, C.; Baas-Maynard, S.; Min, D.; Lower, H.; Doyle, A.; Tang, Y.; Rodriguez, J.; Chen, I.; Read de Alaniz,

J.; Maynard, H. Biosourced Functional Hydroxybenzoate-co-Lactide Polymers with Antimicrobial Activity. *Journal of the American Chemical Society* **2025**.

<https://doi.org/10.1021/jacs.5c04624>

- Peng, H.; **Vexler, S.**; Xu, S.; Chen, I. A. Real-time in vivo bacterial imaging by CT and fluorescence using phage-gold nanorod bioconjugates as contrast agents. *ACS Biomaterials Science & Engineering* **2025**. <https://doi.org/10.1021/acsbiomaterials.4c02190>
- Salas-Ambrosio, P.; **Vexler, S.**; P S, R.; Chen, I. A.; Maynard, H. D. Caffeine and Cationic Copolymers with Antimicrobial Properties. *ACS Bio Med Chem Au* **2023**. <https://doi.org/10.1021/acsbiomedchemau.2c00077>.

Fellowships and Awards:

- Herbert Newby McCoy Commencement Award (2026)
- Herbert Newby McCoy Mentorship Award (2025)
- UCLA Research Showcase Fellowship Award (2025)
- 3rd Place in UCLA Grad SLAM Competition (2024)
- BioPACIFIC MIP Fellow (2022 – 2025)
- NIH T32 Cellular and Molecular Biology Trainee, UCLA (2022 – 2023)

Presentations:

- “M13 Bacteriophage as an Antimicrobial Polymer Delivery Platform”, Talk at UCLA’s Department of Chemical and Biomolecular Engineering Fourth-Year Graduate Student Symposium (2025)
- “Antimicrobial Polylysine-Phage Conjugates for Improved Biocompatibility”, Poster at ACS Spring (2025), Seaborg Symposium Poster Session (2025), and Biophysical Society 69th Annual Meeting (2025)
- “Phage Therapy Targeting Bacterial Polysaccharide Poly-*N*-acetylglucosamine”, Talk at UC Chemical Symposium (2024)
- “From Foe to Friend: Viruses that Fight Bacteria”, Talk at UCLA Grad SLAM Finals (2024)
- “Phage Therapy Targeting Biofilm Polysaccharide Poly-*N*-acetylglucosamine”, Poster at Seaborg Symposium Poster Session (2022)
- “Behind the Lab Door”, Talk at Bruin Engineers Reunion (2022)

Chapter 1. Introduction

1.1 Antibiotic resistance and pathogenic *Staphylococcus aureus*

Prior to the widespread use of antibiotics, bacterial infections posed a substantial threat to human health; in 1900, pneumonia and tuberculosis were the leading cause of death in the United States.¹ Alexander Fleming's discovery of penicillin in 1928,² along with Howard Florey's and Ernst Chain's advancements in purification,³ ushered in a new era of public health.⁴ Their discoveries, which shared the 1945 Nobel Prize in Physiology or Medicine,⁵ reduced deaths from infections from 12% during World War I to 3% during World War II, saving thousands of lives.⁶ This also marked the start of the Golden Age of Antibiotic Discovery, with roughly half of all antibiotics currently in use discovered before the end of this period in 1970.⁷ Despite these advancements, the first instance of penicillin resistant *Staphylococcus aureus* was documented as early as 1942.⁸ Antimicrobial resistance (AMR) has since become widespread, with one out of every six laboratory-confirmed bacterial infections presenting antibiotic resistance in 2021.⁹ In the same year, 1.27 million deaths were directly caused by bacterial AMR, with the global annual mortality is forecasted to reach roughly 10 million by 2050 (**Figure 1.1**).^{10,11}

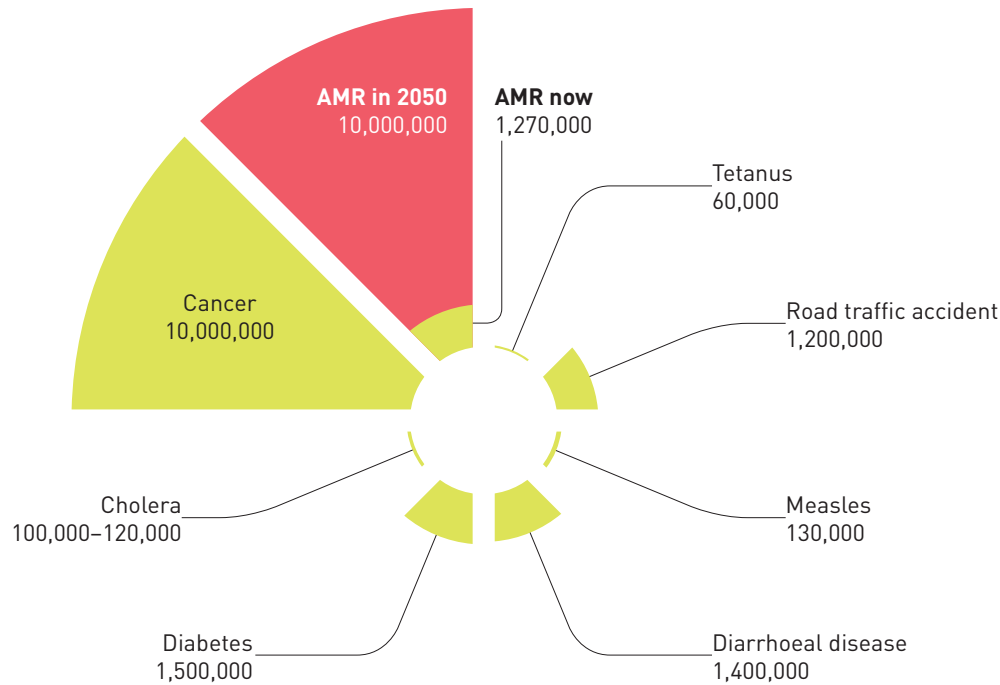


Figure 1.1. Predicted mortality from AMR compared with current common causes of death. By 2050, the number of deaths resulting from AMR are expected to be equivalent to those caused cancer, the current leading cause of death. This figure is adapted from published work.¹¹

While AMR is found across the bacterial tree of life, *S. aureus* is one of the most notorious pathogens, with 27.1% of the species exhibiting methicillin-resistance in 2023.⁹ Resistance to this antibiotic is typically acquired by horizontal gene transfer of the *mecA* gene, which encodes for a penicillin-binding (PBP2a) with low affinity for β -lactams.¹² Currently, methicillin-resistant *S. aureus* (MRSA) is classified as a serious threat by the Centers for Disease Controls and Prevention.¹³ It is one of the main species responsible for healthcare associated infections (HAI)¹⁴ and resulted in an estimated 1.7 billion dollars in attributable healthcare costs in 2017.¹³

S. aureus is readily spread in hospitals and communities by skin-to-skin contact; it is commensal on the skin and mucous membranes, like other members of the *Staphylococcus* genus, with upwards of 20% of the human population acting as persistent carriers.¹⁵ However, distinct structural and biochemical features contribute to greater virulence than other

staphylococci.¹⁶ The genetic plasticity of *S. aureus* readily facilitates its acquisition of resistance,^{17,18} leading to its designation as an “ESKAPE” pathogen, which refers to microbes that frequently “escape” the mechanism of antibiotics.¹⁹ Notably, this group is largely comprised of gram-negative bacteria (*Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter spp*) with *Enterococcus faecium* joining *S. aureus* as the only other gram positive organism. Additionally, *S. aureus* possesses an arsenal of traits to evade the immune system, further complicating treatment.¹⁶ This includes secreting an impressive array of virulence factors such as protein A, which binds to antibodies, and α -toxin, which forms pores in the membranes of host cells.²⁰ The formation of biofilms facilitates the colonization of medical devices, such as catheters, while forming a protective shield from phagocytes and antibiotics.²¹ *S. aureus* can also switch to alternate phenotype, the small colony variant (SCV), characterized by slow growth and reduced metabolism. This leads to a reduced uptake of certain antibiotics, like aminoglycosides, and limits the effectiveness for those that target the cell wall of rapidly dividing bacteria, like β -lactams.²² Both SCV and wild-type *S. aureus* can further evade the immune system by internalizing in host cells.²⁰ This intrinsic virulence, combined with antibiotic resistance, establishes *S. aureus* as a particularly challenging pathogen to treat.

Currently, the gold standard in treating severe MRSA infections is intravenous vancomycin or daptomycin, but mortality for MRSA bacteremia remains between 20 and 30%.²³ Although full resistance is still rare, with only 16 cases of vancomycin resistance identified in the US,²⁴ levels of intermediate and heterogenous resistance are increasing.²⁵ Despite a wider range of treatment options for soft skin and tissue infections (SSTIs), recurrence is common, with upwards of 70% of patients experiencing a second SSTI within one year of their first treatment.²⁶ Prolonged use of antibiotics when treating chronic MRSA substantially increases the risk of

generating resistance.²⁷ This gap in effective treatments highlights the need for alternatives with lower potentials for generating resistance, such as phage therapy and antimicrobial polymers, which are discussed in the following sections.

1.2 Phage therapy

Bacteriophages, or phages, are viruses specific to bacteria. Although they exist in a state of “almost life”, as they cannot survive without a host, they are the most abundant organism on the planet.²⁸ They display incredibly diverse morphology (**Figure 1.2A**), with genomes of single or double stranded DNA or RNA ranging from 3.4 kb to 500 kb packaged inside a protein capsid.²⁹ The capsid structure can generally be categorized as icosahedral, filamentous, or tailed.³⁰ In addition to their capsid proteins, phages possess a receptor binding protein (RBP) that is used to bind to the host and initiate infection; the RBP is therefore the primary determinant of the host range.³¹ The replication cycle that follows is usually categorized as lysogenic, where the viral genome is maintained inside the bacterium, or lytic, where the release of progeny phage damages the host cell’s membrane, resulting in death (**Figure 1.2B**).³² Lytic phages are therefore an attractive antimicrobial being bactericidal, self-amplifying, and highly specific, which minimizes the threat to beneficial microbes in comparison to traditional antibiotics.³³

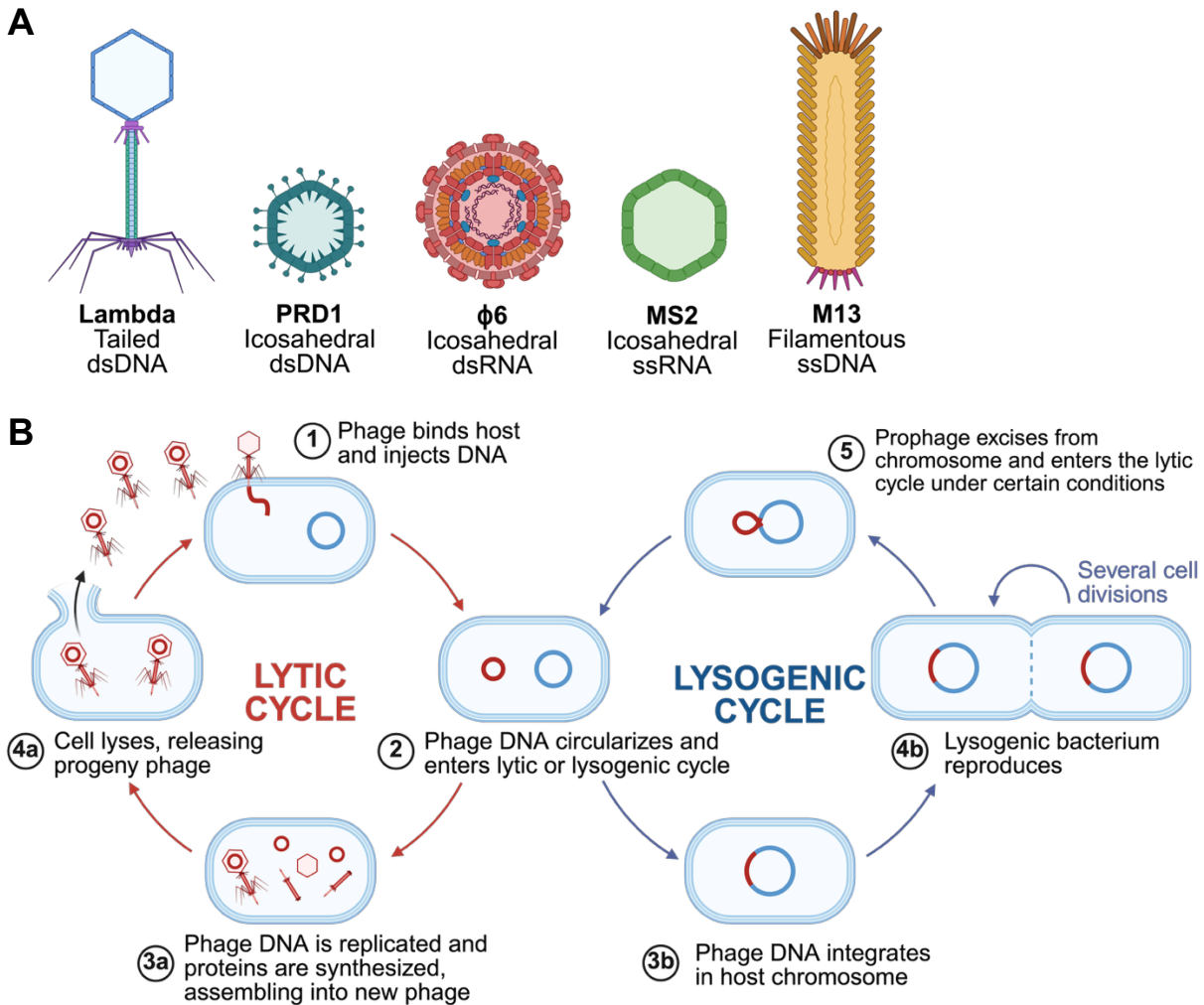


Figure 1.2. Phage morphology and life cycles. A) Examples of phages classified by structure and genome. B) Lytic and lysogenic replication cycles.

Felix d’Herelle is widely considered to be the father of phage therapy, having coined the term “bacteriophage”, meaning “bacteria eater”, in his 1917 paper.³⁴ In 1919, he conducted the first clinical use of phages in attempt to treat dysentery.³⁵ Until the 1940s, phage therapy was pursued globally, but the introduction of antibiotics halted most research in the United States,³⁵ as the broad spectrum of action and easier storage made antibiotics a more attractive treatment than phages. Phage research continued elsewhere, although most extensively in the Soviet Union,

where it went largely unrecognized by western nations.³⁶ But, the emergence of AMR has led to a re-evaluation of phage therapy in the United States.

In 2016, the successful treatment of a multi-drug resistant *Acinetobacter baumannii* infection with phages³⁷ attracted widespread attention, leading to the creation of the first official center for phage therapy in North America, Innovative Phage Applications and Therapeutics (IPATH) at the University of California, San Diego.³⁸ In the following two year period, IPATH successfully treated an additional 17 patients.³⁹ However, less desirable outcomes illustrate the shortcomings of personalized phage therapy resulting from a narrow host range; out of 99 cases where a phage hunt was initiated, 10 patients died before an appropriate phage was found, and an additional 6 died before treatment commenced (**Figure 1.3**).³⁹ To accelerate identifying an appropriate phage in critical cases, much effort has been devoted to creating banks of characterized phages.⁴⁰ An alternative approach is to bypass the screening process altogether and administer a premixed “cocktail” of phages to maximize the chance of success; a phage cocktail also reduces the likelihood of bacteria evolving resistance to a single phage.⁴⁰ However, both personalized and pre-mixed cocktail approaches face regulatory barriers, challenges in manufacturing, and additional concerns with safety.⁴¹

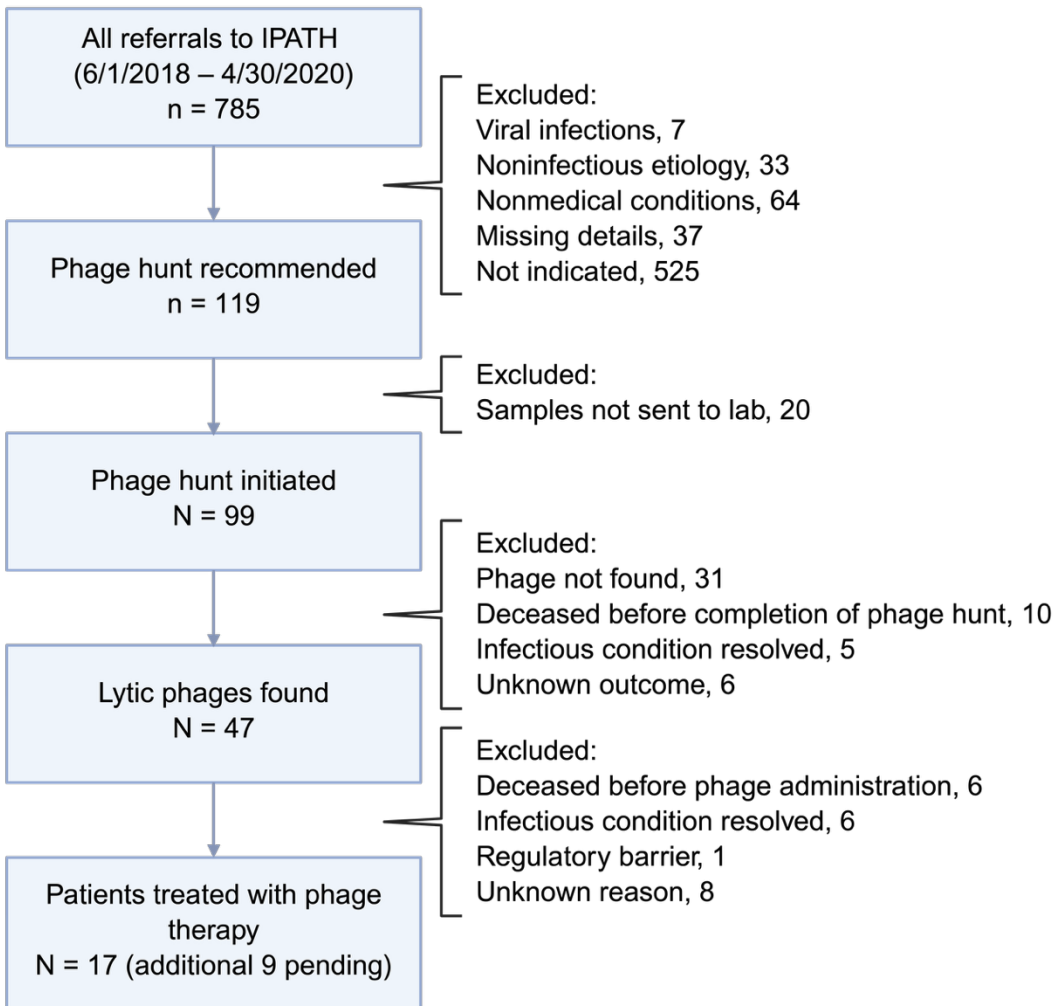


Figure 1.3. Flowchart depicting outcome of all bacteriophage therapy requests at the Center for Innovative Phage Applications and Therapeutics (IPATH). This figure is adapted from published work.³⁹

Much effort has been devoted to expanding the host range of bacteriophages. One approach is “phage training”, where multiple rounds of selection drive evolution towards the desired host range.⁴² However, this protocol is time consuming and may result in a diverse population of phages, rather than a single genotype, complicating isolation and production;⁴² additionally, the host range is subject to instability.⁴³ Alternatively, phages can be genetically engineered through a variety of methods, such as homologous recombination, CRISPR-Cas9, or cell-free

transcription-translation systems.⁴⁴ These techniques can be used to generate a library of phages with varying RBPs and identify viable phages by selection, or to rationally design RBPs based on previous knowledge.⁴⁵ Genetic engineering, however, is not applicable to all phages, and success varies with the technique and species used.^{44,46} Additionally, certain genetic modifications can result in unintended consequences for phage infectivity or development.⁴⁶ Despite their potential for increasing the host range, modified phages face many of the same regulatory barriers and concerns as their wild-type counter parts.

1.3 Antimicrobial polymers

As the Golden Age of Antibiotics drew to an end, Cornell and Donaruma reported the production of the first synthetic antimicrobial polymer based on 2-methacryloxytropone in 1965.⁴⁷ As interest in this synthetic class of macromolecules grew, scientists also investigated the antimicrobial properties of naturally occurring polymers, such as chitosan, a polysaccharide produced by fungi,⁴⁸ and ϵ -poly-L-lysine, a homopoly(amino acid) produced by the bacterium *Streptomyces albulus*.⁴⁹ Synthetic and natural discovery research continued through the 21st century, leading to the incredibly large and chemically diverse class of macromolecules we now consider antimicrobial polymers.^{50,51}

Antimicrobial polymers can be classified as passive, where they prevent bacterial adhesion to a surface, or active, if they are biocidal.⁵² Active polymers fall into one of three categories (**Figure 1.4**), the first being polymeric biocides.⁵³ Here, monomers with intrinsic bioactivity, such as penicillin, are polymerized, although the polymer may exhibit reduced activity compared to the monomer due to steric hinderance.⁵⁴ The second category is biocidal polymers, where activity is derived from the entire macromolecule, and is greater than the

activity of any individual monomer.⁵² Biocidal polymers are plentiful in nature, including the aforementioned chitosan⁵⁵ and ϵ -poly-L-lysine.⁵⁶ Despite their small size and chemical diversity, antimicrobial peptides (AMPs), can technically be classified as biocidal polymers due to the repeated architecture of their polypeptide backbones.^{57,58} The final category is biocide-releasing polymers, where an inert polymer either releases biocidal molecules or degrades into active monomers.⁵³

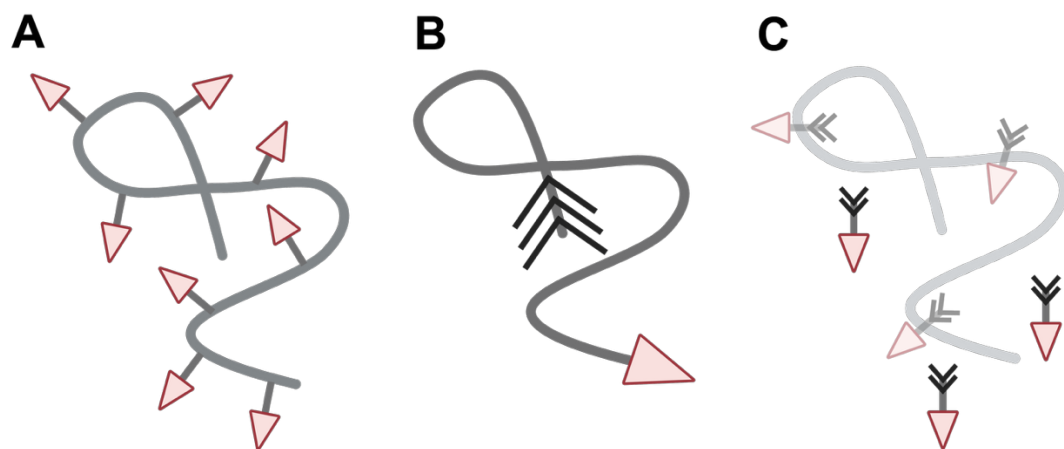


Figure 1.4. General working principles of antimicrobial polymers. Antimicrobial polymers can be categorized as A) polymeric biocides, where a polymer is comprised of bioactive units, B) biocidal polymers, where antimicrobial activity is derived from the entirety of the macromolecule, or C) biocide-releasing polymers, where a polymer with no intrinsic activity releases active units.

Antimicrobial polymers vary widely in their biocidal mechanism, with some specifically designated to act on multiple pathways, but the most prevalent mode of action is membrane disruption.^{59,60} The specific mode of membrane disruption depends on the chemical composition of the polymer, but in general, cationic residues on the polymer facilitate attraction to the negatively charged bacterial membrane. If the polymer possesses hydrophobic residues, this region will insert into the lipid bilayer, leading to the formation of holes.⁵⁸ If the polymer lacks

hydrophobic residues, electrostatic interactions cause the polymer to coat the membrane like a “carpet” and upon reaching a critical concentration, induce membrane distortion.⁵⁹

Other mechanisms include targeting intracellular macromolecules, such as ribosomes or DNA, or inhibiting cell wall synthesis.⁵⁹ In general, this action is more common for AMPs than synthetic polymers, but many cationic polymers have been documented to bind DNA. For example, polyhexamethylene biguanide (PHMB) interferes with cell division by condensing DNA,⁶¹ while poly(2-oxazoline) induces the production of Reactive Oxygen Species (ROS) by binding to DNA.⁶² Using a polycondensation of *p*-ethylphthalimide and 1,4-bis(3-aminopropyl)piperazine, Bai et al. synthesized polymers capable of disrupting bacterial membranes and binding DNA.⁶³ Additionally, antimicrobial polymers can also disrupt the electron transport chain (ETC).⁶⁰ The interaction of poly(guanyurea)–piperazine with the mycobacterial membrane results in a dissipation of membrane potential, ultimately leading to the collapse of the proton motive force, a drop in ATP levels, and cell death.⁶⁴ Some synthetic polymers, but mostly AMPs, exhibit a secondary mechanism by acting as immunomodulators.^{59,60} Notably, Lam et al. synthesized star-shaped peptide polymer nanoparticles consisting of lysine and valine residues capable of both membrane disruption and neutrophil recruitment.⁶⁵ However, membrane disruption has two key advantages compared to other mechanisms: first, it is challenging to renew a damaged membrane, and second, it occurs through a non-receptor-mediated pathway, minimizing the chance of bacteria developing resistance.^{59,60} Previous works have demonstrated that even after exposing bacteria to antimicrobial polymers for 274 generations, the minimum concentration necessary to prevent bacterial growth did not increase, and therefore resistance did not develop.⁶³

However, many membrane-active polymers display a high affinity for mammalian cells in addition to bacterial cells, resulting in poor biocompatibility.⁶⁰ Furthermore, many synthetic polymers are not biodegradable, resulting in cytotoxic accumulation, even if they are selective.⁶⁶ The only antimicrobial polymers with membrane-activity approved by the FDA for systemic administration are in fact, AMPs.⁶⁷ Much effort has been devoted to designing synthetic polymers with higher selectivity for bacterial cells and biodegradable properties,^{60,68} often inspired by AMPs.⁶⁹ Currently, the use of synthetic antimicrobial polymers is restricted to coatings on medical devices and implants⁵⁹ or as a part of wound dressings⁶⁶, rather than for systemic treatment of infections.

1.4 M13 phage as a polymer delivery platform

Phage therapy⁷⁰ and antimicrobial polymers⁷¹ represent two potential solutions to the AMR crisis due to their lower potential for inducing resistance. However, they face opposite drawbacks, as phages tend to be highly specific and are thus restricted to an overly narrow host range,⁷² while polymers generally lack specificity for bacterial cells over mammalian cells, resulting in poor biocompatibility.⁷³ Cocktails of polymers mixed with phages or phage endolysins have been explored as a strategy to improve antibacterial efficacy,⁷⁴⁻⁷⁶ but this strategy does not fundamentally alter the respective individual shortcomings. To circumvent this, we pursued a synergistic approach by engineering the non-lytic M13 bacteriophage into a delivery platform for antimicrobial polymers.

M13 bacteriophage is a well-studied, non-lytic filamentous phage.²⁹ It consists of a single stranded DNA genome packaged in a protein capsid comprised of one major coat protein and four minor coat proteins (**Figure 1.5**).⁷⁷ Of the minor coat proteins, the most significant is the

receptor binding protein, g3p, which binds the F pilus of *Escherichia coli* and initiates a chronic infection.⁷⁸ An important determinant of specificity, the g3p of M13 is highly amenable to modification; using the technology of “phage display”, which won the Nobel Prize in 2018,⁷⁹ peptide fragments can be expressed on the N-terminal of g3p enabling M13 to bind new targets.⁸⁰ M13 displays five copies of g3p, while the remaining bulk of the capsid is comprised of approximately 2,700 copies of the major coat protein, g8p.⁸¹ The solvent-accessible functional groups on the g8p proteins therefore provide thousands of potential sites for the bioconjugation of antimicrobial cargo.⁸²

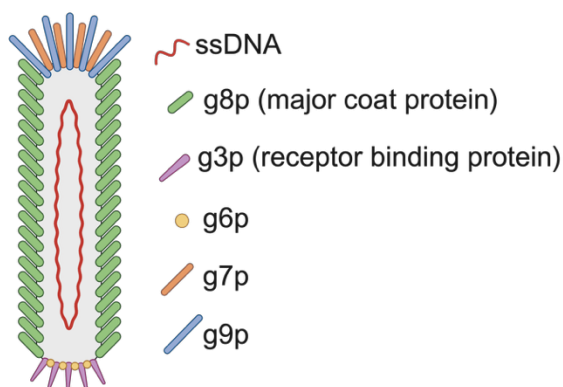
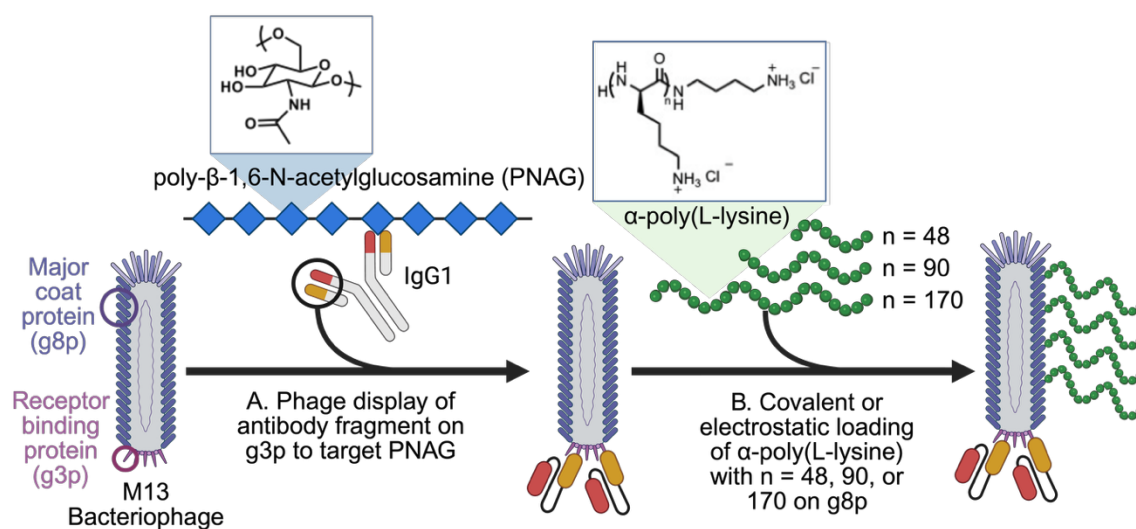


Figure 1.5. Structure of M13 bacteriophage. The single stranded DNA genome of M13 is packaged inside a capsid consisting of four different minor coat proteins (g3p, g6p, g7p, g9p) and one major coat protein (g8p).

Previous works in our group have explored engineering M13 into a drug delivery vehicle through modifications to g3p and g8p.⁴⁵ Peng et al. expressed receptor binding proteins from other phages on the M13 g3p to target other gram-negative species, such as *Pseudomonas aeruginosa* or *Vibrio cholera*, then loaded the major coat protein with gold nanorods, creating “phage gold nanorods”;⁸³ when exposed to near infrared light, the excited gold nanorods release energy as heat, effectively killing any bacteria bound by the phanorod. Using this approach, they

successfully treated drug-resistant *P. aeruginosa* wounds in a mouse model.⁸⁴ Yang et al. expressed an anti-lipopolysaccharide antibody fragment on g3p to target a broader range of gram-negative bacteria, then crosslinked a membrane-disruptive antimicrobial lipopeptide, polymyxin B, to the phage.⁵⁸ They successfully treated *P. aeruginosa* keratitis and multi-drug resistant lung infections in a mouse model, with phage bioconjugates inducing substantially less toxicity than the free peptide. Additionally, Yang et al. screened a library of inovirus receptor binding proteins to target *P. aeruginosa*, then cross-linked a similar peptide, colistin, to the phage, finding phage-based delivery reduced the necessary dosage of peptide in vitro treatment.⁸⁵

Building on these advances, this dissertation aims to modify M13 phage into a polymer delivery platform to successfully target the gram-positive pathogen *S. aureus* (**Scheme 1.1**). First, an antibody fragment specific to a conserved surface polysaccharide, poly-*N*-acetylglucosamine, was expressed on the receptor-binding protein of M13, then binding to *S. aureus* was confirmed. Second, three libraries of bio-inspired polymers were screened for antimicrobial activity, identifying the cationic amino acid homopolymer α -poly(L-lysine) as the best candidate for phage-bioconjugation. Lastly, polylysines varying by degrees of polymerization (48, 90, or 170) were loaded onto the recombinant phage through electrostatic interactions or EDC crosslinking; the impact of degrees of polymerization and method of loading on the physiochemical properties, antimicrobial activity, and biocompatibility of the phage bioconjugates was investigated.



Scheme 1.1. Engineering M13 bacteriophage as an antimicrobial polymer delivery platform. A) The receptor-binding protein g3p was genetically engineered to display an scFv (red and orange) that binds to the common *S. aureus* surface polysaccharide poly-β-(1,6)-N-acetylglucosamine (PNAG). B) After screening bioinspired polymers for antimicrobial activity, α-poly(L-lysine) was attached to the major coat protein (g8p) (green), resulting in phage conjugates or complexes.

Chapter 2. A poly-*N*-acetylglucosamine binding phage targets *Staphylococcus aureus*

Adapted from:

- **Vexler, S.**; Morrison, C.; Jain, S.; Yang, Y.; Tsunemoto, H.; Zhang, N.; McCarthy, K.; Mihalache, B.; Kutach, M.; Yang, S.; Pogliano, J.; Annabi, N.; Deming, T.J.; Chen, I.A. Phage-Polymer conjugates inhibit *S. aureus* with high cytocompatibility and low bacterial resistance. (under review)
- Yang, Y.; **Vexler, S.**; Jordan, M.C.; Abbondante, S.; Kang, D.; Peng, H.; Marshall, M.; Naini, B.V.; Jain, S.; Lai, Y.; Annabi, N.; Roos, K.P.; Chen, I.A. Phage-peptide conjugates as potent, broad-range antibacterial agents for gram-negative infections. *ACS Central Science* 2025. <https://doi.org/10.1021/acscentsci.5c00562>

The well-studied, filamentous M13 phage (order *Inoviridae*) normally infects F⁺ strains of *Escherichia coli*. The receptor binding protein, or g3p, binds to the F pilus of *E. coli*, initiating a chronic infection that decreases the fitness of the host strain.^{78,86,87} While the receptor-binding protein is an important determinant of specificity, the N-terminal domain of g3p is amenable to the display of receptor-binding domains from other filamentous phages or antibody fragments to alter binding specificity.⁸⁰ In addition to its utility in phage display technology, this approach has been used to engineer M13 to bind *Pseudomonas aeruginosa* and other gram-negative organisms and deliver antimicrobial cargo;^{85,88,89} notably, M13 has not been previously engineered to target gram-positive organisms. Additionally, the host range of *Inoviridae* phages is restricted to gram-negative organisms, due to the absence of appropriate receptors and particularly secretion

systems, including the TolQRA system, in gram-positive organisms like *Staphylococcus aureus*.^{90,91}

The surface polysaccharide poly-*N*-acetylglucosamine (PNAG) is common across *S. aureus* and is actively being investigated as a promising vaccine candidate, making PNAG an ideal antigen to target.⁹² Additionally, PNAG is also found in the biofilms of several other bacterial pathogens, but is crucially absent from mammalian cells. Of the many antibodies documented to bind PNAG and provide protective activity against *S. aureus*, the human IgG1 mAb (F598) is notable as it can bind the surface associated deacetylated form (dPNAG) with high affinity, in addition to the secreted, acetylated form.^{93–95} To target *S. aureus*, the g3p of M13 was fused to a short-chain variable fragment (scFv) of F598 and the binding affinity of the resulting anti-PNAG phage was characterized primarily by enzyme-linked immunosorbent assays (ELISAs).

2.1 Methodology

Anti-PNAG phagemid design. The published amino acid sequences^{92,94,95} of the variable light (V_L) and heavy (V_H) regions of mAb F598 were reverse-translated into DNA and codon-optimized for expression in *E. coli*. Two single-chain constructs were designed with the order V_L -linker- V_H or V_H -linker- V_L (**Figure S2.1**), where the linker had sequence (GGGGS)₃. Gene fragments for the two scFv constructs were synthesized by Twist Biosciences, amplified by PCR, run on a 1% agarose gel, and recovered using a Zymoclean™ Gel DNA Recovery Kit. Phagemid vector pADL-10bS239T was grown in *E. coli* DH5 α and purified with a ZymoPURE™ Plasmid Miniprep Kit.⁸⁸ Phagemid and inserts were digested with NotI-HF and AgeI-HF restriction enzymes (NEB), run on a 1% agarose gel, and purified using a Zymoclean™ Gel DNA Recovery

Kit. Digested inserts were ligated into digested phagemid using ElectroLigase® (NEB), then transformed into Mix & Go! Competent Cells-DH5 Alpha (Zymo Research). Single colonies were selected and grown in LB media with ampicillin, and phagemid was isolated with a ZymoPURE™ Plasmid Miniprep Kit. The purified phagemid vectors were sequenced to confirm successful cloning of pADL-10bS239T-scFv598-V_H-L-V_L and pADL-10bS239T-scFv598-V_L-L-V_H (L = linker).

E. coli TG1 cells were made chemically competent with a 100 mM CaCl₂ solution and transformed with either pADL-10bS239T-scFv598-V_H-L-V_L or pADL-10bS239T-scFv598-V_L-L-V_H plasmids, for production of M13^{aPNAG(HL)} or M13^{aPNAG(LH)} phage, respectively.

Recombinant phage production. 9 mL of 2xYT media containing 40% w/v glucose and 100 µg/mL ampicillin was inoculated with *E. coli* transformed by the phagemid construct and then incubated overnight at 37 °C with shaking at 225 rpm. The following day, 8 mL of overnight culture was diluted in 152 mL fresh 2xYT with no supplements and incubated under the same conditions as before. When the OD₆₀₀ reached approximately 0.5, cells were transduced with 160 µL of CM13d3 pIII-Defective Helper Phage (Antibody Design Labs). After one hour, ampicillin, kanamycin, and IPTG were added to final concentrations of 100 µg/mL, 50 µg/mL, and 200 µM respectively. The culture was incubated at 30 °C and 225 rpm overnight.

Phage purification. To purify phage, cultures containing *E. coli* and phage were centrifuged at 7000 rcf for 10 minutes. Pellets were discarded and the supernatant was filtered through a 0.22 µm PES filter, then concentrated with using Amicon® Ultra Centrifugal Filters (50 kDa MWCO) to approximately 30 mL. 40 mg of PEG8000 and 30 mg NaCl were added per milliliter of

concentrated supernatant, and the suspension was incubated on ice for at least four hours to precipitate phages. The solution was centrifuged at 7000 rcf for 30 minutes to pellet phage. The supernatant was discarded, and phage pellets were resuspended in PBS. Phage concentration was quantified by measuring the absorbance at 269 nm and 320 nm, using the equation $\frac{\text{virions}}{\text{mL}} =$

$$\frac{(A_{269} - A_{320}) \times 6 \times 10^{16}}{\frac{\text{number of bases}}{\text{phage genome}}}$$

⁹⁶ Phage solutions were stored at 4°C.

Biofilm polysaccharide purification and ELISA. *S. aureus* (Newman, ATCC 25904) biofilm polysaccharides were purified based on a previously described protocol.⁹⁷ Glycerol stocks were streaked on tryptic soy agar and grown overnight at 37 °C. Tryptic soy broth (TSB, 5 mL) was inoculated with a single colony, and the culture was grown overnight at 37°C with shaking (225 rpm). The following day, overnight cultures were diluted 1:100 with fresh TSB and grown in petri dishes without shaking at 37 °C. After five days, biofilm sludge was removed from the petri dishes. To 10 mL of sludge, 60 µL of formaldehyde (36.5% solution) was added and incubated for one hour with shaking. 4 mL of 1 M NaOH was added and incubated for three hours with shaking, then centrifuged for thirty minutes at 7000 rcf. The supernatant was filtered with a 0.22 µm filter, then concentrated to 1 to 2 mL with a Centrifugal Ultrafiltration Device (3 kDa MWCO, Pall). 20% trichloroacetic acid was added to the concentrated supernatant, and the mixture was incubated on ice for thirty minutes, then centrifuged at 16800 rcf for one hour at 4 °C. 1.5 volumes of 95% ethanol were added to the supernatant containing the polysaccharides and incubated overnight at -20°C. The following day, the solution was centrifuged at 16800 rcf for one hour at 4 °C, and the polysaccharide pellet was resuspended in 0.1 M NaHCO₃, pH 8.6. Polysaccharide content was quantified by the phenol-sulfuric acid method.⁹⁸

For ELISA, 200 μ L of purified polysaccharides or blocking buffer (5 mg/mL BSA, 0.1 M NaHCO_3 , pH 8.6) per well were added to 96-well flat bottom Nunc plates with MultiSorp coating (ThermoFisher) and incubated overnight at 4 $^{\circ}\text{C}$. The following day, the solution was shaken out and replaced with 200 μ L of blocking buffer, then incubated for 2 hours at 4 $^{\circ}\text{C}$. Plates were washed thoroughly by pipetting 200 μ L of Tris-Buffered Saline with Tween 20 (TBST) into each well with a multi-channel pipette, shaking out excess solution, and slapping face down on a paper towel. This process was repeated six times. A series of dilutions of $\text{M13}^{\text{aPNAG(HL)}}$ or $\text{M13}^{\text{aPNAG(LH)}}$ phage, ranging from 10^8 to 10^{11} virions/mL, in PBS were prepared. 100 μ L of phage at the appropriate concentration was added to each well, then incubated for 2 hours at room temperature. Plates were washed again before adding 200 μ L of anti-M13-g8p-HRP antibody (Antibody Design Labs) at a concentration of 0.2 $\mu\text{g/mL}$ in PBS supplemented with 5 mg/mL BSA and incubated for 1 hour at room temperature. A final wash step was followed by adding 100 μ L of TMB substrate solution (ThermoFisher) and incubating for 15 minutes at room temperature. The reaction was stopped by adding 50 μ L of 2 N sulfuric acid and absorbance at 450 nm was read using a Tecan Infinite $\text{\textcircled{R}}$ M200 PRO.

Cell-Based Enzyme-Linked Immunosorbent Assay (ELISA) for Phage Binding. 5 mL cultures of the following strains were grown overnight in LB broth: *E. coli* strains DH5 α or ER2738 (New England Biolabs), *S. aureus* (Newman), *Staphylococcus epidermidis* (clinical isolate), and *P. aeruginosa* (ATCC 25102). Tetracycline added at 10 $\mu\text{g/mL}$ for ER2738. The following day, 1 mL of each culture was centrifuged for 5 minutes at 5000 rpm. The supernatant was discarded, and cells were resuspended in 1 mL of PBS. Cells were centrifuged under the same conditions and resuspended in fresh PBS. The OD600 of the cell resuspension was adjusted

to 1, and the cells were diluted 100-fold in PBS. 200 μ L of diluted cells or blocking buffer (5 mg/mL BSA, 0.1 M NaHCO₃, pH 8.6) per well were added to 96-well flat bottom Nunc plates with MultiSorp coating (ThermoFisher) and incubated overnight at 4 °C to allow cell attachment.

The following day, the solution was shaken out and replaced with 200 μ L of blocking buffer and then incubated for 2 hours at 4 °C. Plates were washed thoroughly by pipetting 200 μ L of Tris-Buffered Saline with Tween 20 (TBST) into each well with a multichannel pipet, shaking out excess solution, and slapping face down on a paper towel. This washing process was repeated six times. A series of phage dilutions from 5×10^{11} to 10^8 virions/mL in PBS were prepared. 100 μ L of phage at the appropriate concentration was added to each well, then incubated for 2 h at room temperature. Plates were washed again before adding 200 μ L of anti-M13-g8p-HRP antibody (Antibody Design Laboratories) at a concentration of 0.2 μ g/mL in PBS supplemented with 5 mg/mL BSA and incubated for 1 hour at room temperature. Plates were washed again before adding 100 μ L of TMB substrate solution (ThermoFisher) and incubating for 15 minutes at room temperature. The reaction was stopped by adding 50 μ L of 2 N sulfuric acid, and absorbance at 450 nm was read using a Tecan Infinite M200 PRO plate reader.

SYBR Gold binding assay. 10,000 $\times$ SYBR Gold Nucleic Acid Gel Stain was added at a final concentration of 2.5 $\times$ to 1 mL of phage at a final concentration of 10^{13} virions/mL, then mixed on a benchtop rotator at room temperature in the dark for 60 minutes to allow phage staining. Labeled phage were purified by concentrating down to 1 mL in Amicon 50 kDa Ultra Centrifugal Filter membranes via successive centrifugation at 5,000 $\times$ g. Labeled phage were then washed 3 times in 1 mL of PBS to remove excess SYBR Gold and resuspended in 200 μ L PBS. The concentration of the recovered phage was quantified by UV-Vis absorbance

spectroscopy as described above. Fluorescence labeling was verified via fluorescence reading on a Tecan Infinite 200 Pro microplate reader with an excitation wavelength of 495 nm and emission wavelength of 537 nm.

Bacteria from overnight cultures were pelleted and resuspended in PBS, then diluted to an OD₆₀₀ of 0.1. In triplicate, varying amounts of bacteria were mixed with fluorescently stained phage to a final concentration of 10^{11} virions/mL in a total volume of 1 mL. Each bacteria-phage mixture was incubated on a benchtop rotator at room temperature in the dark for 30 minutes to allow binding. Bound bacteria and phage were centrifuged for 10 minutes at 5,000 rpm, washed and resuspended in PBS, centrifuged again for 10 minutes at 5,000 rpm, and resuspended in 200 mL of PBS. Each resuspended sample was added to a well of a 96-well plate. Fluorescence was measured on a Tecan Infinite 200 Pro microplate reader with an excitation wavelength of 495 nm and emission wavelength of 537 nm. Fluorescence of bacteria without phage was subtracted from samples with phage to account for background, then normalized by the number of nucleotides in each phage genome to estimate virion concentration.

Glycan microarrays. M13^{aPNAG} phage was labeled with SYBR Gold as described above and sent to the Center for Functional Glycomics (Boston, MA, USA). Microarray assays were performed at 10^{12} or 10^{13} virions/mL on the CFG Glycan microarray (version 5.5) and Microbial Glycan microarray (MGM, version 2).⁹⁹

2.2 Results

Design and construction of anti-PNAG phage. The N1 and N2 domains of the receptor binding protein (RBP) of M13 was replaced with a short chain variable fragment (scFv) of

antibody F598, which is known to bind poly-*N*-acetylglucosamine (PNAG). Two scFv fragments were designed with different orders of the heavy and light variable regions, consisting of a PelB1 leader sequence, first variable region, a flexible linker with sequence (GGGGS)₃, second variable region, and appropriate restriction sites. The construct with V_H appearing first is designated as “HL” and the construct with V_L appearing first is designated as “LH” (**Figure S2.1**). Gene fragments were cloned into a phagemid vector and verified with sequencing. After transforming *E. coli* with either of the two phagemids, the cells were infected with CM13d3 helper phage, which has a defective g3p RBP. Thus, only scFv-g3p fusion proteins would be incorporated into the progeny phage. Propagation using standard methods resulted in two different anti-PNAG phages: M13^{aPNAG(HL)} having the HL construct and M13^{aPNAG(LH)} having the LH construct.

Binding of anti-PNAG phages to bacterially derived polysaccharide extracts. An enzyme-linked immunosorbent assay (ELISA) was used to determine whether each construct was capable of binding biofilm polysaccharides purified from known PNAG producer *S. aureus*.

Polysaccharides were extracted from bacterial culture and adsorbed to ELISA plates, and phage binding was detected using an anti-M13 g8p antibody. M13^{aPNAG(HL)} demonstrated binding, whereas M13^{aPNAG(LH)} showed no apparent binding signal to the polysaccharides (**Figure 2.1**).

The M13^{aPNAG(HL)} binding curve was fit to the Hill-Langmuir equation giving an apparent dissociation constant of 600 pM (95% CI: 370 pM to 2.9 nM) (**Table S2.1**). The previously reported K_D value for F598 IgG was 500 pM for PNAG (the secreted form), and 16 pM for dPNAG (the surface associated form), indicating that M13^{aPNAG(HL)} exhibited comparable binding to extracted *S. aureus* polysaccharides as the IgG binding to PNAG.^{95,100} M13^{aPNAG(HL)} was used for all downstream applications and also designated as M13^{aPNAG} hereon.

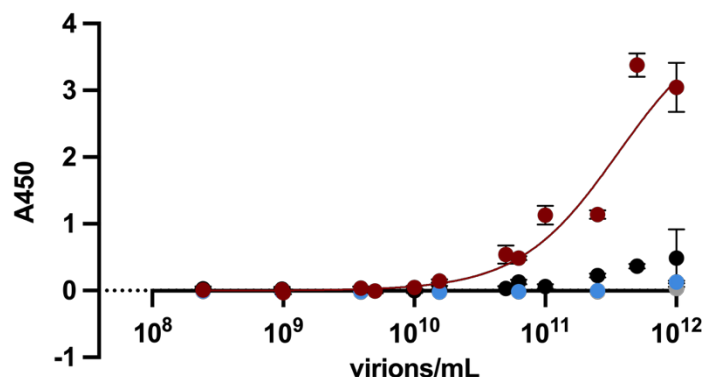


Figure 2.1. Engineered M13 binding to *S. aureus* biofilm polysaccharides. Bound phages were detected by ELISA using an HRP-conjugated anti-g8p antibody. ELISA signals are shown at different virion concentrations of anti-PNAG phages. The HL construct (M13^{aPNAG(HL)}) bound purified *S. aureus* biofilm polysaccharides in a concentration-dependent fashion (red) at a level significantly higher than its binding to the negative control (BSA; black). In comparison, the LH construct (M13^{aPNAG(LH)}; blue) showed a similar signal as the negative control (BSA; gray). Error bars indicate standard deviation between triplicate wells. The binding curve of M13^{aPNAG(HL)} to *S. aureus* polysaccharides was fit to the Hill-Langmuir equation [$Y = B_{\max} * X^h / (K_D^h + X^h)$], where K_D is the dissociation constant, $B_{\max}$ is the maximum binding, and h is the Hill coefficient. K_D was determined to be 3.6×10^{11} virions/mL (600 pM) with $R^2 = 0.9337$. Full fitting parameters with 95% confidence intervals are listed in Table S2.1.

A defined microbial glycan microarray (MGM, National Center for Functional Glycomics) was used to assess the binding specificity of M13^{aPNAG} by testing binding to 313 glycan samples from multiple microbial species. The MGM panel includes 285 extracts from gram-negative strains from various species and 23 extracts from gram-positive species, as well as 5 other glycans; the panel does not include PNAG or any other polysaccharides from *S. aureus*. M13^{aPNAG} did not significantly bind the MGM glycans, with the exception of weak binding to glycans isolated from *E. coli* serotype O128-B12 (**Figure S2.2A**), consistent with the expected lack of PNAG among these samples.⁹⁹ The ELISA and microarray studies indicate that M13^{aPNAG} had high affinity and binding specificity, in accordance with its design to bind PNAG biofilm polysaccharides.

Binding of anti-PNAG phage M13^{aPNAG} to gram-negative and gram-positive bacterial cells.

Since the isolation procedure of polysaccharides prepared for ELISA or microarrays may alter their binding properties, M13^{aPNAG} binding to whole bacterial cells was measured. A modified ELISA was employed by immobilizing bacteria on 96-well plates through adsorption and formaldehyde fixation. Phage binding was detected by an anti-M13 g8p antibody. Wild-type M13 was observed to bind *E. coli* (ER2738, an F⁺ strain) but not *S. aureus* or BSA (**Figure 2.2A**), as expected, providing initial validation of the whole-cell ELISA method. M13^{aPNAG} was observed to bind both *S. aureus* and *E. coli* cells, along with *S. epidermis* and *P. aeruginosa* (**Figure 2.2B**). The binding curves were fit to the Hill-Langmuir equation, with apparent dissociation constants of 340 pM, 470 pM, 1.1 nM, and 5.1 nM for *S. aureus*, *S. epidermidis*, *E. coli*, and *P. aeruginosa*, respectively (**Table S2.1**). These K_D values are within an order of magnitude of the value measured for polysaccharides isolated from *S. aureus* and the previously reported affinity for PNAG, supporting binding according to the design. The whole-cell ELISAs also demonstrated greater M13^{aPNAG} binding to species where PNAG is a tightly associated surface polysaccharide (in *S. aureus* and *S. epidermidis*) rather than a more loosely associated exopolysaccharide (e.g., in *E. coli*). Greater binding to *S. aureus* is also consistent with greater PNAG production by this species.^{92,94,101} While *P. aeruginosa* is not known to produce PNAG, it does produce the structurally similar polysaccharide Pel, which PNAG-specific antibodies are capable of binding.¹⁰²

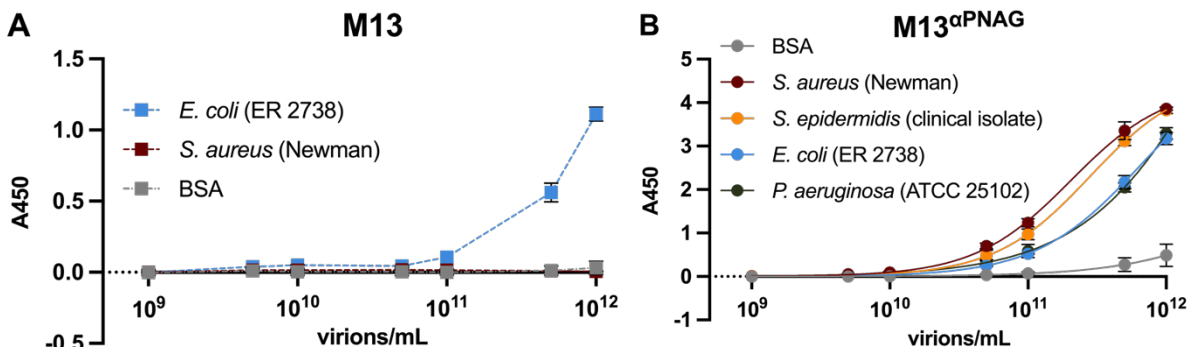


Figure 2.2. Engineered M13 binding to *E. coli*, *S. aureus*, *S. epidermidis*, and *P. aeruginosa* cells. Whole-cell ELISA was used to measure binding of A) wild-type M13 and B) M13^{αPNAG} phage to different bacterial species. As expected, wild-type M13 bound significantly to *E. coli* (ER2738) but not *S. aureus* (Newman) or the negative control (BSA), validating the assay. M13^{αPNAG} bound strongly to *S. aureus* (Newman) and *S. epidermidis* (clinical isolate), and to *E. coli* (ER2738) and *P. aeruginosa* (ATCC 25102) to a lesser extent. Error bars indicate standard deviation between duplicate wells. Binding curves were fit to the Hill-Langmuir equation [$Y = B_{\max} * X^h / (K_D^h + X^h)$]. Fitting parameters with 95% confidence intervals are listed in Table S2.1.

Binding to bacterial cells was further confirmed by staining phages with SYBR Gold, mixing with cells, washing, and measuring the fluorescence of the cell-phage pellet. The SYBR Gold assay confirmed that the engineered M13^{αPNAG} phage showed significantly greater binding than wild-type M13 to *S. aureus* (**Figure 2.3A**). Both wild-type M13 and M13^{αPNAG} phages bound *E. coli* (**Figure 2.3B**). Thus, multiple techniques indicated that M13^{αPNAG} bound to *S. aureus* polysaccharides and whole cells, as designed.

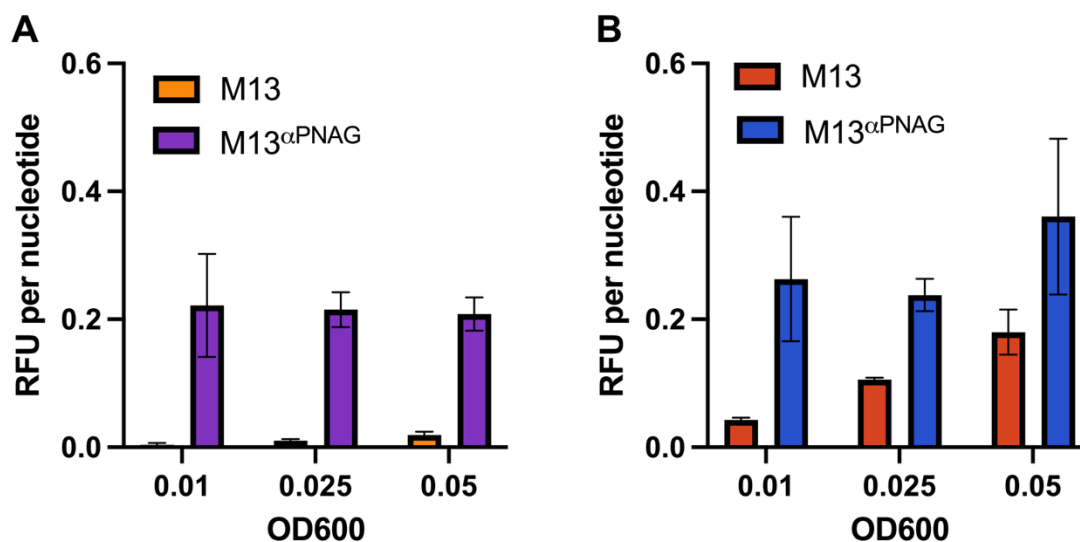


Figure 2.3. Binding of SYBR Gold-labeled M13 and M13^{αPNAG} phages to A) *S. aureus* Newman and B) *E. coli* ER 2738. Phage at a concentration of 10^{11} virions/mL were incubated with bacteria at varying OD600 in a final volume of 1 mL. Unbound phages were removed by centrifugation, and bacterial pellets were resuspended in 200 μ L of PBS. RFU of bacteria without phage was subtracted from samples with phage to account for background, then normalized by the number of nucleotides in each phage genome.

Lack of M13^{αPNAG} binding to mammalian cells and glycans. Since phage conjugation or complexation was intended to provide specificity, particularly to avoid binding to mammalian cells, the whole-cell ELISA method was used to evaluate binding of M13^{αPNAG} to mammalian cell line HEK 293. Neither wild-type M13 nor M13^{αPNAG} bound to HEK 293 cells, as expected (**Figure 2.4A**). M13^{αPNAG} did exhibit some binding to *S. cerevisiae* (**Figure 2.4B**), indicating some potential for eukaryotic cell binding, but with 50-fold lower affinity than the affinities measured for *Staphylococcus* species (**Table S2.1**). These results confirmed that M13^{αPNAG} exhibits superior binding to *S. aureus* glycans compared to eukaryotic, especially mammalian, cells.

To further probe binding to mammalian glycans, M13^{αPNAG} was tested against a diverse panel of 562 mammalian N- and O-glycans (CFG Glycan microarray). No binding was observed

on the microarray, supporting the expected lack of binding between M13^{aPNAG} and mammalian glycans (Figure S2.2B).

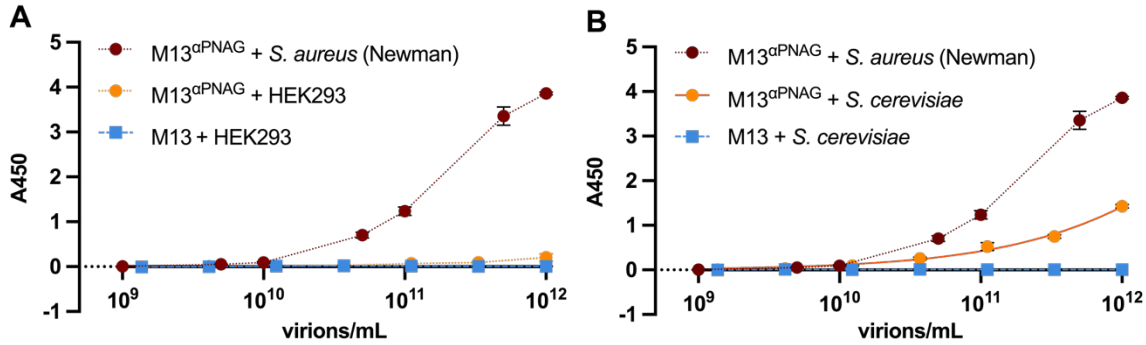


Figure 2.4. Engineered M13 binding to eukaryotic cells. Whole-cell ELISA was used to measure binding of wild-type M13 and M13^{aPNAG} phage to A) HEK 293 and B) *S. cerevisiae*. Neither phage exhibiting substantial binding to HEK 293 compared to M13^{aPNAG} target *S. aureus*. M13^{aPNAG} exhibited weak binding to *S. cerevisiae* compared to *S. aureus*. Error bars indicate standard deviation between triplicate wells. Fits to the Hill-Langmuir equation are shown. Full fit parameters with 95% confidence intervals are listed in Table S2.1.

2.3 Discussion

Here, we demonstrate the successful engineering of M13 bacteriophage to bind *S. aureus* by antibody fragment expression. M13^{aPNAG} exhibited strong binding to *S. aureus* biofilm polysaccharides while the other phage construct, M13^{aPNAG(LH)}, exhibited no binding, highlighting the importance of scFv orientation. The apparent dissociation constant of M13^{aPNAG} to the biofilm polysaccharides, being rich in acetylated PNAG, was 600 pM, similar to the report K_D of the monoclonal antibody to acetylated PNAG, 500 pM.^{95,100}

M13^{aPNAG} exhibited the strongest binding affinities for prolific PNAG producers, such as *S. aureus* and *S. epidermidis*,^{92,94,101} and no binding to mammalian cells, as determined by the whole cell ELISA. While the K_D of F598 to *S. aureus* has not been determined, the apparent dissociation constant of M13^{aPNAG} to *S. aureus* falls in between the K_D of the antibody to PNAG

(500 pM) and dPNAG (16 pM), validating the whole cell ELISA developed here. These results were also consistent with the SYBR Gold binding assay and glycans microarrays. Additionally, the whole cell ELISA method was used to confirm the binding of an anti-lipopolysaccharide (LPS) phage to *E. coli*.⁸⁸ Thus, the whole cell ELISA methodology developed here therefore presents an attractive method for characterizing the binding affinity of phage to bacteria.

As they presented similar binding affinities, the recombinant phage can serve as a suitable replacement for the targeting molecule in antibody-drug-conjugates, but M13 phage has the additional advantage of being quicker and more-cost effective to produce than monoclonal antibodies.¹⁰³ Additionally, the phage major coat protein, g8p, provides solvent-accessible functional groups for potential bioconjugation of antimicrobial cargo and is present in thousands of copies per virion;^{45,81} a typical IgG has roughly 20 potential solvent accessible residues for bioconjugation.¹⁰⁴ Thus, engineering the phage's binding specificity expands the host range to include target pathogens, like *S. aureus*, and the natural antibacterial effect of phage infection can next be replaced by the lytic activity of a synthetic polymer conjugated to the coat protein.

2.4 Supplementary figures and tables

VH-L-VL

GCGGCCGCTGGATTGTTATTACTCGCGGCCAGCCGGCCATGGCGCAAGTCCAGCT
GCAAGAAAGCGGGCCGGGCTTGGTGAAGCCAAGTGAAACACTGTCGTAAACCTGTA
CCGTGAGTGGCGGCAGCATTCTGTTATTATTGGTCCTGGATCCGTCAGCCCCCGG
GTAAAGGCCTCGAGTGGATCGGATATATTCACCTATTCACGCTCGACGAATAGTAACC
CAGCCCTGAAAAGCCGAGTCACGATTAGCTCTGACACCTCCAAGAATCAGTTGAGC
CTTCGCCTTAGTTCAGTTACCGCCGCGGATACCGCTGTTTACTATTGCGCGCGTGATA
CCTACTACTACGATTCAGGTGACTACGAGGATGCATTTGATATCTGGGGCCAGGGGA
CTATGGTGACTGTAAGTTCTGGTGGCGGTGGTTCAGGTGGGGGAGGTTTCGGGCGGC
GGCGGCAGCCAACCTCGTGCTGACCCAGTCCCCGAGCGCGTCCGCATCCTTGGGTGCC
AGCGTGAACTGACCTGTACTCTGAGCTCAGGCCACTCCAACCTATGCGATCGCTTGG
CATCAGCAGCAACCGGGGAAAGGTCCTAGATATTTAATGAAAGTAAACCGTGACGG
TAGTCATATTCGTGGAGACGAATACCGGATCGGTTCTCGGGCTCGACAAGCGGTGC
AGAACGCTATCTGACAATTTCTTCGCTGCAGTCTGAAGATGAAGCGGATTATTACTG
CCAAACGTGGGGCGCCGGCATTTCGCGTCTTTGGGGGCGGAACGAACTGACGGTTC
TAGGGCAGGGCGGGCGGCACCGGT

VL-L-VH

GCGGCCGCTGGATTGTTATTACTCGCGGCCAGCCGGCCATGGCGCAACTGGTCTT
GACCCAGAGCCCTAGTGCCAGCGCGAGCCTGGGTGCATCAGTTAAGCTGACCTGCA
CACTATCAAGCGGCCACTCGAATTATGCAATCGCCTGGCATCAGCAGCAACCGGGG
AAAGGACCGCGATATCTCATGAAAGTTAACCGTGATGGCTCTCACATCCGCGGTGAT
GGTATTCGGATCGTTTCAGCGGCTCAACCTCGGGCGCCGAGAGATATTTGACTATT
TCTTCTTTACAATCGGAAGATGAAGCGGACTATTACTGCCAGACGTGGGGTGCTGGC
ATCCGTGTGTTTGGTGGTGGCACCAAAGTACAGTTCTCGGTTCAGGGGGGGGGGAGG
CTCAGGCGGTGGCGGCTCCGGCGGGGGCGGTAGTCAAGTCCAGCTGCAGGAGTCTG
GTCCGGGACTGGTGAAACCGAGCGAACTTTATCGCTTACCTGTACGGTCTCCGGGG
GTAGCATTAGTGGCTATTACTGGTCCTGGATTCCGCAACCCCCAGGAAAAGGCCTGG
AATGGATAGGATATATTCATTACTCCCGTTCAACCAACTCCAACCCAGCGCTGAAGT
CGCGCGTAACAATCTCGAGCGACACGAGTAAAAATCAGCTTAGTCTGCGCTTGAGC
AGTGTGACGGCTGCGGACACCGCAGTGTATTATTGTGCGCGGGGATACTTATTACTAC
GACTCTGGTGATTACGAAGATGCCTTTGATATTTGGGGGCGAGGGCACGATGGTAACG
GTGTCCAGCGGCGGGCGGCACCGGT

Figure S.2.1. Sequences of VH-L-VL and VL-L-VL inserts from NotI (GCGGCCGC) to AgeI (ACCGGT) restriction sites. Successful cloning was verified by Sanger sequencing.

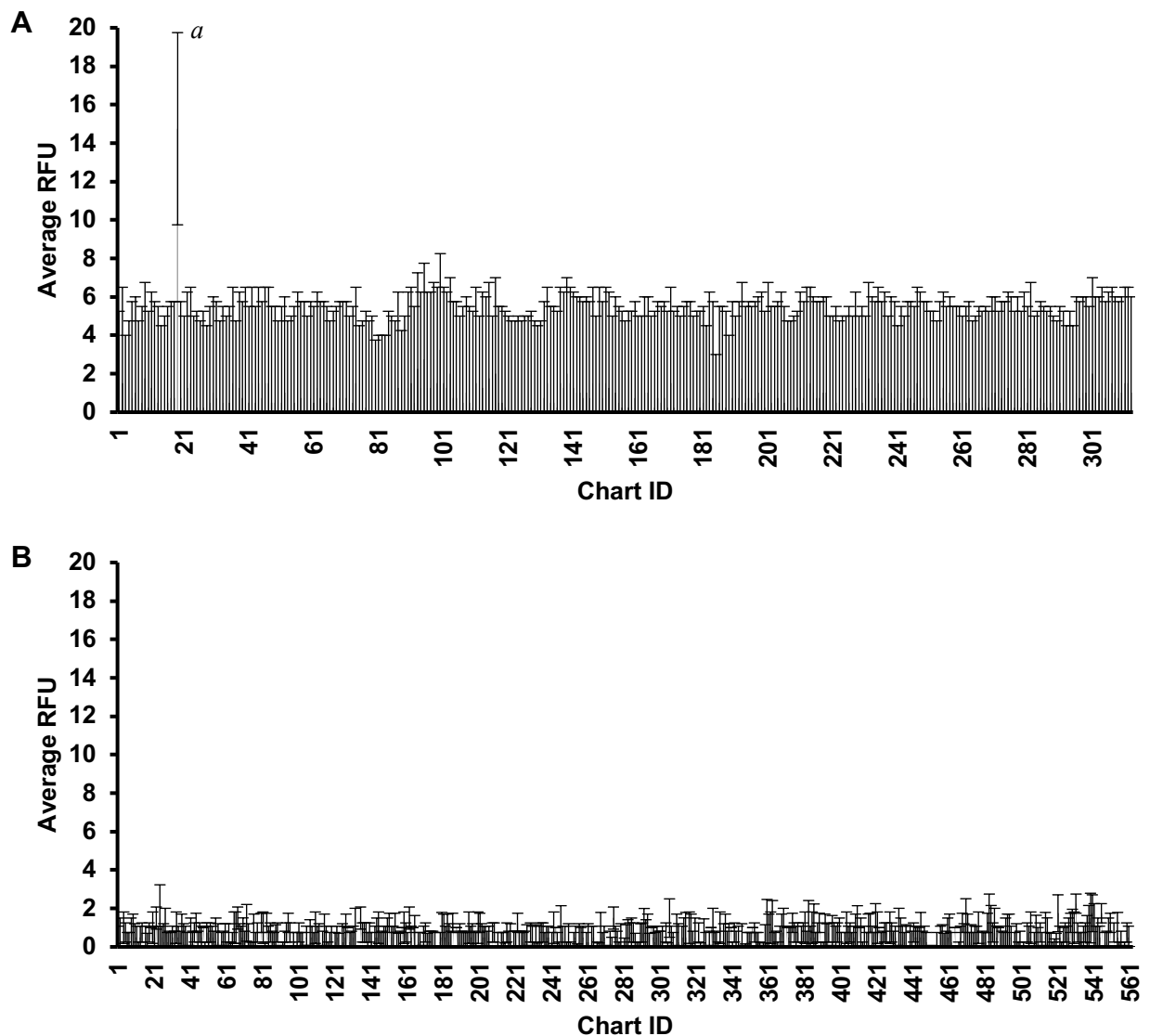


Figure S.2.2. Binding of M13^αPNAG to glycan microarrays from the National Center for Functional Glycomics. A) Microbial Glycan Microarray (MGM) and B) CFG mammalian type microarray, version 5.5. Typical binding hits show a >10-fold increase in fluorescence over background based on prior MGM experiments. *Escherichia coli* lipopolysaccharide O128-B12 is indicated by “a”. A full list of all glycans screened and their indices is found on the National Center for Functional Glycomics website: <https://research.bidmc.org/ncfg/microarrays>.

Substrate	K_D in virions/mL	K_D in pM	B_{max}	h	R^2
<i>S. aureus</i> polysaccharide	3.6×10^{11} (2.2×10^{11} to 1.8×10^{12})	600 (370 to 2900)	4.1 (3.3 to 8.1)	1.2 (0.75 to 1.7)	0.9337
<i>S. aureus</i> (Newman)	2.0×10^{11} (1.7×10^{11} to 2.5×10^{11})	340 (280 to 420)	4.4 (4.1 to 4.8)	1.2 (1.1 to 1.4)	0.9973
<i>S. epidermidis</i> (clinical isolate)	2.8×10^{11} (2.4×10^{11} to 3.4×10^{11})	470 (400 to 560)	4.6 (4.3 to 5.0)	1.3 (1.1 to 1.4)	0.9975
<i>E. coli</i> (ER 2738)	6.5×10^{11} (4.6×10^{11} to 1.1×10^{12})	1100 (760 to 1900)	5.1 (4.2 to 6.9)	1.2 (0.99 to 1.3)	0.9977
<i>P. aeruginosa</i> (ATCC 25102)	3.0×10^{12} (NC)	5100 (NC)	12 (NC)	0.87 (NC)	0.9970
<i>S. cerevisiae</i>	1.5×10^{13} (NC)	25 nM	8.3 (NC)	0.59 (NC)	0.9864

Table S.2.1. Fitting parameters of binding curves. Binding curves of M13^{aPNAG} as determined by ELISA were fit to the Hill-Langmuir equation, $[Y = B_{max} * X^h / (K_D^h + X^h)]$, where K_D is the dissociation constant, B_{max} is the maximum binding, and h is the Hill coefficient. 95% confidence intervals are listed in parentheses, where NC indicates the interval could not be calculated. K_D is expressed as virions/mL and also as pM.

Chapter 3. Antimicrobial properties of bio-inspired polymers

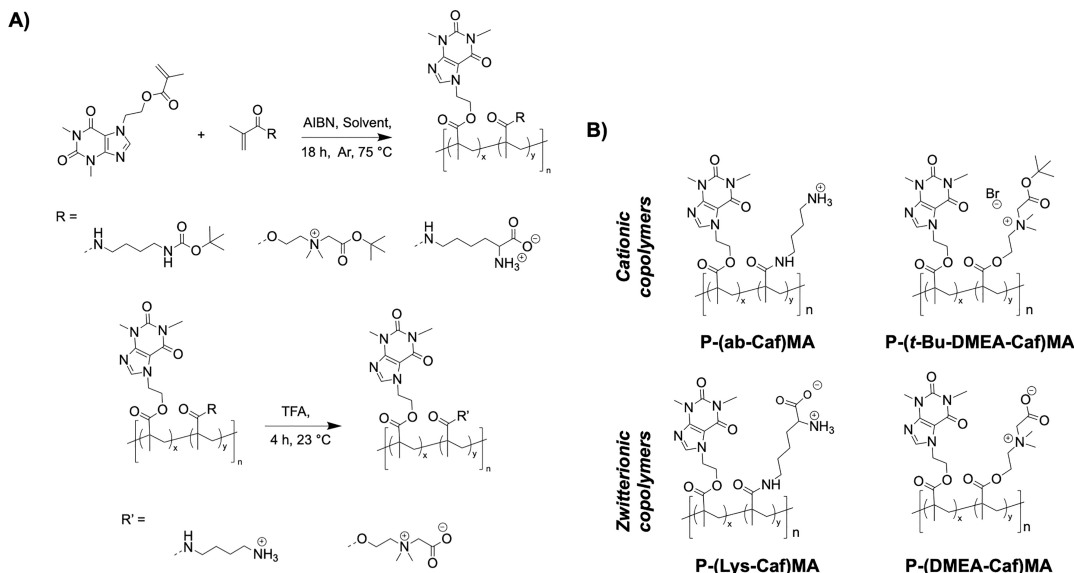
Adapted from:

- Salas-Ambrosio, P.; **Vexler, S.**; P S, R.; Chen, I. A.; Maynard, H. D. Caffeine and Cationic Copolymers with Antimicrobial Properties. *ACS Bio Med Chem Au* **2023**.
<https://doi.org/10.1021/acsbioimedchemau.2c00077>.
- Salas-Ambrosio, P.; **Vexler, S.**; P Sivasankaran, R.; Vlahakis, N.; Lai, R.; Johnson, C.; Baas-Maynard, S.; Min, D.; Lower, H.; Doyle, A.; Tang, Y.; Rodriguez, J.; Chen, I.; Read de Alaniz, J.; Maynard, H. Biosourced Functional Hydroxybenzoate-co-Lactide Polymers with Antimicrobial Activity. *Journal of the American Chemical Society* **2025**.
<https://doi.org/10.1021/jacs.5c04624>
- **Vexler, S.**; Morrison, C.; Jain, S.; Yang, Y.; Tsunemoto, H.; Zhang, N.; McCarthy, K.; Mihalache, B.; Kutach, M.; Yang, S.; Pogliano, J.; Annabi, N.; Deming, T.J.; Chen, I.A. Phage-Polymer conjugates inhibit *S. aureus* with high cytocompatibility and low bacterial resistance. (under review)

Antimicrobial polymers can be prepared from a variety of different polymerization techniques depending on the nature of the monomers. Among the different polymeric backbones with antimicrobial properties, it is possible to find polyacrylates,¹⁰⁵ polynorbornenes,¹⁰⁶ polypeptides,¹⁰⁷ their analogues polypeptoids,^{108,109} or synthetic polybetapeptides.¹¹⁰ Different parameters can be tuned during the preparation of antimicrobial polymers, such as architecture, molecular weight, monomer arrangement, nature of the cation, and hydrophobic group.^{111,112} Additional modifications include zwitterionic groups as side chains and satellite-active

groups.^{113,114} In this study, we synthesized three different libraries of polymers, each differing in their backbone, and investigated their antimicrobial activity.

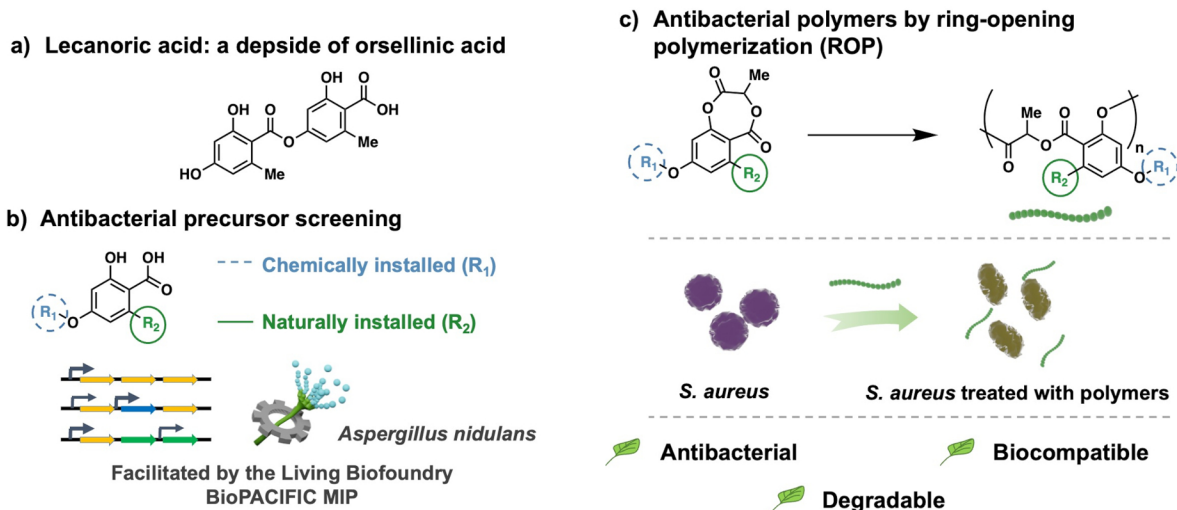
For poly(methacrylates), two key properties are their primary cationic group, which can be tuned at the side chain to mimic cationic amino acids,^{115,116} and the hydrophobic side chain; increased length of the hydrophobic side chain allows for greater hydrophobic interactions with the lipid components of the membranes but comes at the cost of higher cytotoxicity.¹¹⁷ Therefore, evaluating different ratios of cationic and hydrophobic content is crucial to select molecules with optimal antimicrobial activity and minimal cytotoxicity. Moreover, to the best of our knowledge, heterocyclic side chains, such as purine derivatives, have not been reported as the hydrophobic side chain of antimicrobial polymers, whereas polymers having heterocyclic cationic side chains are already known.^{118–121} Potential purine derivatives include caffeine, which could provide antibacterial properties by itself^{122,123} or improve the biological activity of other drugs.^{124,125} In the first library, we prepared a series of methacrylate copolymers bearing cationic or zwitterionic groups and ethyl caffeine by free-radical polymerization and tested their potential as antimicrobial agents (**Scheme 3.1**).



Scheme 3.1. General strategy to prepare methacrylate copolymers with caffeine and cationic or zwitterionic side chains. A) Synthetic route of preparation and B) copolymer Structures.

A lesser studied backbone is hydroxybenzoate-co-lactide, which can be prepared from depside precursors. Depsides are natural compounds produced by condensation between hydroxybenzoic acids and have shown antimicrobial activity as a class.¹²⁶ For example, lecanoric acid, which is a depside of orsellinic acid (**Scheme 3.2A**), possesses antibacterial activity against gram-negative and gram-positive bacteria.^{127,128} Depsides are found in lichen, plants, and fungi.^{129,130} Interestingly, they possess a range of other biological activities such as antitumor,¹³¹ anti-inflammatory,^{132,133} anti-fungal,¹³⁴ and antiviral^{128,135} properties. They have also been found active against drug-resistant bacteria including methicillin-resistant *Staphylococcus aureus* (MRSA).^{134,136} Depsides are linked by ester bonds, making them good candidates for preparing active and degradable molecules. However, the specificity that living organisms possess to link them at the 4-hydroxy position and the versatility of different carbon chain lengths at the 6-position pose synthetic challenges for researchers. Thus, preparing synthetic polymer analogs of

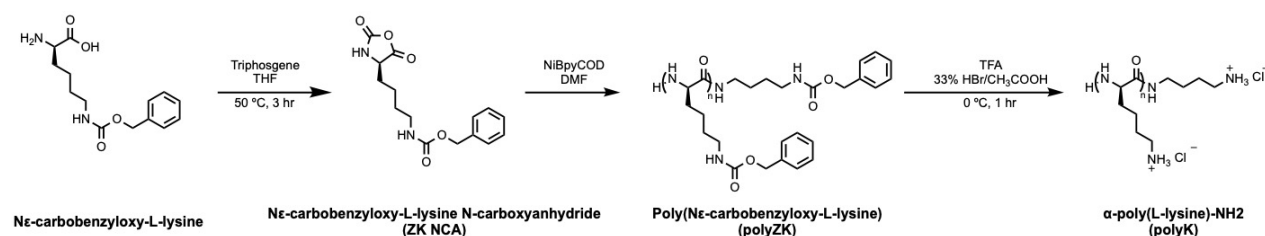
depsides is an area of interest for drug discovery and biomedical applications. Inspired by the promising potential of depsides, the second library investigates the antimicrobial activity of hydroxybenzoate oligomers and polymers obtained using a combination of synthetic biology and chemical synthesis to enable access to a range of functionalities (**Scheme 3.2**). Substituted hydroxybenzoic acids (such as resorcylic acids) are widely found in nature and are biosynthesized from polyketide pathways. Variations in the structure of these natural products arise through polyketide synthase substrate specificity and have been leveraged for engineered biosynthesis from model microbial hosts.¹³⁷ First, 2-hydroxybenzoic acid monomer precursors with 4-O (R1) and 6-alkyl (R2) functionality were heterologously produced and purified from an engineered fungal host.¹³⁸ The biosynthetically sourced natural products were studied for activity against gram-positive *S. aureus* and gram-negative *Escherichia coli*. Next, these compounds were derivatized with methyl, allyl, and benzyl groups at the 4-O position and converted into the corresponding lactides prior to polymerization. The antimicrobial activity of the resulting polymers was investigated, determining the importance of the peripheral functional group and molecular weight on antimicrobial efficacy; this library represents the first successful design of functionalized poly(hydroxybenzoate-co-lactide) derivatives for antimicrobial activity.



Scheme 3.2. Polymeric antimicrobial agents against *S. aureus* inspired by depsides. A) Structure of a depside from orsellinic acid, B) 4-(methyl/allyl/benzyl)oxy and 6-(H/alkyl)-2-hydroxybenzoates from synthetic and biological processes, and C) their functional hydroxybenzoate-co-lactide polymers prepared by ROP.

The polypeptide backbone represents a significant class of antimicrobial macromolecules, largely comprised of antimicrobial peptides (AMPs), in addition to more “polymer-like” polypeptides.⁵⁸ Many AMPs are produced by nonribosomal peptide synthetases (NRPS) and contain substantial modifications, such as a cyclic peptide structure, D-amino acids, and fatty acid tails.¹³⁹ Notably, several have been approved for systemic use, such as vancomycin and polymyxin b, unlike synthetic antimicrobial polymers.¹⁴⁰ Like other membrane active antimicrobial polymers, their activity is usually derived from a mix of cationic and hydrophobic residues,¹⁴¹ although cationic homopolymers of lysine or arginine exhibit strong activity despite the absence of hydrophobicity.¹⁴² These cationic homopolymers represent the more “polymer-like” class of antimicrobial polypeptides, as they consist of repeating units and can exceed 50 residues. Poly(lysine) is a particularly well studied antimicrobial polypeptide, as the naturally produced epsilon form is approved as a food preservative, and the synthetic alpha form is utilized for gene delivery and cell culture.^{49,143,144} For the final library, we used a ring-opening

polymerization of N-carboxyanhydrides (NCAs) to synthesize α -poly(L-lysine)-NH₂, referred to as “polyK”, ranging from 40 to 200 residues to determine the impact of degrees of polymerization on antimicrobial activity (**Scheme 3.3**).



Scheme 3.3. Synthesis of α -poly(L-lysine)-NH₂ (called “polyK” here) by NCA polymerization.

3.1 Methodology

3.1.1 Preparation of methacrylate copolymers with caffeine and cationic or zwitterionic side chains

All the chemicals and solvents in this work were purchased from Sigma-Aldrich and TCI and, unless otherwise described, were used without any purification. AIBN was recrystallized from acetone before use. DMSO was dried using molecular sieves and kept under an inert atmosphere. MilliQ water was obtained from a Purelab Prima, ELGA.

NMR spectra were obtained on a Bruker AV 400 MHz instrument (UCLA, CA, USA), and the data was analyzed using MestRenova v12 software. Size exclusion chromatography (SEC) measurements in the trifluoroethanol (TFE) phase were performed on a JASCO BS 4000-1 HPLC System, equipped with a UV-4075 UV/vis detector. The system also included a differential refractive index detector RI-4030 RI detector. Polymers were separated on a mixed-column system equipped with two PSS PFG columns (8 × 300 mm, 5 μm) at a flow rate of 0.5

mL/min. Column temperatures were held at 23 °C in TFE 20 mM NaTFA. Molar mass was calculated from a calibration curve of poly(methyl methacrylate). Size exclusion chromatography (SEC) measurements in DMF were performed on an Infinity 1260 II HPLC system from Agilent equipped with a diode array detector DAD from Wyatt technology. The system also included a multiangle light scattering detector MALS and differential refractive index detector dRI from Wyatt technology. Polymers were separated on two PLgel Mixed-D gel columns PL1110–6504 (300 × 7.5 mm) (exclusion limits from 200 to 400 000 Da) at a flow rate of 0.6 mL min⁻¹. Column temperatures were held at 40 °C in DMF with LiBr (0.1 M). Molar masses were calculated from the dn/dc of the different polymers. Size exclusion chromatography (SEC) measurements in the aqueous phase were performed on a Waters Alliance HPLC System, 2695 Separation Module equipped with photodiode array detector 2998 PDA from Watters technology. The system also included a multiangle light scattering detector MALS and differential refractive index detector dRI from Wyatt technology. Polymers were separated on Tosoh TSKgel G3000PWXL (7 μm) + TSKgel G5000PWXL (10 μm) at a flow rate of 1.0 mL/min. Column temperatures were held at 23 °C in MilliQ Water with 0.2 M NaNO₃ + 0.1% TFA. Molar masses were calculated from the dn/dc of the different polymers.

Monomer preparation

Synthesis of 2-(1,3-Dimethyl-2,6-dioxo-1,2,3,6-tetrahydro-7H-purin-7-yl)ethyl

Methacrylate; Ethofylline-methacrylate (CafMA). In a round-bottom flask etofylline 99% (1g, 4 mmol, 1 equiv) was dissolved in 15 mL of dry DCM, placed in an ice bath, and mixed with triethylamine 97% (Et₃N 0.7 mL, 5 mmol, 1.1 equiv). Then, methacryloyl chloride 90% (0.6 mL, 5 mmol, 1.2 equiv) was added slowly. The reaction was stirred at 23 °C for 16 hours.

Upon completion, as attested by TLC (DCM:Acetone 8:2, $R_{f\text{ product}} = 0.76$, $R_{f\text{ caffeine OH}} = 0.1$), the solution was filtered, and the organic phase was dried using rotavapor. Then, the powder was resolubilized in 10 mL of DCM, placed in a separation funnel, and followed by the addition of 30 mL of water and 2 mL of HCl. The organic phase was separated, and the aqueous phase was extracted twice using 10 mL of DCM. The organic phase was dried using MgSO_4 , filtered, and dried in a rotavapor. The product was isolated as a slightly yellowish powder in a 56% yield. ^1H NMR signals agreed with the previous report.¹⁴⁵

Synthesis of *tert*-Butyl (4-Methacrylamidobutyl)carbamate (Boc-ab-MA). In a round-bottom flask *tert*-butyl(4-aminobutyl) carbamate 95% (5 mL, 24.82 mmol, 1 equiv) and Et_3N 97% (4.28 mL, 29.79 mmol, 1.2 equiv) were dissolved in 41 mL of DCM and placed them in an ice bath. Then, methacryloyl chloride 90% (3.34 mL, 29.79 mmol, 1.2 equiv) was added slowly. The reaction was stirred reaching 23 °C conditions for 16 hours. Then, the solution was washed with 10 mL of HCl 3 M and 50 mL of water three times. The organic phase was dried with magnesium sulfate, filtered off and the solvent was evaporated using the rotavapor. The solid was washed with cyclohexane and isolated in a 65% yield as a white powder.

Synthesis of 2-(*tert*-Butoxy)-*N*-(2-(methacryloyloxy)ethyl)-*N,N*-dimethyl-2-oxoethan-1-aminium (*t*-Bu-DMEA-MA). The monomer was prepared as reported elsewhere with slight modification.¹⁴⁶ In a round-bottom flask 2-(dimethylamino) ethyl methacrylate 97% (5.15 mL, 30.85 mmol, 1 equiv) and *tert*-butyl 2-bromoacetate 97% (5.64 mL, 37 mmol, 1.2 equiv) were mixed in 50 mL of ACN. The mixture was stirred at 50 °C for 16 hours. Then the solvent was evaporated. The crude oil was mixed with 15 mL of DCM and precipitated in 200 mL of Et_2O

twice. The powder was isolated as a white powder in an 83% yield. ^1H NMR signals agreed with the previous report.

Synthesis of Lysine Methacrylate (Lys-MA). The monomer was prepared as reported elsewhere with slight modification.¹⁴⁷ In a round-bottom flask, CbzLys-OH 99% (2 g, 7.13 mmol, 1 equiv), methacryloyl chloride 90% (1.6 mL, 14.2 mmol, 2 equiv), and NaCO_3 97% (1.24 g, 14.2 mmol, 2 equiv) were dissolved in 5 mL of water and 24 mL of THF. The mixture was stirred in an ice bath for 30 min and then for 3 hours at 23 °C. Then, 150 mL of water and 10 mL of 3 M HCl were added and extracted with DCM 50 mL three times. The organic phase was dried with magnesium sulfate, filtered off, and then, 10 μL of MEHQ (20 mg/mL) was added to prevent autopolymerization, and the solvent was evaporated in the rotavapor. The crude oil was then solubilized in TFA 10 mL and deprotected with HBr 33% (2.5 mL, 14.2 mmol, 2 equiv) for 4 hours. Then it was precipitated in Et_2O 200 mL and kept in the freezer at -20 °C for 3 hours. The mixture was centrifuged at 4000 rpm for 4 min. The supernatant was discarded, and the powder was resolubilized in 5 mL of ethanol followed by the addition of triethylamine in excess. The mixture was centrifuged at 400 rpm for 4 min. The solid was dried at a high vacuum affording a yellowish solid in 30% yield. ^1H NMR signals agreed with the previous report.

Polymer Preparation

Synthesis of Poly(etofylline)methacrylate (CafMA). AIBN (90 mg, 6.6 μmol , 1 equiv) was dissolved in 220 μL of DMSO (dried with molecular sieves) and transferred to a dried Schlenk tube, and CafMA (5 mg, 0.2 mmol, 30 equiv) was added. The mixture was degassed four times using the freeze–thawing technique. The reaction was stirred at 75 °C for 1.5 hours after

confirming the absence of monomer. The polymer was precipitated in 20 mL of Et₂O and centrifuged at 4000 rpm for 5 min, repeating this action three times. The polymer was dried *in vacuo* and recovered as a white powder (yield 75%). $M_n = 25.4$ kg/mol $\bar{D} = 2.77$.

Synthesis of Poly[(*tert*-butyl(4-methacrylamidobutyl)carbamate)-(etofylline-methacrylate)];

P(Boc-ab-Caf50%)MA. The monomer Boc-ab-MA (88.6 mg, 3.42×10^{-4} mol, 15 equiv) was transferred to a Schlenk vessel followed by the addition of CafMA (100 mg, 3.42×10^{-4} mol, 15 equiv) in 530 μ L of DMF, the monomers were solubilized with gentle heat, and then 72.4 μ L of AIBN (2.28×10^{-5} mol, 1 equiv) was added (solution of 20.75 mg in 400 μ L of DMSO). The mixture was degassed four to five times using the freeze–thawing technique. The reaction was stirred at 75 °C overnight for 16 hours. The polymers were precipitated in 25 mL of Et₂O, and the solid was resolubilized in approximately 1 mL DCM and reprecipitated in 25 mL of Et₂O. The solid was dried *in vacuo* and isolated as a yellowish powder in a 69% yield. $M_n = 27.7$ kg/mol $\bar{D} = 1.75$. The caffeine content was calculated by correlating the protons in methyl groups of the polymer backbone (0.49 ppm, CH₃) and the protons of the caffeine side chain (8.05 ppm, CH) using the following equation: $\text{Caf}\% = \frac{\text{Int}_{\text{CH}}}{2} \times 100\%$

Synthesis of Poly[(*tert*-butyl(4-methacrylamidobutyl)carbamate)-(etofylline-methacrylate)];

P(Boc-ab-Caf30%)MA. The copolymer was prepared similarly to P(Boc-ab-Caf50%)MA varying the equivalent ratio of Boc-ab-MA and CafMA. Upon isolation, the copolymer was obtained as a yellowish powder in a 73% yield. $M_n = 21.8$ kg/mol $\bar{D} = 1.74$.

Synthesis of Poly[(*tert*-butyl(4-methacrylamidobutyl)carbamate)-(etofylline-methacrylate)]; P(Boc-ab-Caf10%)MA. The copolymer was prepared similarly to P(Boc-ab-Caf50%)MA varying the equivalent ratio of Boc-ab-MA and CafMA. Upon isolation, the copolymer was obtained as a yellowish powder in a 68% yield. $M_n = 25.8$ kg/mol $D = 1.50$.

Synthesis of Poly(*tert*-butyl(4-methacrylamidobutyl)carbamate); P(Boc-ab)MA. The copolymer was prepared similarly to P(Boc-ab-Caf50%)MA using only Boc-ab-MA. Upon isolation, the copolymer was obtained as a yellowish powder in a 67% yield. $M_n = 23.2$ kg/mol $D = 1.52$.

Synthesis of Poly[(*N*-(4-aminobutyl)-methacrylamide)-(etofylline-methacrylate)]; P(ab-Caf50%)MA. An amount of 100 mg of P(Boc-ab-Caf50%)MA polymer was dissolved in 1 mL of TFA and stirred for 4 hours at 23 °C. Then the product was precipitated in 25 mL of Et₂O, centrifuged at 4500 rpm for 5 min, reprecipitated, centrifuged, dried in vacuo, and dialyzed with deionized water (MWCO 3.5 kDa). Upon isolation, the copolymer was isolated as a yellowish powder in a 69% yield.

Synthesis of Poly[(*N*-(4-aminobutyl)-methacrylamide)-(etofylline-methacrylate)]; P(ab-Caf30%)MA. The copolymer was deprotected similarly as described for P(ab-Caf50%)MA. Upon isolation, the copolymer was isolated as a yellowish powder in a 70% yield.

Synthesis of Poly[(*N*-(4-aminobutyl)-methacrylamide)-(etofylline-methacrylate)]; P(ab-Caf10%)MA. The copolymer was deprotected similarly as described for P(ab-Caf50%)MA. Upon isolation, the copolymer was isolated as a yellowish powder in an 81% yield.

Synthesis of Poly(*N*-(4-aminobutyl)-methacrylamide); P(ab-MA). The copolymer was deprotected similarly as described for P(ab-Caf50%)MA. Upon isolation, the copolymer was isolated as a yellowish powder in a 73% yield.

Synthesis of Poly[(2-(*tert*-butoxy)-*N*-(2-(methacryloyloxy)ethyl)-*N,N*-dimethyl-2-oxoethan-1-aminium)-(etofylline-methacrylate)]; P(*t*-Bu-DMEA-Caf30%)MA. The monomer *t*-Bu-DMEA-MA (170.4 mg, 4.79×10^{-4} mol, 21 equiv) was transferred to a Schlenk tube followed by the addition CafMA (60 mg, 2.05×10^{-4} mol, 9 equiv) in 530 μ L of DMSO. The monomers were solubilized with gentle heat and then 35.4 μ L of AIBN (2.28×10^{-5} mol, 1 equiv) was added (AIBN solution = 21.19 mg in 200 μ L of DMSO). The mixture was degassed four to five times using the freeze–thawing technique. The reaction was stirred at 75 °C for 16 hours. The polymer was then precipitated in 20 mL of Et₂O and centrifuged at 4500 rpm for 5 min. The solid was resolubilized in DCM 1 mL, reprecipitated in 25 mL Et₂O, centrifuged, and dried in vacuo. Upon isolation, the copolymer was obtained as a yellowish powder in an 80% yield.

Synthesis of Poly[(2-(*tert*-butoxy)-*N*-(2-(methacryloyloxy)ethyl)-*N,N*-dimethyl-2-oxoethan-1-aminium)-(etofylline-methacrylate)]; P(*t*-Bu-DMEA-Caf10%)MA. The copolymer was prepared similarly to P(Boc-ab-Caf30%)MA varying the equivalent ratio of *t*-Bu-DMEA-MA

and CafMA. Upon isolation, the copolymer was obtained as a yellowish powder in an 83% yield. $M_n = 21.1$ kg/mol $\bar{D} = 2.03$.

Synthesis of Poly(2-(*tert*-butoxy)-*N*-(2-(methacryloyloxy)ethyl)-*N,N*-dimethyl-2-oxoethan-1-aminium); P(*t*-Bu-DMEA)MA. The copolymer was prepared similarly to P(Boc-ab-Caf30%)MA using only *t*-Bu-DMEA-MA. Upon isolation, the copolymer was obtained as a yellowish powder in a 92% yield. $M_n = 32.0$ kg/mol $\bar{D} = 2.06$.

Synthesis of Poly[(2-((2-(methacryloyloxy)ethyl)dimethylammonio)acetate)-(etofylline-methacrylate)]; P(DMEA-Caf30%)MA. Deprotection was performed by dissolving 100 mg of polymer in 1 mL of TFA. The solution was stirred for 4 hours at 23 °C and then the polymer was precipitated in 20 mL Et₂O, centrifuged at 4500 rpm for 5 min, reprecipitated, centrifuged, and dialyzed in water (MWCO = 3.5 kDa). The polymer was freeze-dried and obtained as a yellowish powder in a 91% yield. $M_n = 16.0$ kg/mol $\bar{D} = 2.24$.

Synthesis of Poly[(2-((2-(methacryloyloxy)ethyl)dimethylammonio)acetate)-(etofylline-methacrylate)]; P(DMEA-Caf10%)MA. The copolymer was deprotected similarly as described for P(DMEA-Caf30%)MA. Upon isolation, the copolymer was isolated as a yellowish powder in an 86% yield. $M_n = 26.7$ kg/mol $\bar{D} = 2.53$.

Synthesis of Poly(2-((2-(methacryloyloxy)ethyl)dimethylammonio)acetate); P(DMEA)MA. The copolymer was deprotected similarly as described for P(DMEA-Caf30%)MA. Upon

isolation, the copolymer was isolated as a yellowish powder in a 91% yield. $M_n = 27.7$ kg/mol $\bar{D} = 3.24$.

Synthesis of Poly((lysine-methacrylate)-(etofylline-methacrylate)); P(Lys-Caf50%)MA. The monomer LysMA (55.5 mg, 2.57×10^{-4} mol, 15 equiv) was transferred to a Schenck vessel followed by the addition CafMA (75 mg, 2.57×10^{-4} mol, 15 equiv) in 0.6 mL of DMSO and 0.2 mL of HCl 6 M. The monomers were solubilized with gentle heat, and then 35.8 μ L of AIBN (1.71×10^{-5} mol, 1 equiv) was added (solubilizing 16.04 mg in 200 μ L of DMSO). The mixture was degassed four times using the freeze–thawing technique. The reaction was stirred at 75 °C for 16 hours. The polymers were precipitated in 20 mL of Et₂O, centrifuged at 4500 rpm for 5 min, reprecipitated, centrifuged, and dialyzed in deionized water (MWCO 3.5 kDa). The polymer was obtained as a yellowish powder in a 4% yield.

Synthesis of Poly((lysine-methacrylate)-(etofylline-methacrylate)); P(Lys-Caf30%)MA. The copolymer was prepared similarly to P(Lys-Caf50%)MA varying the equivalent ratio of LysMA and CafMA. Upon isolation, the copolymer was obtained as a yellowish powder in a 39% yield. $M_n = 18.0$ kg/mol $\bar{D} = 2.24$.

Synthesis of Poly((lysine-methacrylate)-(etofylline-methacrylate)); P(Lys-Caf10%)MA. The copolymer was prepared similarly to P(Lys-Caf50%)MA varying the equivalent ratio of LysMA and CafMA. Upon isolation, the copolymer was obtained as a yellowish powder in a 38% yield. $M_n = 17.8$ kg/mol $\bar{D} = 2.25$.

Synthesis of Poly(lysine-methacrylate); P(Lys)MA. The copolymer was prepared similarly to P(Lys-Caf50%)MA using only LysMA. Upon isolation, the copolymer was obtained as a yellowish powder in a 17% yield. $M_n = 25.2$ kg/mol $\bar{D} = 2.28$.

Synthesis of Poly[(2-(*tert*-butoxy)-*N*-(2-(methacryloyloxy)ethyl)-*N,N*-dimethyl-2-oxoethan-1-aminium)-(etofylline-methacrylate)]; P(*t*-Bu-DMEA-Caf30%)MA DP30. The monomer *t*-Bu-DMEA-MA (204.5 mg, 5.75×10^{-4} mol, 21 equiv) was transferred to a Schlenk tube followed by the addition CafMA (72 mg, 2.46×10^{-4} mol, 9 equiv) in 630 μ L of DMSO. The monomers were solubilized with gentle heat, and then AIBN (1.12 mg, 6.84×10^{-6} mol, 0.25 equiv) and 4-cyano-4-(phenyl carbonothioyllthio)pentanoic acid 99% (CTA, 7.72 mg, 2.74×10^{-5} mol, 1 equiv) were added. The mixture was degassed four times using the freeze–thawing technique. The reaction was stirred at 75 °C for 16 hours. The polymers were precipitated in 25 mL of Et₂O and centrifuged at 4500 rpm for 5 min. The solid was resolubilized in DCM 1 mL, reprecipitated in 25 mL of Et₂O, centrifuged, and dried in vacuo. Upon isolation, the copolymer was obtained as a yellowish powder in a 70% yield. $M_n = 6.4$ kg/mol $\bar{D} = 1.16$. The polymerization degree (DP) was calculated using the methyl group in the polymer backbone (signal 0.78 ppm, CH₃) according to the following equation: $DP = \frac{Int_{CH_3}}{3}$. The caffeine content was calculated by correlating the polymerization degree and the protons of the caffeine side chain (8.30 ppm, CH) using the following equation: $Caf\% = \frac{Int_{CH}}{DP} \times 100\%$

Synthesis of Poly[(2-(*tert*-butoxy)-*N*-(2-(methacryloyloxy)ethyl)-*N,N*-dimethyl-2-oxoethan-1-aminium)-(etofylline-methacrylate)]; P(*t*-Bu-DMEA-Caf10%)MA DP30. The copolymer was prepared similarly to P(*t*-Bu-DMEA-Caf30%)MA DP30 varying the equivalent ratio of *t*-

Bu-DMEA-MA and CafMA. Upon isolation, the copolymer was obtained as a yellowish powder in a 71% yield. $M_n = 6.3$ kg/mol $D = 1.06$.

Synthesis of Poly(2-(*tert*-butoxy)-*N*-(2-(methacryloyloxy)ethyl)-*N,N*-dimethyl-2-oxoethan-1-aminium); P(*t*-Bu-DMEA)MA DP30. The copolymer was prepared similarly to P(*t*-Bu-DMEA-Caf30%)MA DP30 using only *t*-Bu-DMEA-MA. Upon isolation, the copolymer was obtained as a yellowish powder in a 76% yield. $M_n = 4.7$ kg/mol $D = 1.03$.

Synthesis of Poly[(2-((2-(methacryloyloxy)ethyl)dimethylammonio)acetate)-(etofylline-methacrylate))]; P(DMEA-Caf30%)MA DP30. Deprotection was performed by dissolving 100 mg of polymer in 1 mL of TFA. The solution was stirred for 4 hours at 23 °C, and then the polymer was precipitated in 20 mL Et₂O, centrifuged at 4500 rpm for 5 min, reprecipitated, centrifuged, and dialyzed in water (MWCO = 3.5 kDa). The polymer was freeze-dried and obtained as a yellowish powder in a 72% yield.

Synthesis of Poly[(2-((2-(methacryloyloxy)ethyl)dimethylammonio)acetate)-(etofylline-methacrylate))]; P(DMEA-Caf10%)MA DP30. The copolymer was deprotected similarly as described for P(DMEA-Caf30%)MA DP30. Upon isolation, the copolymer was isolated as a yellowish powder in a 73% yield. $M_n = 4.2$ kg/mol $D = 1.16$.

Synthesis of Poly(2-((2-(methacryloyloxy)ethyl)dimethylammonio)acetate); P(DMEA)MA DP30. The copolymer was deprotected similarly as described for P(DMEA-Caf30%)MA DP30.

Upon isolation, the copolymer was isolated as a yellowish powder in a 74% yield. $M_n = 5.1$ kg/mol $\bar{D} = 1.18$.

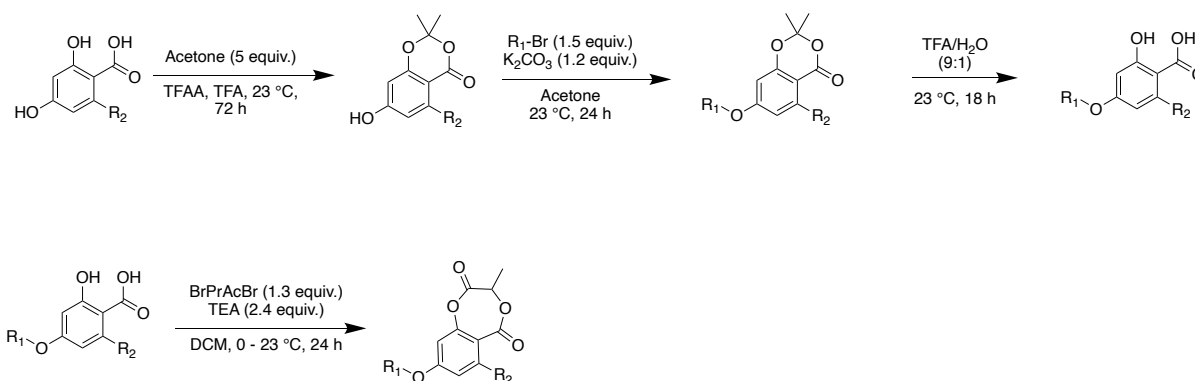
3.1.2 Preparation of hydroxybenzoate-co-lactide polymers

All chemicals and solvents in this work were purchased from Sigma Aldrich and TCI and, unless otherwise described, were used without any purification. Salicylic acid, MTBD, DCM, THF, allyl bromide, benzyl bromide, methyl iodide, 2-bromo propionyl bromide, P(LLA-*alt*-GA) were purchased from Sigma-Aldrich, P(DLLA-*r*-GA) was purchased from polysciences. Orsellinic acid was purchased from Ambeed chemicals. THF, Et₂O, and hexanes were dried over molecular sieves at least 24 hours before use. 3-O urea Catalyst was synthesized according to the previous protocol.¹⁴⁸ P(DLLA) was prepared with 3-O urea catalyst and MTBD ($M_n = 6$ kg/mol). P(DLLA-*r*-GA) (IV 0.2 dl/g) and P(LLA-*alt*-GA) ($M_n = 10$ kg/mol) were purchased from Polysciences Inc. and Sigma Aldrich, respectively. MilliQ water was obtained from a Purelab Prima (ELGA) system.

Polymer molecular weights were determined by SEC analysis using an SEC-MALS instrument, equipped with Shimazu Prominence-I LC 2030 C 3D autosampler, two MZ Analysentechnik MZ-Gel SDplus LS 5 μ m, 300 $\times$ 8 mm linear columns, Wyatt DAWN HELOS-II, and a Wyatt Optilab T-rEX. The column possesses a linear porosity of $\approx 5000\text{\AA}$ according to the vendor (<https://www.mz-at.de/en/mz5005-300080-mz-gel-sdplus-linear-5mym-300x8-0mm-sec-gpc-columns.html>). Thus, the molecular weight of polymers with $M/I < 30$ was determined based on ¹H NMR spectroscopy. The column temperature was set to 40 °C, flow rate to 0.7 mL/min, and all samples were dissolved in HPLC grade THF at 5 mg/mL. The dn/dc values, molar mass, and dispersity values were calculated from the RI vs signal by using the 100% mass

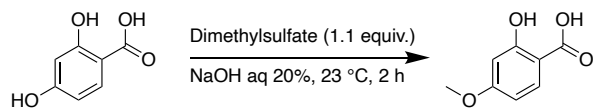
recovery method in the Astra software and a known sample concentration. High-resolution mass spectra were obtained from an Agilent LC-Q-TOF 6545 at the UCLA Molecular Instrumentation Center.

Preparation of Precursors. Most of the precursors and monomers were prepared as it is illustrated in **Scheme 3.4**. Otherwise, the synthesis is presented accordingly.



Scheme 3.4. General scheme of semi-aromatic lactide monomers (TFFA = trifluoroacetic anhydride, TFA=trifluoroacetic acid, TEA= triethylamine).

Synthesis of 2-hydroxy-4-methoxybenzoic acid, “Compound 3”. Compound 5 was prepared according to **Scheme 3.5**. In a round bottom flask 2.0 g of 2,4-dihydroxybenzoic acid (15×10^{-3} mol, 1 equiv.) were dissolved in 6 mL of 20% NaOH under argon. Then, 1.4 mL of dimethyl sulfate (16.5×10^{-3} mol, 1.1 equiv.) was added dropwise and stirred at room temperature for 2 h. The solvent was evaporated, and the powder was recrystallized in MeOH-water (2:5). The product was isolated as a white powder in 44% yield (950 mg). NMR signals are a match to previously reported.¹⁴⁹



Scheme 3.5. Preparation of 2-hydroxy-4-methoxybenzoic acid, “Compound 3”.

Synthesis of 7-hydroxy-2,2-dimethyl-4*H*-benzo[*d*][1,3]dioxin-4-one. In a round bottom flask, 6 g of 2,4-dihydroxybenzoic acid (97%, 3.78×10^{-2} mol, 1 equiv.) and 22.5 mL of trifluoroacetic anhydride were mixed, followed by the addition of 15 mL of trifluoroacetic anhydride and 15 mL of acetone (95%, 1.93×10^{-1} mol, 5 equiv.). The mixture was stirred from 0 °C to 23 °C for 72 hours. Then, the mixture was diluted in 50 mL of EtOAc, neutralized with saturated NaHCO₃ at 0 °C, extracted with EtOAc extractions 50 mL three times, dried over anhydrous MgSO₄, filtered, and concentrated in a rotatory evaporator. The product was purified by column chromatography using Biotage® (Hexane-EtOAc 20:80). The solvent was evaporated, and the product was recovered as a yellowish powder in 81% yield (5.91 g). NMR signals matched with previous work.¹⁵⁰

Synthesis of 7-(allyloxy)-2,2-dimethyl-4*H*-benzo[*d*][1,3]dioxin-4-one. In a round bottom flask, 10 g of 7-hydroxy-2,2-dimethyl-4*H*-benzo[*d*][1,3]dioxin-4-one (5.12×10^{-2} mol, 1 equiv.) was dissolved in 100 mL acetone, followed by the addition of 6.8 mL of allyl bromide (7.65×10^{-2} mol, 1.5 equiv.) and 8.54 g of potassium carbonate (99%, 6.12×10^{-2} mol, 1.2 equiv.). The reaction mixture was stirred for 24 hours at 23 °C. Then, the solvent was evaporated, resuspended with 100 mL of EtOAc, mixed with 300 mL of water, and extracted with 100 mL of EtOAc three times. The organic phase was dried over MgSO₄, filtered, and concentrated in a rotatory evaporator. The product was isolated using Biotage® (Hexane-EtAcO, 80:20), and the

solvent was evaporated. The product was recovered as a yellowish powder in a 77% yield (9.2 g).

Synthesis of 4-(allyloxy)-2-hydroxybenzoic acid, “Compound 4”. In a round bottom flask 7-(allyloxy)-2,2-dimethyl-4*H*-benzo[*d*][1,3]dioxin-4-one (9.2 g, 3.2×10^{-2} mol) and 72 mL of TFA and 8 mL of deionized water, the mixture was stirred for 18 hours at 23 °C. Then the product was precipitated in 150 mL of deionized water and the solid was filtered and washed with water. The product was freeze-dried and recovered as a white powder in an 83% yield (6.3 g).

Synthesis of 7-(benzyloxy)-2,2-dimethyl-4*H*-benzo[*d*][1,3]dioxin-4-one. In a round bottom flask, 10 g of 7-hydroxy-2,2-dimethyl-4*H*-benzo[*d*][1,3]dioxin-4-one (5.1×10^{-2} mol, 1 equiv.) was dissolved in 100 mL of acetone, followed by the addition of 9.36 mL of benzyl bromide (97%, 7.65×10^{-2} mol, 1.5 equiv.), and 8.54 g of potassium carbonate (99%, 6.12×10^{-2} mol, 1.2 equiv.). The reaction mixture was stirred for 24 hours at 23 °C. Then, the solvent was evaporated, resuspended with 100 mL of EtOAc, mixed with 300 mL of water, and extracted with 100 mL of EtOAc three times. The organic phase was dried over MgSO₄, filtered, and concentrated in a rotatory evaporator. The product was isolated using Biotage® (Hexane-EtAcO, 80:20), the solvent was evaporated and recovered as a yellowish powder in a 69% yield (10.0 g).

Synthesis of 4-(benzyloxy)-2-hydroxybenzoic acid, “Compound 5”. In a round bottom flask 3 g of 7-(benzyloxy)-2,2-dimethyl-4*H*-benzo[*d*][1,3]dioxin-4-one was mixed with 25 mL of TFA and 5 mL of deionized water, the mixture was stirred for 18 hours at 23 °C. Then the product was precipitated in 150 mL of deionized water and the solid was filtered and washed with water.

The product was freeze-dried and recovered as a yellowish powder in 87% yield (2.2 g). NMR signals matched with previous work.¹⁵¹

Synthesis of 2-hydroxy-4-methoxy-6-methylbenzoic acid, “Compound 7”. The product was prepared following the protocol for **Compound 3** using 2,4-dihydroxy-6-methylbenzoic acid (1.5 g, 8.9×10^{-3} mol, 1 equiv.) and dimethyl sulfate (1.6 mL, 11.1×10^{-3} mol, 1.1 equiv.). The product was isolated as a white powder in 36% yield (580 mg). NMR signals are similar to previously reported.¹⁵²

Biosynthesis of 2-heptyl-4,6-dihydroxybenzoic acid, “Compound 8”. 2-Heptyl-4,6-dihydroxybenzoic acid, **Compound 8**, was purified from large-scale cultures of the model fungus *Aspergillus nidulans* A1145 Δ ST Δ EM heterologously expressing Ma_OvaA-C on episomal vectors as previously described.^{138,153} Briefly, 5 L of liquid CD-ST medium were inoculated with 1×10^4 spores/mL and cultured at 28 °C with shaking (250 rpm). Following 5 days of growth in CD-ST, the cultures were filtered, and the media extensively extracted with EtOAc which were concentrated under reduced pressure. The dried residue was subjected to purification by RediSep Rf Gold reversed-phase C18 column on a Teledyne Combi-Flash system using a linear gradient of 30-100% MeOH-H₂O with 0.1% formic acid in 45 min at 60 min/mL. The collected fractions were analyzed by LC-MS using a Shimadzu 2020 EV LC-MS (Phenomenex Kinetex, 1.7 μ m, 2.0 x 100 mm, C18 column) using positive- and negative-mode electrospray ionization with a linear gradient of 5–95% CH₃CN-H₂O with 0.1% formic acid (v/v) in 15 min followed by 95% CH₃CN for 3 min with a flow rate of 0.3 mL/min. Pure

fractions as determined by LC-MS were pooled and concentrated giving 200 mg of **Compound 8** as a white amorphous solid.

Synthesis of 2-heptyl-6-hydroxy-4-methoxybenzoic acid, “Compound 9”. The product was prepared following the protocol for **Compound 3** using 2-heptyl-4,6-dihydroxybenzoic acid (50 mg, 1.75×10^{-4} mol, 1 equiv.) and dimethyl sulfate (18.6 μ L, 1.92×10^{-4} mol, 1.1 equiv.). The product was isolated as a white creamy powder in 45% yield (20 mg).

Synthesis of 5-heptyl-7-hydroxy-2,2-dimethyl-4H-benzo[d][1,3]dioxin-4-one. The product was prepared following a similar protocol to prepare **Compound 1** using: 214 mg of 2-heptyl-4,6-dihydroxybenzoic acid (8.23×10^{-4} mol). Upon isolation, the product was recovered as a yellow viscous oil in a 65% yield (200 mg, calculated yield takes into account a deprotected form that was observed as an impurity by ^1H NMR).

Synthesis of 7-(benzyloxy)-5-heptyl-2,2-dimethyl-4H-benzo[d][1,3]dioxin-4-one]. The product was prepared following a similar protocol for **Compound 2**, using: 200 mg of 5-heptyl-7-hydroxy-2,2-dimethyl-4H-benzo[d][1,3]dioxin-4-one (6.77×10^{-4} mol, 1 equiv.), 124.4 μ L of benzyl bromide (97%, 1.02×10^{-3} mol, 1.5 equiv.) and 113.4 mg of potassium carbonate (8.13×10^{-4} mol, 1.2 equiv.). The product was recovered as a yellow viscous oil in a 48% yield (124 mg).

Synthesis of 4-(benzyloxy)-2-heptyl-6-hydroxybenzoic acid, “Compound 10”. The product was prepared following a similar protocol for **Compound 2**: 124 mg of 7-(benzyloxy)-5-heptyl-2,2-dimethyl-4H-benzo[d][1,3]dioxin-4-one (3.21×10^{-4} mol), 1.1 mL TFA and 0.14 mL of

deionized water. Upon isolation, the product was recovered as a red viscous oil in an 87% yield (96 mg).

Monomer preparation.

Synthesis of 8-(benzyloxy)-3-methyl-5*H*-benzo[*e*][1,4]dioxepine-2,5(3*H*)-dione, “Compound 13”. In a round bottom flask, 7.4 g of **5** (3.0×10^{-2} mol, 1 equiv.) were dissolved in 100 mL of anhydrous acetonitrile, followed by the addition of 4.74 mL of dry triethylamine (3.3×10^{-2} mol, 1.1 equiv.), cooled down to 0 °C and then slowly added 4.29 mL of 2-bromopropionyl bromide 95% (3.9×10^{-2} mol, 1.3 equiv.). The mixture was stirred at 0 °C for 2 hours, and then 5.6 mL of dry triethylamine (3.9×10^{-2} mol, 1.3 equiv.) were added. The mixture was stirred from 0 °C to 23 °C for 24 hours, then the solvent was evaporated in a rotatory evaporator, resuspended in 100 mL of EtOAc and 300 mL of water (keeping the pH = 5 using HCl 1 M), followed by three extractions of EtOAc. The organic phase was dried over MgSO₄, filtered, and evaporated in a rotatory evaporator. The solid was recrystallized using DCM and hexane (1:10). The fine yellowish crystals were recovered in a 49% yield (4.4 g).

Synthesis of 8-methoxy-3-methyl-5*H*-benzo[*e*][1,4]dioxepine-2,5(3*H*)-dione, “Compound 11”. The product was prepared with the same protocol of **Compound 13** using: 1.3 g of **Compound 3** (7.65×10^{-3} mol, 1 equiv.), 1.09 mL of bromopropionyl bromide (9.95×10^{-3} mol, 1.3 equiv.) and 2.64 mL of TEA (1.84×10^{-2} mol, 2.4 equiv.). Upon isolation, the product was recovered as a white powder in a 65% yield (1.1 g).

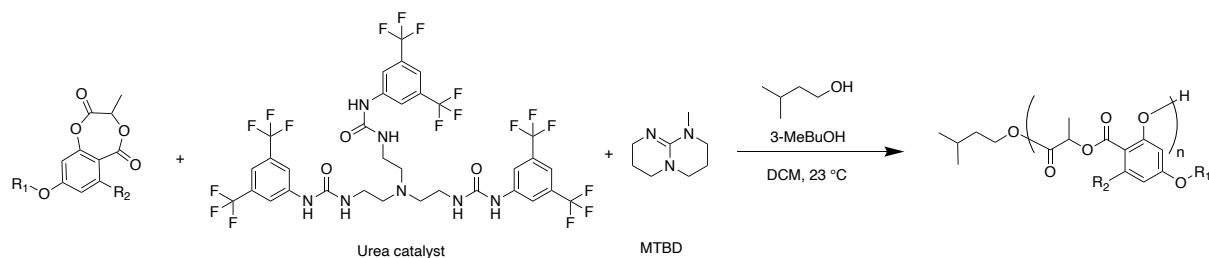
Synthesis of 8-(allyloxy)-3-methyl-5*H*-benzo[*e*][1,4]dioxepine-2,5(3*H*)-dione, “Compound 12”. The product was prepared with the same protocol of **Compound 13** using: 6.8 g of **Compound 4** (2.76×10^{-2} mol, 1 equiv.), 3.95 mL of bromopropionyl bromide (3.58×10^{-2} mol, 1.3 equiv.) and 4.36 mL + 5.15 mL of TEA (6.6×10^{-2} mol, 2.4 equiv.). Upon isolation, the product was recovered as a yellowish powder in a 73% yield (5.0 g).

Synthesis of 8-methoxy-3,6-dimethyl-5*H*-benzo[*e*][1,4]dioxepine-2,5(3*H*)-dione, “Compound 14”. The product was prepared with the same protocol of **Compound 13** using: 0.6 g of **Compound 7** (3.26×10^{-3} mol, 1 equiv.), 516 μ L of bromopropionyl bromide (4.24×10^{-3} mol, 1.3 equiv.) and 1.13 mL of TEA (7.83×10^{-2} mol, 2.4 equiv.). Upon isolation, the product was recovered as a white powder in a 37% yield (284 mg).

Synthesis of 8-(benzyloxy)-6-heptyl-3-methyl-5*H*-benzo[*e*][1,4]dioxepine-2,5(3*H*)-dione, “Compound 15”. The product was prepared with the same protocol of **Compound 13** using: 100 mg of **Compound 10** (2.89×10^{-4} mol, 1 equiv.), 41.4 μ L of bromopropionyl bromide (3.76×10^{-4} mol, 1.3 equiv.) and 99.8 μ L of TEA (6.94×10^{-4} mol, 2.4 equiv.). The product was isolated by evaporation of the solvent, redissolution with 6 mL of dried THF, filtration under anhydrous conditions, and evaporation. The product contained TEA salt and it was used as it during the ROP. The resultant product was a viscous yellowish liquid in a 17% yield (20 mg, the yield takes into account the TEA salt).

Polymer preparation by ring-opening polymerization (ROP).

Synthesis of poly(1-oxopropan-2-yl 4-(benzyloxy)-2-hydroxybenzoate), [DP 50]. The polymers were prepared as depicted in **Scheme 3.6** and as follows: In a scintillation vial, and using a glovebox under N₂ atmosphere, the **Compound 13** (50 mg, 1.68×10^{-5} mol, 50 equiv.) was weighted and 250 μ L of DCM were added. Separately, a solution was prepared using 16.7 μ L of 3-methylbutan-1-ol(3-MeBuOH) 0.2M in THF (3.35×10^{-6} mol, 1 equiv.), 16.7 μ L of 3-O urea catalyst (1,1',1''-(nitrilotris(ethane-2,1-diyl))tris(3-(3,5-bis(trifluoromethyl)phenyl)urea)) 0.2M in THF (3.35×10^{-6} mol, 1 equiv.) and 16.7 μ L of MTBD (1-methyl-2,3,4,6,7,8-hexahydro-1H-pyrimido[1,2-a]pyrimidine) 0.2M in THF (3.35×10^{-6} mol, 1 equiv.). The monomer-catalyst solution was added quickly to the monomer one and after 30 min of reaction, the polymer was precipitated in hexanes, dried under vacuum for 24 hours, and obtained as a white powder in 75% yield (37.5 mg). SEC in THF: $M_n = 16.6$ kg/mol, $D = 1.14$. The DP or M/I was calculated by correlating the intensity of the signal CH of the polymer backbone at 5.51 ppm with the intensity of the 2CH₃ initiator signal at 0.89 ppm. DP = 66.



Scheme 3.6. Ring-opening polymerization of the monomers using 3-O urea catalyst, MTBD and 3-MeBuOH as initiator.

Synthesis of poly(1-oxopropan-2-yl 2-hydroxy-4-methoxybenzoate), “Polymer 16” [DP5].

The polymer was prepared following similar methodology as described above using: **Compound 11** (31.1 mg, 1.4×10^{-4} mol, 5 equiv.), 3-MeBuOH (28 μ L 1M, 2.8×10^{-5} mol, 1 equiv.), 3-O urea

catalyst (25.5 mg, 2.8×10^{-5} mol, 1 equiv.) and MTBD (28 μ L 1M, 2.8×10^{-5} mol, 1 equiv.). After silica column purification (hexanes - ethyl acetate gradient 10-100%), the polymer was recovered as a white powder in 84% yield (26 mg). The DP or M/I was calculated by correlating the intensity of the signal CH of the polymer backbone at 5.51 ppm with the intensity of the 2CH_3 initiator signal at 0.86 ppm. Calculated DP = 5.

Synthesis of poly(1-oxopropan-2-yl 2-hydroxy-4-methoxybenzoate), “Polymer 17” [DP15].

The polymer was prepared following similar methodology as described above using: **Compound 11** (31.1 mg, 1.4×10^{-4} mol, 15 equiv.), 3-MeBuOH (9.3 μ L 1M, 9.3×10^{-6} mol, 1 equiv.), 3-O urea catalyst (8.5 mg, 9.3×10^{-6} mol, 1 equiv.) and MTBD (9.3 μ L 1M, 9.3×10^{-6} mol, 1 equiv.). After silica column purification (hexanes - ethyl acetate gradient 10-100%), the polymer was recovered as a white powder in 91% yield (28.2 mg). The DP or M/I was calculated by correlating the intensity of the signal CH of the polymer backbone at 5.51 ppm with the intensity of the 2CH_3 initiator signal at 0.86 ppm. Calculated DP = 13.

Synthesis of poly(1-oxopropan-2-yl 2-hydroxy-4-methoxybenzoate), “Polymer 18” [DP30].

The polymer was prepared following similar methodology as described above using: **Compound 11** (31.1 mg, 1.4×10^{-4} mol, 30 equiv.), 3-MeBuOH (4.6 μ L 1M, 4.6×10^{-6} mol, 1 equiv.), 3-O urea catalyst (4.2 mg, 4.6×10^{-6} mol, 1 equiv.) and MTBD (4.6 μ L 1M, 4.6×10^{-6} mol, 1 equiv.). After silica column purification (hexanes - ethyl acetate gradient 10-100%), the polymer was recovered as a white powder in 84% yield (26.2 mg). The DP or M/I was calculated by correlating the intensity of the signal CH of the polymer backbone at 5.51 ppm with the intensity of the 2CH_3 initiator signal at 0.86 ppm. Calculated DP = 30.

Synthesis of poly(1-oxopropan-2-yl 4-(allyloxy)-2-hydroxybenzoate), “Polymer 19” [DP5].

The polymer was prepared following similar methodology as described above using: **Compound 12** (34.7 mg, 1.4×10^{-4} mol, 5 equiv.), 3-MeBuOH (28 μ L 1M, 2.8×10^{-5} mol, 1 equiv.), 3-O urea catalyst (25.5 mg, 2.8×10^{-5} mol, 1 equiv.) and MTBD (28 μ L 1M, 2.8×10^{-5} mol, 1 equiv.). After silica column purification (hexanes - ethyl acetate gradient 10-100%), the polymer was recovered as a white powder in 59% yield (20.4 mg). The DP or M/I was calculated by correlating the intensity of the signal CH of the polymer at 6.11 – 5.90 ppm with the intensity of the 2CH₃ initiator signal at 0.86 ppm. Calculated DP = 5.

Synthesis of poly(1-oxopropan-2-yl 4-(allyloxy)-2-hydroxybenzoate), “Polymer 20” [DP15].

The polymer was prepared following similar methodology as described above using: **Compound 12** (34.7 mg, 1.4×10^{-4} mol, 15 equiv.), 3-MeBuOH (9.3 μ L 1M, 9.3×10^{-6} mol, 1 equiv.), 3-O urea catalyst (8.5 mg, 9.3×10^{-6} mol, 1 equiv.) and MTBD (9.3 μ L 1M, 9.3×10^{-6} mol, 1 equiv.). After silica column purification (hexanes - ethyl acetate gradient 10-100%), the polymer was recovered as a white powder in 81% yield (28 mg). The DP or M/I was calculated by correlating the intensity of the signal CH of the polymer at 6.11 – 5.90 ppm with the intensity of the 2CH₃ initiator signal at 0.86 ppm. Calculated DP = 11.

Synthesis of poly(1-oxopropan-2-yl 4-(allyloxy)-2-hydroxybenzoate), “Polymer 21” [DP30].

The polymer was prepared following similar methodology as described above using: **Compound 12** (34.7 mg, 1.4×10^{-4} mol, 30 equiv.), 3-MeBuOH (4.6 μ L 1M, 4.6×10^{-6} mol, 1 equiv.), 3-O urea catalyst (4.2 mg, 4.6×10^{-6} mol, 1 equiv.) and MTBD (4.6 μ L 1M, 4.6×10^{-6} mol, 1 equiv.). After

silica column purification (hexanes - ethyl acetate gradient 10-100%), the polymer was recovered as a white powder in 58% yield (20.0 mg). The DP or M/I was calculated by correlating the intensity of the signal CH of the polymer at 6.11 – 5.90 ppm with the intensity of the 2CH₃ initiator signal at 0.86 ppm. Calculated DP = 22.

Synthesis of poly(1-oxopropan-2-yl 4-(benzyloxy)-2-hydroxybenzoate), “Polymer 22”

[DP5]. The polymer was prepared following similar methodology as described above using: **Compound 13** (41.7 mg, 1.4×10^{-4} mol, 5 equiv.), 3-MeBuOH (28 μ L 1M, 2.8×10^{-5} mol, 1 equiv.), 3-O urea catalyst (25.5 mg, 2.8×10^{-5} mol, 1 equiv.) and MTBD (28 μ L 1M, 2.8×10^{-5} mol, 1 equiv.). After silica column purification (hexanes - ethyl acetate gradient 10-100%), the polymer was recovered as a white powder in 61% yield (25.4 mg). The DP or M/I was calculated by correlating the intensity of the signal CH of the polymer at 5.72 – 5.40 ppm with the intensity of the 2CH₃ initiator signal at 0.86 ppm. Calculated DP = 5.

Synthesis of poly(1-oxopropan-2-yl 4-(benzyloxy)-2-hydroxybenzoate), “Polymer 23”

[DP15]. The polymer was prepared following similar methodology as described above using: **Compound 13** (41.7 mg, 1.4×10^{-4} mol, 15 equiv.), 3-MeBuOH (9.3 μ L 1M, 9.3×10^{-6} mol, 1 equiv.), 3-O urea catalyst (8.5 mg, 9.3×10^{-6} mol, 1 equiv.) and MTBD (9.3 μ L 1M, 9.3×10^{-6} mol, 1 equiv.). After silica column purification (hexanes - ethyl acetate gradient 10-100%), the polymer was recovered as a white powder in 49% yield (20.6 mg). The DP or M/I was calculated by correlating the intensity of the signal CH of the polymer at 5.72 – 5.40 ppm with the intensity of the 2CH₃ initiator signal at 0.86 ppm. Calculated DP = 10.

Synthesis of poly(1-oxopropan-2-yl 4-(benzyloxy)-2-hydroxybenzoate), “Polymer 24”

[DP30]. The polymer was prepared following similar methodology as described above using:

Compound 13 (41.7 mg, 1.4×10^{-4} mol, 30 equiv.), 3-MeBuOH (4.6 μ L 1M, 4.6×10^{-6} mol, 1 equiv.), 3-O urea catalyst (4.2 mg, 4.6×10^{-6} mol, 1 equiv.) and MTBD (4.6 μ L 1M, 4.6×10^{-6} mol, 1 equiv.). After silica column purification (hexanes - ethyl acetate gradient 10-100%), the polymer was recovered as a white powder in 89% yield (37 mg). The DP or M/I was calculated by correlating the intensity of the signal CH of the polymer at 5.72 – 5.40 ppm with the intensity of the 2CH₃ initiator signal at 0.86 ppm. Calculated DP = 30.

Synthesis of poly(1-oxopropan-2-yl 2-hydroxy-4-methoxy-6-methylbenzoate), “Polymer 25”

[DP5]. The polymer was prepared following similar methodology as described above using:

Compound 14 (33.1 mg, 1.4×10^{-4} mol, 5 equiv.), 3-MeBuOH (28 μ L 1M, 2.8×10^{-5} mol, 1 equiv.), 3-O urea catalyst (25.5 mg, 2.8×10^{-5} mol, 1 equiv.) and MTBD (28 μ L 1M, 2.8×10^{-5} mol, 1 equiv). After silica column purification (hexanes - ethyl acetate gradient 10-100%), the polymer was recovered as a white powder in 53% yield (17.4 mg). The DP or M/I was calculated by correlating the intensity of the signal CH of the polymer at 5.63 – 5.12 ppm with the intensity of the 2CH₃ initiator signal at 0.86 ppm. Calculated DP = 7.

Synthesis of poly(1-oxopropan-2-yl 2-hydroxy-4-methoxy-6-methylbenzoate), “Polymer 26”

[DP15]. The polymer was prepared following similar methodology as described above using:

Compound 14 (33.1 mg, 1.4×10^{-4} mol, 15 equiv.), 3-MeBuOH (9.3 μ L 1M, 9.3×10^{-6} mol, 1 equiv.), 3-O urea catalyst (8.5 mg, 9.3×10^{-6} mol, 1 equiv.) and MTBD (9.3 μ L 1M, 9.3×10^{-6} mol, 1 equiv.). After silica column purification (hexanes - ethyl acetate gradient 10-100%), the

polymer was recovered as a white powder in 44% yield (14.5 mg). The DP or M/I was calculated by correlating the intensity of the signal CH of the polymer at 5.63 – 5.12 ppm with the intensity of the 2CH₃ initiator signal at 0.86 ppm. Calculated DP = 12.

Synthesis of poly(1-oxopropan-2-yl 2-hydroxy-4-methoxy-6-methylbenzoate), “Polymer 27”

[DP30]. The polymer was prepared following similar methodology as described above using:

Compound 14 (33.1 mg, 1.4×10^{-4} mol, 30 equiv.), 3-MeBuOH (4.6 μ L 1M, 4.6×10^{-6} mol, 1 equiv.), 3-O urea catalyst (4.2 mg, 4.6×10^{-6} mol, 1 equiv.) and MTBD (4.6 μ L 1M, 4.6×10^{-6} mol, 1 equiv.). After silica column purification (hexanes - ethyl acetate gradient 10-100%), the polymer was recovered as a white powder in 78% yield (25.9 mg). The DP or M/I was calculated by correlating the intensity of the signal CH of the polymer at 5.63 – 5.12 ppm with the intensity of the 2CH₃ initiator signal at 0.86 ppm. Calculated DP = 28.

Synthesis of poly(1-oxopropan-2-yl 2-hydroxy-4-benzyloxy-6-heptylbenzoate), “Polymer 28”

[DP5]. The polymer was prepared following similar methodology as described above using:

Compound 14 (15.8 mg, 4.0×10^{-5} mol, 5 equiv.), 3-MeBuOH (80 μ L 0.1M, 8.0×10^{-6} mol, 1 equiv.), 3-O urea catalyst (80 μ L 0.1M, 8.0×10^{-6} mol, 1 equiv.) and MTBD (80 μ L 0.1M, 8.0×10^{-6} mol, 1 equiv.). After silica column purification (hexanes - ethyl acetate gradient 10-100%), the polymer was recovered as a white powder in 63% yield (10 mg). The DP or M/I was calculated by correlating the intensity of the signal CH of the polymer at 5.37 – 5.20 ppm with the intensity of the 2CH₃ initiator signal at 0.86 ppm. Calculated DP = 6.

3.1.3 Preparation of polylysine

Tetrahydrofuran (THF) and hexanes were each degassed with dinitrogen and passed through an alumina column before use. In-house deionized water (DI H₂O) was used for all aqueous chemistry, sample preparation and dialysis unless otherwise specified. NiBpyCOD initiator, α -methoxy- ω -isocyanatoethyl-poly(ethylene glycol), and mPEG-NCO (number-average molecular weight M_n = 1 kDa) were prepared according to published procedures.^{154,155} All other reagents and solvents were used as received. NCA purifications and polymerizations were performed in an N₂-filled glovebox, unless otherwise specified.

Monomer preparation. N ϵ -carbobenzyloxy-L-lysine N-carboxyanhydride (ZK NCA) was synthesized from N ϵ -carbobenzyloxy-L-lysine following published procedures (**Scheme 3.3**).¹⁵⁵ Under N₂, N ϵ -carbobenzyloxy-L-lysine amino acid (5.0 g, 1 equiv) was suspended in 100 mL THF. Triphosgene (2.64 g, 0.5 equiv) was quickly added and the reaction vessel sealed.

WARNING: Triphosgene is an extremely dangerous chemical, and proper precautions must be taken to avoid exposure. The reaction vessel was heated to 50 °C and stirred for 3 h, after which the reaction vessel was removed from heat, allowed to cool, and all solvent was removed under vacuum. The crude ZK NCA was transferred to an N₂-filled glovebox and crystallized 3 times by dissolving the crude material in minimal THF and suspending it under 3x (v/v) hexanes. The final product was obtained as white fluffy crystals (Yield: 4.8 g, 88%).

Polymer preparation. Poly(N ϵ -carbobenzyloxy-L-lysine) (polyZK) was synthesized using NiBpyCOD initiator following published protocols (**Scheme 3.3**).^{154,156} Samples of polyZK were prepared at *ca.* 500 mg scale in a N₂ filled glove box. NiBpyCOD initiator solution was quickly

added to a solution of ZK NCA (10 mL, 50 mg/mL in DMF). To synthesize polyZK₄₈, polyZK₉₀, and polyZK₁₇₀, 2.64 mL, 0.84 mL, or 105 μ L of NiBpyCOD were added, respectively. After *ca.* 120 min, complete consumption of ZK NCA was confirmed by FTIR spectroscopy.

In order to determine polypeptide chain lengths, a small aliquot of the polymerization reaction mixture (*ca.* 200 μ L) was removed for end-group analysis. Active chain-ends were reacted with a solution of mPEG-NCO (M_n = 1000 Da, 50 mg/mL in THF, 4 eq per NiBpyCOD) in an N₂-filled glove box overnight. The following day, the sample was removed from the glove box, and the polypeptide was precipitated by addition to 0.1 M HCl. The sample was centrifuged at 3000 rpm, and the supernatant was discarded. The pellet was washed 3 times with DI H₂O and centrifuged to remove unconjugated mPEG-NCO, and the resulting pellet was then lyophilized to yield the PEG-polypeptide conjugate as a white solid (typical yields = 90 to 95%). To determine the number-average molecular weight (M_n) of the polypeptide, a ¹H NMR spectrum was obtained in deuterated trifluoroacetic acid (TFA-d).^{156,157}

The remainder of the reaction mixture was precipitated by addition to 0.1 M HCl (50 mL) and isolated by centrifugation. The solid was then washed two additional times with DI H₂O and dried under reduced pressure to yield polyZK as a white powder. polyZK was deprotected to give α -poly(L-lysine)-NH₂ (polyK) following published protocols (**Scheme 3.3**).¹⁵⁸ Briefly, polyZK was dissolved in TFA (50 mg/mL) and cooled to 4 °C in an ice bath. 33 wt% HBr in acetic acid was added (1 mL per 100 mg of polyZK) and the reaction was allowed to stir for 1 hr. PolyK was precipitated with diethyl ether, decanted, and washed with diethyl ether 2x before redissolving the polyK in 0.01 M HCl and transferred to regenerated cellulose tubing (Spectrum Laboratories) with 2000 Da molecular weight cutoffs (MWCO). The solutions were dialyzed against 150 mM NaCl in pH 5 DI H₂O (4 dialyzate changes, 48 hours) followed by DI H₂O

(2 dialyzate changes, 24 hours) and then lyophilized to yield a white fluffy powder (Typical yield: 79%).

3.1.4 Antimicrobial assays

Minimum Inhibitory Concentration (MIC). A standard broth-microdilution protocol was used to determine the minimum inhibitory concentration based on Clinical and Laboratory Standards Institute (CLSI) recommendations.¹⁵⁹

Caffeine-based copolymers were prepared as follows: An amount of 50 μ L of sterile Mueller Hinton Broth was added to every well in a 96-well plate, except for the last column, where 100 μ L was added. Copolymers and antibiotics were resuspended in MilliQ water to a concentration four times greater than the highest amount to be tested. 50 μ L of the copolymers or antibiotics to be assayed was added to the first column, then two-fold serial dilutions were carried out across the next nine wells in the row.

2-hydroxybenzoic acid-based precursors and hydroxybenzoate-co-lactide polymers were prepared as followed: Small molecules were resuspended in DMSO to a concentration of 20,000 μ g/mL. A series of 10 two-fold dilutions in DMSO were prepared. Each serial dilution was then diluted ten-fold in Mueller Hinton broth, 50 μ L of which was added to a 96-well plate.

Ampicillin, at a concentration of 100 mg/mL in MilliQ water, was diluted to a concentration of 1 mg/mL in Mueller Hinton broth. A series of ten two-fold dilutions in Mueller Hinton broth were prepared, 50 μ L of which was added to a 96-well plate. The same protocol was repeated for the polymers by dissolving in DMSO to a concentration of 20,000 μ g/mL using six two-fold serial dilutions instead of ten. All MIC assays were performed in triplicate with two replicates of each polymer/small molecule concentration per 96-well plate.

PolyK was prepared as followed: Lyophilized polyK was resuspended in Mueller Hinton Broth (MHB) and two-fold serial dilutions in MHB were prepared. 50 μ L of each dilution was added to a 96-well round bottom plate. Assays were performed in duplicate.

For all polymers, single colonies were picked and resuspended in MHB from cultures grown overnight on Mueller Hinton agar at 37 °C. The OD₆₂₅ was measured and adjusted between 0.08 and 0.13, corresponding to the optical density of McFarland standard 0.5, then diluted 100-fold. 50 μ L of bacterial suspension was added to each well containing diluted antimicrobial, resulting in approximately 5×10^5 CFU/mL per well.¹⁵⁹ A growth control, consisting of 50 μ L of bacterial suspension and 50 μ L of broth, and sterility control, consisting of 100 μ L broth only, were also prepared. For the hydroxybenzoate polymers, ten-fold dilutions of the growth control in 1x PBS were prepared and plated on Mueller Hinton Agar. Covered plates were incubated at 37 °C without shaking.

After 24 hours, the MIC was determined as the lowest concentration with no visible growth. For hydroxybenzoate polymers, each well was plated using the same dilution that provided a countable number of colonies for the growth control. MIC was confirmed the following day as the concentration with no more than one standard deviation greater than the average number of colonies of the growth control.

Minimum Bactericidal Concentration (MBC). To determine the MBC of polyK, 50 μ L from each well of the MIC assay with no visible growth was plated on Mueller Hinton agar and incubated overnight at 37 °C. The MBC was determined as the lowest concentration of treatment yielding fewer than 25 colonies per plate, corresponding to survival of $\leq 0.1\%$ of the original inoculum.

Biofilm. Quantification of *S. aureus* biofilm inhibition and disruption by 2-hydroxybenzoic acid-based precursors and hydroxybenzoate-co-lactide polymers was performed as previously reported, with the following modifications.^{160,161}

S. aureus isolates were grown overnight at 37 °C on a brain heart infusion (BHI) agar plate. A single colony from the BHI agar plate was cultured in BHI broth at 37 °C for 24 hours to establish the primary culture. Following incubation, a secondary culture was prepared by adding 100 µL of the primary culture to 50 mL of fresh BHI broth and incubating at 37 °C until the optical density at 650 nm (OD₆₅₀) reached 0.8 – 1. The culture was then diluted in BHI broth supplemented with 1% glucose and 1% sucrose to an OD of 0.1 (~10⁸ CFU/mL), and 200 µL was added to each well. For biofilm inhibition studies, polymers and small molecules at varying concentrations (MIC concentration, half the MIC concentration and double the MIC concentration) were added to the wells along with the bacterial culture and incubated for 72 hours.

For biofilm disruption studies, *S. aureus* was cultured under stationary conditions for 34 hours in a 96-well plate to form biofilms. After 34 hours (once the biofilm was formed), each well was treated with different concentrations of the synthesized polymers and small molecules (MIC concentration, half the MIC concentration and double the MIC concentration) and incubated for an additional 38 hours. After 72 hours incubation, the media was removed, and the wells were washed with PBS. The effect on biofilm inhibition and disruption was observed by staining the well with crystal violet dye (1%), which binds the extracellular polymeric substance (EPS) of the biofilm. Biofilm inhibition and disruption were quantified by diluting the adherent dye with 70% ethanol and measuring the absorbance at 650 nm.

3.2 Results

3.2.1 Methacrylate copolymers with caffeine and cationic or zwitterionic side chains.

Preparation of copolymers bearing caffeine and cationic or zwitterionic side chains.

To investigate the antibacterial properties of caffeine copolymers with hydrophilic charged groups, we prepared libraries of random copolymers through free-radical polymerization. Caffeine polymer derivatives are known to be accessible by free radical polymerization through methacrylate functionalization at the 8-position¹⁶² or 7-position¹⁶³ of the caffeine heterocycle or by polymerization of lipoylated caffeine.¹⁶⁴ In the current study, we used etofylline, a derivative of caffeine bearing an ethyl alcohol side chain at the 7-position and functionalized with a methacrylate (Caf-MA) by nucleophilic substitution of methacryloyl chloride in a 56% yield (**Figures S3.1 and S3.2**). Free-radical homopolymerization of Caf-MA (30 equiv) was performed using azobis(isobutyronitrile) (AIBN, 1 equiv) in dimethyl sulfoxide (DMSO) under argon at 75 °C. After 100 min, the polymer poly(etofylline methacrylate) (P(Caf-MA)) was isolated by precipitation in ethyl acetate and characterized by ¹H NMR and size exclusion chromatography (SEC) in trifluoroethanol (TFE), showing a molecular weight by number $M_n = 25.4$ kg/mol and molecular weight dispersity $\bar{D} = 2.77$ (**Figures S3.3 and S3.4**). The insolubility in phosphate buffer saline (PBS) at 1 mg/mL, and the calculated logP of monomer and small oligomers, demonstrated its hydrophobic character (**Figure S3.5**).

It is known that the cationic-hydrophobic ratio as well as the nature of these side chains are important parameters during the preparation of polymers to display antimicrobial activity.^{111,112} Therefore, we prepared copolymer libraries of P(Caf-MA) through a similar methodology, varying the caffeine content from 50% to 0% and using monomers with hydrophilic side chains. To obtain copolymers with cationic side chains and caffeine, the

monomer tert-butyl (4-methacrylamidobutyl) carbamate (Boc-ab-MA) was prepared by nucleophilic substitution of methacryloyl chloride and tert-butyl(4-aminobutyl) carbamate in a 65% yield (**Figure S3.6 and S3.7**). Then, free-radical polymerization was performed in dimethylformamide (DMF) under inert atmosphere conditions at 75 °C using AIBN and varying the content of CafMA from 50 to 0%. The library of P-(Boc-ab-Caf)MA was successfully prepared in a 67–73% yield and the synthesized polymers were characterized by ^1H NMR and SEC in DMF (**Figures S3.8–S3.12**).

The caffeine content was determined using the signal corresponding to the proton in the heterocyclic moiety (signal *a*, 8.05 ppm) and the protons of the CH₃-group (signal *h, h'*, 0.49 ppm) as illustrated for the copolymer bearing N-Boc aminobutyl and caffeine side chains having a caffeine content of 50% (P-(Boc-ab-Caf 50%)MA, **Figure S3.8**). The caffeine content of the copolymers corroborated the theoretical values, according to the ^1H NMR analyses and had $M_n = 21.8\text{--}27.7$ kg/mol with a polymer dispersity of $\text{Đ} = 1.50\text{--}1.75$ (**Table 3.1, Figures S3.8 and S3.13**). A shoulder peak was observed at a lower retention time in the SEC that we attributed to aggregation under the analytic conditions. This interpretation was further confirmed by the light scattering signal from SEC in DMF (**Figure S3.14**).

Further, N-Boc deprotection using trifluoroacetic acid (TFA) permitted isolation of the P-(ab-Caf)MA copolymers bearing caffeine and primary ammonium side chains in 69–81% yields. The characterization by SEC was not performed since the polymers were not soluble in the aqueous eluent system. The caffeine content was determined using the signal corresponding to the proton in the heterocyclic moiety (signal *a*, 8.05 ppm) and the protons of the CH₃- group (signal *h, h'*, 0.49 ppm) as illustrated for the copolymer bearing aminobutyl and caffeine side chains with a 50% of caffeine content (P-(ab-Caf 50%)MA, **Figures S3.15–S3.18**). The caffeine

content was consistent with the values before deprotection showing that the deprotection did not cause side chain cleavage.

With the purpose of comparing the activity of the cationic side chain, we prepared copolymers bearing permanent cationic groups, specifically quaternary ammonium, and caffeine side chains P-(t-Bu-DMEA-Caf)MA. First, the monomer 2-(tert-butoxy)-N-(2-(methacryloyloxy)ethyl)-N,N-dimethyl-2-oxoethan-1-aminium (t-Bu-DMEA-MA) was prepared by quaternization of 2-(dimethylamino) ethyl methacrylate with tert-butyl 2-bromoacetate in acetonitrile at 50 °C for 16 hours in an 83% yield (**Figure S3.19**). Then, free radical copolymerization with AIBN in DMSO was performed using t-Bu-DMEA-MA and CafMA while varying the caffeine content from 30 to 0% under inert atmosphere conditions at 75 °C. The copolymers were isolated in 80–92% yield. The copolymers with higher content (>50%) of caffeine were not prepared because the reaction mixture became heterogeneous, and no conversion was observed by ¹H NMR. ¹H NMR and SEC were used to characterize the copolymers in water (**Figures S3.20–S3.23**). The caffeine content was determined in a similar way as P-(Boc-ab-Caf)MA and correlated with the theoretical values (**Table 3.1**), having $M_n = 32.0\text{--}21.1$ kg/mol and $\bar{D} = 2.03\text{--}2.06$. Further, *tert*-butyl ester (t-Bu) deprotection in TFA at 23 °C for 4 hours allowed us to isolate the copolymers bearing zwitterionic and caffeine side chains P-(DMEA-Caf)MA in 86–91% yields. We characterized the copolymers by ¹H NMR and SEC in water and the caffeine content was determined in a similar way as for P-(ab-Caf)MA; the values agreed with the values before deprotection, having $M_n = 16.0\text{--}26.7$ kg/mol and $\bar{D} = 2.24\text{--}3.24$ (**Table 3.1, Figures S3.24 – S3.27**).

In addition, we prepared another copolymer library bearing zwitterionic groups from amino acid derivatives and caffeine side chains, specifically P-(Lys-Caf)MA. The monomer was

prepared by nucleophilic substitution of methacryloyl chloride with N-Cbz-Lysine and further N-Boc deprotection using HBr and TFA at 23 °C for 4 hours, without isolation of the intermediate, which allowed us to obtain lysine methacrylate (LysMA) in a 30% yield (**Figure S3.28**). We attributed the low yield to autopolymerization encountered during the preparation of the intermediate. Then we prepared copolymers by free radical polymerization using AIBN in DMSO-water (1:1 v/v) under inert atmosphere conditions at 75 °C for 16 hours. We synthesized the library of copolymers in 4–39% yields and characterized the copolymers by ¹H NMR and SEC in water (**Figures S3.29 – S3.33**). We determined the caffeine content similarly to P-(ab-Caf)MA, and the caffeine content agreed with the values before deprotection having $M_n = 18.0 - 25.2$ kg/mol with a polymer dispersity of $\bar{D} = 2.24 - 2.28$ (**Table 3.1**).

Polymer	Theoretical caffeine content (%)	Caffeine content from ¹ H NMR (%)	M_n (kg/mol)	$\bar{D}$	Yield (%)
P(Caf)MA	100	100	25.4 ^a	2.77 ^a	77
P-(ab-Caf 50%)MA	55	55	27.7 ^b	1.75 ^b	69
P-(ab-Caf 30%)MA	30	34	21.8 ^b	1.74 ^b	70
P-(ab-Caf 10%)MA	10	10	25.8 ^b	1.50 ^b	81
P-(ab)MA	0	0	23.2 ^b	1.52 ^b	73
P-(t-Bu -DMEA-Caf 30%)MA ^c	30	26			80
P-(t-Bu -DMEA-Caf 10%)MA	10	8	21.1 ^d	2.03 ^d	83
P-(t-Bu-DMEA)MA	0	0	32 ^d	2.06 ^d	92
P-(DMEA-Caf 30%)MA	30	37	16 ^d	2.24 ^d	91
P-(DMEA-Caf 10%)MA	10	11	26.7 ^d	2.53 ^d	86
P-(DMEA)MA	0	0	22.7 ^d	3.24 ^d	91
P-(Lys-Caf 50%)MA ^c	55	59			4
P-(Lys-Caf 30%)MA	30	38	18 ^d	2.24 ^d	39
P-(Lys-Caf 10%)MA	10	10	17.8 ^d	2.25 ^d	38
P-(Lys)MA	0	0	25.2 ^d	2.28 ^d	17

Table 3.1. Characterization of copolymers bearing caffeine and cationic or zwitterionic side chains by ¹H-NMR, SEC, and yields. ^a Determined from SEC in trifluoroethanol. ^b Determined

from the Boc-protected polymer through SEC in DMF. ^c Polymers were not possible to analyze due to poor solubility under the conditions of analysis. ^d Determined from SEC in water.

Antimicrobial evaluation of copolymers bearing caffeine and cationic or zwitterionic side chains. Once the libraries of copolymers were prepared, their activities were tested against the gram-positive bacteria *S. aureus* (ATCC 25094, Newman strain) and the gram-negative bacteria *E. coli* (ATCC 27065). A microdilution method in a 96-well plate was used to determine the minimum inhibitory concentration (MIC), the minimum concentration where no bacterial growth was detectable.¹⁶⁵ Copolymers bearing the primary cationic group and caffeine (P-(ab-Caf)MA) did not present antimicrobial activity at 50% to 0% of caffeine content (MIC > 500 µg/mL, **Table 3.2**) against *S. aureus*. In contrast, an increase in cationic copolymers were capable of inhibiting the growth of exclusively gram-negative bacteria, which is related to the simplicity of the membranes as compared to gram-positive microorganisms.

Polymer	<i>S. aureus</i> (ATCC 25904) MIC		<i>E. coli</i> (ATCC 27065) MIC		MRSA MIC	
	($\mu\text{g/mL}$)	(μM) ^a	($\mu\text{g/mL}$)	(μM) ^a	($\mu\text{g/mL}$)	(μM) ^a
P-(ab-Caf 50%)MA	>500	>18	125	4.5	-	-
P-(ab-Caf 30%)MA	>500	>18	26 $\pm$ 2	2 $\pm$ 0.1	-	-
P-(ab-Caf 10%)MA	>500	>18	19 $\pm$ 7	7 $\pm$ 0.1	-	-
P-(ab)MA	>500	>18	18 $\pm$ 7	7 $\pm$ 0.3	-	-
P-(t-Bu-DMEA-Caf 30%)	>500	>25	125	6.3	-	-
P-(t-Bu-DMEA-Caf 10%)MA	60 $\pm$ 39	2.9 $\pm$ 1.9	39 $\pm$ 11	1.9 $\pm$ 0.5	237 $\pm$ 83 ^b 124 $\pm$ 94 ^c	11.3 $\pm$ 4.0 5.9 $\pm$ 4.5
P-(t-Bu-DMEA)MA	15 $\pm$ 7	0.5 $\pm$ 0.2	41 $\pm$ 13	1.3 $\pm$ 0.4	197 $\pm$ 108 ^b 128 $\pm$ 97 ^c	6.2 $\pm$ 3.4 4.0 $\pm$ 3.0
P-(DMEA-Caf 30%)MA	>500	-	>500	-	-	-
P-(DMEA-Caf 10%)MA	>500	-	>500	-	-	-
P-(DMEA)MA	>500	-	>500	-	-	-
P-(Lys-Caf 50%)MA	>500	-	>500	-	-	-
P-(Lys-Caf 30%)MA	>500	-	>500	-	-	-
P-(Lys-Caf 10%)MA	>500	-	>500	-	-	-
P-(Lys)MA	>500	-	>500	-	-	-
P-(t-Bu-DMEA-Caf 30%)MA DP30	>500	-	>500	-	-	-
P-(t-Bu-DMEA-Caf 10%)MA DP30	>500	-	>500	-	-	-
P-(t-Bu-DMEA)MA DP30	>500	-	>500	-	-	-
P-(DMEA-Caf 30%)MA DP30	>500	-	>500	-	-	-
P-(DMEA-Caf 10%)MA DP30	>500	-	>500	-	-	-
P-(DMEA)MA DP30	>500	-	>500	-	-	-
Ampicillin	1	2.9	15.6	44.6	625	1290
Kanamycin	2	4.1	2	4.1	19.5	56

Table 3.2. Antimicrobial evaluation of the caffeine copolymers against *S. aureus* (ATCC 25904, strain Newman), *E. coli* (ATCC 27065), and clinical isolates of methicillin-resistant *S. aureus* (MRSA) through the broth microdilution method (n=6). ^a Calculated from the molecular weight obtained from SEC. ^b and ^c Two different MRSA strains isolated from patients.

The anti-infective activity of the methacrylate copolymers series bearing caffeine and quaternary ammonium side chains (P-(t-Bu-Caf)MA) was evaluated using the same methodology (**Table 3.2**). The anti-infective activity against *S. aureus* increased as the caffeine content decreased, with MIC = 15 ± 7 $\mu\text{g/mL}$ for the homopolymer (P-(t-Bu-DMEA)MA). However, the copolymer with 10% of caffeine (P-(t-Bu-DMEA-Caf10%)MA) also demonstrated activity against *S. aureus* having a MIC = 60 ± 39 $\mu\text{g/mL}$. These values were superior to the activity of P(ab-Caf30%)MA because of the permanent quaternary ammonium group.¹¹¹ A similar trend was noted against *E. coli*; copolymers displayed the best activity with 10% or 0% caffeine, having MIC = 39 ± 11 or 41 ± 13 $\mu\text{g/mL}$, respectively. The copolymers at 50% caffeine content presented MIC = 125 $\mu\text{g/mL}$, which was comparable to that of P-(abCaf50%)MA. The copolymer series bearing zwitterionic groups, P-(DMEA-Caf)MA and P-(Lys-Caf)MA, did not display anti-infective activity against *S. aureus* or *E. coli*, confirming the necessity of a cationic side chain to inhibit the bacteria growth (**Table 3.2**).¹⁶⁶ To summarize, the copolymers P-(t-Bu-DMEA-Caf)MA possess a broad spectrum of action, while primary cationic copolymers were only active against *E. coli*.

It has been shown for other polymethacrylates that the extent of antimicrobial activity depends on molecular weight and that the optimal degree of polymerization is 30.¹¹¹ Therefore, we prepared a random copolymer library having a DP of 30 by reversible addition–fragmentation chain-transfer (RAFT) polymerization. 4-Cyano-4-(phenyl carbonothioylthio) pentanoic acid (CTA) was employed as the RAFT agent and AIBN as the initiator in DMSO at 75 °C for 16 hours. The copolymer library containing caffeine from 30 to 0% was isolated in a 70–76% yield. Upon isolation, the copolymers were characterized by ¹H

NMR and SEC in water (**Figures S3.34–S3.37**). The polymerization degree was determined by ^1H NMR in DMSO using the signals of the aromatic protons in the CTA (signal *o*, 7.46–7.86 ppm) and the signals of the CH_3 group of the polymer backbone (signal *c*, 0.78 ppm). Then, the caffeine content was determined by correlating the signal of the proton of the caffeine (signal *f*, 8.3 ppm) and the polymerization degree. The polymerization degree and caffeine content correlated with the theoretical value for all the copolymers and displayed $M_n = 4.7\text{--}6.4$ kg/mol and narrow polymer dispersity $\text{Đ} = 1.03\text{--}1.16$ (**Table S3.1**). Further N-Boc deprotection in TFA allowed the isolation of P(DMEA-Caf)MA zwitterionic copolymers in a 72–74% yield. The copolymers were characterized by ^1H NMR and SEC, and the caffeine content and polymerization degree were determined similarly to before deprotection. We observed that the polymers correlated with the DP and the caffeine content before deprotection, and they presented $M_n = 4.2\text{--}5.1$ kg/mol and narrow polymer dispersity $\text{Đ} = 1.16\text{--}1.18$ (**Table S3.1, Figures S3.38–S3.41**). These results demonstrated that caffeine methacrylate copolymers bearing cationic or zwitterionic side chains are also accessible through RAFT polymerization with control of the molecular weight and polymer dispersity. Then, the antimicrobial activity was evaluated against *S. aureus* and *E. coli* using the microdilution methodology as previously discussed. Surprisingly, none of the copolymers were active against the bacteria tested ($\text{MIC} > 500$ $\mu\text{g/mL}$), suggesting that activity is only present at the higher molecular weight ($\text{DP} > 30$). A full molecular weight dependence on antimicrobial activity will need to be studied in the future. The polymerization kinetics of certain methacrylates and methacrylamides have been reported to be similar,¹⁶⁷ and thus, it is possible that the polymers reported here are random copolymers; this will also need to be investigated in future studies, particularly because sequence could be a major factor in activity.

We selected the two copolymers with the best activity against the Newman strain for further testing against *S. aureus*. As copolymers can be effective against drug-resistant pathogens,^{122,123} we obtained two clinical isolates of methicillin-resistant *S. aureus* (MRSA)^{125,162} and applied the same microdilution method to determine the MIC: 194 µg/mL (P-(t-Bu-DMEA-Caf 10%)MA) and 170 µg/mL (P-(t-Bu-DMEA)MA, **Table 3.2**). Notably, the MICs differed greatly between the two isolates, resulting in a high standard deviation (101 and 105, respectively), yet both copolymers still proved more effective than ampicillin (MIC = 625 µg/mL). While further testing is necessary to assess the clinical relevancy, our initial findings establish the copolymers with caffeine side chains and a bulky hydrophobic side chain on the quaternary ammonium groups as interesting antimicrobial materials.

3.2.2 Hydroxybenzoate-co-lactide polymers

Antimicrobial screening of small molecules. While a few compounds such as salicylic acid (**1**), 2, 4-dihydroxybenzoic acid (resorcylic acid, **2**), and orsellinic acid (**6**) can be sourced commercially, those with longer 6-alkyl substitutions are not available (**Figure 3.1**). We previously described the discovery of biosynthetic pathways that produce the heptyl-substituted sphaerophorolcarboxylic acid (**8**).¹³⁸ Here, the microbial biosynthesis of **8** was scaled up from engineered *Aspergillus nidulans* for use as a building block. To study the effects of substitution on antimicrobial activity, we used resorcylic (**2**), orsellinic (**6**), and sphaerophorolcarboxylic (**8**) acids and introduced methyl, allyl, and benzyl groups at the 4-hydroxy position. This resulted in a diverse series of 2-hydroxybenzoic acids with varying substitutions at the 4-O (R₁) and 6 (R₂) positions (**Figure 3.1**, **compounds 1 – 10**, **Figures S3.42 – S3.53**). The small library of synthesized compounds was tested for activity against the gram-positive organism *S. aureus* (strain Newman) and the gram-negative organism *E. coli* (**Table 3.3**).

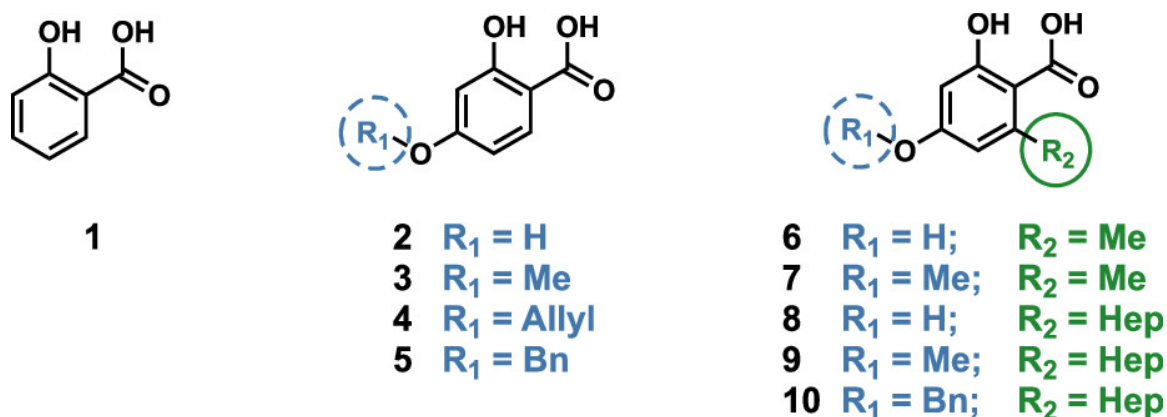


Figure 3.1. Compounds tested for antimicrobial evaluation with different R_1 and R_2 groups on the aromatic ring.

The effect of the substituents with H-, methyl-, allyl-, or benzyl- groups at the 4-O position and methyl or heptyl groups at the 6- position on the aromatic ring was evaluated using a standard broth microdilution method to determine the MIC.¹⁵⁹ 4-O substituents (**2–5**) resulted in generally lower activity compared to **1**, although an increase in substituent size or hydrophobicity gave relatively higher antimicrobial activity. Specifically, small 4-O substituents generally decreased activity relative to salicylic acid, but a benzyl substituent retained antimicrobial activity (compound **5**, MIC = 125 $\mu\text{g/mL}$ against *S. aureus*). On the other hand, when varying the alkyl side chain at the 6- position and keeping the 4- position constant (**8** vs **6** and **9** vs **7**), the 6-heptyl compounds (**8** and **9**) showed 16- to 32-fold lower MICs on *S. aureus* compared to the 6-methyl compounds (**6** and **7**), indicating that greater hydrophobicity at this position resulted in greater activity. The combination of a benzyl group at the 4-O position and a heptyl group at the 6- position (**10**) exhibited the highest antibacterial activity (MIC = 3.9 $\mu\text{g/mL}$), comparable to that of the positive control, ampicillin. Moreover, compound **10** showed antimicrobial activity against two other strains of *S. aureus*, including a methicillin-resistant *S. aureus* strain (MRSA; MIC = 15.6 $\mu\text{g/mL}$, **Table 3.3**). These compounds showed low or negligible activity against *E. coli*, suggesting that their effect may be related to the cell

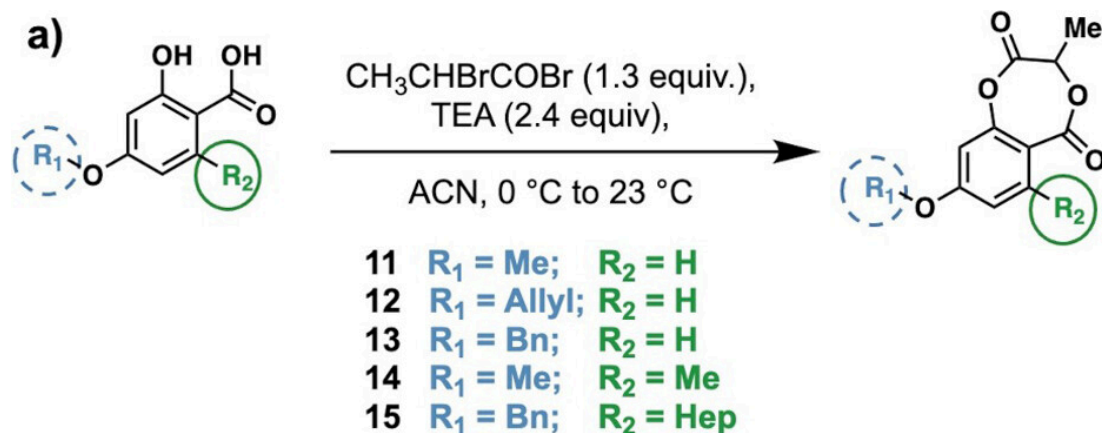
membrane.^{168,169} With these results, monomers were then synthesized to enable the ring-opening polymerization of these adducts.

Compound	<i>S. aureus</i> (ATCC 25904)		<i>S. aureus</i> (ATCC 29213)		<i>S. aureus</i> (ATCC 43300)		<i>E. coli</i> (ATCC 27065)	
	(µg/mL)	(µM)	(µg/mL)	(µM)	(µg/mL)	(µM)	(µg/mL)	(µM)
1	62.5	453	1000	7240	1000	7240	375	2715
2	1000	6488	>1000	>6488	>1000	>6488	187.5	1217
3	1000	5947	>1000	>5947	>1000	>5947	>1000	>5947
4	500	2575	1000	5150	1000	5150	>1000	>5150
5	125	557	333	1485	500	2230	>1000	>4459
6	1000	5947	>1000	>5947	>1000	>5947	1000	5947
7	500	2745	1000	5489	>1000	>5489	1000	5489
8	62.5	248	62.5	248	62.5	248	250	991
9	15.6	59	31.3	118	31.3	118	250	939
10	3.9	11	15.6	46	15.6	46	>1000	>2920
Ampicillin	1.0	3	1.6	5	5.9	17	31.3	61

Table 3.3. Antimicrobial screening of 4-(methyl/allyl/benzyl)oxy, 6-(H/alkyl)-2-hydroxybenzoates. Data shown are the average of $n = 3$; when no standard deviation is given, all replicates gave identical MIC values.

Monomer preparation. One way to prepare oligomeric and polymeric analogs of depsides could be through polymerization from hydroxybenzoates. The preparation of the monomer was inspired by recent works dealing with the synthesis of alternate polymers consisting of salicylic acid and lactide. In the studies, the authors have prepared 3-methyl-5*H*-benzo[*e*][1,4]dioxepine-2,5(3*H*)-dione and 5*H*-benzo[*e*][1,4]dioxepine-2,5(3*H*)-dione, which were further ring-opened using catalytic systems employing tin(II) 2-ethylhexanoate (SnOct₂),¹⁷⁰ 4-dimethylaminopyridine (DMAP),¹⁷¹ or tetrabutylammonium halides¹⁷² at a variety of temperatures (50 °C, 110–140 °C). Thus, the monomers were prepared similarly to previous works^{170–175} with some modifications by reacting 4-(methyl/allyl/benzyl)oxy-6-alkyl-2-hydroxybenzoates with 2-bromo-propionyl-bromide (1.3 equiv) and triethylamine (TEA, 2.4 equiv) (Scheme 3.7). Upon isolation, the

monomers were obtained in 17–73% yields and characterized by ^1H and ^{13}C NMR (Figures S3.54 – S3.58).



Scheme 3.7. Preparation of lactide monomers (compounds 11–15) from 4-(methyl/allyl/benzyl)oxy-6-(H/alkyl)-2-hydroxybenzoates.

Polymer preparation. Although lactide monomers from salicylic acid are known to undergo ring-opening polymerization using SnOct_2 , DMAP, tetrabutylammonium chloride,^{170–175} and organometallic catalysts,¹⁷⁶ ROP using organocatalysts such as urea has not yet been reported. In this study, we explored the ROP of lactide monomers with 3-methylbutan-1-ol using the 3-O urea catalyst (1,1',1''-(nitrilotris(ethane-2,1-diyl))tris(3-(3,5-bis(trifluoromethyl)phenyl)urea)) and MTBD (1-methyl-2,3,4,6,7,8-hexahydro-1H-pyrimido[1,2-*a*]pyrimidine) as a cocatalyst in dichloromethane (DCM) under an inert atmosphere. An initial successful attempt using monomer **13** was conducted while targeting a ratio of 50:1:1:1 (monomer:3-O urea:MTBD:initiator), as confirmed by SEC and NMR spectroscopic studies (**Figure 3.2** and **Figures S3.59 – S3.61**).

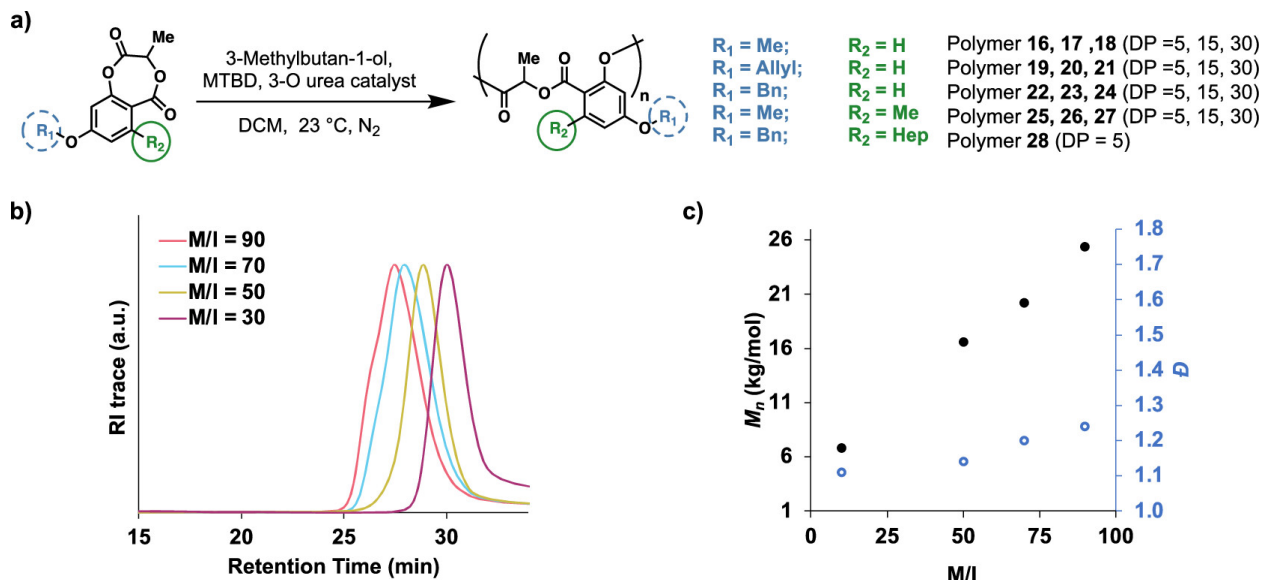


Figure 3.2. Preparation and characterization of polymers derived from 4-(methyl/allyl/benzyl)-oxy-6-alkyl-2-oxybenzoate lactide monomers by ring-opening polymerization. A) Synthetic route to prepare polymers with different M/I. Variation of the [monomer]/[initiator] (M/I) during ROP of monomer 13: B) refractive index (RI) trace SEC in THF and C) plot of M_n (black) and dispersity (D , blue) versus M/I.

The polymers analyzed by SEC in THF exhibited an $M_n = 16.6$ kg/mol (**Figure 3.2B**), which was slightly lower than the NMR calculation (20 kg/mol, monomer/initiator concentration ratio, M/I = 66, **Figure S3.59B**), and the molar-mass dispersity was found to be $D = 1.14$. On the other hand, using only MTBD or the 3-O urea catalyst, with or without DMAP, resulted in higher dispersities, and no polymerization was observed in the absence of a catalyst (**Table S3.2**). The results suggested that it is feasible to prepare polymers with a targeted M/I by controlling the stoichiometric ratio of the monomer, catalyst, cocatalyst, and initiator. To validate this, the monomer/initiator concentration ratio (M/I) for **13** was varied from 30 to 90 (**Figure 3.2B**), and a linear relationship was observed from this assay while preserving low dispersities ($D < 1.3$, **Figure 3.2C**, **Table S3.3**). Thus, it was possible to precisely control the M/I by varying the monomer/initiator ratio using the organocatalytic system.

Molecular weight is an important parameter in the design of antimicrobial polymers, particularly depsides are found as oligomeric compounds.¹³⁰ In the literature, most active polymers have an $M/I \leq 30$, although high molecular weight polymers can also exhibit antimicrobial activity.^{177–179} Thus, after the successful polymerization of **13**, a series of polymers were prepared with different substituents and degrees of polymerization ($M/I = 5, 15$, and 30) (**Figure 3.2A**). The polymerizations went to completion, and the polymers were isolated in 53–91% yields and analyzed by NMR spectroscopy and SEC (**Table 3.2** and **Figures S3.62 – S3.75**). The majority of the obtained polymers aligned well with the targeted polymerization degree. At low polymerization degrees, we observed multiple peaks in both the ^1H NMR and ^{13}C NMR spectra attributed to the oligomeric forms and uncontrolled stereoregularity. Furthermore, polymers synthesized with an $M/I = 30$ were examined by ^{13}C NMR spectroscopy, and no evidence of transesterification was observed,¹⁷⁰ although this cannot be completely ruled out. Moreover, the M_n obtained from SEC analysis was in full agreement with the calculated M/I from ^1H NMR analysis for $M/I = 30$ and possessed $M_n = 5.6\text{--}6.8$ kg/mol and $D = 1.11\text{--}1.23$. For $M/I < 30$, it was not possible to conduct SEC analysis due to instrument limitations. Therefore, the degree of polymerization was determined by ^1H NMR spectroscopy.

Polymer	M/I _{theo}	M _n (kg/mol) ^a	<i>D</i>	M/I _{NMR}	Yield (%)
16	5			5	84
17	15			13	91
18	30	6.1	1.23	30	84
19	5			5	59
20	15			11	81
21	30	5.5	1.10	22	58
22	5			6	61
23	15			10	49
24	30	6.8	1.11	30	89
25	5			7	53
26	15			12	44
27	30	5.6	1.21	28	78
28	5			3	63

Table 3.4. Characterization of functional hydroxybenzoate-co-lactide polymers with different M/I. ^a Determined from SEC in THF.

Antimicrobial assays of polymers. Next, we evaluated the antimicrobial potency of the polymer library against *S. aureus* by determining the MIC using the same broth microdilution protocol as that for the small molecules. Interestingly, some polymers displayed enhanced antibacterial activity (Table 3.5) compared with their small molecule forms (Table 3.3). Comparison of the substituent variation on the 4-O position with M/I_{NMR} < 10 showed significant differences among the methyl, allyl, or benzyl side chains when assessing the MIC values of polymers 16 (MIC = 333 ± 144 µg/mL), 19 (MIC = 1000 µg/mL), and 22 (MIC = 83 ± 36 µg/mL), with the benzyl substituent resulting in the greatest activity for this position, similar to the small molecules. Interestingly, when a methyl group was introduced at the 6-position in 25, the polymer displayed a higher antimicrobial effect (MIC = 31.3 µg/mL), compared to its small molecule counterpart (Table 3.3, compound 7). Moreover, introducing R₁ = Bn, and R₂ = heptyl, which had given the greatest activity among the small molecules (compound 10), led to polymer 28 with antimicrobial potency similar to that of 25 (MIC = 31.3 µg/mL). Thus, small molecule activity was not always a good predictor of polymer activity.

Polymer	R ₁	R ₂	M/I _{NMR}	<i>S. aureus</i> MIC (µg/mL)	<i>S. aureus</i> MIC (µM)
16	Me	H	5	333 ± 144	278 ± 98
17	Me	H	13	>1000	>336
18	Me	H	30	>1000	>148
19	allyl	H	5	1000	752
20	allyl	H	11	>1000	>355
21	allyl	H	22	>1000	>180
22	Bn	H	6	83 ± 36	44 ± 16
23	Bn	H	10	>1000	>326
24	Bn	H	30	>1000	>111
25	Me	Me	7	31.3	18
26	Me	Me	12	>1000	>342
27	Me	Me	28	>1000	>149
28	Bn	Hep	3	31.3	25
Ampicillin				1.0	3

Table 3.5. Antimicrobial activity on *S. aureus* of polymers M/I = 5 – 30 (*n* = 3). A broth microdilution assay was used to determine the MIC of the polymers against *S. aureus*, Newman strain. The MIC in molarity (µM) was calculated using the M_w from ¹H NMR.

The second comparison assessed the effect of M/I on antimicrobial activity (**Table 3.5**). It was observed that increasing the M/I above 5 increased the MIC values for all polymers. A representative example is the loss of activity on polymer **25**, whose MIC significantly increased from 31 µg/mL at M/I = 7 to >1000 µg/mL at M/I = 28 (polymer **27**). Together, these results demonstrate the antibacterial activity against gram-positive microorganism *S. aureus*, highlighting the importance of the substituents at 4-O and 6- positions and the size of the hydroxybenzoate polymers.

One major concern with bacteria is their ability to form biofilms, which are complex matrices that confer an extra layer of protection and prevent the penetration of antibiotics.^{180,181} Thus, disruption and inhibition of biofilms are important targets for combating bacteria. We hypothesized that the active polymers **22**, **25**, and **28** prepared in this work could also inhibit and disrupt biofilms. To assess this hypothesis, the inhibition of biofilm was assessed in *S. aureus* cultures under stationary conditions with different concentrations of the polymers for 72

hours in a 96-well plate. For biofilm disruption studies, *S. aureus* was cultured under stationary conditions for 34 hours in a 96-well plate to form biofilms. After 34 hours (once the biofilm was formed), each well was treated with different concentrations of the synthesized polymers and incubated for an additional 38 hours. After 72 hours, the effect on biofilm inhibition and disruption was observed by staining the well with crystal violet dye, which binds the extracellular polymeric substance (EPS) of the biofilm. Polymers **22**, **25**, and **28** were tested along with their small molecule precursor **5**, **7**, and **10**, respectively (**Figure 3.3**). Interestingly, inhibition of biofilm and disruption were observed for polymer **22** at 250 µg/mL (**Figure 3.3G and H**) and for **28** at 15.6 µg/mL (**Figure 3.3K and L**). However, polymer **25** did not display biofilm inhibition or disruption at 2- or 3-fold higher concentrations (**Figure 3.3G and H**) even though it exhibited antimicrobial activity in liquid culture.

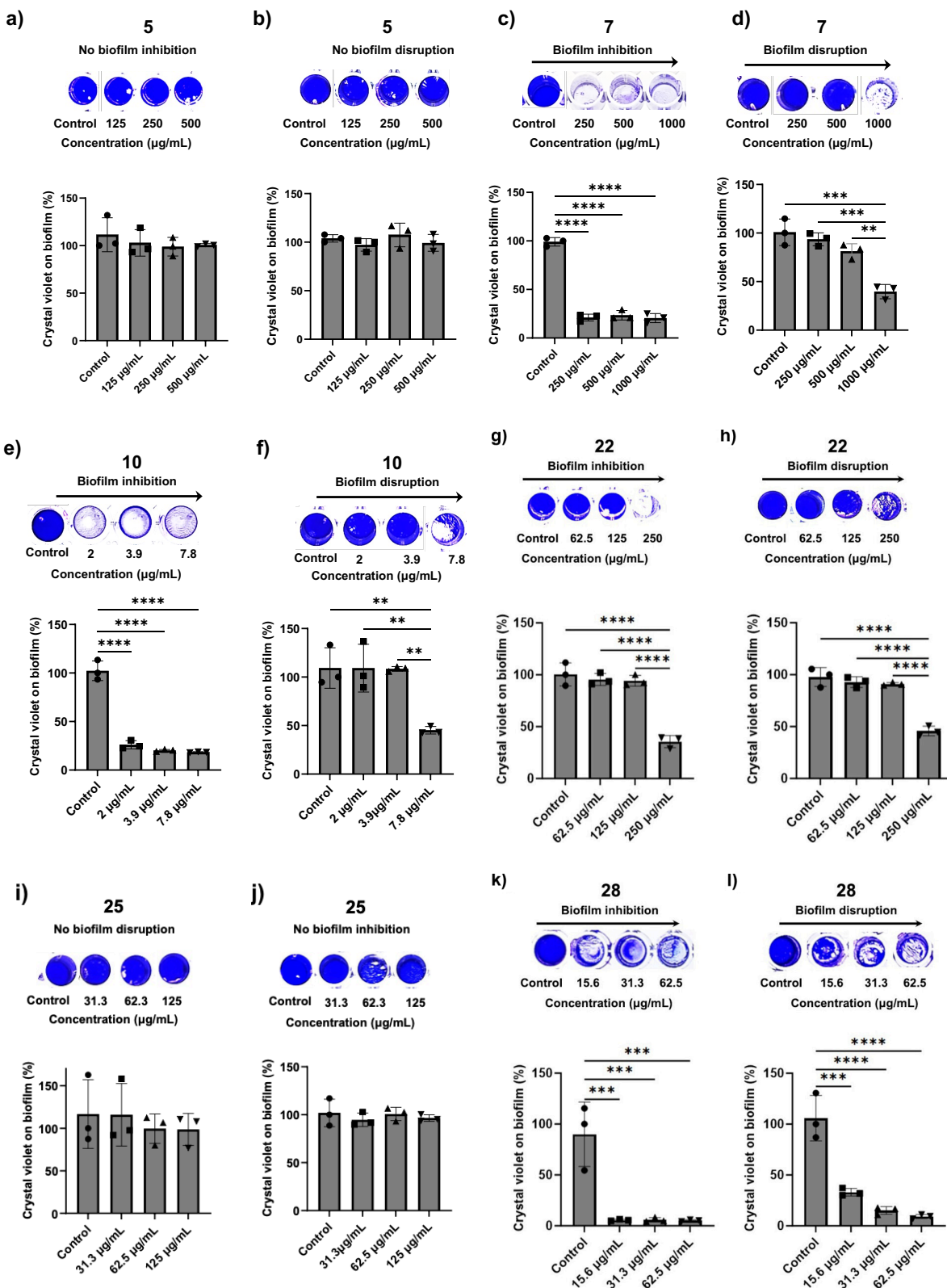


Figure 3.3. Biofilm inhibition and disruption of small molecules (5, 7, and 10) and functional hydroxybenzoate-co-lactide polymers (22, 25, and 28) on *S. aureus*. Antimicrobial

properties were determined by evaluating the biofilm inhibition and disruption by staining with 1% crystal violet. The figure shows the 96-well plate picture accompanied by the UV-vis quantitation of the biofilm assays. Statistical significance was determined via a one-way ANOVA with multiple comparisons ($p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****)) ($n = 3$).

Antibiofilm activity was also compared between the corresponding small molecules and polymers. Compound **5** (precursor of **22**) did not cause biofilm disruption or inhibition, and compounds **7** (precursor of **25**) and **10** (precursor of **28**) displayed both inhibition and disruption of the *S. aureus* biofilm (**Figure 3.3A – F**). Of these, compound **7** showed a weaker effect, with inhibition at 250 $\mu\text{g/mL}$ and disruption at 1000 $\mu\text{g/mL}$; while compound **10** showed relatively potent inhibition at 2 $\mu\text{g/mL}$ and disruption of biofilm at 7.8 $\mu\text{g/mL}$.

We also evaluated the particle size formation of the polymers by dynamic light scattering (DLS) as they formed turbid solutions during the activity tests described above at 1000 $\mu\text{g/mL}$ (**Table S3.4**). Despite the formation of self-assemblies within the range of 117–686 nm, no correlation was found between antimicrobial activity and particle size. These results demonstrated that the inhibition and disruption of biofilms depend on the molecular weight and chemical structure.

3.2.3 Polylysine

Antibacterial activity of α -poly(L-lysine)-NH₂ (polyK). A series of α -poly(L-lysine)-NH₂ polymers (called ‘polyK’ here) with varying degrees of polymerization (DP) ($n = 40$ to 200) were synthesized by NCA polymerization¹⁵⁵ (**Scheme 3.3**). ¹H NMR spectra of intermediates and deprotected polypeptides agreed with literature data (**Figures S3.76 – S3.78**).¹⁵⁸ The polymers were tested for antimicrobial activity against *S. aureus* (ATCC 25904, strain Newman), *E. coli* (ATCC 25922), and *P. aeruginosa* (ATCC 25102) by determination of the MIC through

broth microdilution (**Table 3.6**). The polymers were active against all three species. When polymer concentration was expressed as mass-per-volume, all measured MIC values for different DP were found to be within a factor of 3 of one another. That is, while the MIC expressed in molarity decreased consistently as DP increased, the MIC in terms of mass-per-volume was essentially constant. All polymers were bactericidal, preventing colony-forming units, with the minimum bactericidal concentration (MBC) being equal to the MIC (**Table S3.5**).

DP	<i>S. aureus</i> (ATCC 25904)		<i>E. coli</i> (ATCC 25922)		<i>P. aeruginosa</i> (ATCC 25102)	
	μM	$\mu\text{g/mL}$	μM	$\mu\text{g/mL}$	μM	$\mu\text{g/mL}$
40	5	33	10	67	20	130
48	5	40	5	40	5	40
67	5	55	2.5	28	5	55
75	5	62	2.5	31	5	62
90	2.5	37	2.5	37	5	74
110	2.5	45	1.3	23	5	91
128	2.5	53	1.3	26	5	110
148	2.5	61	0.63	15	5	120
155	2.5	64	0.63	16	2.5	64
160	1.3	33	0.63	16	2.5	66
170	1.3	35	0.63	18	2.5	70
200	1.3	41	0.63	21	2.5	82

Table 3.6. Minimum Inhibitory Concentration (MIC) of synthetic polyK with various degrees of polymerization (DP). The broth microdilution method was used to determine the MIC of each polymer on three bacterial species (concentration expressed as μM and $\mu\text{g/mL}$).

3.3 Discussion

In this study, we synthesized three bio-inspired polymer libraries with different backbones (methacrylate, hydroxybenzoate-co-lactide, and peptide) to investigate their antimicrobial properties.

For the methacrylate copolymers bearing cationic or zwitterionic side chains with caffeine, cationic side chains were necessary to present activity against bacteria, as primary ammonium (P-(ab-Caf)MA) and quaternary ammonium copolymers (P-(*t*-Bu-DMEA-Caf)MA) inhibited bacteria growth. In this sense, primary ammonium copolymers inhibited the growth exclusively for gram-negative, while quaternary ammonium copolymers presented a broad spectrum of action. It was also found that finely tuning the hydrophobic ratio allowed optimization of antimicrobial polymers, consistent with other work on selectivity.¹⁸² It is important to note that P-(*t*-Bu-DMEA)MA is a quaternary ammonium homopolymer, and the antibacterial might be related to a hydrophilic polycation mechanism, rather than to membrane disruption, which might characterize the polymers having caffeine as a hydrophobic side chain.^{113,183} Therefore, the mechanism of these antimicrobial polymers needs further study. Collectively, the data shows that caffeine side chains and *tert*-butyl-protected carboxybetaine as hydrophobic and cationic groups could help to prepare selective polymers against bacteria.

Inspired by active natural products, the antimicrobial potential of functional hydroxybenzoate-co-lactide polymers was explored. The synthesis of the precursors using a systems biology approach and a controlled organocatalytic ROP approach facilitated the rapid production of a diverse library of oligomers and polymers with targeted molecular weights. The structure–activity relationship study of the precursors demonstrated that modifications at the 4-O and 6-positions of the aromatic ring significantly enhanced activity against *S. aureus*, with the 6-

heptyl series exhibiting the lowest MICs (**Figure 3.4**). With regard to antimicrobial activity, the polymers were more active in general at smaller sizes; degrees of polymerization at or below 7 were most active within their respective series. The effect of molecular weight was crucial, as polymers with $M/I > 10$ were inactive. Moreover, the effect of the substituents was important for biofilm inhibition and disruption, as polymers with $R_1 = \text{Bn}$ and $R_2 = \text{H}$ (**22**) or $R_1 = \text{Bn}$ and $R_2 = \text{Hep}$ (**28**) showed both inhibition and disruption of the biofilm, whereas the polymer with $R_1 = \text{Me}$ and $R_2 = \text{Me}$ did not show antibiofilm activity (**25**). Additional studies are needed to elucidate the exact mechanism, but it is clear that a balance of hydrophobic side chains is required.¹⁸⁴ In cases in which the polymers are more active than the monomers, the difference may be due to effects such as cooperativity and avidity, or the polymer and monomer may act through different mechanisms; understanding these mechanisms will require further research. Together, these results confirm the potential of modifying antibacterial properties by designing polymers with easy-to-functionalize hydroxybenzoate groups.

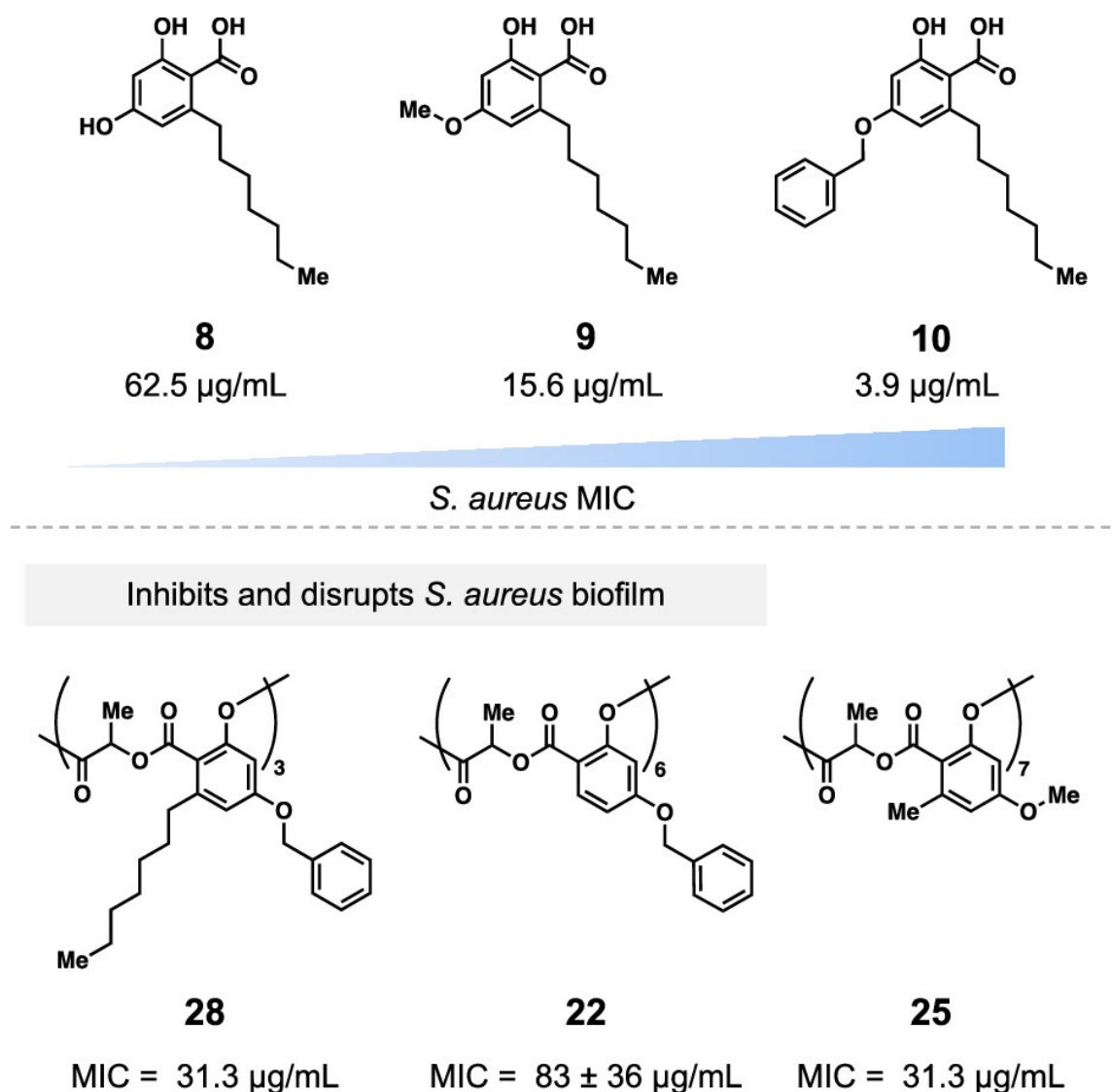


Figure 3.4. Key compounds with antimicrobial activity against *S. aureus*. MIC is the minimal inhibitory concentration against *S. aureus*. Polymers 22 and 28 inhibit and disrupt *S. aureus* biofilms.

Lastly, the library of α -poly(L-lysine)-NH₂, referred to as “polyK”, with varying degrees of polymerization was assessed for antimicrobial activity. As degrees of polymerization increased, the MIC decreased with respect to molarity, although there was no trend with respect to mass-per-volume. Additionally, polyK exhibited broad spectrum activity against both gram-positive and negative organisms, and strong bactericidal action, as the MIC was equivalent to the MBC.

Across the three libraries, differing trends for degrees of polymerization, hydrophobicity, and cationic nature emerged. The poly(hydroxybenzoate-co-lactide)s exhibited lower MICs with lower DP, unlike the polyK and poly(methacrylate) libraries. Additionally, poly(hydroxybenzoate-co-lactide)s displayed activity despite lacking cationic residues, and polymers derived from monomers with higher hydrophobicity exhibited a lower MIC. P-(*t*-Bu-DMEA)MA polymers exhibited lower MIC as the proportion of cationic side chains was increased, although the quaternary ammonium groups presented a bulky hydrophobic side chain. PolyK remains active in the absence of any hydrophobic residues. These differences highlight the chemical complexity of antimicrobial polymers.

When assessed by mass-per-volume, the polymers with the lowest MICs against *S. aureus* for each library were on the same order of magnitude: 15 ± 7 $\mu\text{g/mL}$ for P-(*t*-Bu-DMEA)MA, 60 ± 39 $\mu\text{g/mL}$ for P-(*t*-Bu-DMEA-Caf 10%)MA, 31.3 $\mu\text{g/mL}$ for polymers 25 and 28, and 33 to 64 $\mu\text{g/mL}$ for the entire polyK library. When measured by molarity, P-(*t*-Bu-DMEA)MA had the lowest MIC (0.5 ± 0.2 μM), while P-(*t*-Bu-DMEA-Caf 10%)MA (2.9 ± 1.9 μM) was comparable to the polyK library (5 μM for DP 40 to 75, 2.5 μM for DP 90 to 155, and 1.3 μM for DP 160 to 200). However, P-(*t*-Bu-DMEA)MA and P-(*t*-Bu-DMEA-Caf 10%)MA exhibited substantially higher MIC for clinical isolates with methicillin resistance (170 and 194 $\mu\text{g/mL}$, respectively). Additionally, poly(methacrylate) are nondegradable in body conditions,¹⁸⁵ in comparison to other polymers, making them poor candidates for systemic delivery via phage. The poly(hydroxybenzoate-co-lactide)s possessed the highest MIC when measured by molarity, with respective MICs of 18 and 25 μM against *S. aureus* for polymers 25 and 28, although they displayed key anti-biofilm properties at these concentrations. Additionally, the exact mechanism of action of the poly(hydroxybenzoate-co-lactide)s is unknown, making it difficult to predict

whether they would remain active after conjugation to a phage for delivery. Therefore, the drawbacks of these two polymer libraries established polyK as the leading candidate for bioconjugation.

Additional key attributes make polyK ideal for phage bioconjugation. Its antimicrobial properties and mechanism are well-documented,^{49,186–188} with the data presented here confirming the bactericidal action. The chemical synthesis is well established, and can be performed on the gram scale with control over the degree of polymerization (DP).^{154–156} Lastly, polyK is highly water soluble, unlike the other DMSO soluble polymers tested, and presents ample primary amines, enabling crosslinking to carboxyl groups on the phage by EDC chemistry. Therefore, polyK was selected as a representative cationic polymer for phage-based delivery and future studies.

3.4 Supplementary figures

3.4.1 Methacrylate copolymers with caffeine and cationic or zwitterionic side chains

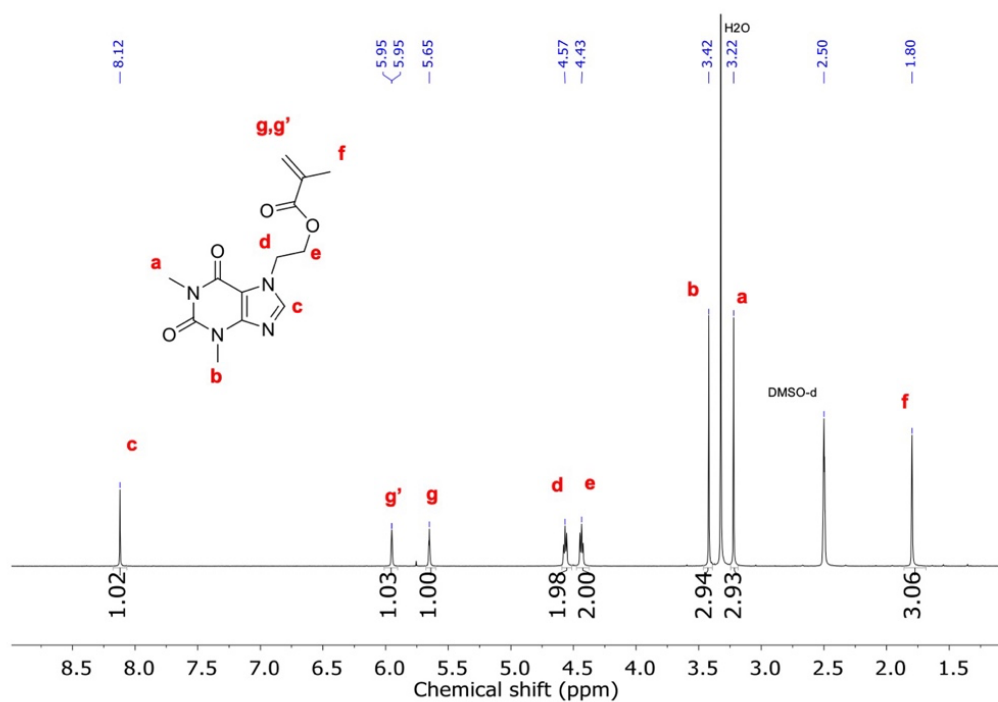


Figure S3.1. ¹H-NMR spectrum of CafMA in DMSO-*d*₆.

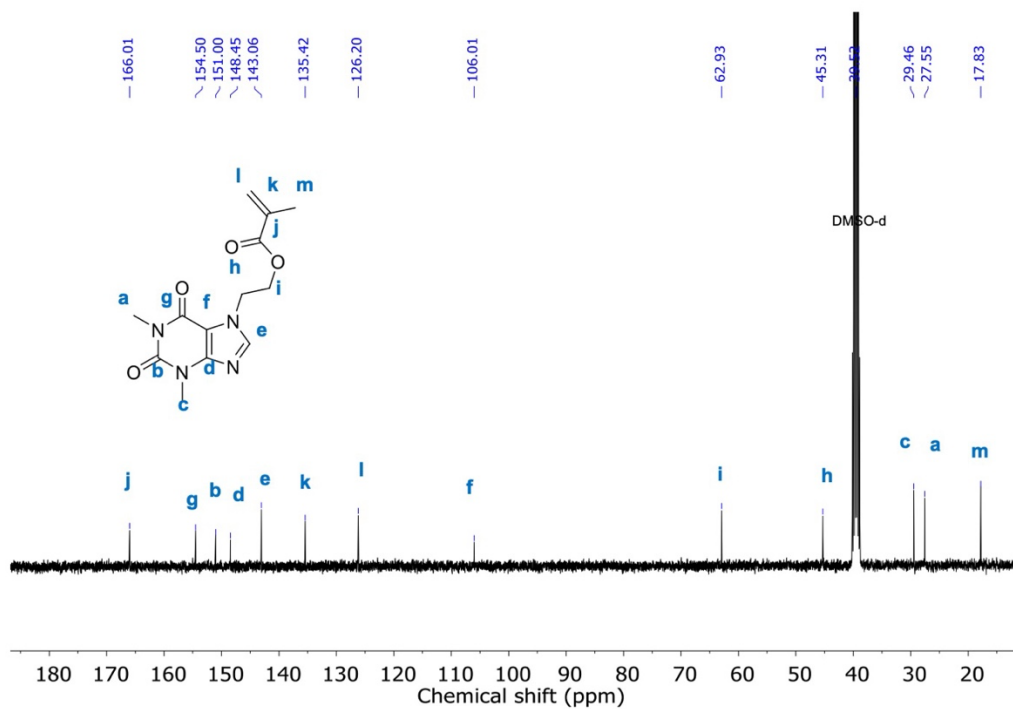


Figure S3.2. ¹³C-NMR spectrum of CafMA in DMSO-*d*₆.

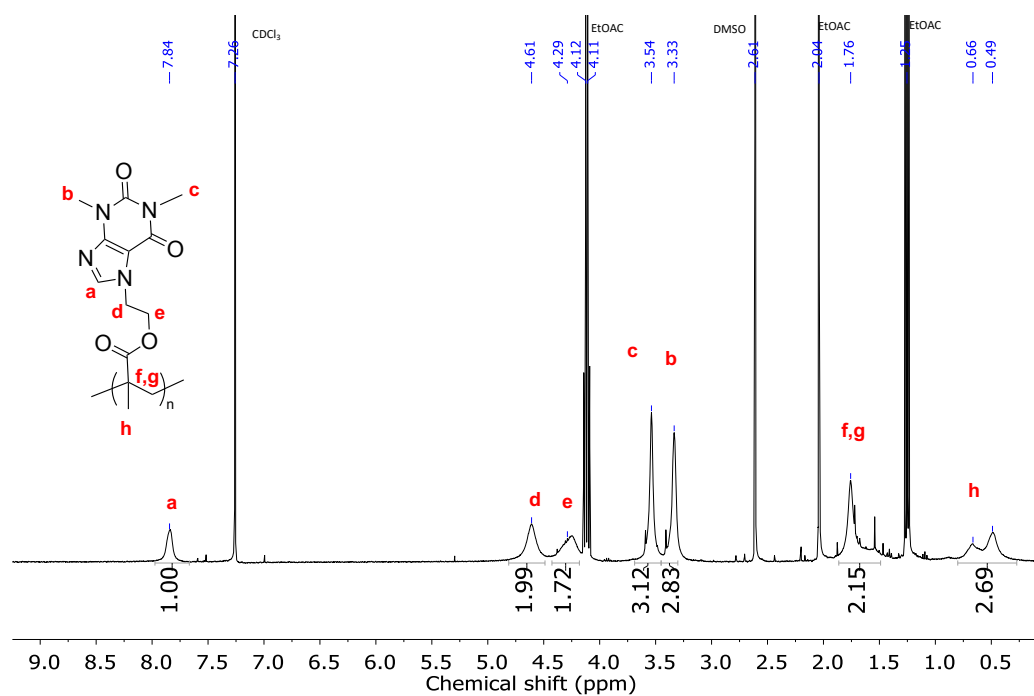


Figure S.3.3. ^1H -NMR spectrum of P(CafMA) in CDCl_3 .

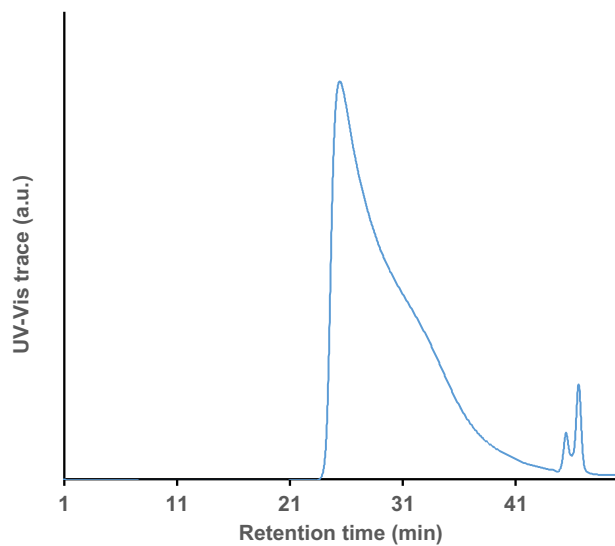
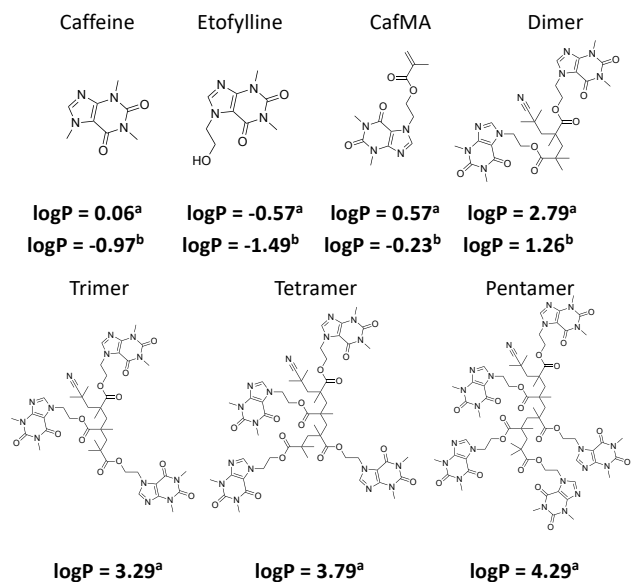
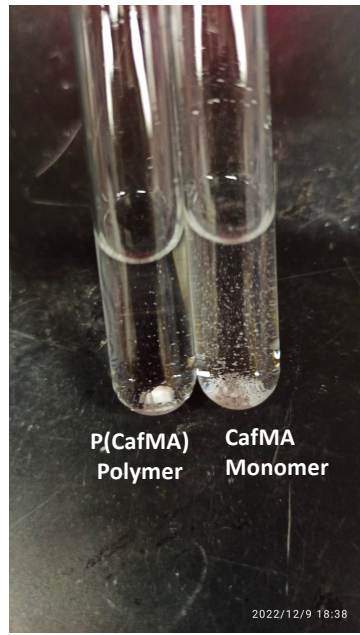


Figure S3.4. SEC chromatogram of P(CafMA) in trifluoroethanol.

a)



b)



a. Using Chemdraw[®]

b. Using molinspiration open source online

Figure S3.5. Solubility of monomer CafMA, oligomers and homopolymer P(CafMA). A) Theoretical study using ChemDraw[®] or molinspiration open source (<https://www.molinspiration.com/services/faq.html>). B) Solubility in PBS at 1 mg/mL.

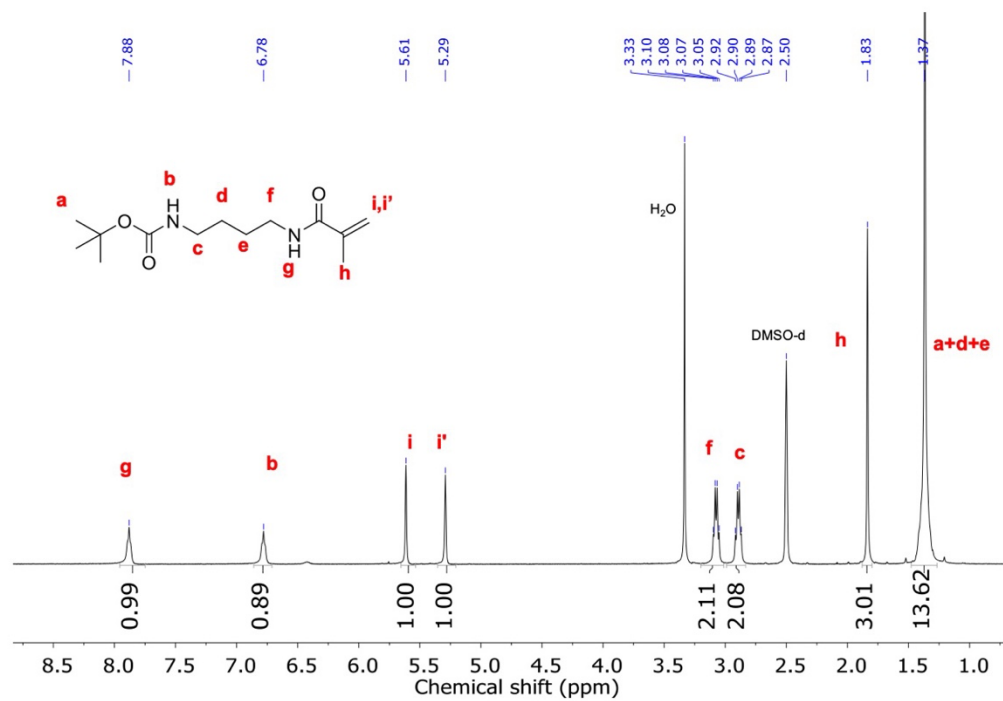


Figure S3.6. ¹H-NMR spectrum of Boc-ab-MA in DMSO-*d*₆.

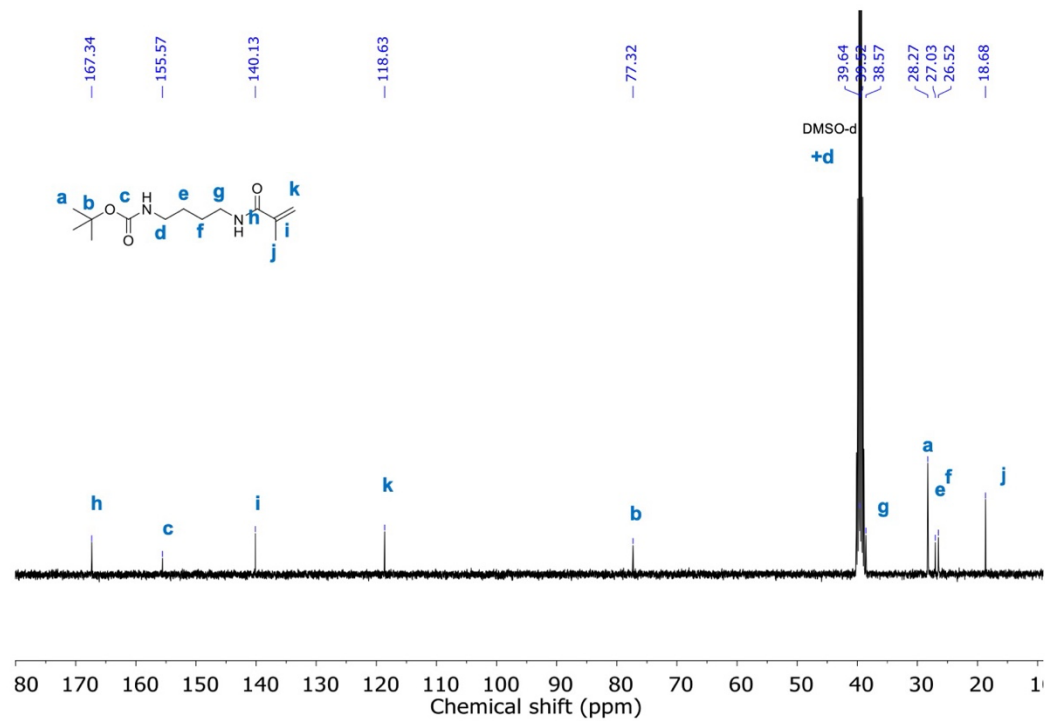


Figure S3.7. ¹³C-NMR spectrum of Boc-ab-MA in DMSO-*d*₆.

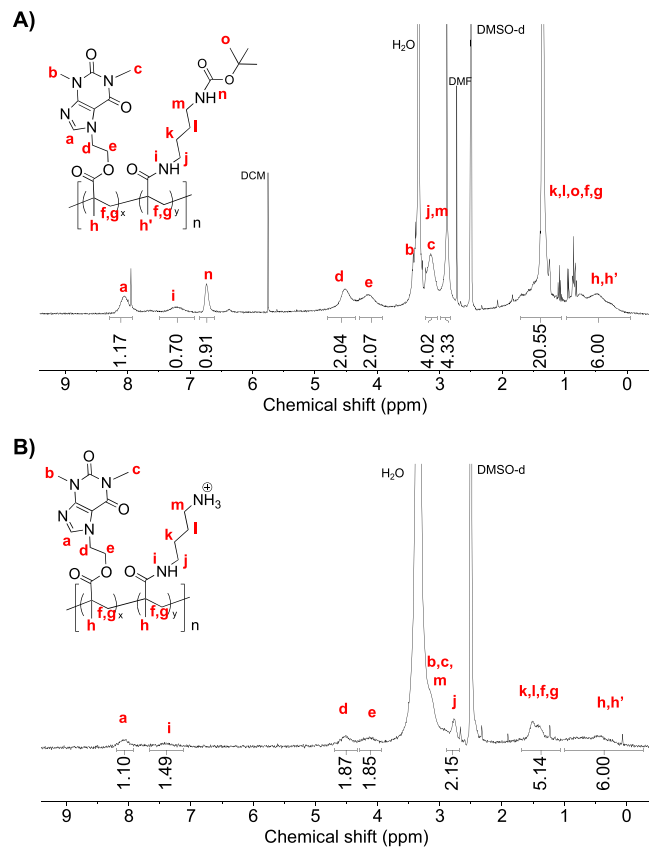


Figure S3.8. ¹H NMR of a representative copolymer bearing caffeine and cationic side chains **A**) before (P-(Boc-ab-Caf 50%)MA) and **B**) upon acidic deprotection (P-(ab-Caf 50%)MA).

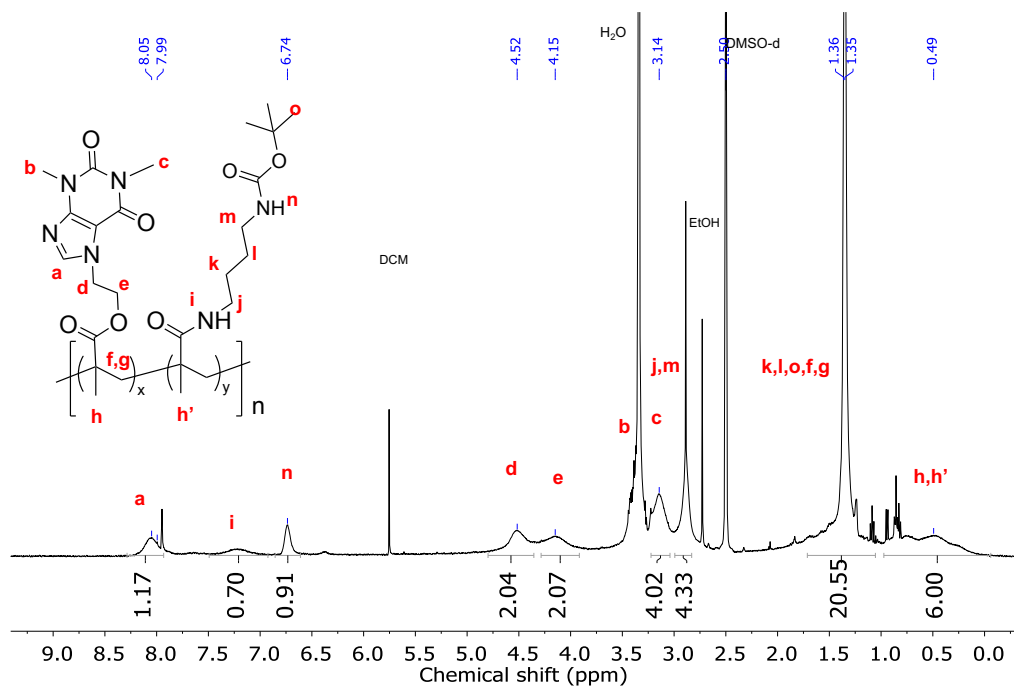


Figure S3.9. ^1H -NMR spectrum of P(Boc-ab-Caf50%)MA in $\text{DMSO}-d_6$.

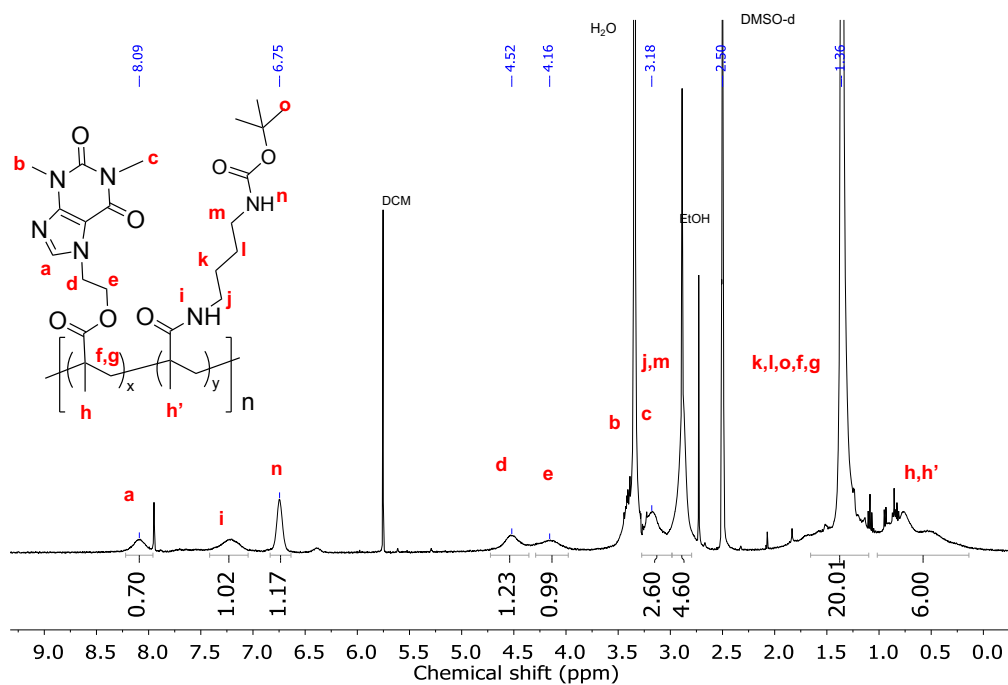


Figure S3.10. ^1H -NMR spectrum of P(Boc-ab-Caf30%)MA in $\text{DMSO}-d_6$.

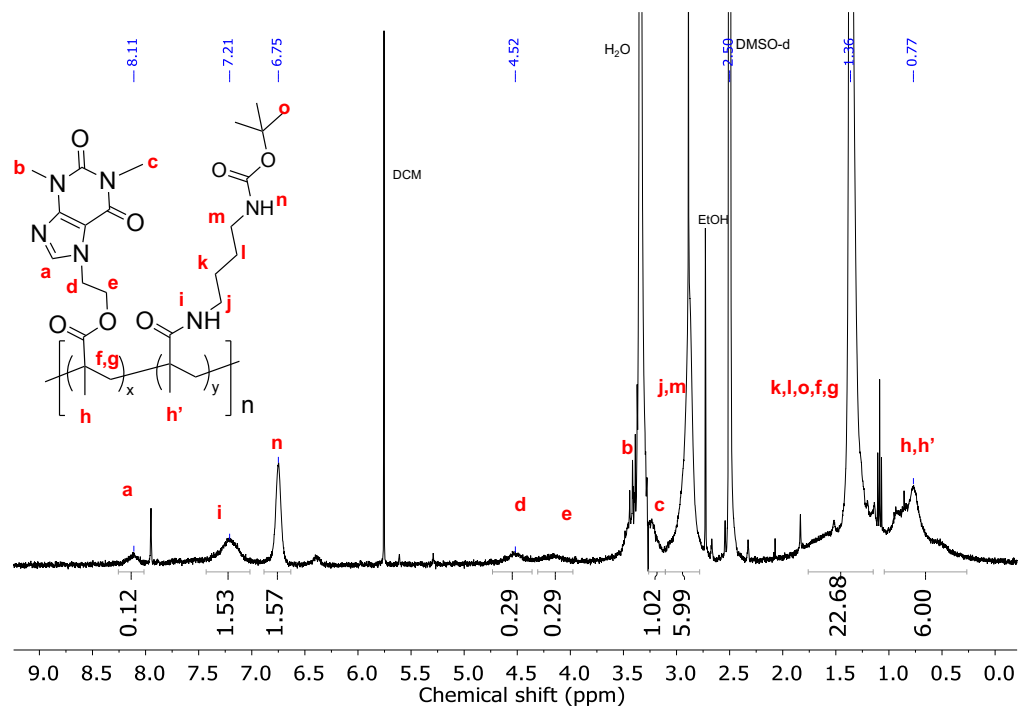


Figure S3.11. ^1H -NMR spectrum of P(Boc-ab-Caf10%)MA in DMSO- d_6 .

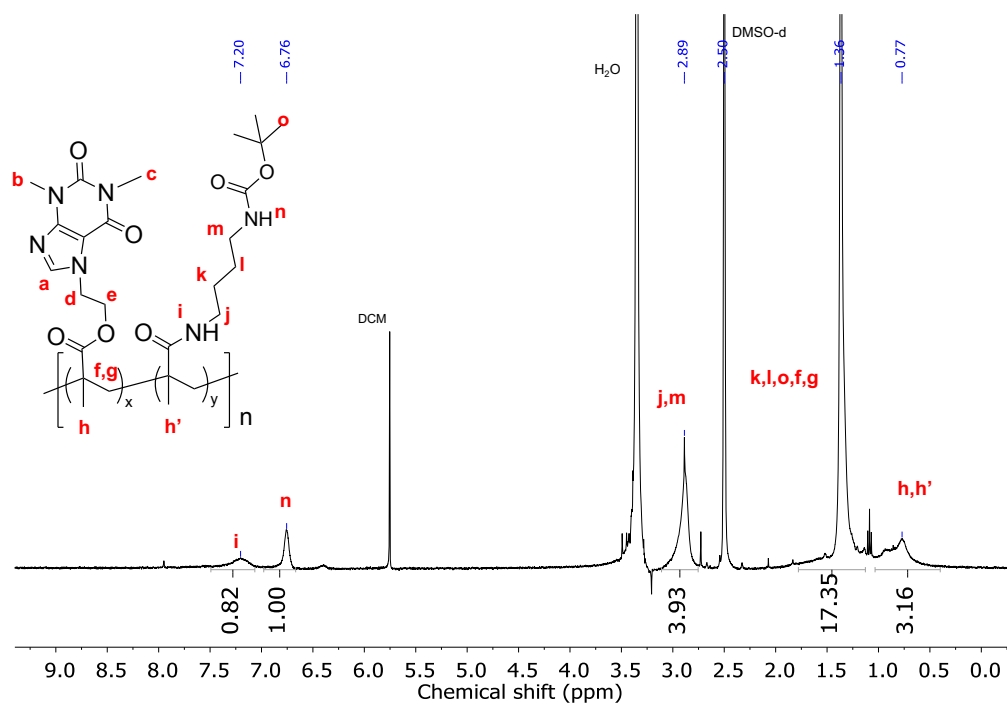


Figure S3.12. ^1H -NMR spectrum of P(Boc-ab)MA in DMSO- d_6 .

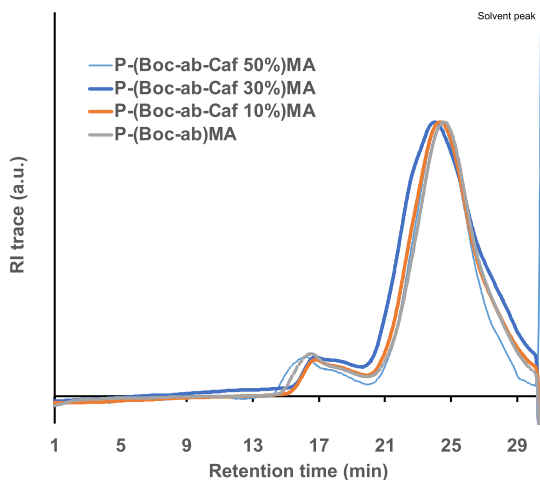


Figure S3.13. Representative chromatograms from refractive index (RI) trace of copolymer bearing caffeine and cationic side chains from SEC in DMF (au = arbitrary units).

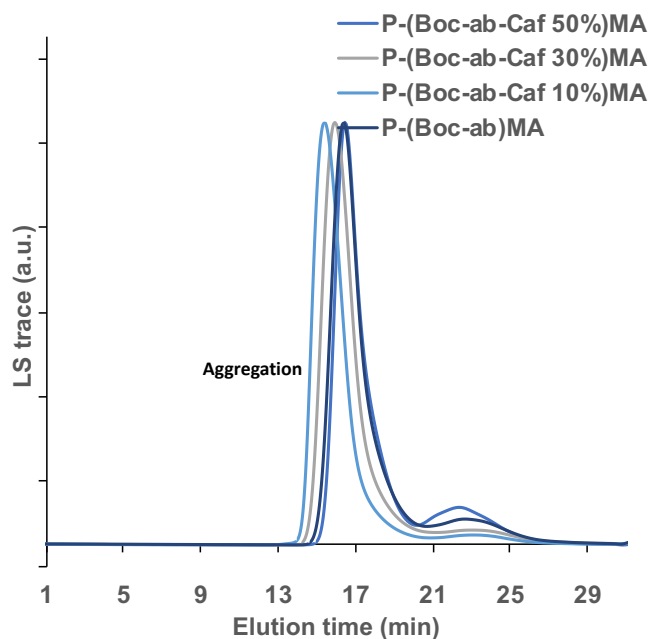


Figure S3.14. Light scattering signal (LS) from the series of copolymers P(Boc-ab-Caf)MA by SEC in DMF. The high signal from LS at lower elution time confirmed aggregation observed from the RI trace.

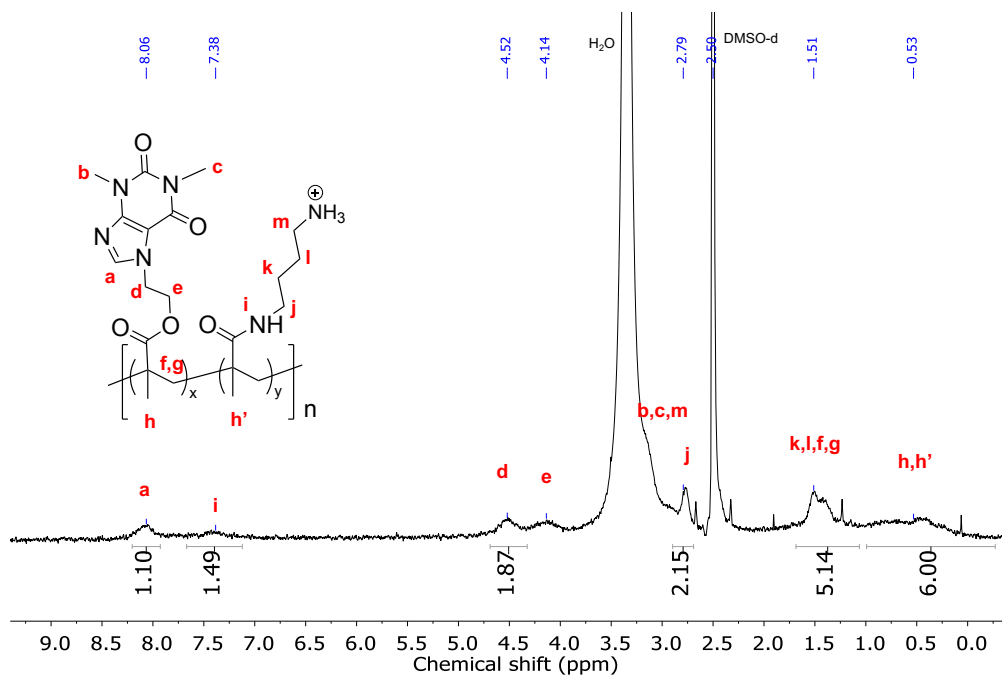


Figure S3.15. ^1H -NMR spectrum of P(ab-Caf50%)MA in $\text{DMSO-}d_6$.

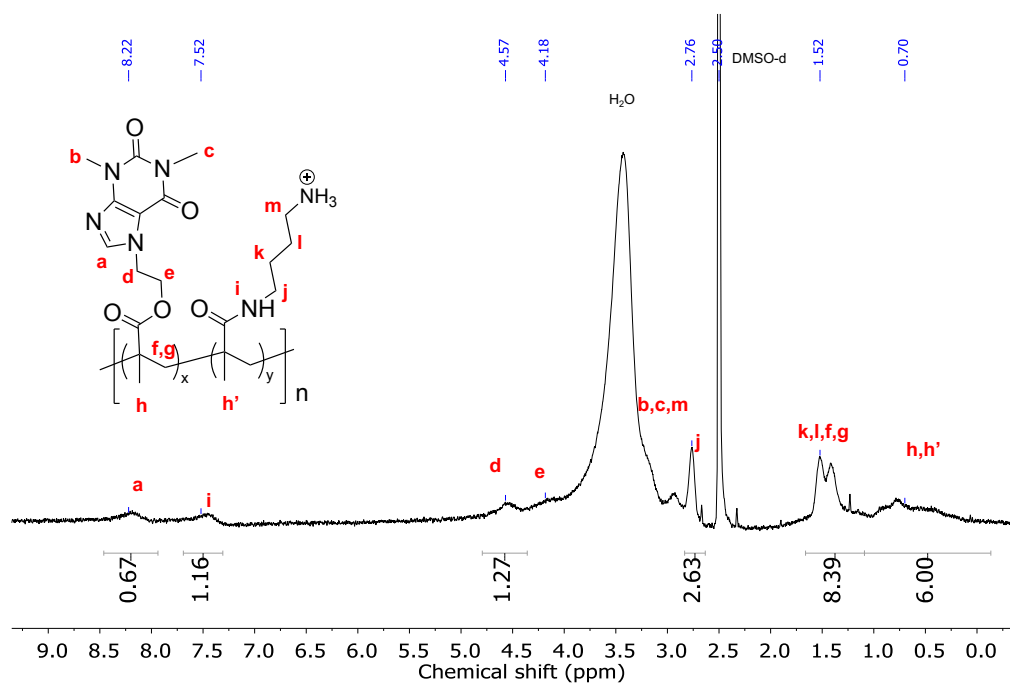


Figure S3.16. ^1H -NMR spectrum of P(ab-Caf30%)MA in $\text{DMSO-}d_6$.

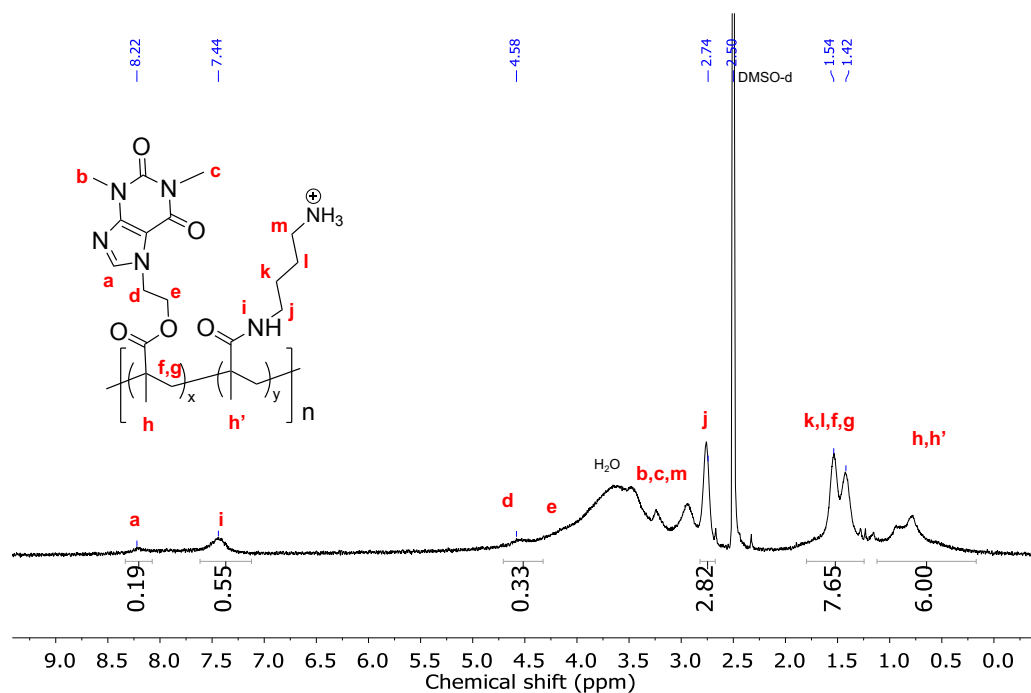


Figure S3.17. ^1H -NMR spectrum of P(ab-Caf10%)MA in $\text{DMSO-}d_6$.

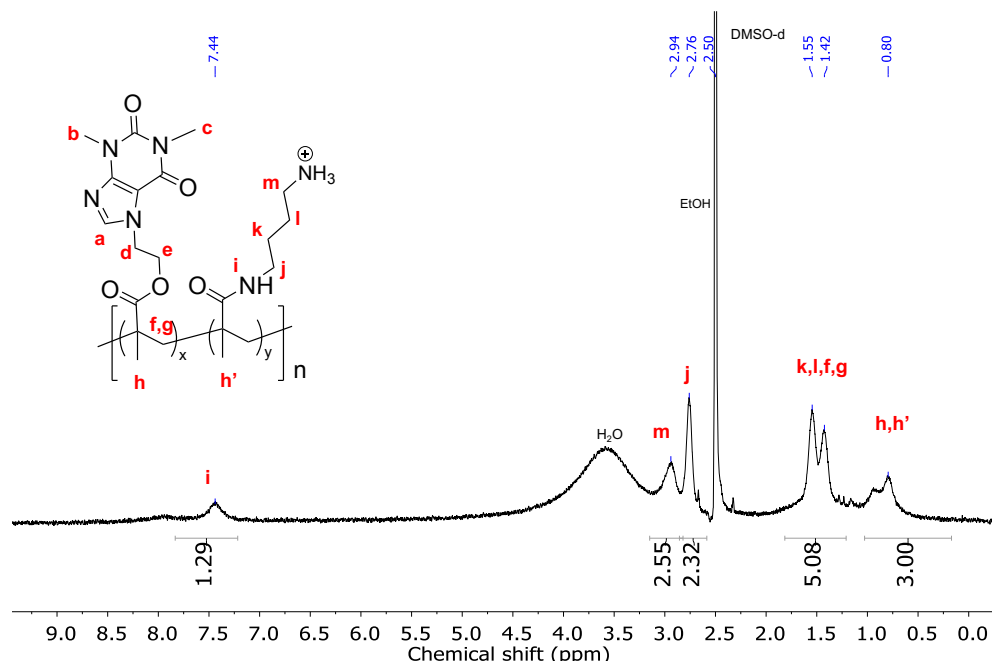


Figure S3.18. ^1H -NMR spectrum of P(ab)MA in $\text{DMSO-}d_6$.

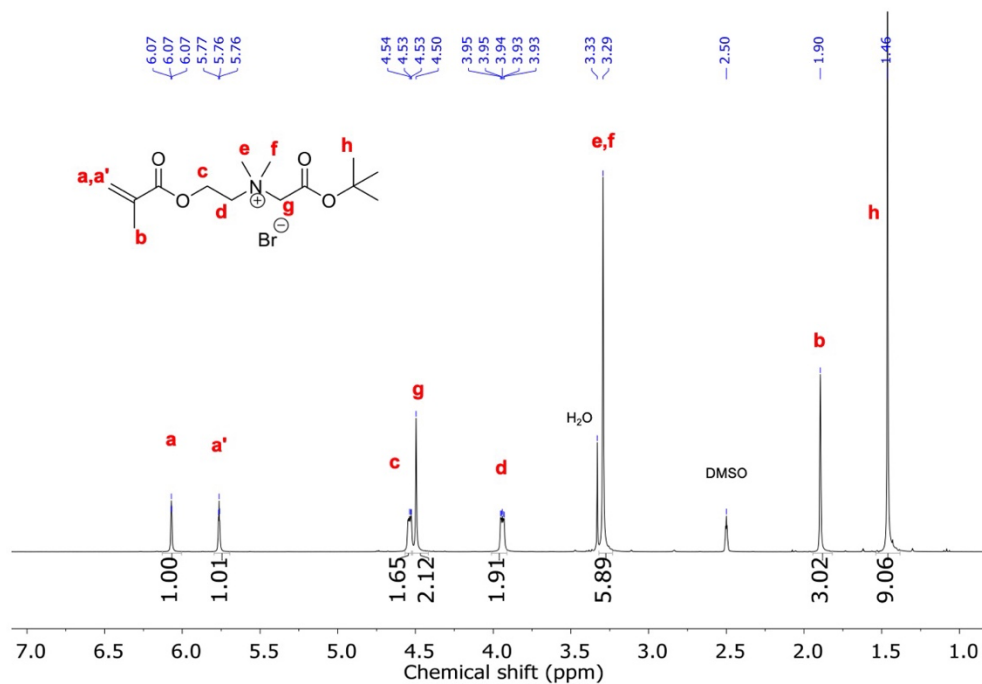


Figure S3.19. ^1H -NMR spectrum of *t*-Bu-DMEA-MA in $\text{DMSO-}d_6$.

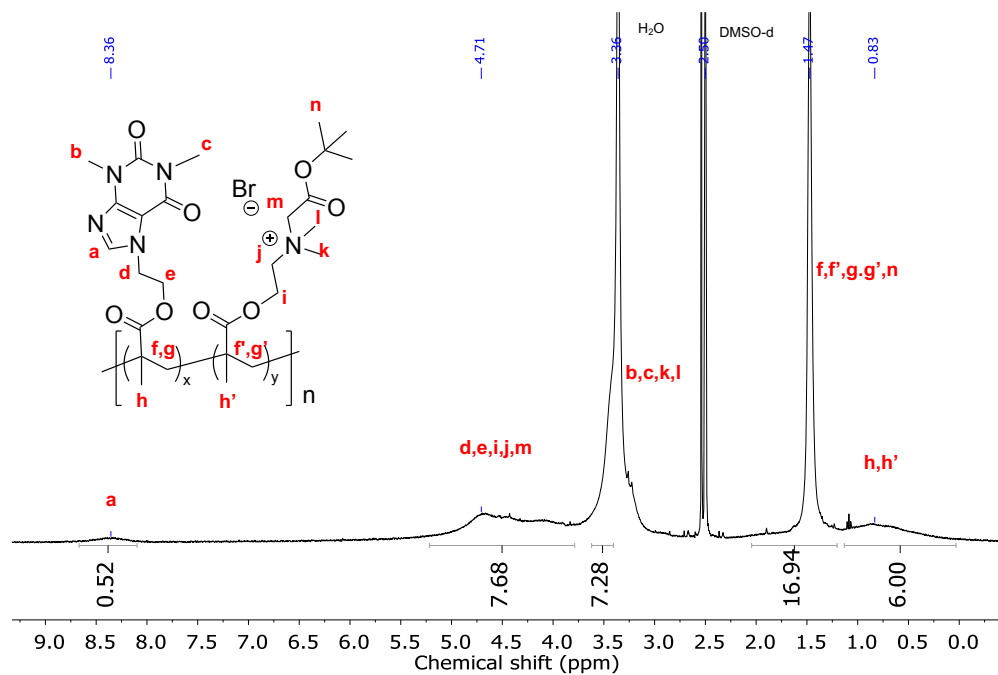


Figure S3.20. ^1H -NMR spectrum of $\text{P}(t\text{-Bu-DMEA-Caf30\%})\text{MA}$ in $\text{DMSO-}d_6$.

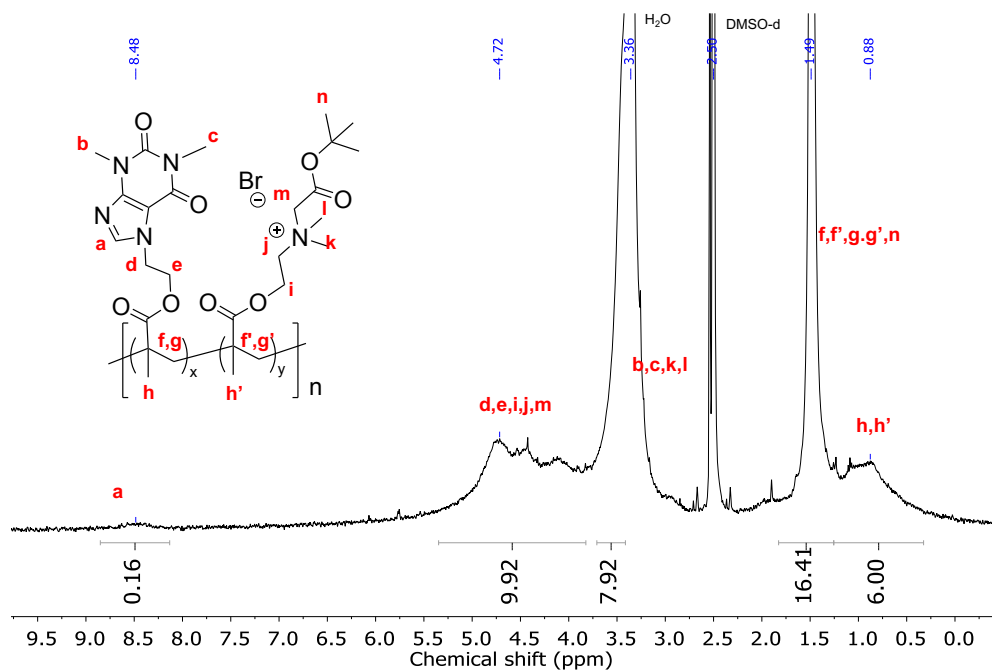


Figure S3.21. ^1H -NMR spectrum of P(*t*-Bu-DMEA-Caf10%)MA in $\text{DMSO-}d_6$.

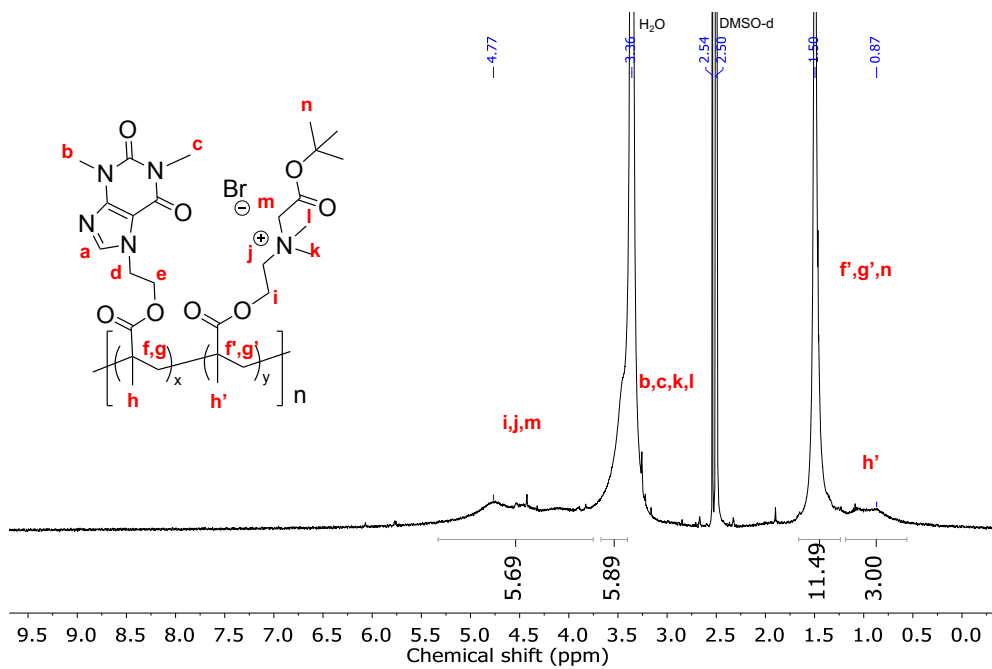


Figure S3.22. ^1H -NMR spectrum of P(*t*-Bu-DMEA)MA in $\text{DMSO-}d_6$.

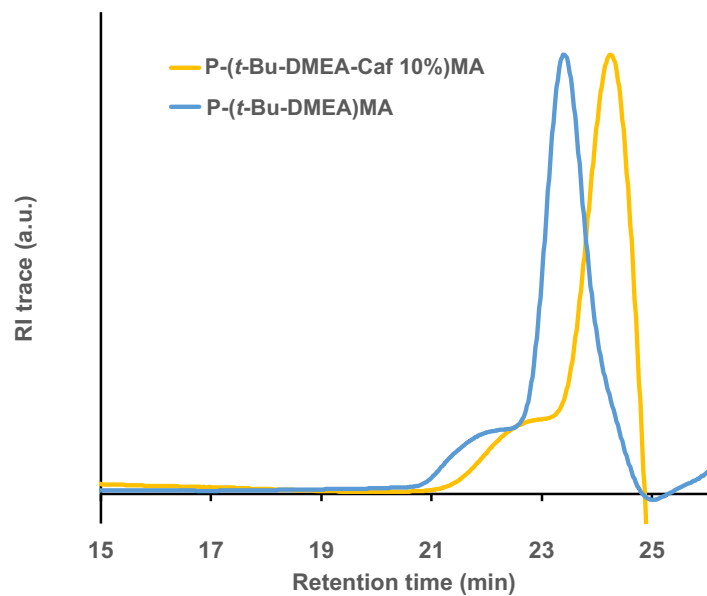


Figure S3.23. Chromatograms from aqueous SEC from RI detector for P(*t*-Bu-DMEA-Caf)MA series of copolymers.

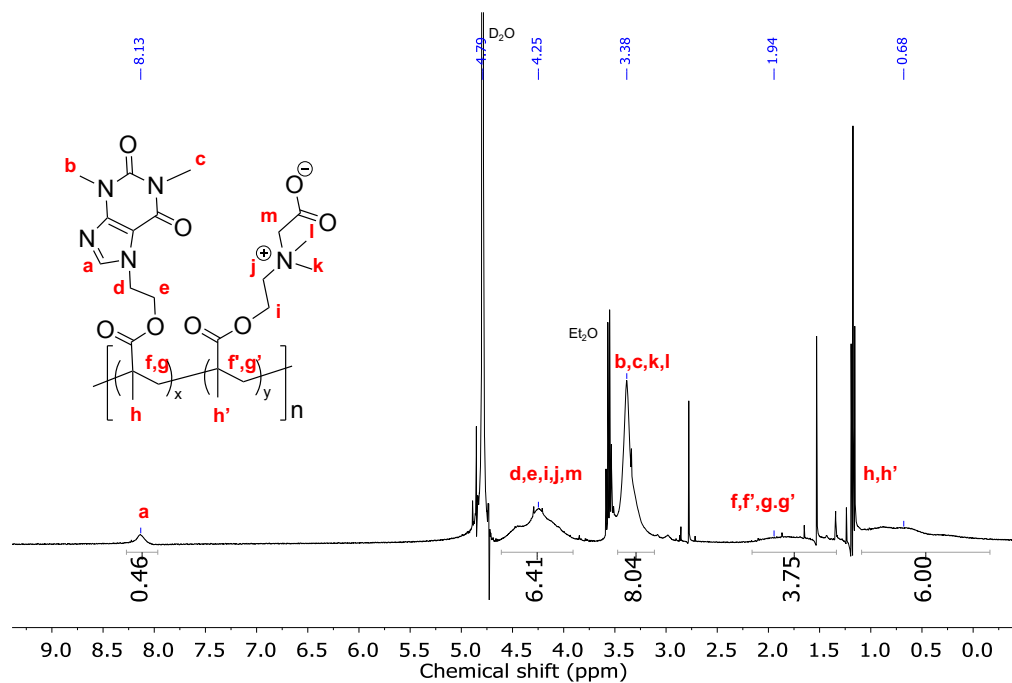


Figure S3.24. ¹H-NMR spectrum of P(DMEA-Caf30%)MA in DMSO-*d*₆.

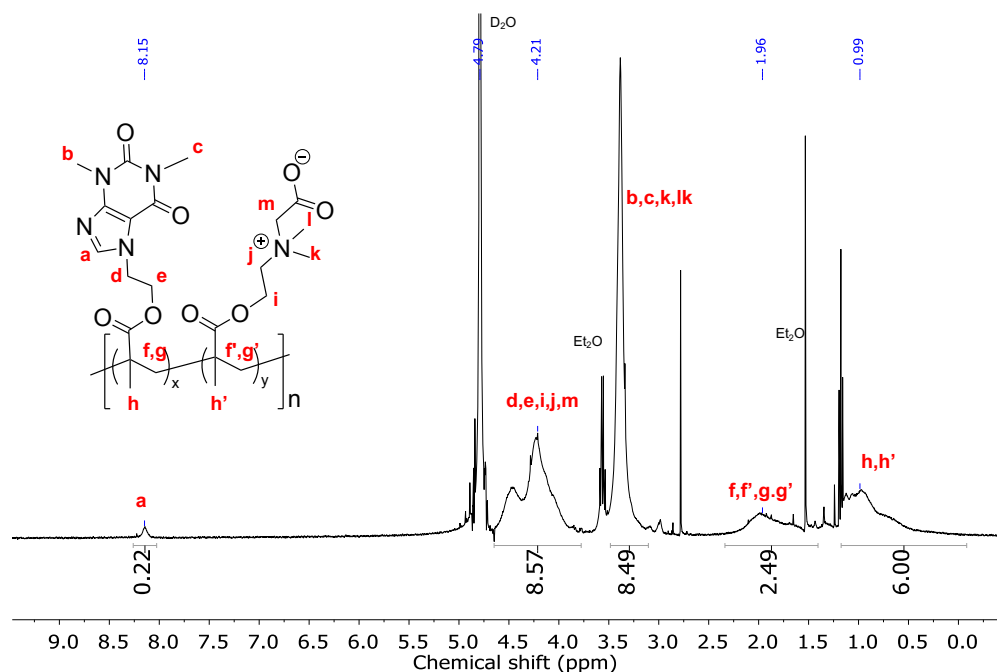


Figure S3.25. ^1H -NMR spectrum of P(DMEA-Caf10%)MA in DMSO- d_6 .

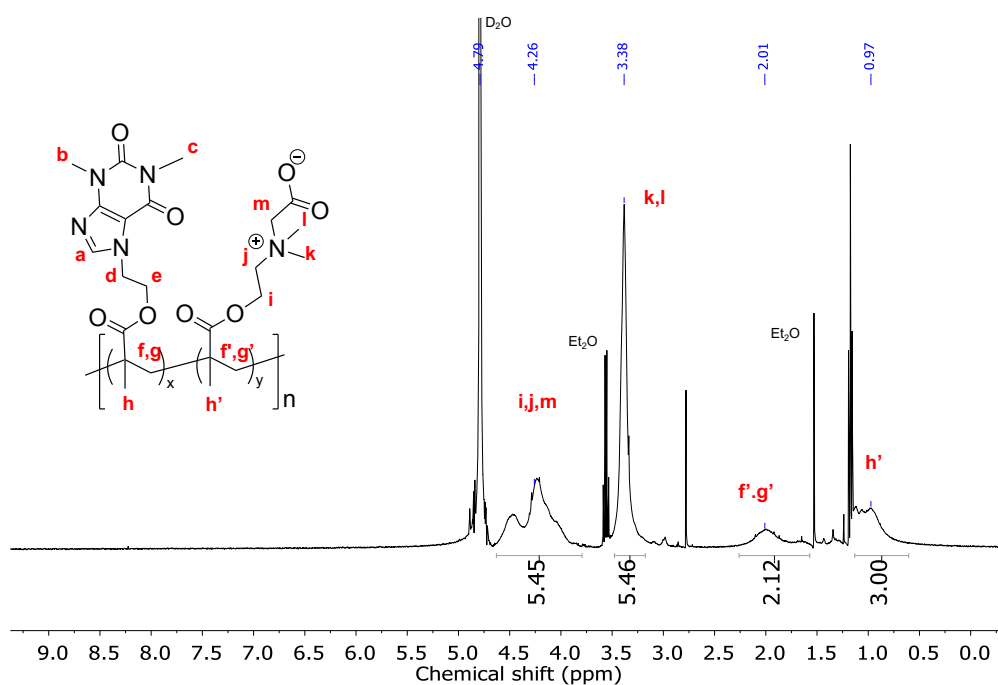


Figure S3.26. ^1H -NMR spectrum of P(DMEA)MA in DMSO- d_6 .

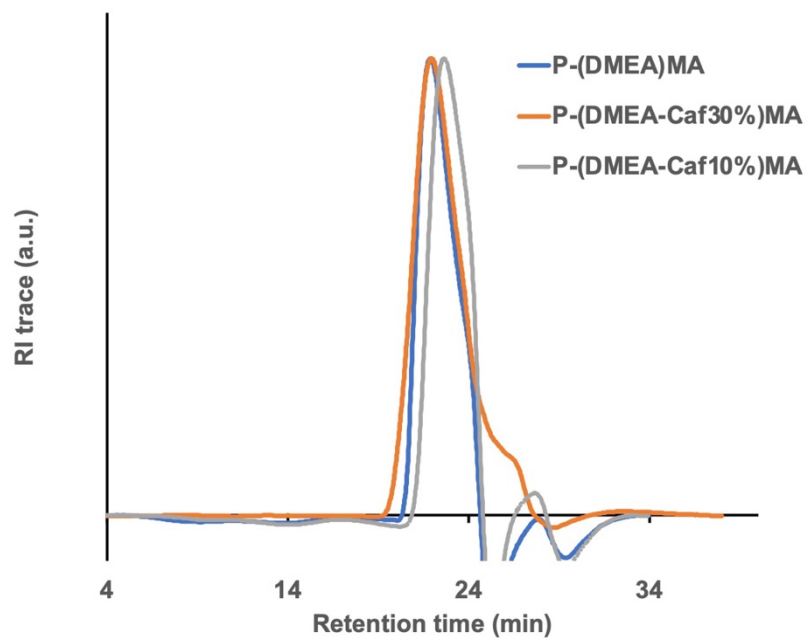


Figure S3.27. Chromatograms from aqueous SEC from RI detector for P(DMEA-Caf)MA series of copolymers upon Boc-deprotection.

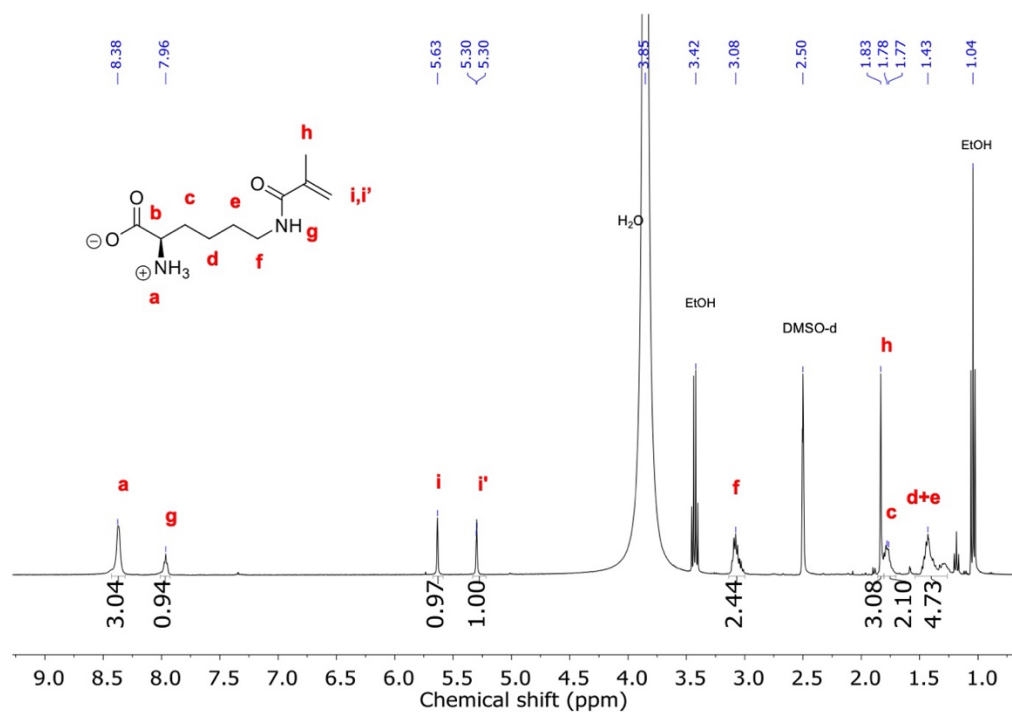


Figure S3.28. ^1H -NMR spectrum of Lys-MA in $\text{DMSO-}d_6$.

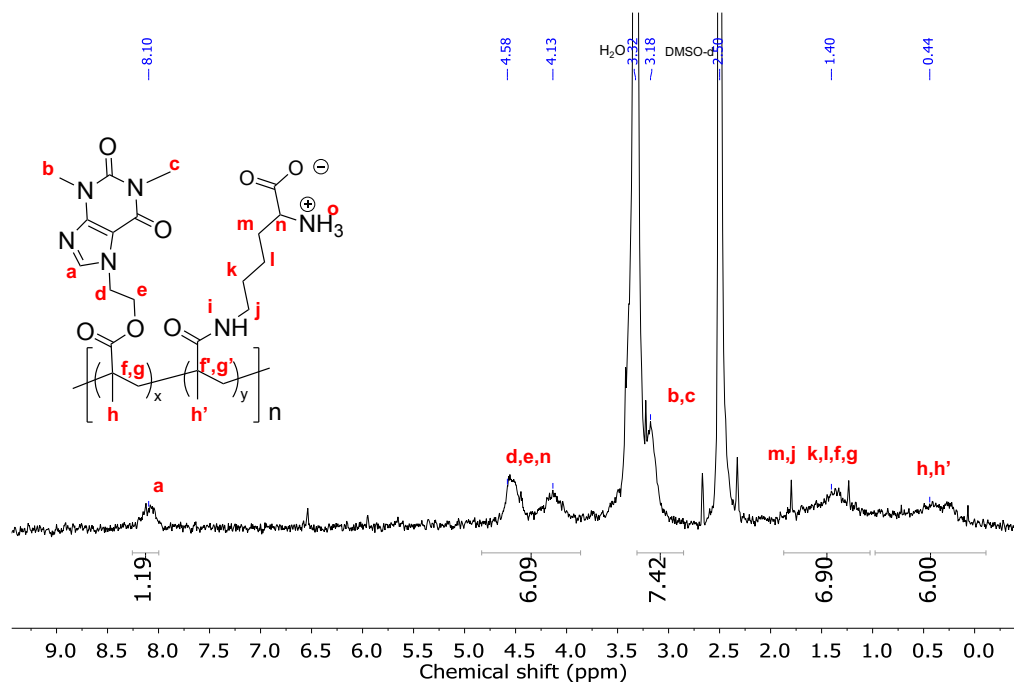


Figure S3.29. ^1H -NMR spectrum of P(Lys-Caf50%)MA in DMSO- d_6 .

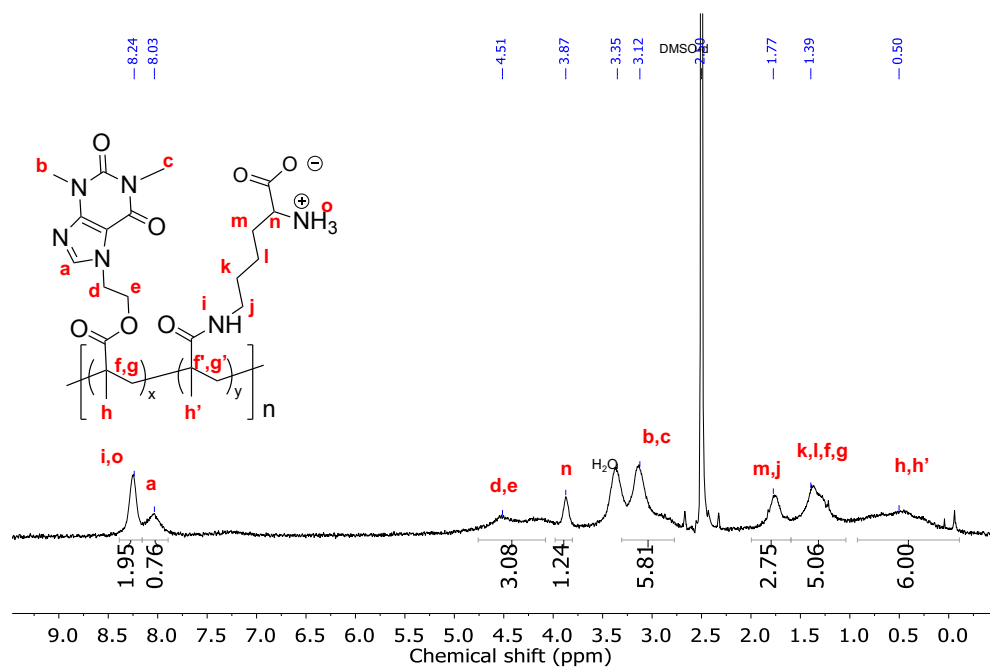


Figure S3.30. ^1H -NMR spectrum of P(Lys-Caf30%)MA in DMSO- d_6 .

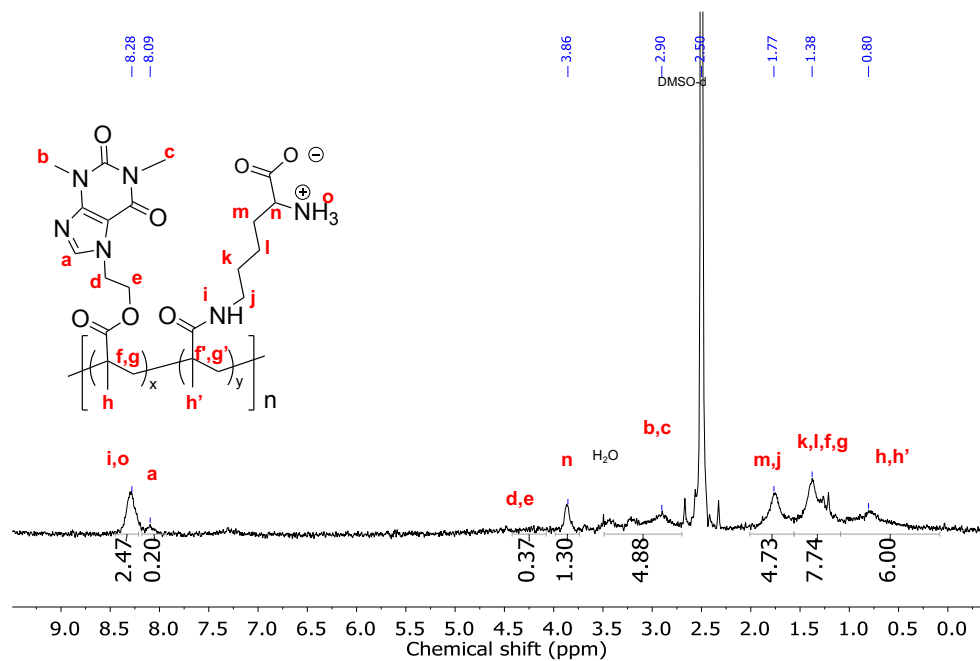


Figure S3.31. ¹H-NMR spectrum of P(Lys-Caf10%)MA in DMSO-*d*₆.

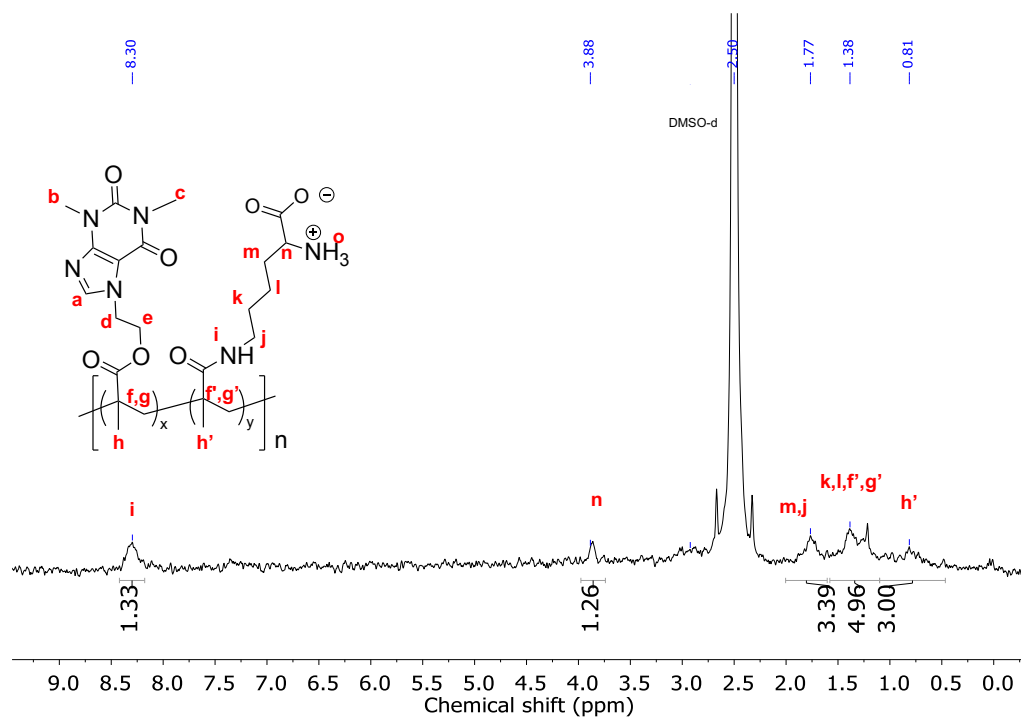


Figure S3.32. ¹H-NMR spectrum of P(Lys)MA in DMSO-*d*₆.

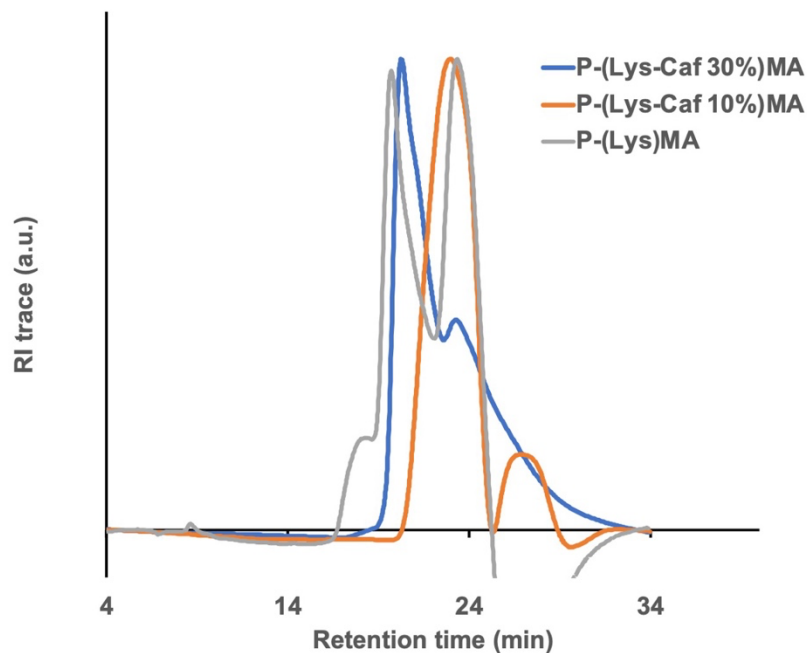


Figure S3.33. Chromatograms from aqueous SEC from RI detector for P(Lys-Caf)MA series of copolymers.

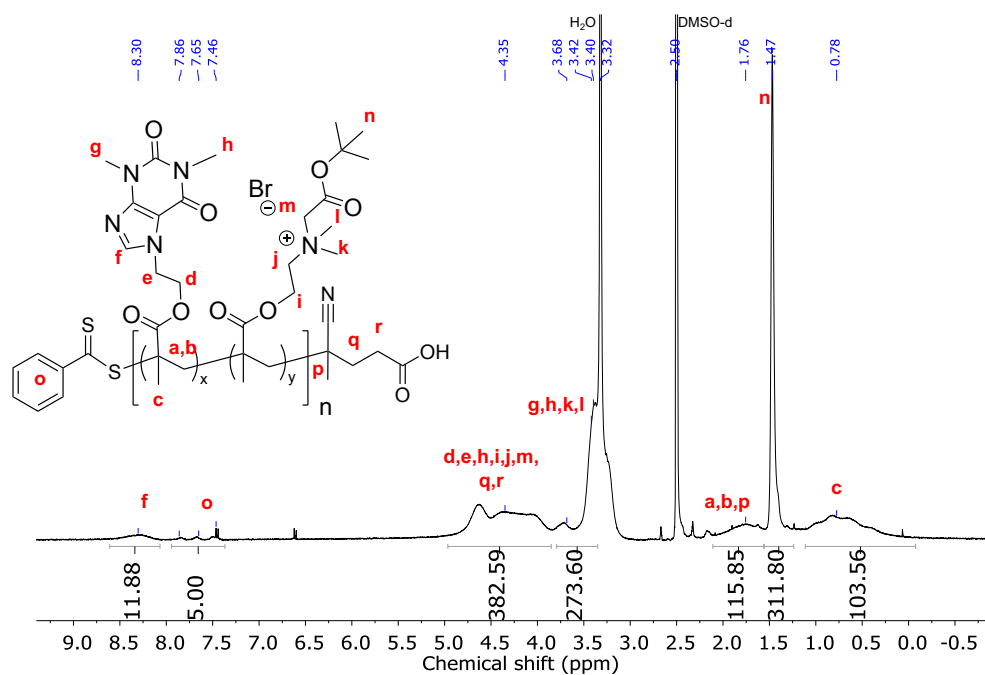


Figure S3.34. ^1H -NMR spectrum of P(*t*-Bu-DMEA-Caf30%)MA DP30 in $\text{DMSO-}d_6$.

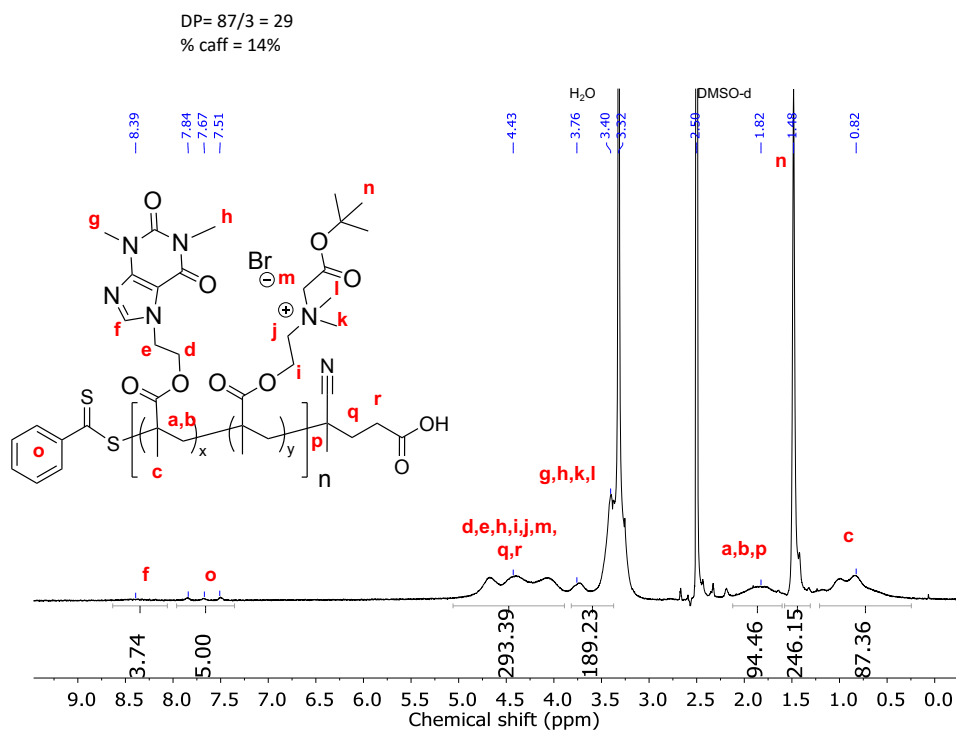


Figure S3.35. ^1H -NMR spectrum of P(*t*-Bu-DMEA-Caf10%)MA DP30 in $\text{DMSO-}d_6$.

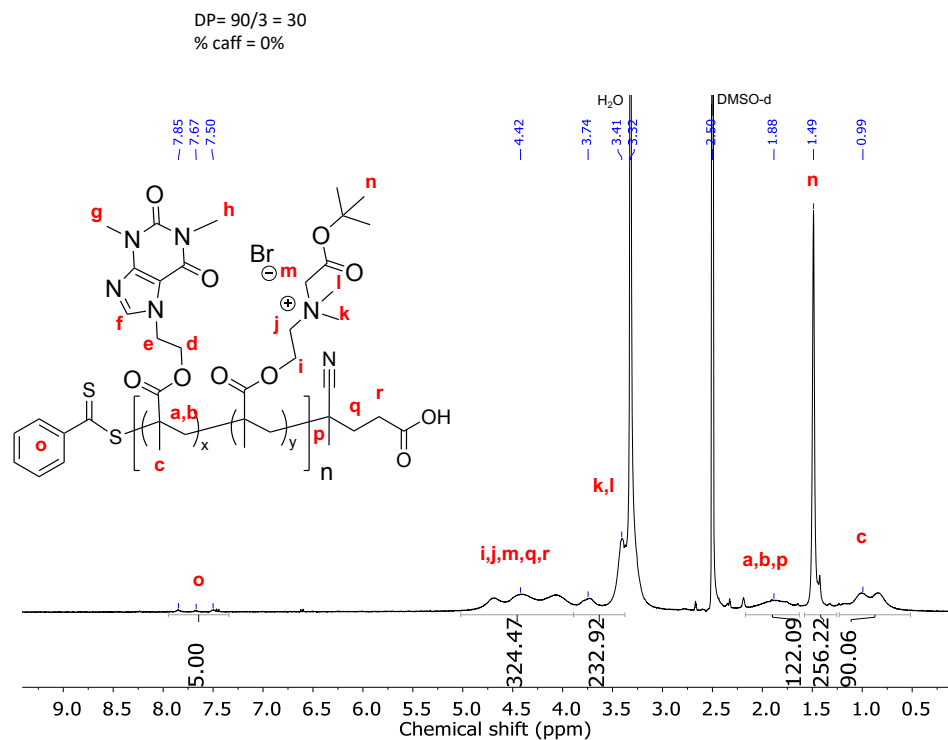


Figure S3.36. ^1H -NMR spectrum of P(*t*-Bu-DMEA)MA DP30 in $\text{DMSO-}d_6$.

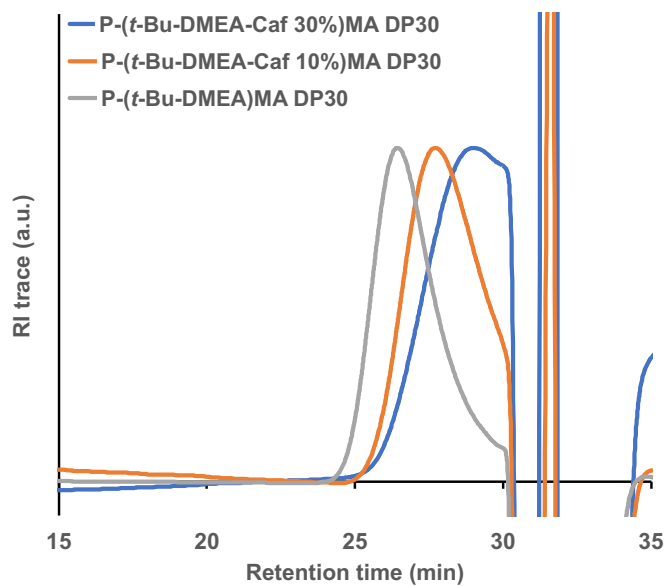


Figure S3.37. Chromatograms from aqueous SEC from RI detector for P(*t*-Bu-DMEA-Caf)MA DP30 series of copolymers.

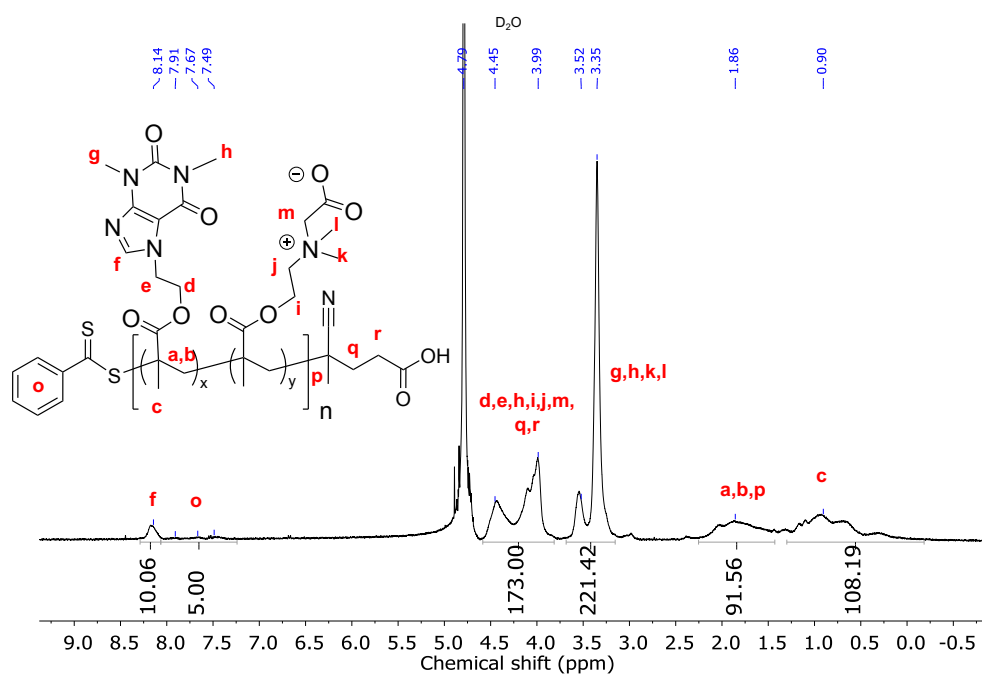


Figure S3.38. ¹H-NMR spectrum of P(DMEA-Caf30%)MA DP30 in DMSO-*d*₆.

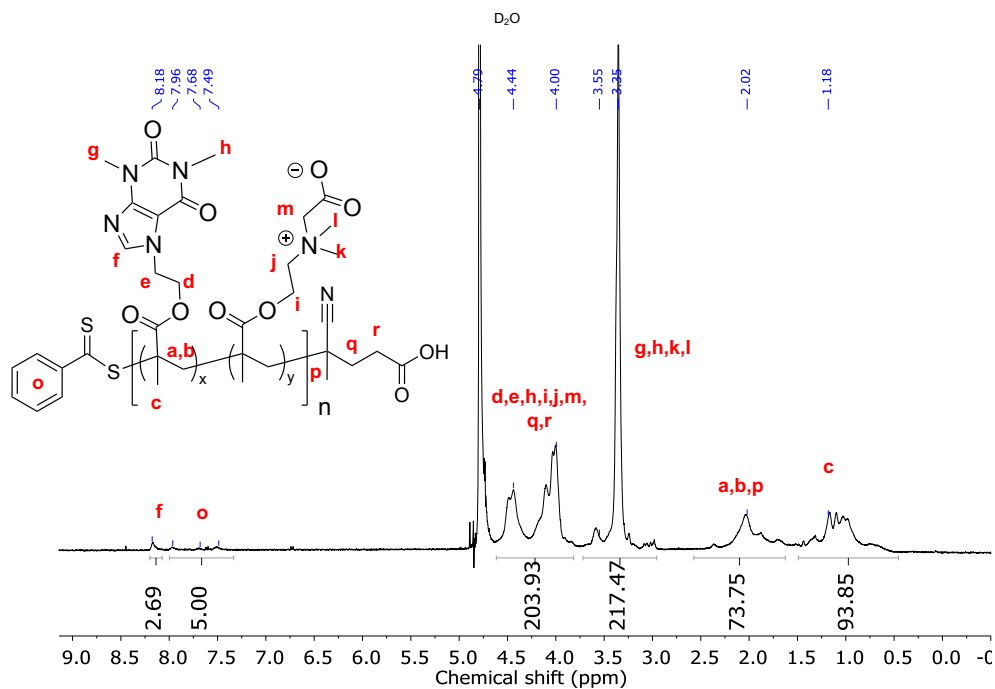


Figure S3.39. ¹H-NMR spectrum of P(DMEA-Caf10%)MA DP30 in DMSO-*d*₆.

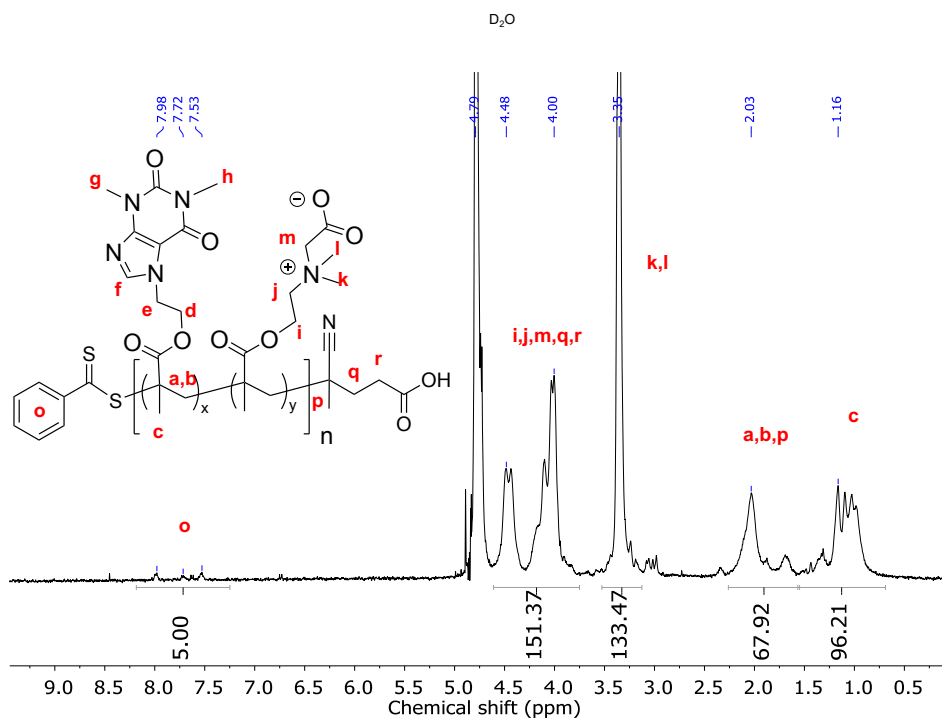


Figure S3.40. ¹H-NMR spectrum of P(DMEA)MA DP30 in DMSO-*d*₆.

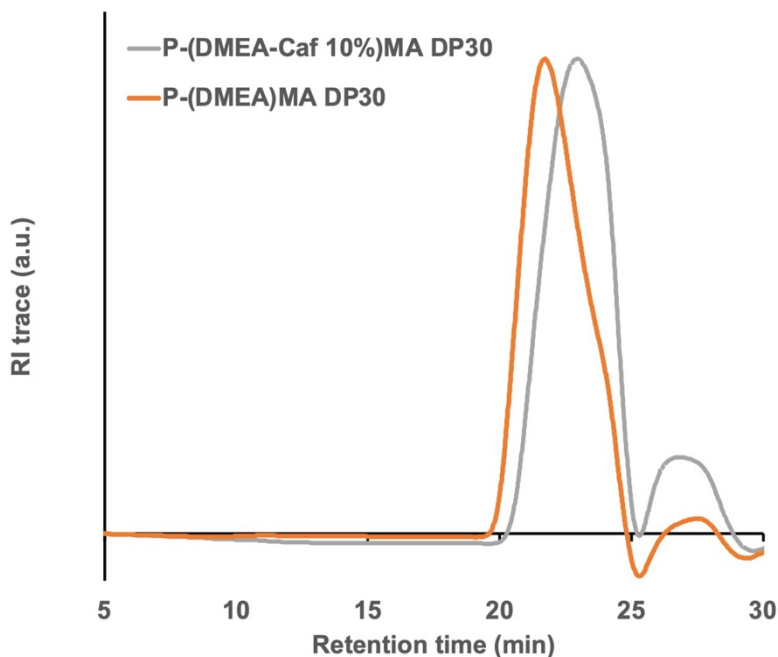


Figure S3.41. Chromatograms from aqueous SEC from RI detector for P(DMEA-Caf)MA DP30 series of copolymers upon Boc-deprotection.

3.4.2. Hydroxybenzoate-co-lactide polymers.

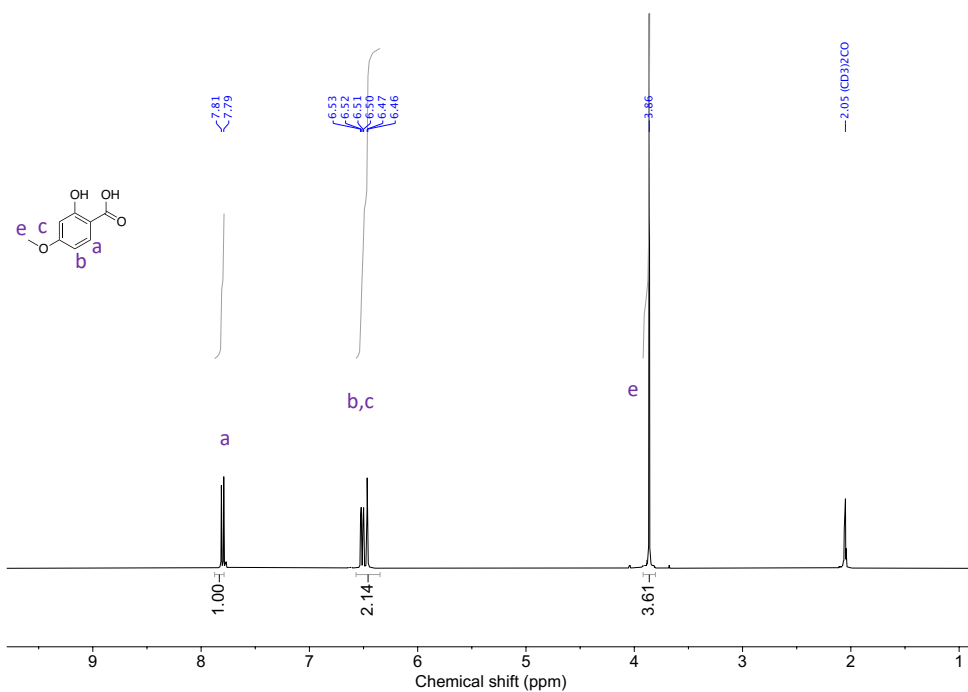


Figure S3.42. ^1H -NMR of 3 in acetone- d_6 .

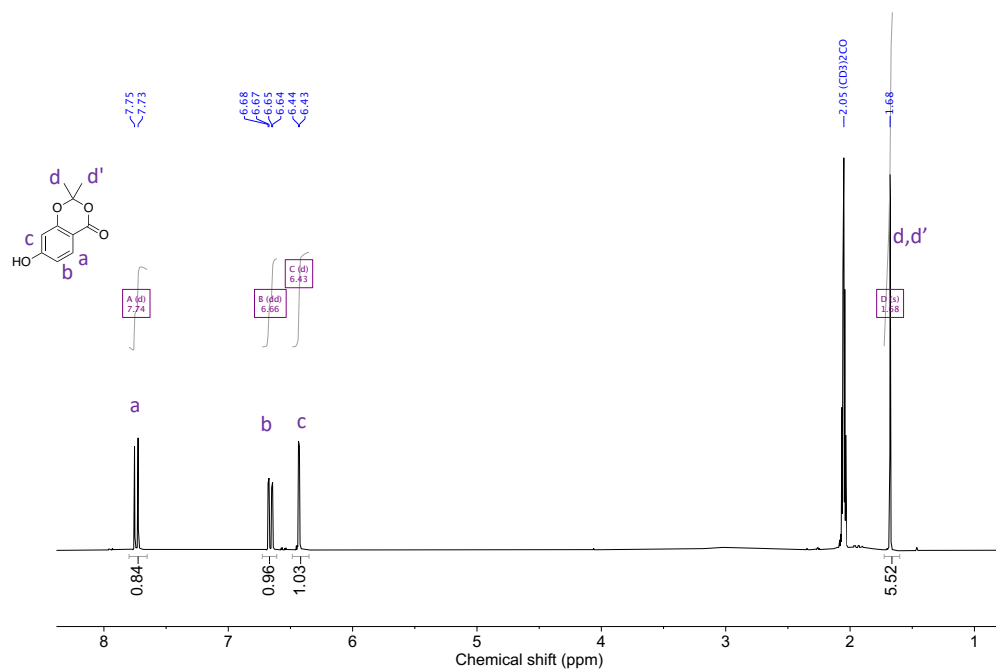
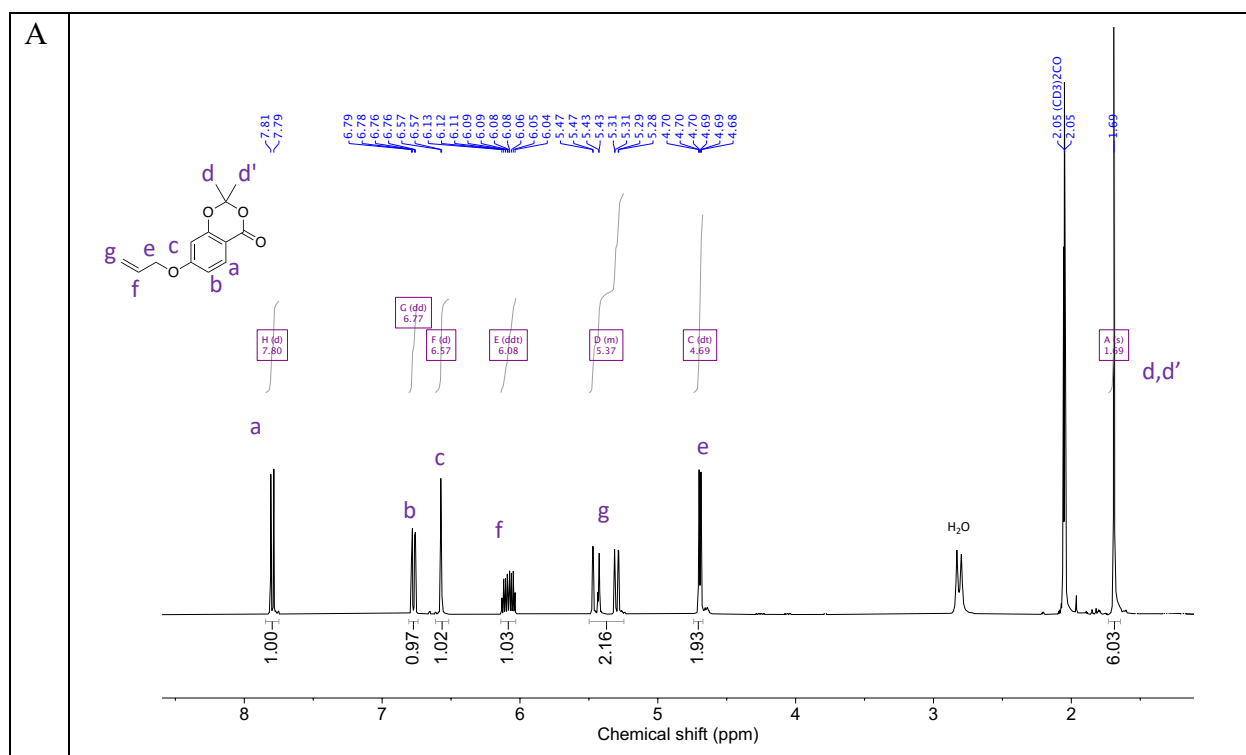


Figure S3.43. ^1H -NMR of 7-hydroxy-2,2-dimethyl-4H-benzo[d][1,3]dioxin-4-one in acetone- d_6 .



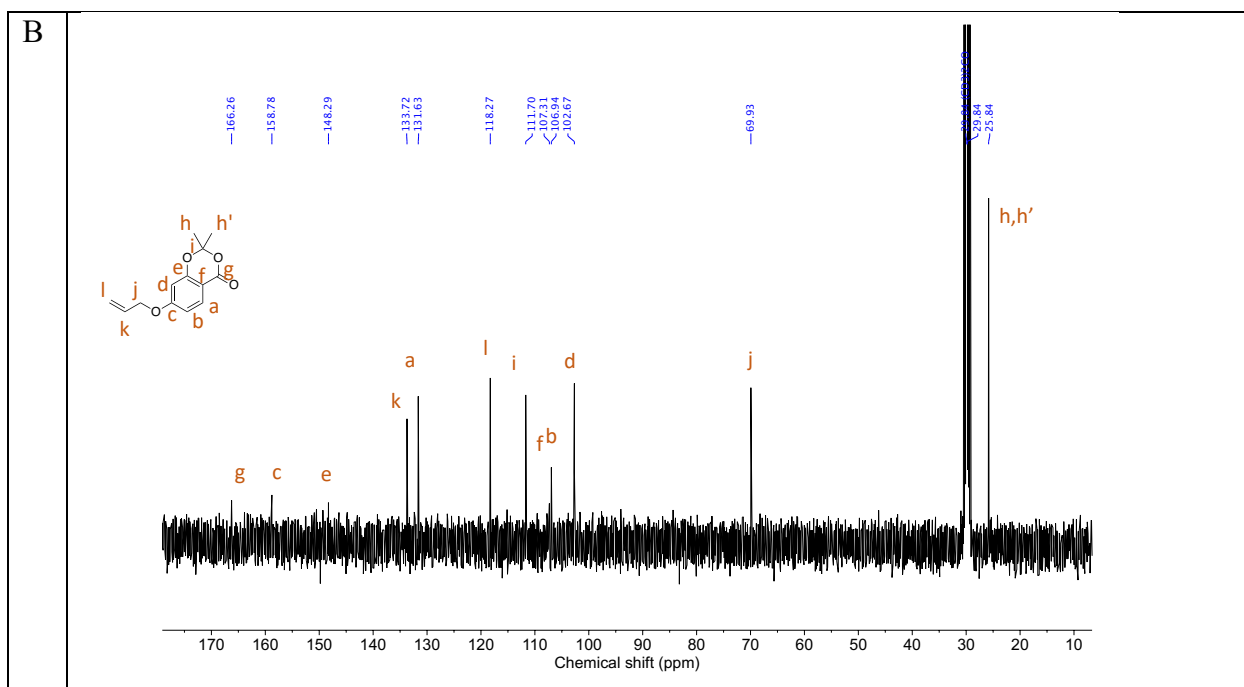
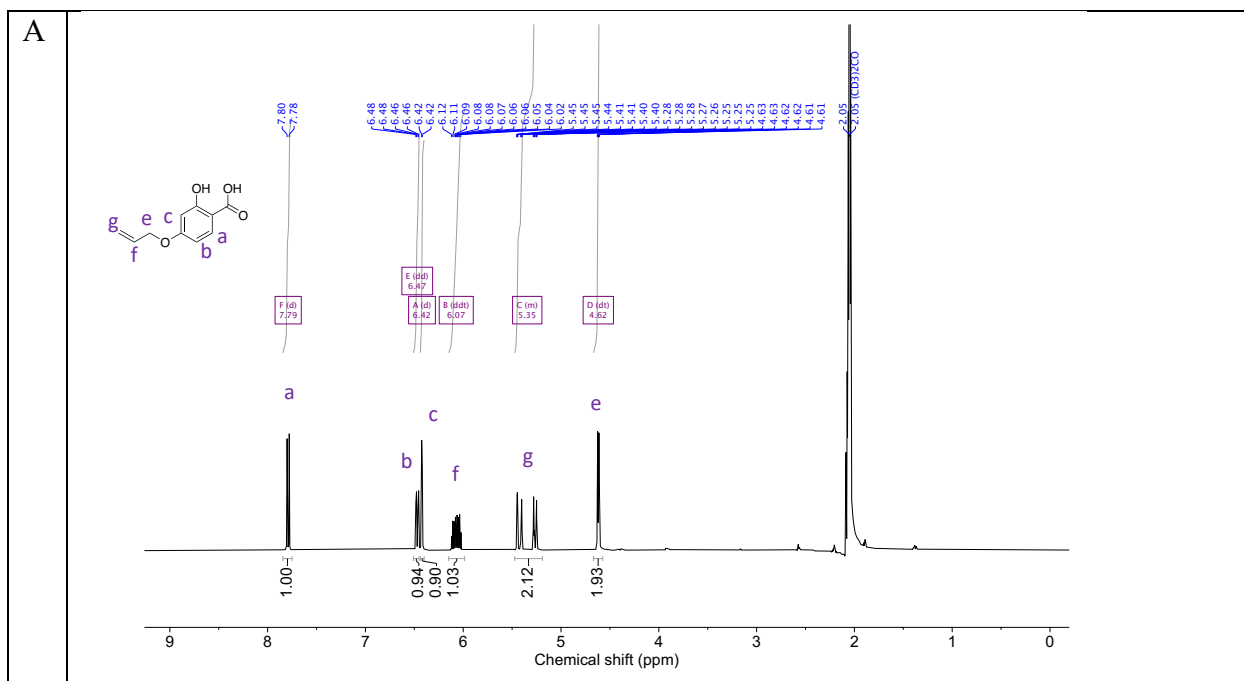


Figure S3.44. A) ^1H -NMR and B) ^{13}C -NMR of 7-(allyloxy)-2,2-dimethyl-4*H*-benzo[*d*][1,3]dioxin-4-one in acetone- d_6 .



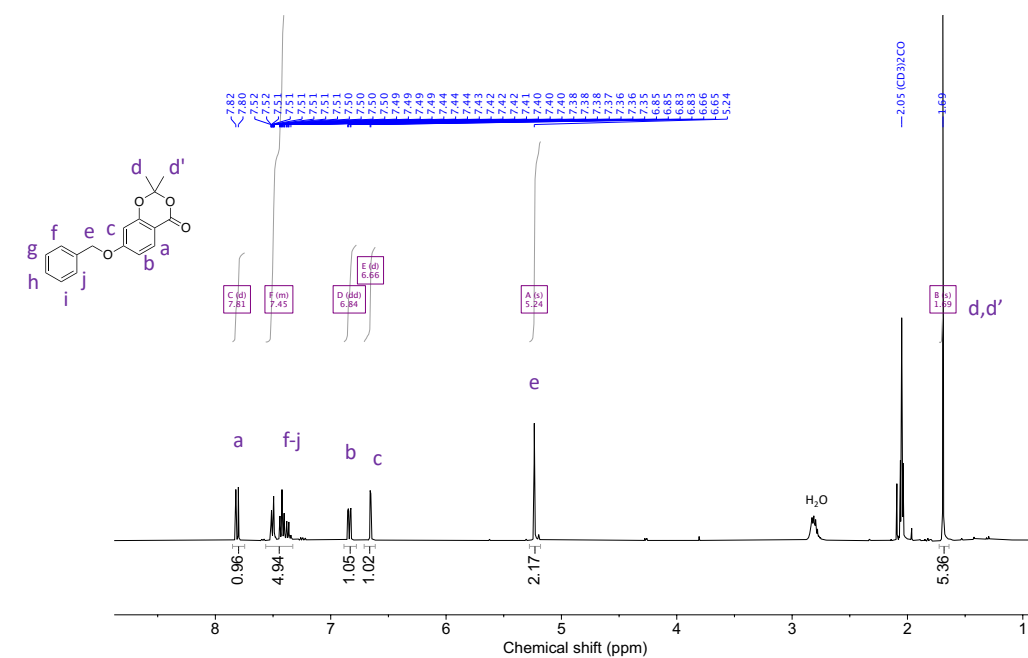
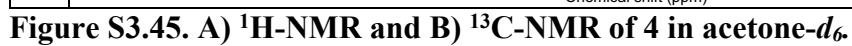
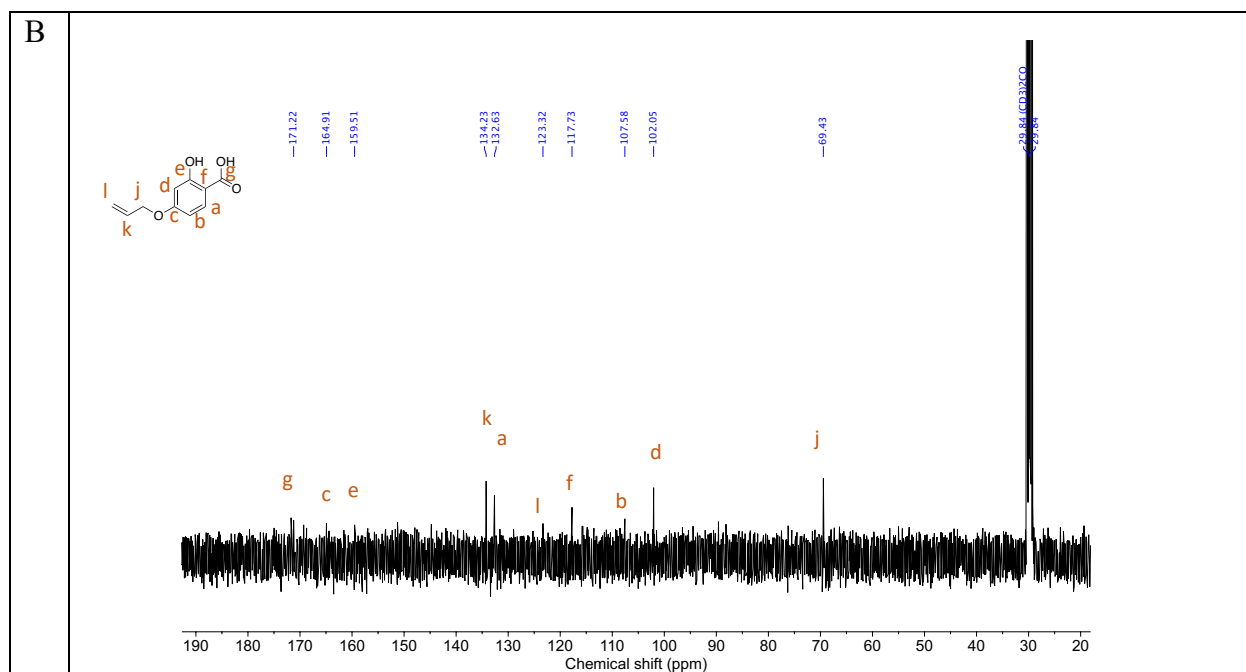


Figure S3.46. ¹H-NMR of 7-(benzyloxy)-2,2-dimethyl-4*H*-benzo[*d*][1,3]dioxin-4-one in acetone-*d*₆.

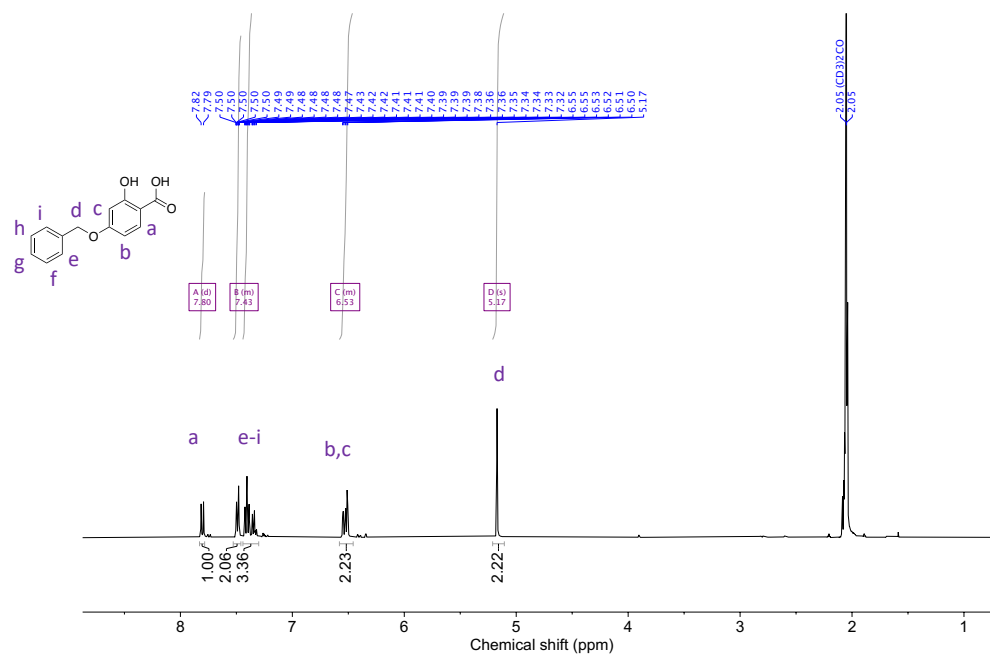


Figure S3.47. ¹H-NMR of 5 in acetone-*d*₆.

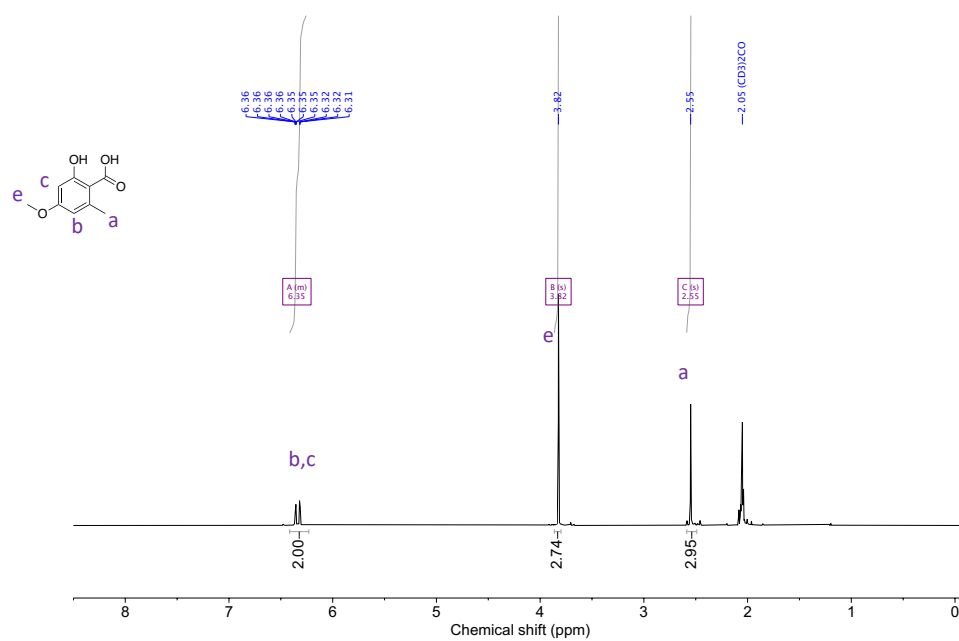


Figure S3.48. ¹H-NMR of 7 in acetone-*d*₆.

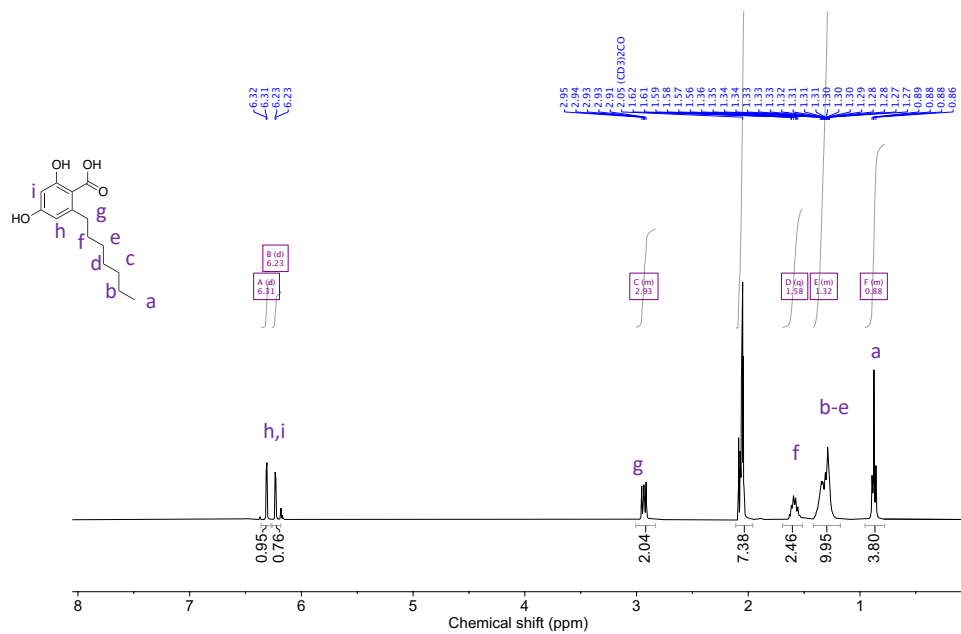
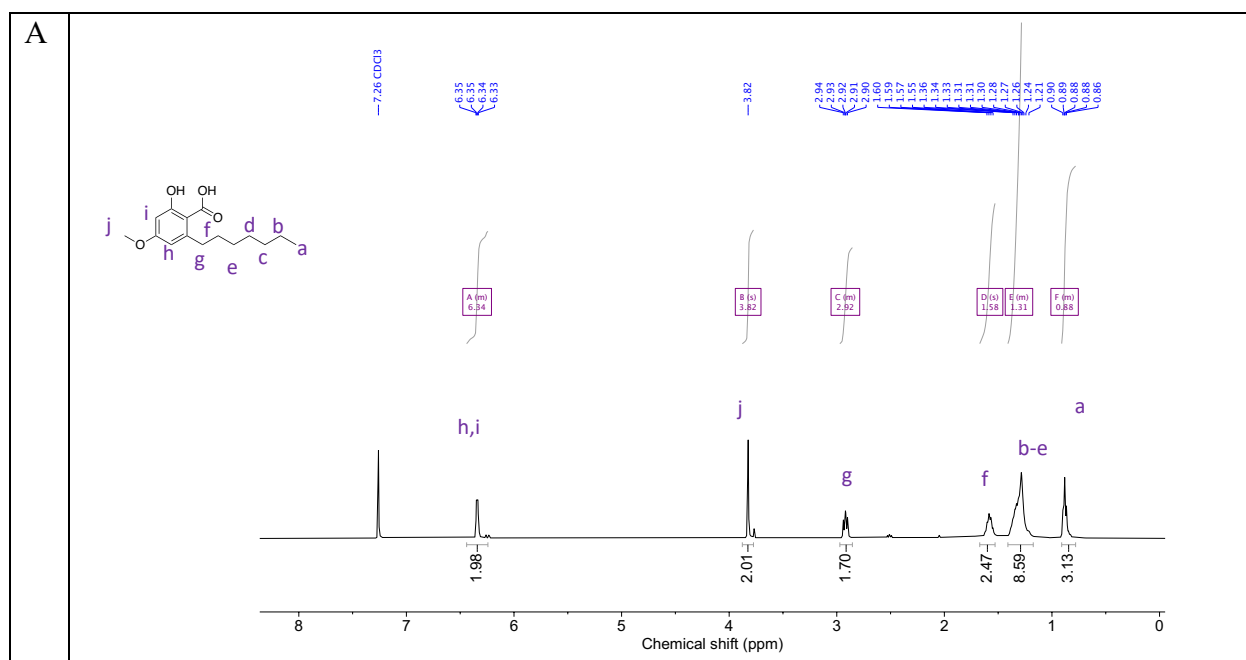


Figure S3.49. ^1H -NMR of 8 in acetone- d_6 .



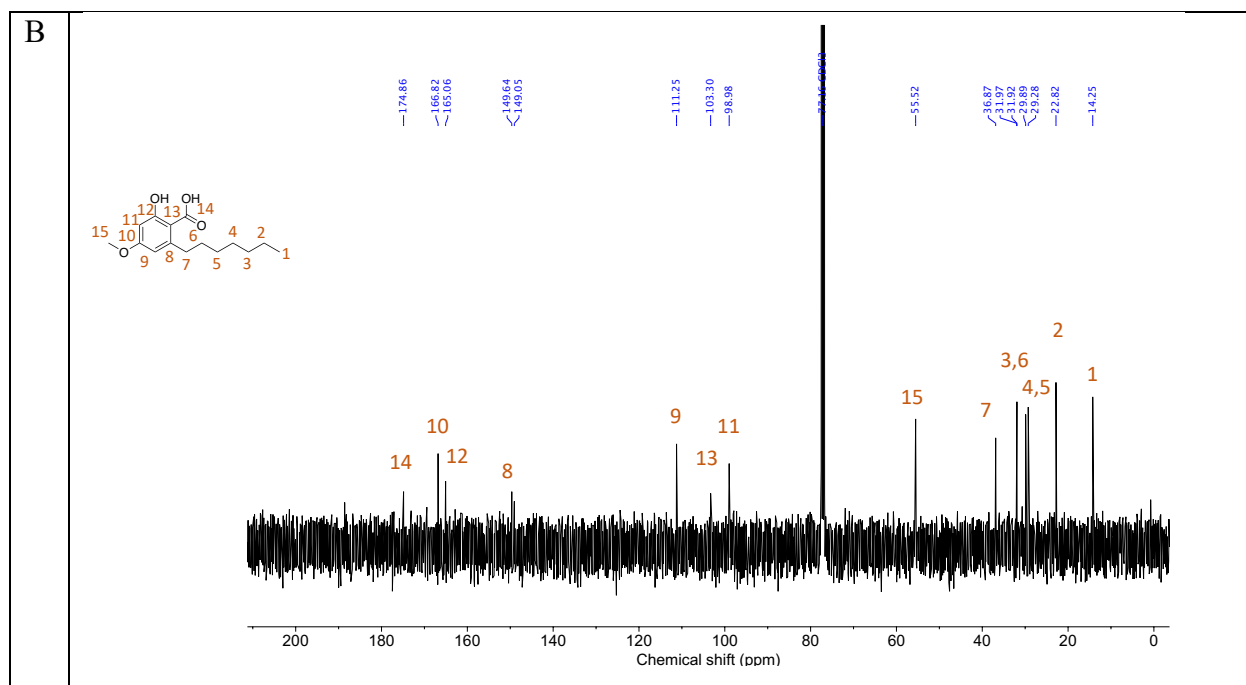


Figure S3.50. A) ^1H -NMR and B) ^{13}C -NMR of 9 in CDCl_3 .

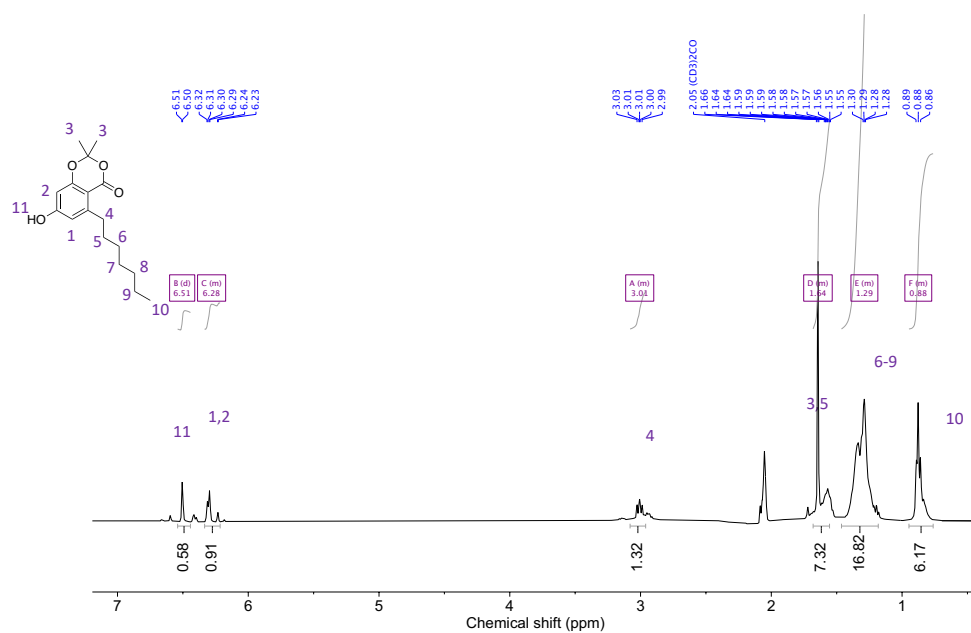


Figure S3.51. ^1H -NMR of 5-heptyl-7-hydroxy-2,2-dimethyl-4H-benzo[d][1,3]dioxin-4-one in acetone- d_6 .

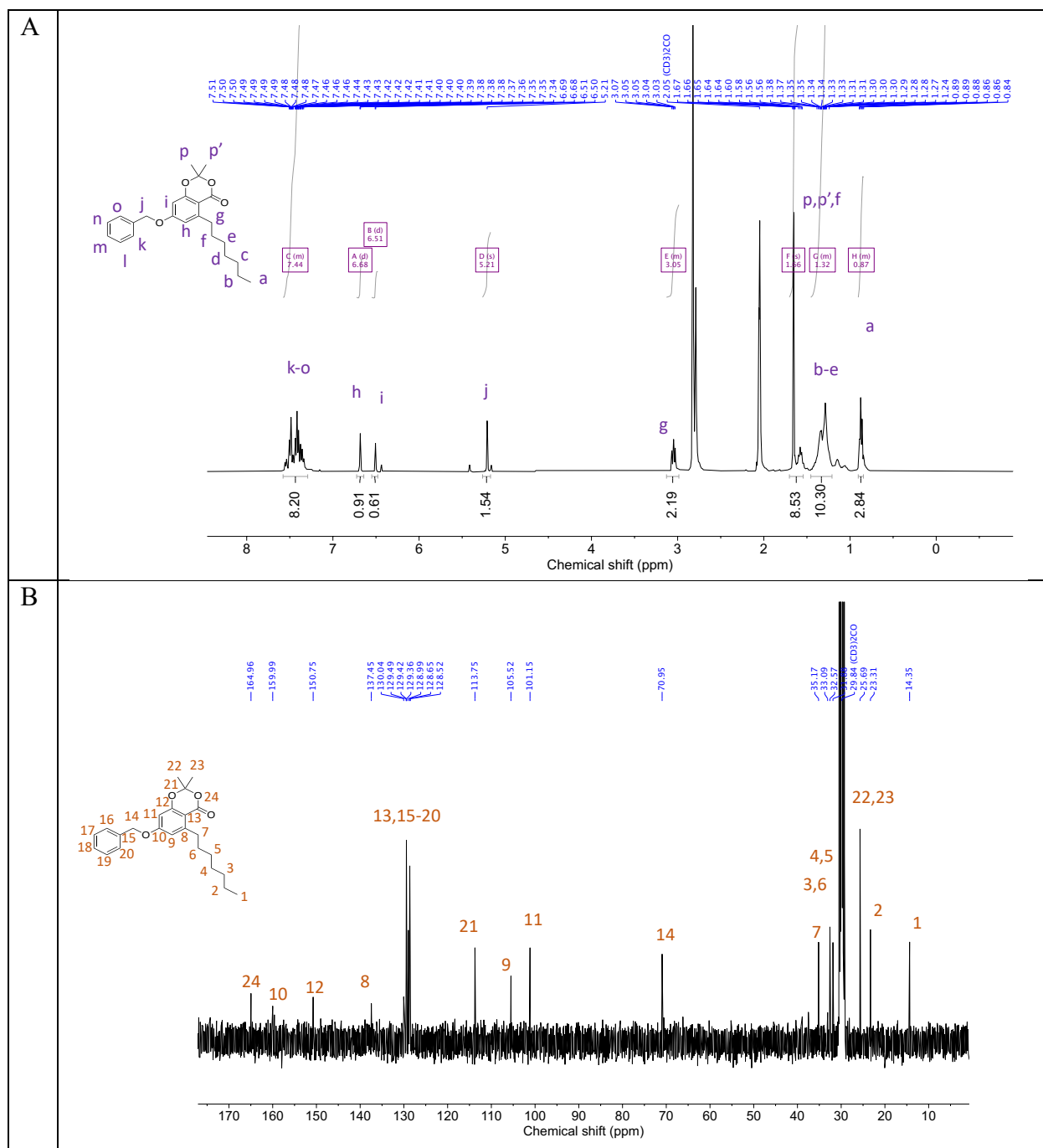


Figure S3.52. A) ^1H -NMR and B) ^{13}C -NMR of 7-(benzyloxy)-5-heptyl-2,2-dimethyl-4*H*-benzo[*d*][1,3]dioxin-4-one in acetone- d_6 .

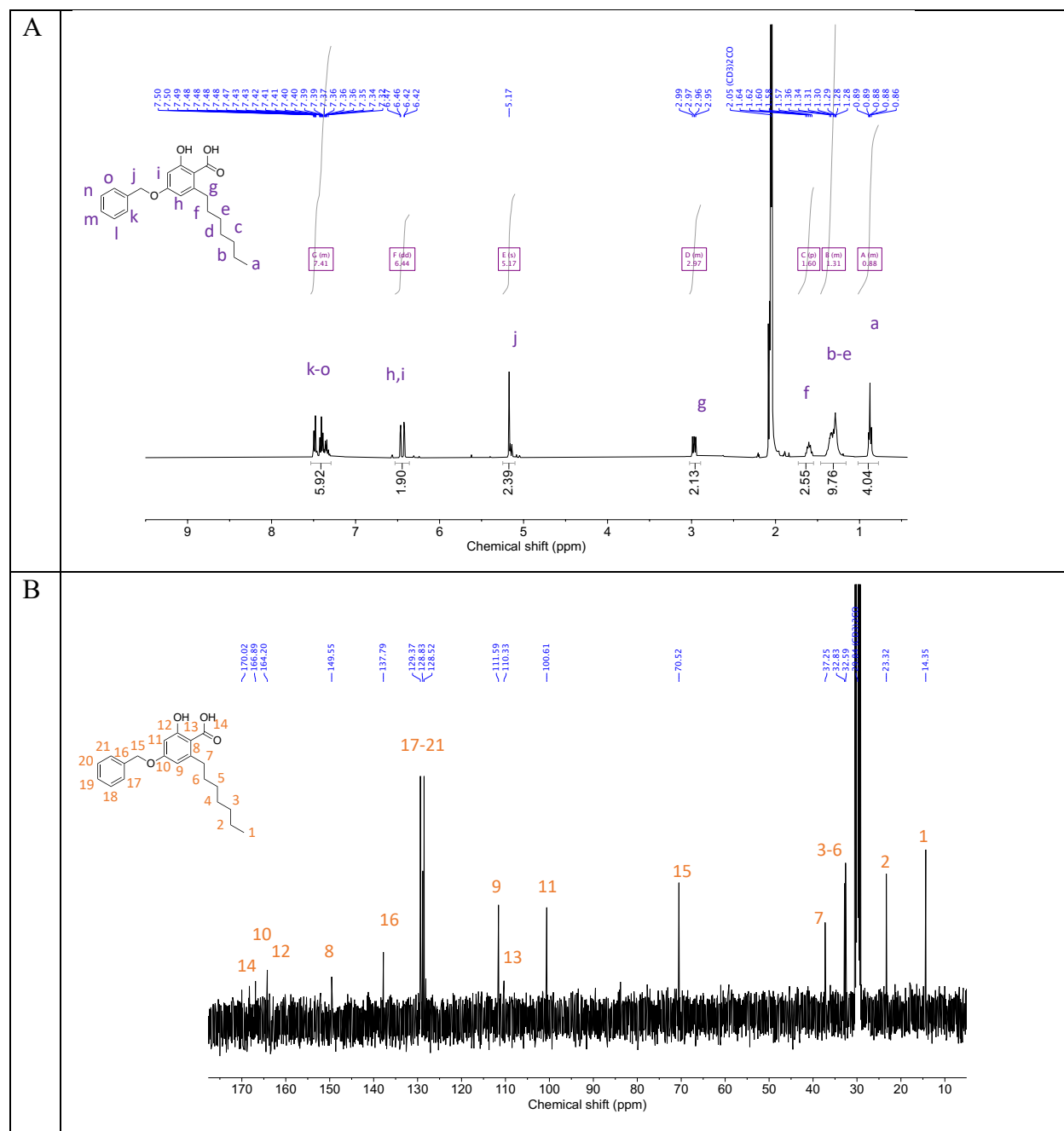
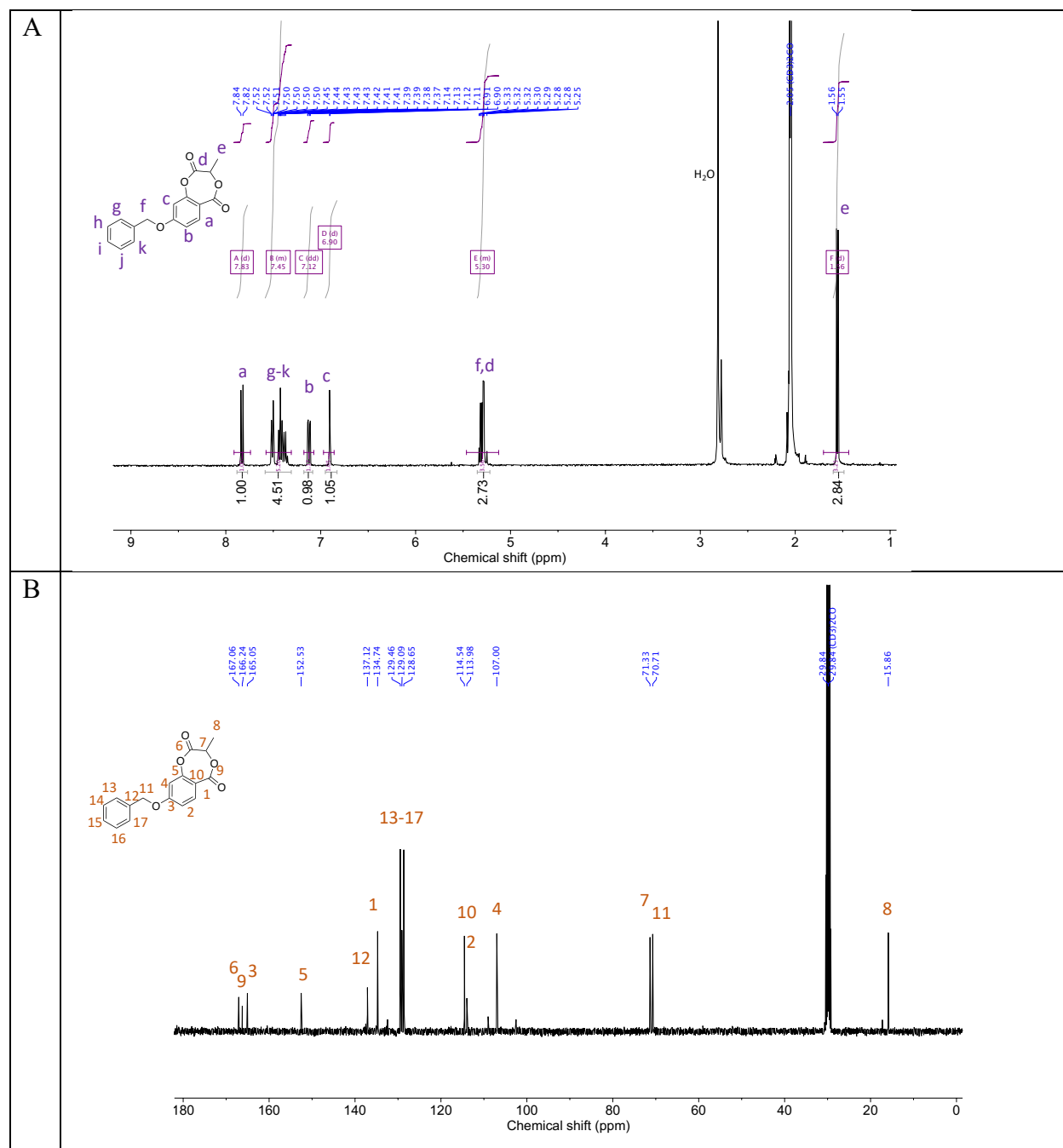


Figure S3.53. A) ^1H -NMR and B) ^{13}C -NMR of 10 in acetone- d_6 .



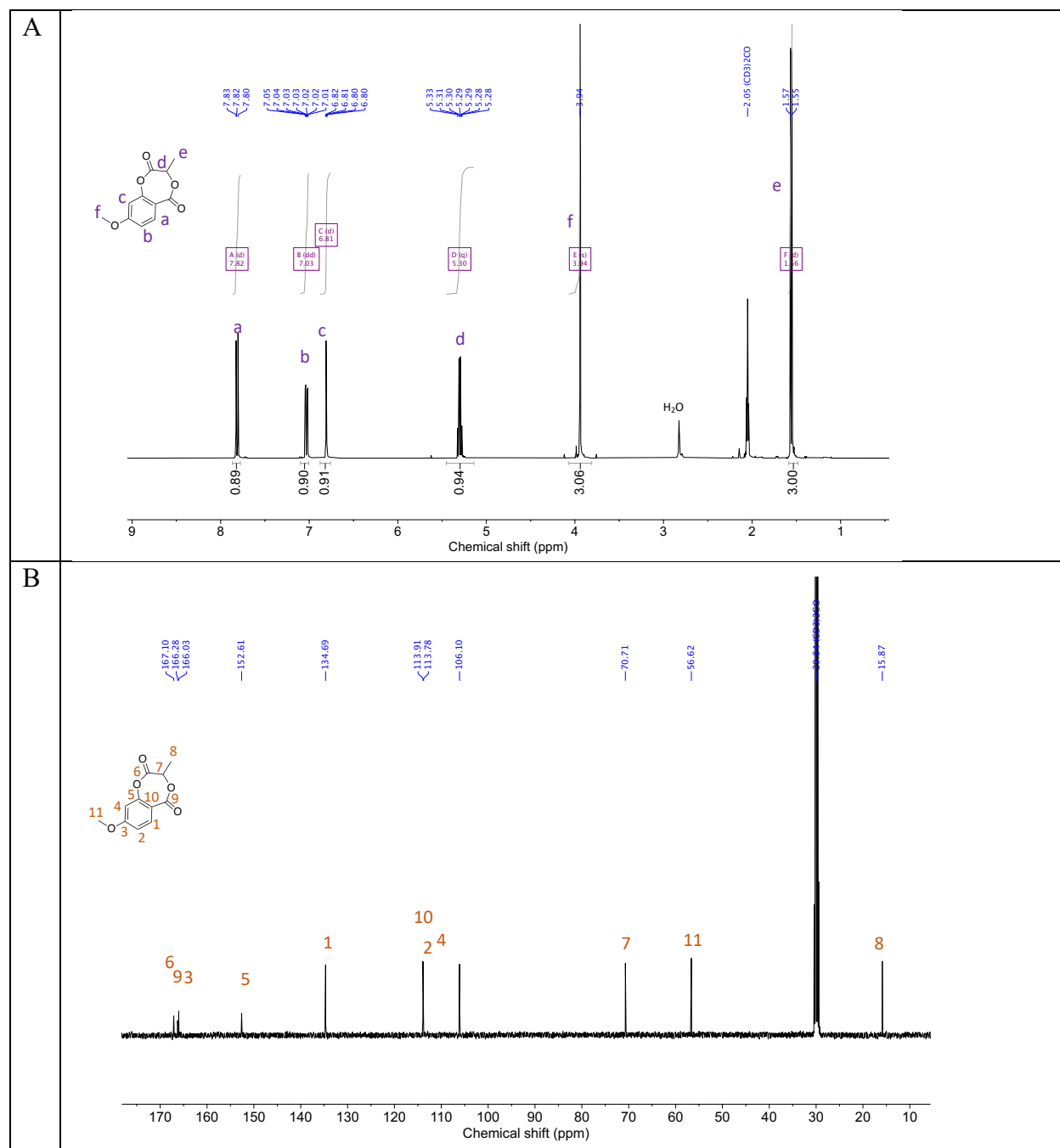


Figure S3.55. A) ^1H -NMR and B) ^{13}C -NMR of 11 in acetone- d_6 .

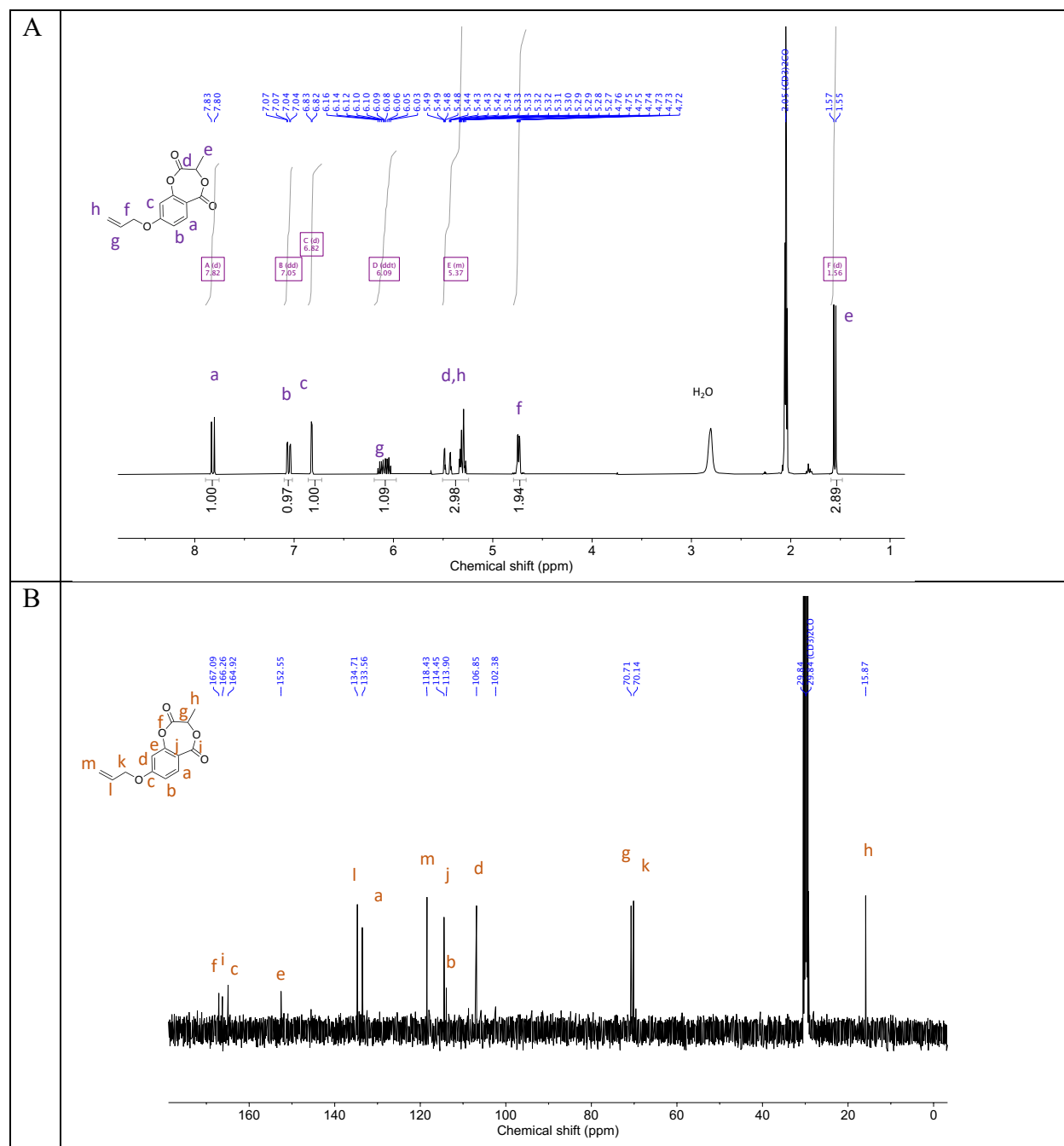


Figure S3.56. A) ^1H -NMR and B) ^{13}C -NMR of 12 in acetone- d_6 .

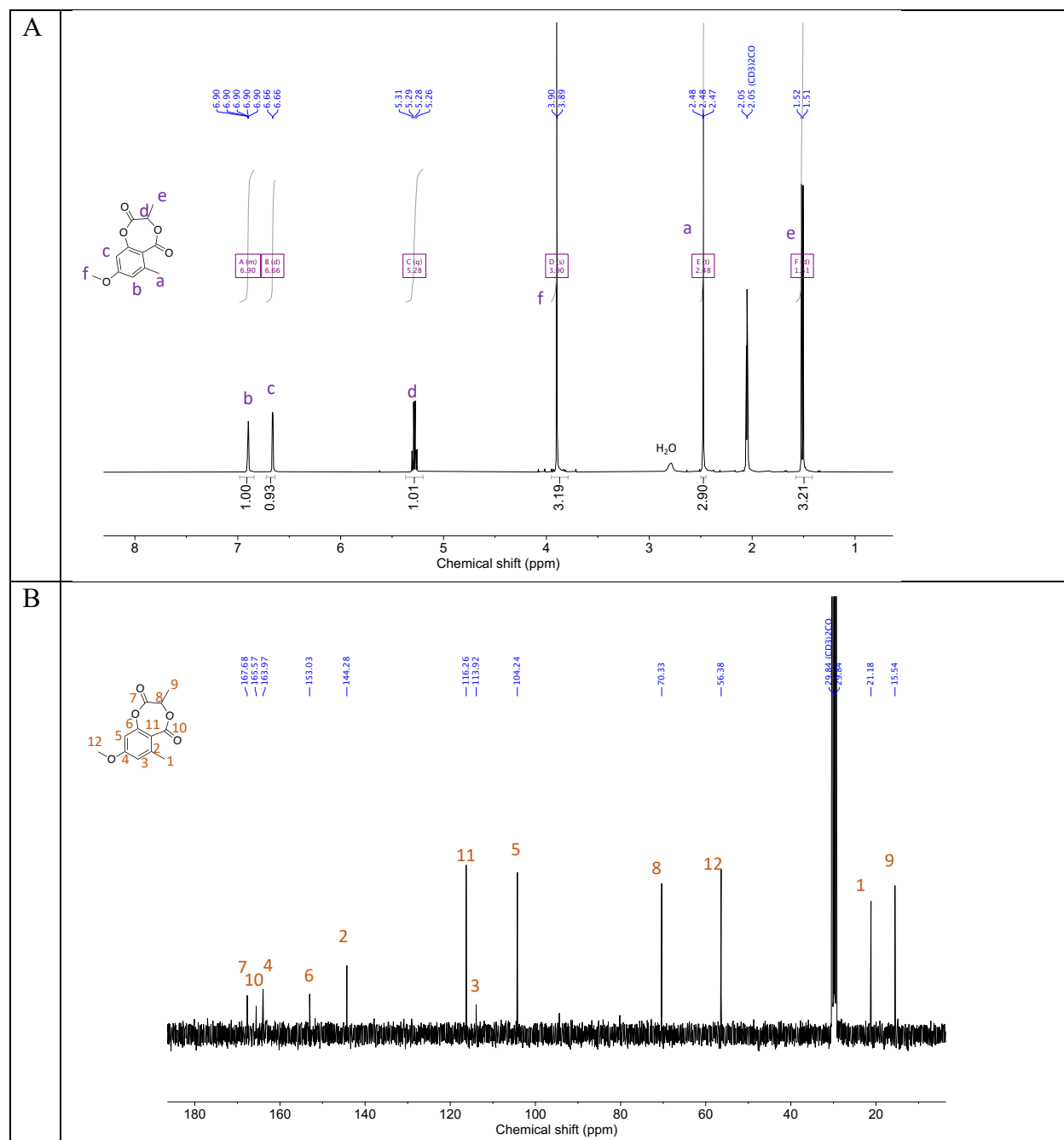


Figure S3.57. A) ^1H -NMR and B) ^{13}C -NMR of 14 in acetone- d_6 .

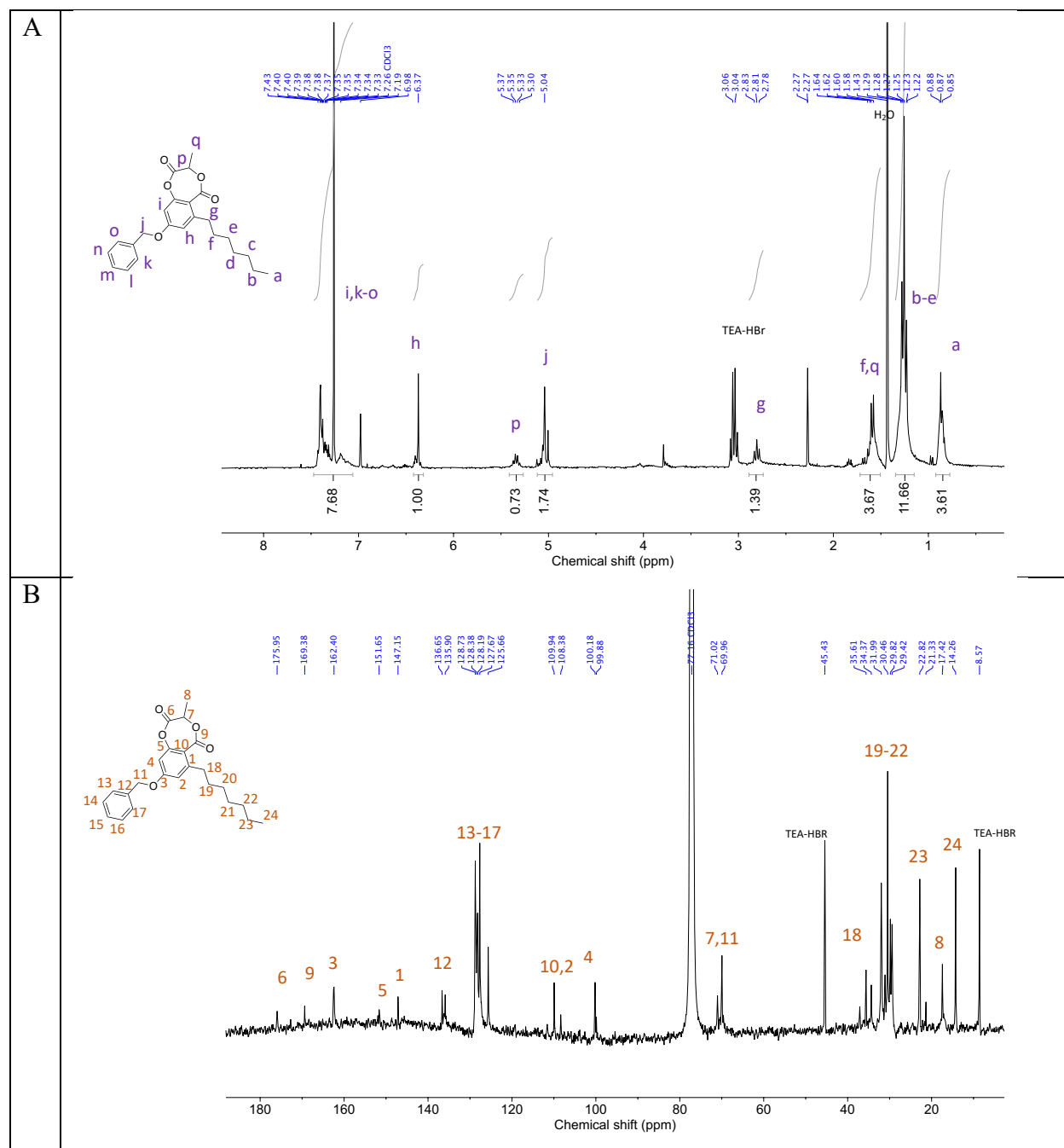


Figure S3.58. A) ^1H -NMR and B) ^{13}C -NMR of **15** in CDCl₃.

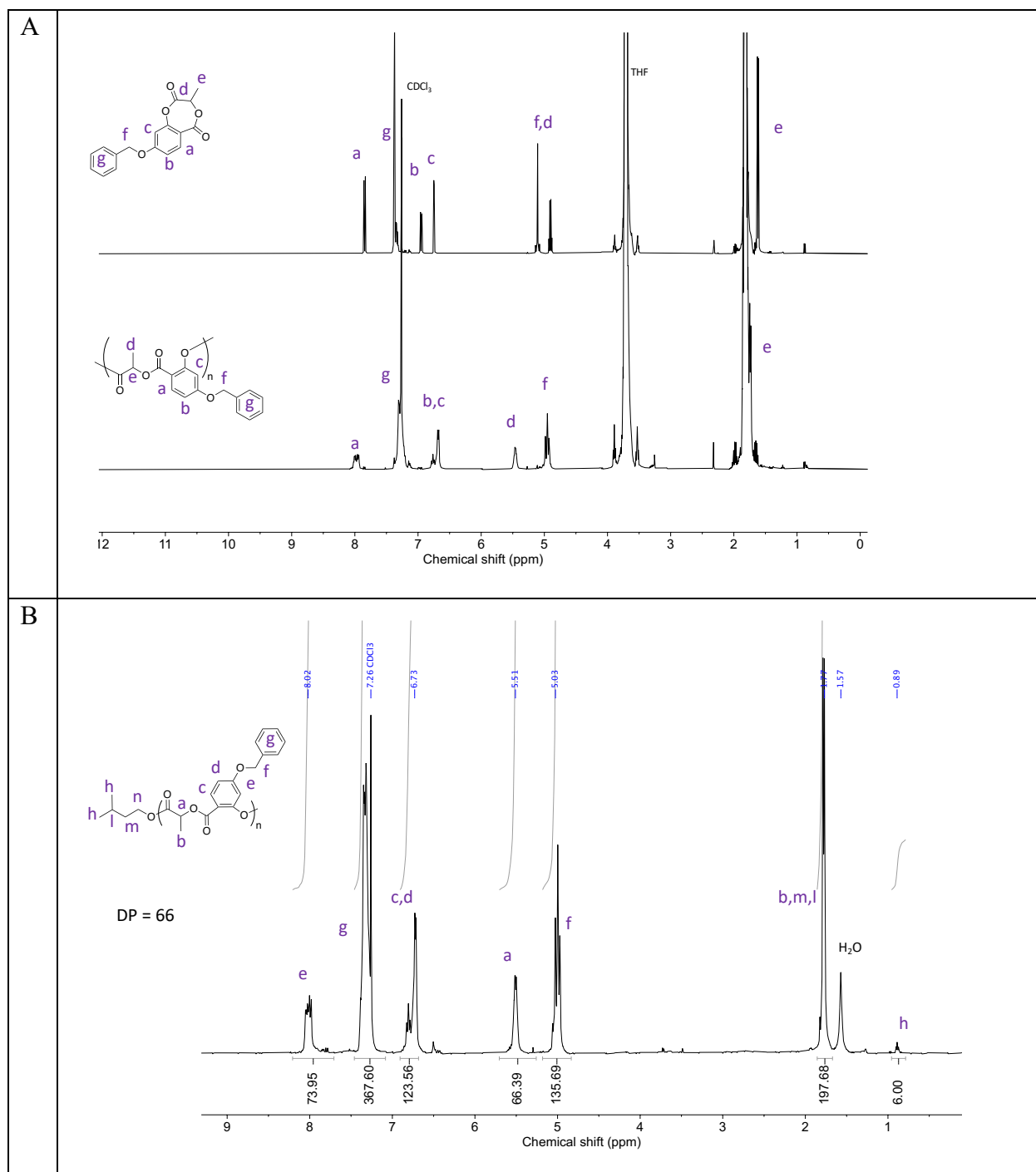


Figure S3.59. Ring-opening polymerization of monomer 13. A) Evolution by ^1H -NMR at the initial time (up) and after 1 hour (down) using the system monomer/3-O urea/MTBD/initiator (50:1:1:1) in CDCl_3 . B) ^1H -NMR spectrum of the resultant polymer upon isolation in CDCl_3 .

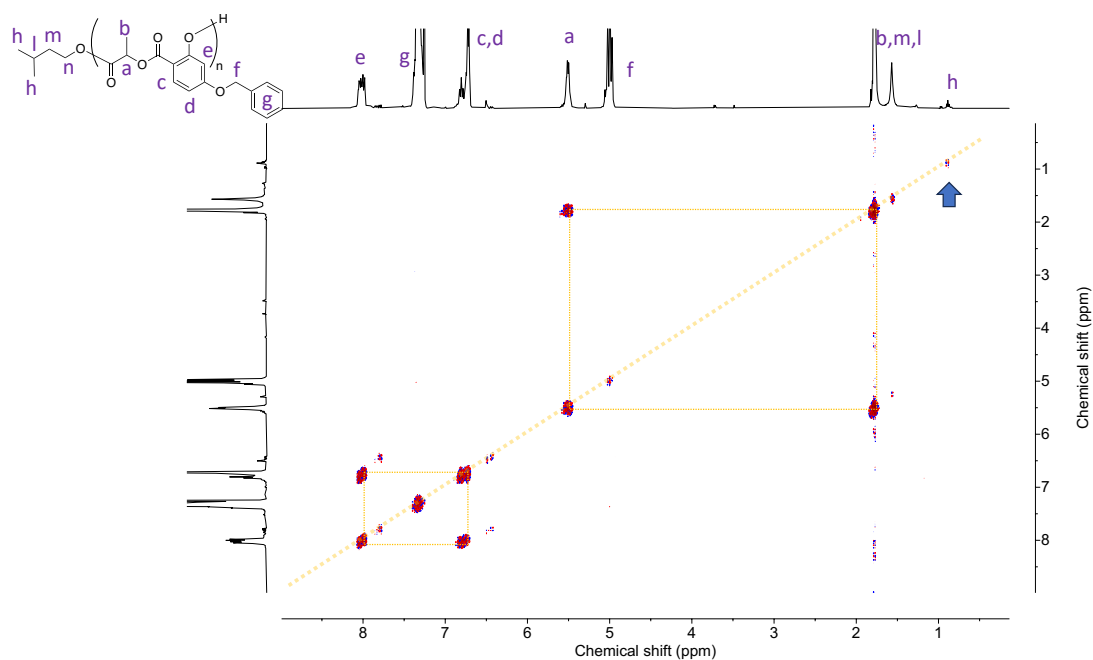


Figure S3.60. COSY 2D-NMR of the polymer from monomer 13 using the system monomer/3-O urea/MTBD/initiator (50:1:1:1). The signals correlate linearly meaning that all of them belong to one macromolecule, including the initiator that appears at 0.88 ppm (peak h).

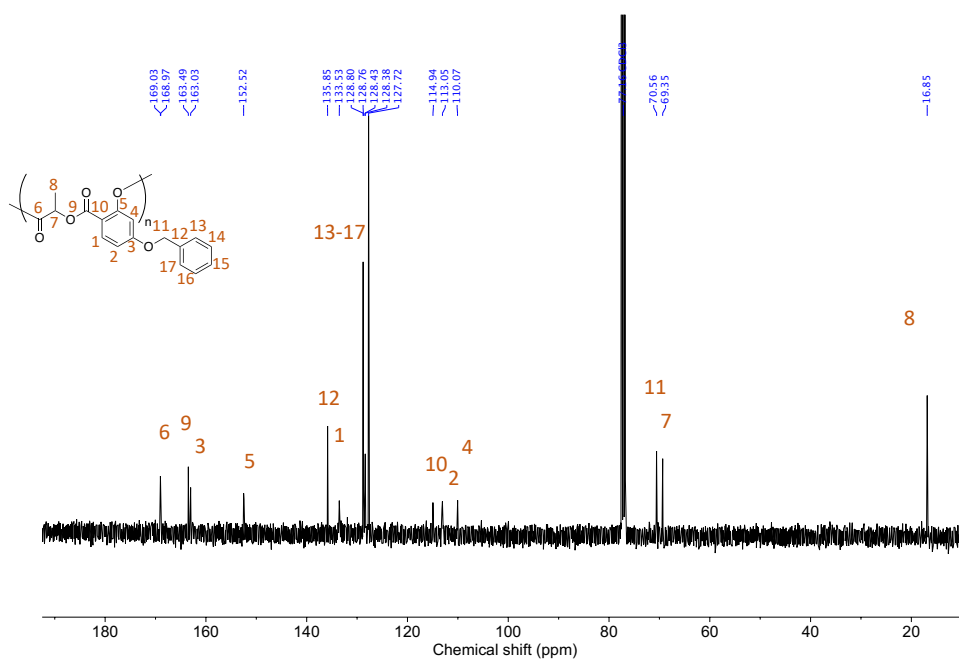


Figure S3.61. ^{13}C -NMR of the polymer from monomer 13 using the system monomer/3-O urea/MTBD/initiator (50:1:1:1) in CDCl_3 .

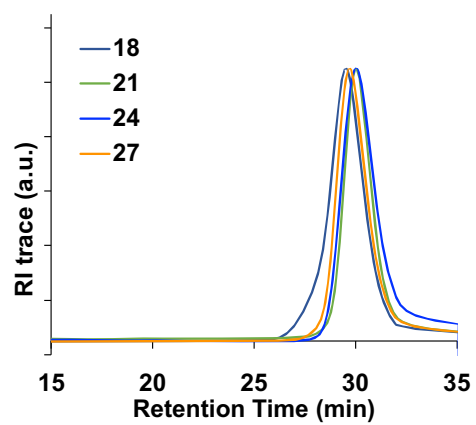


Figure S3.62. Refractive index trace from SEC in THF of the resultant polymers at molar ratio 30:1:1 (polymers 18, 21, 24, 27).

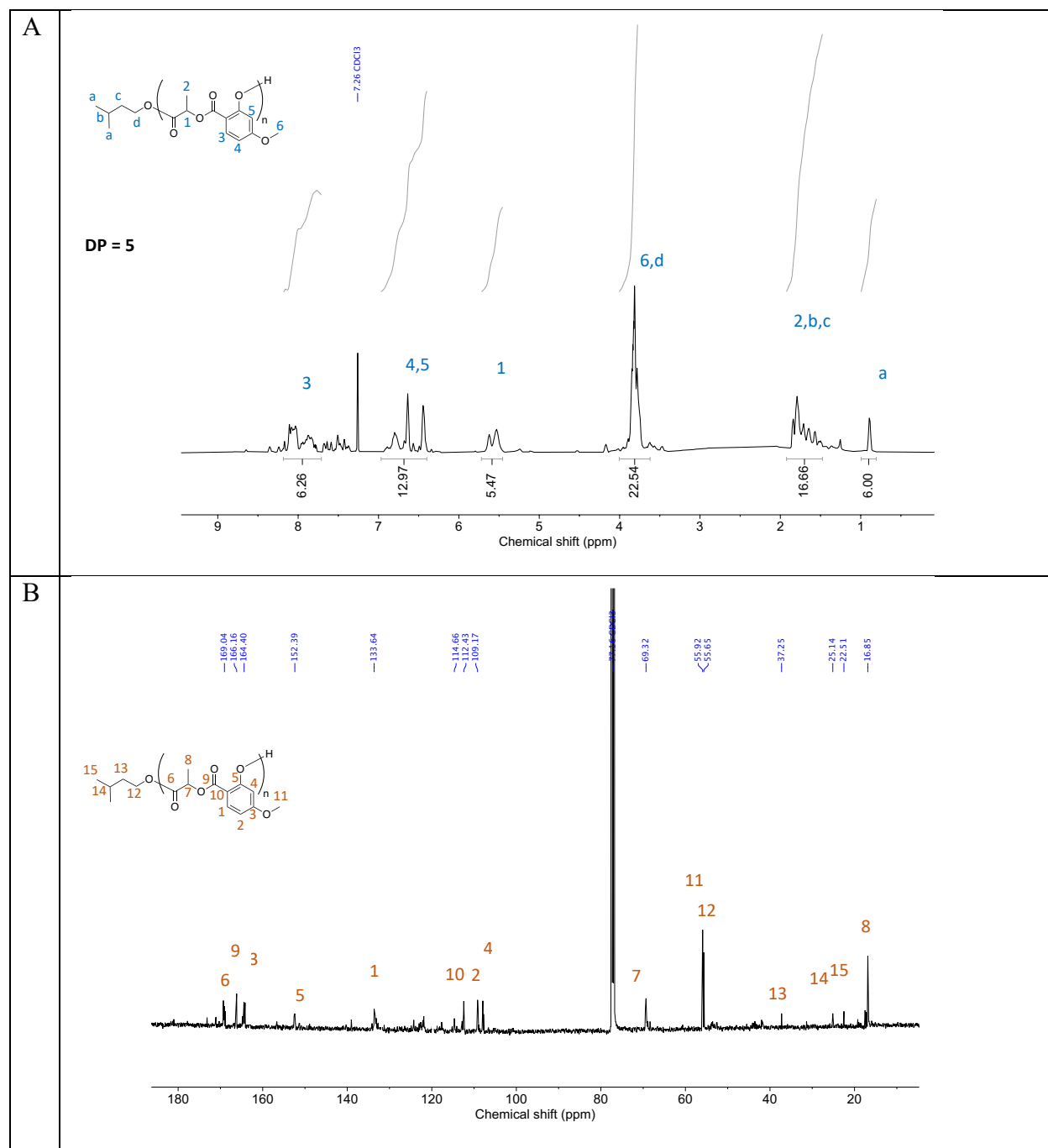


Figure S3.63. A) $^1\text{H-NMR}$ and B) $^{13}\text{C-NMR}$ of 16 in CDCl₃.

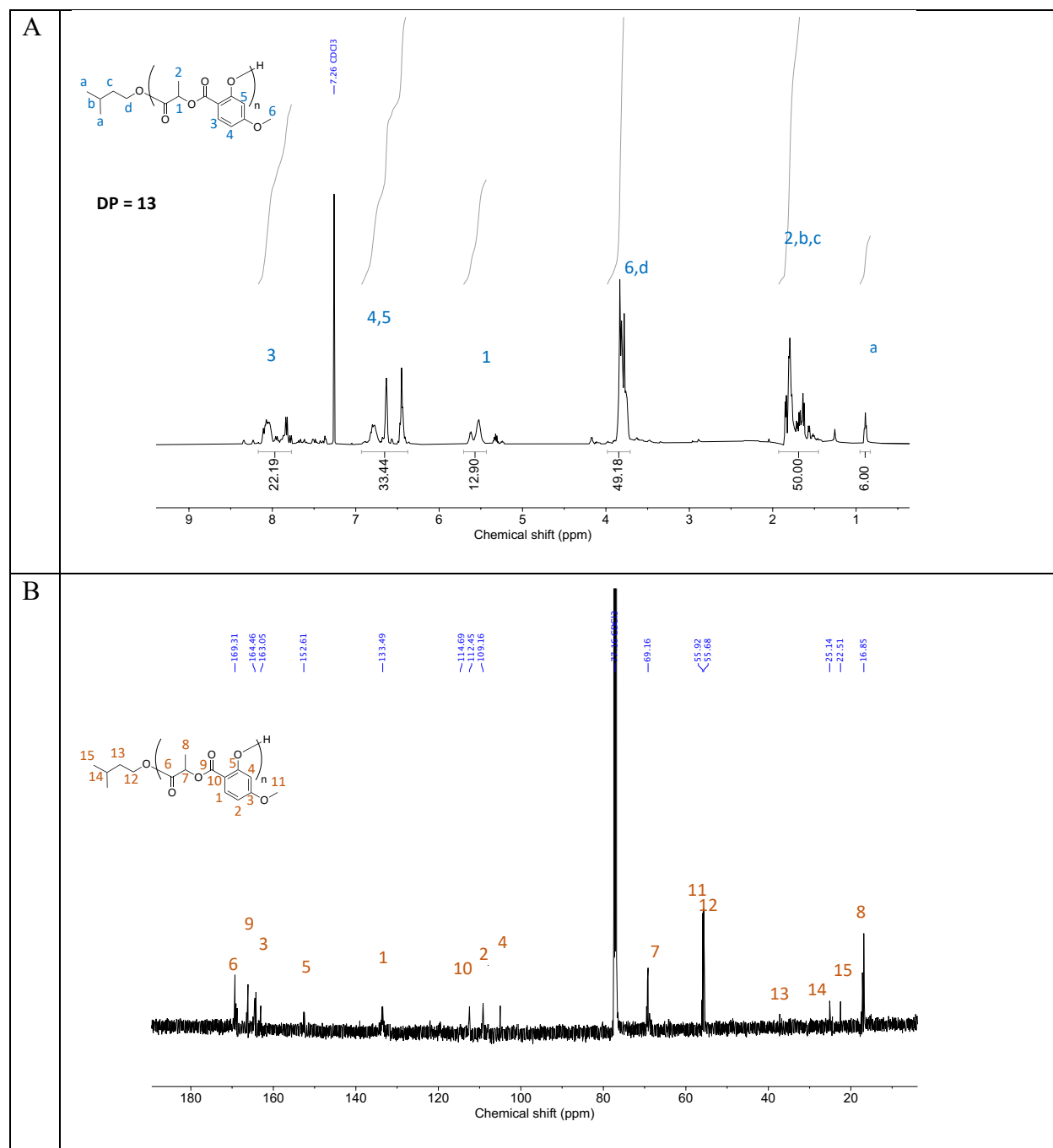


Figure S3.64. A) $^1\text{H-NMR}$ and B) $^{13}\text{C-NMR}$ of 17 in CDCl_3 .

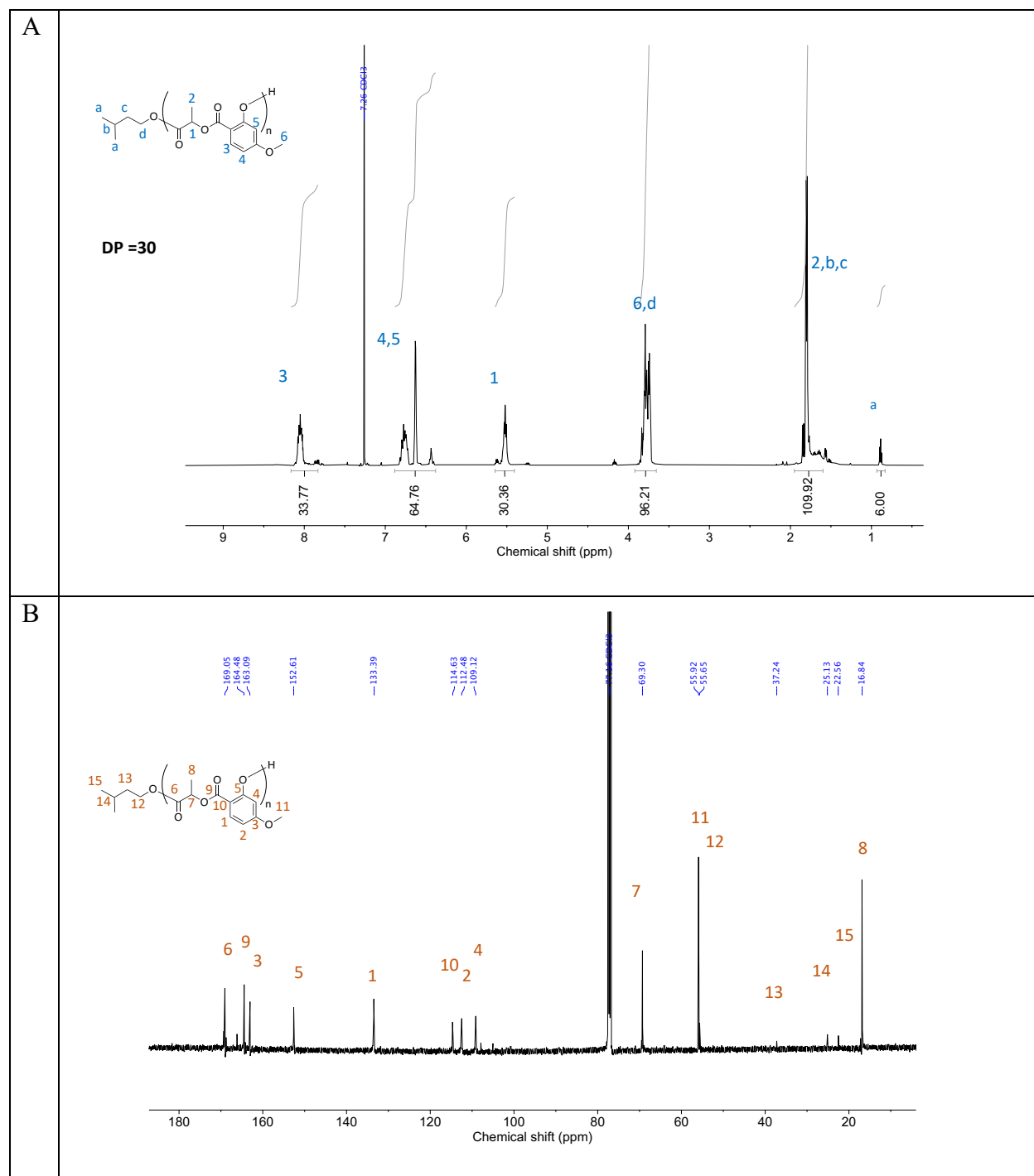


Figure S3.65. A) ¹H-NMR and B) ¹³C-NMR of 18 in CDCl₃.

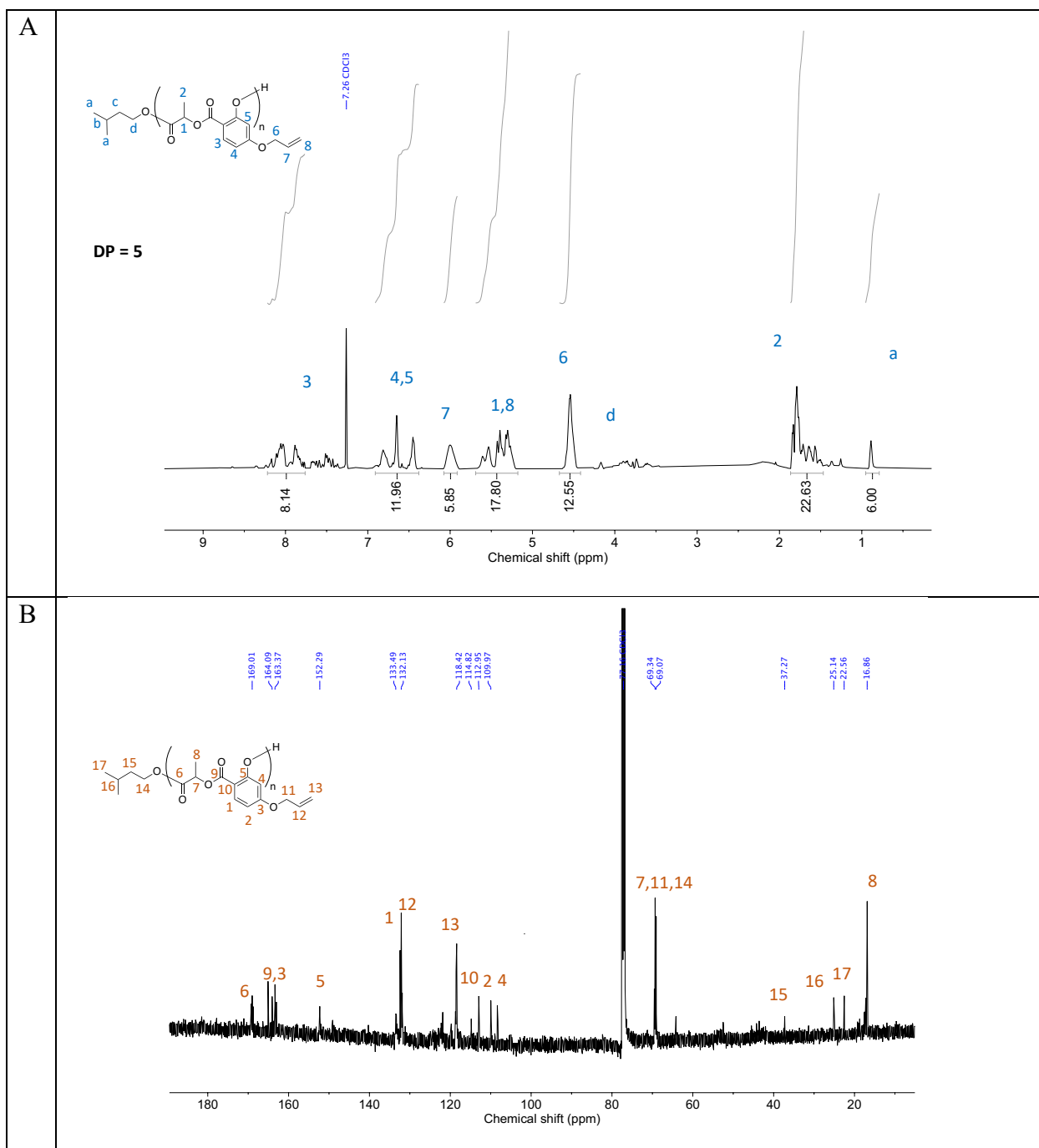


Figure S3.66. A) $^1\text{H-NMR}$ and B) $^{13}\text{C-NMR}$ of 19 in CDCl_3 .

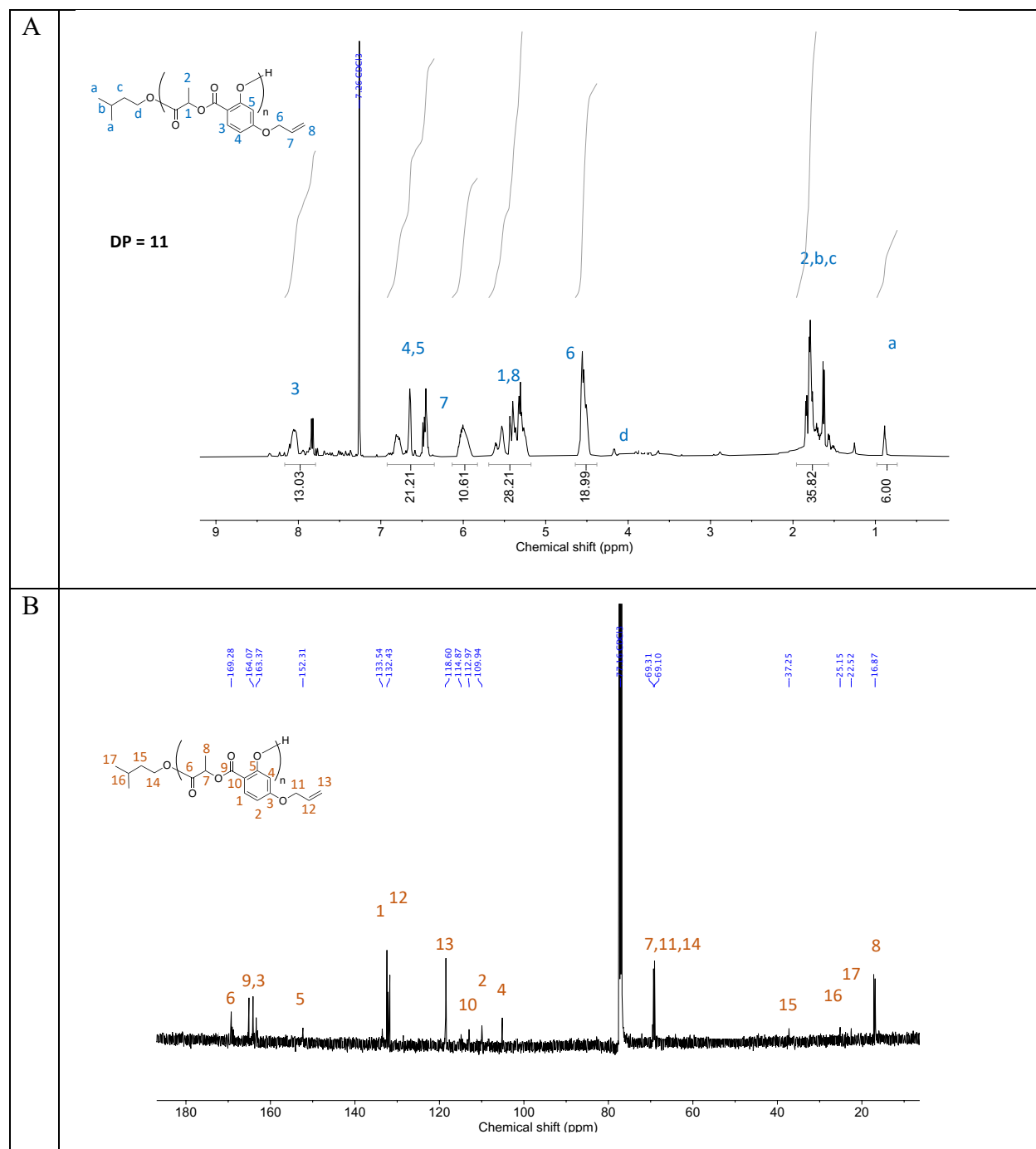


Figure S3.67. A) ¹H-NMR and B) ¹³C-NMR of 20 in CDCl₃.

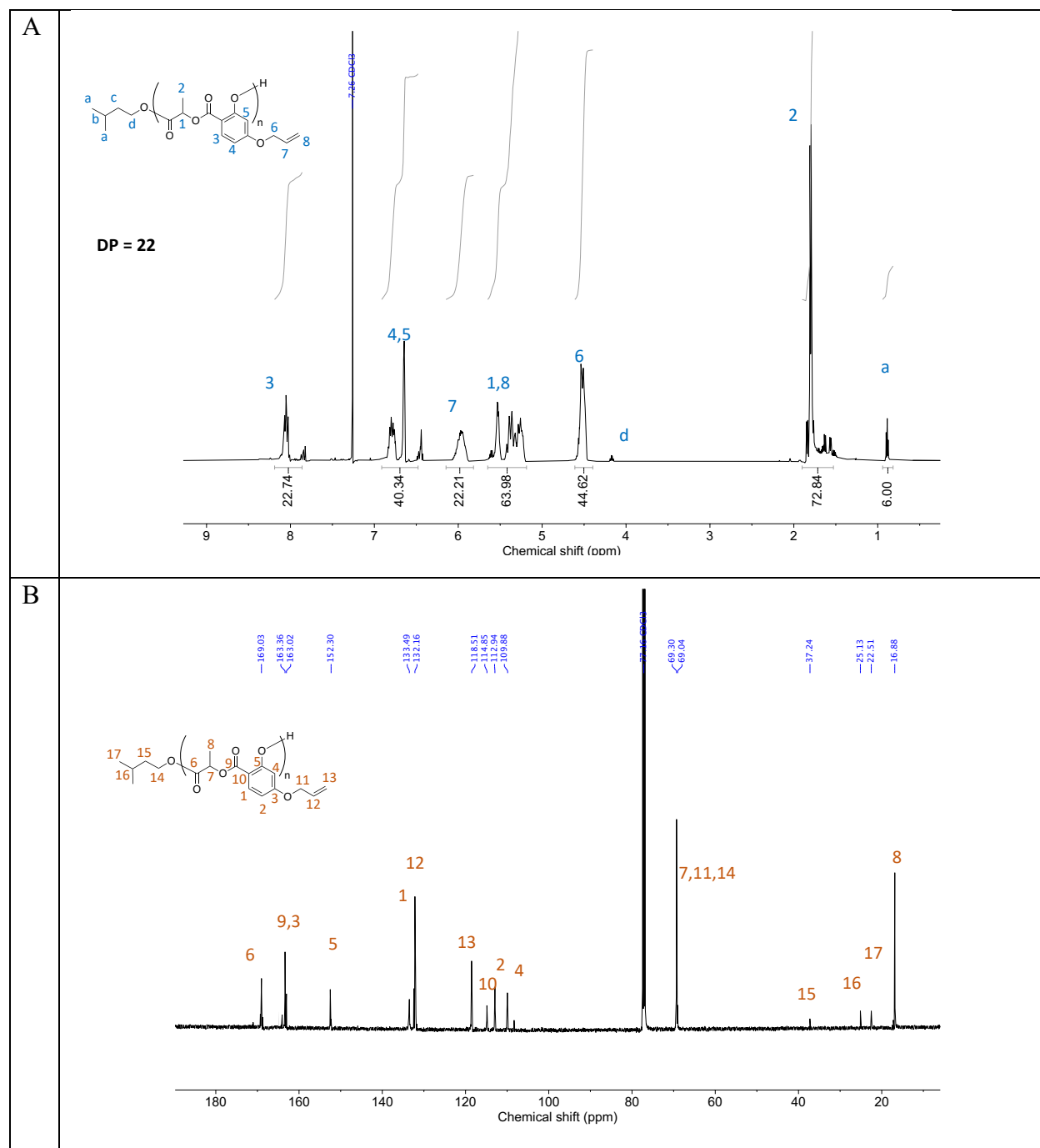


Figure S3.68. A) ¹H-NMR and B) ¹³C-NMR of 21 in CDCl₃.

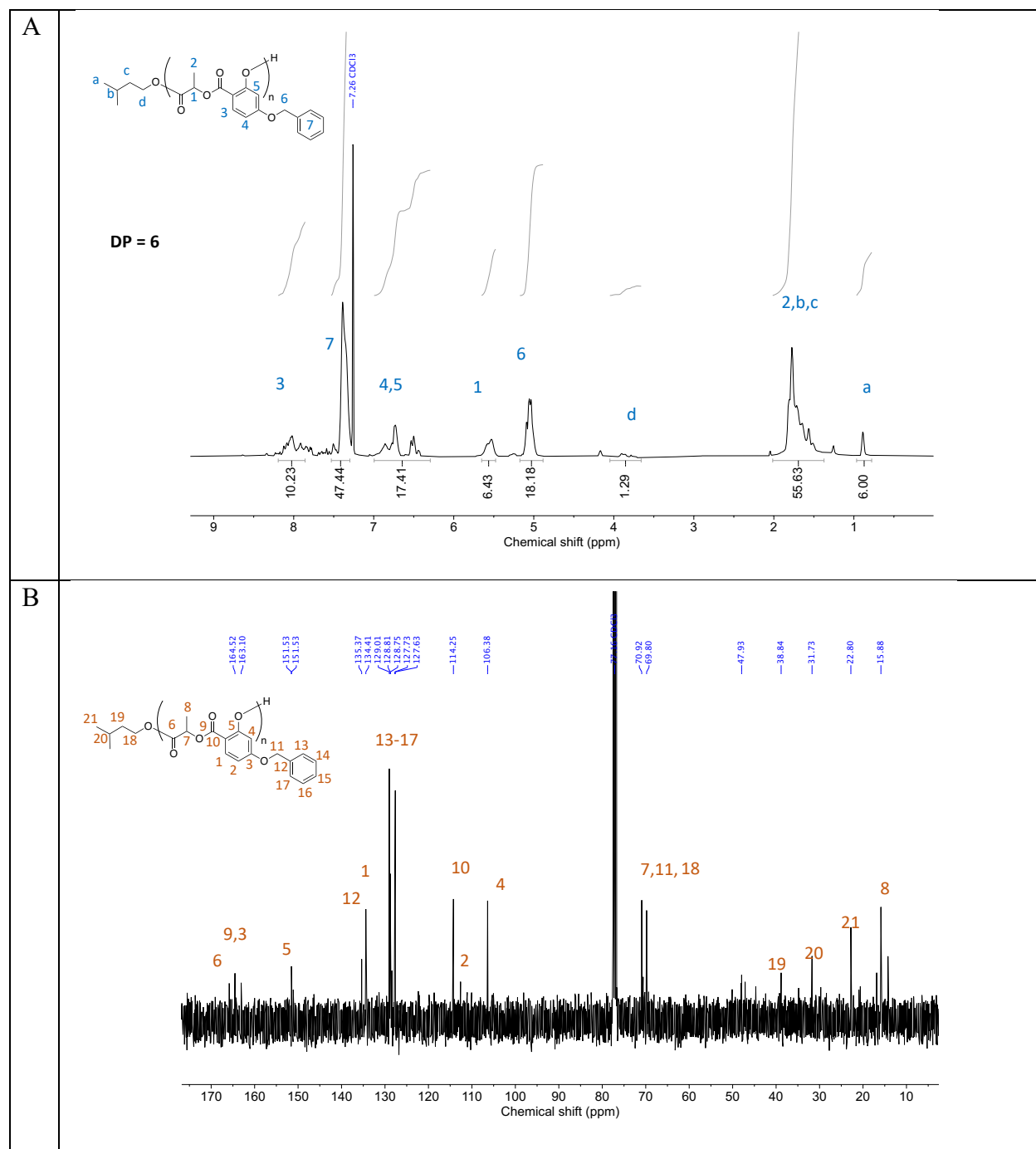


Figure S3.69. A) 1H -NMR and B) ^{13}C -NMR of 22 in CDCl₃.

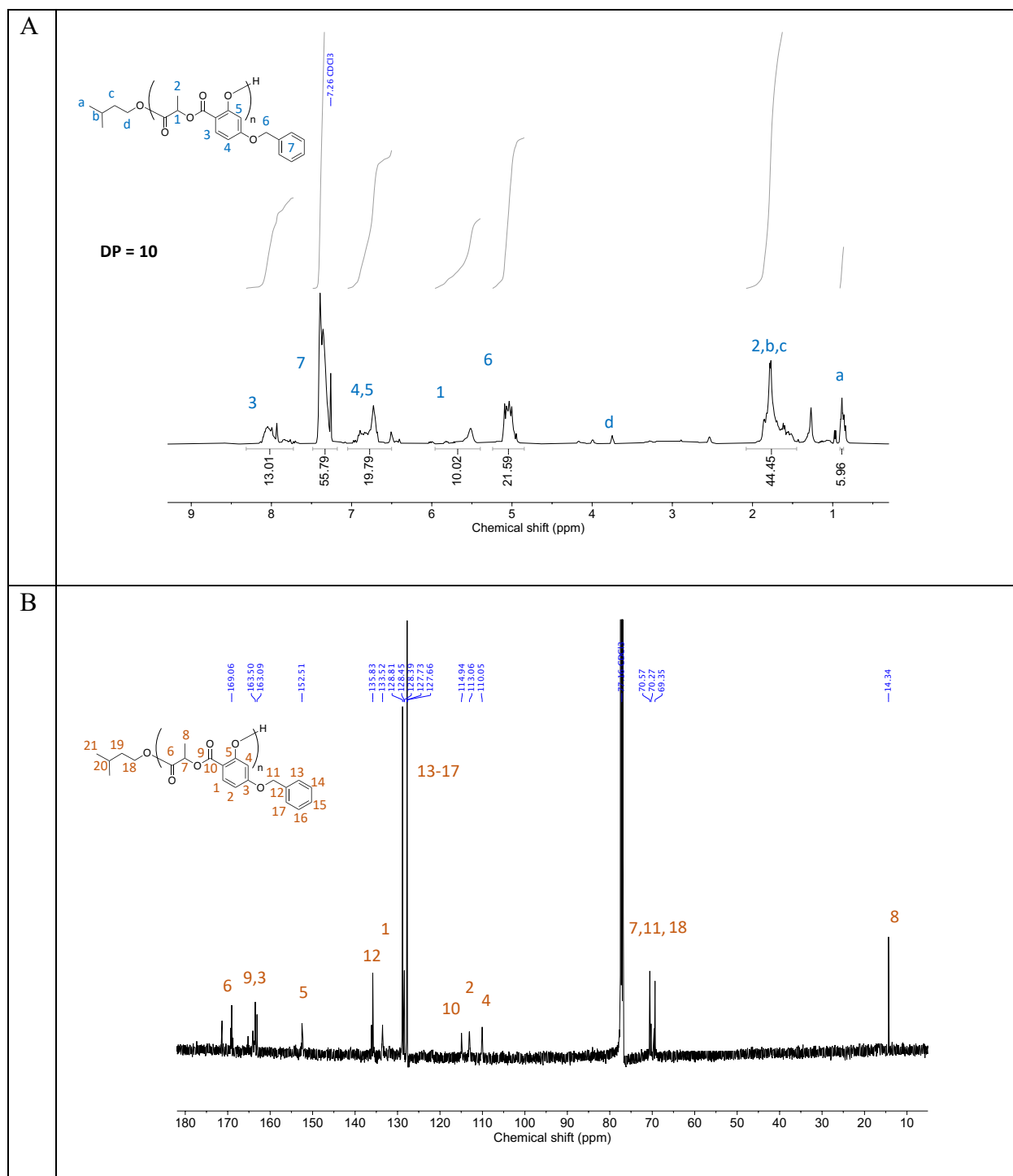


Figure S3.70. A) $^1\text{H-NMR}$ and B) $^{13}\text{C-NMR}$ of 23 in CDCl₃.

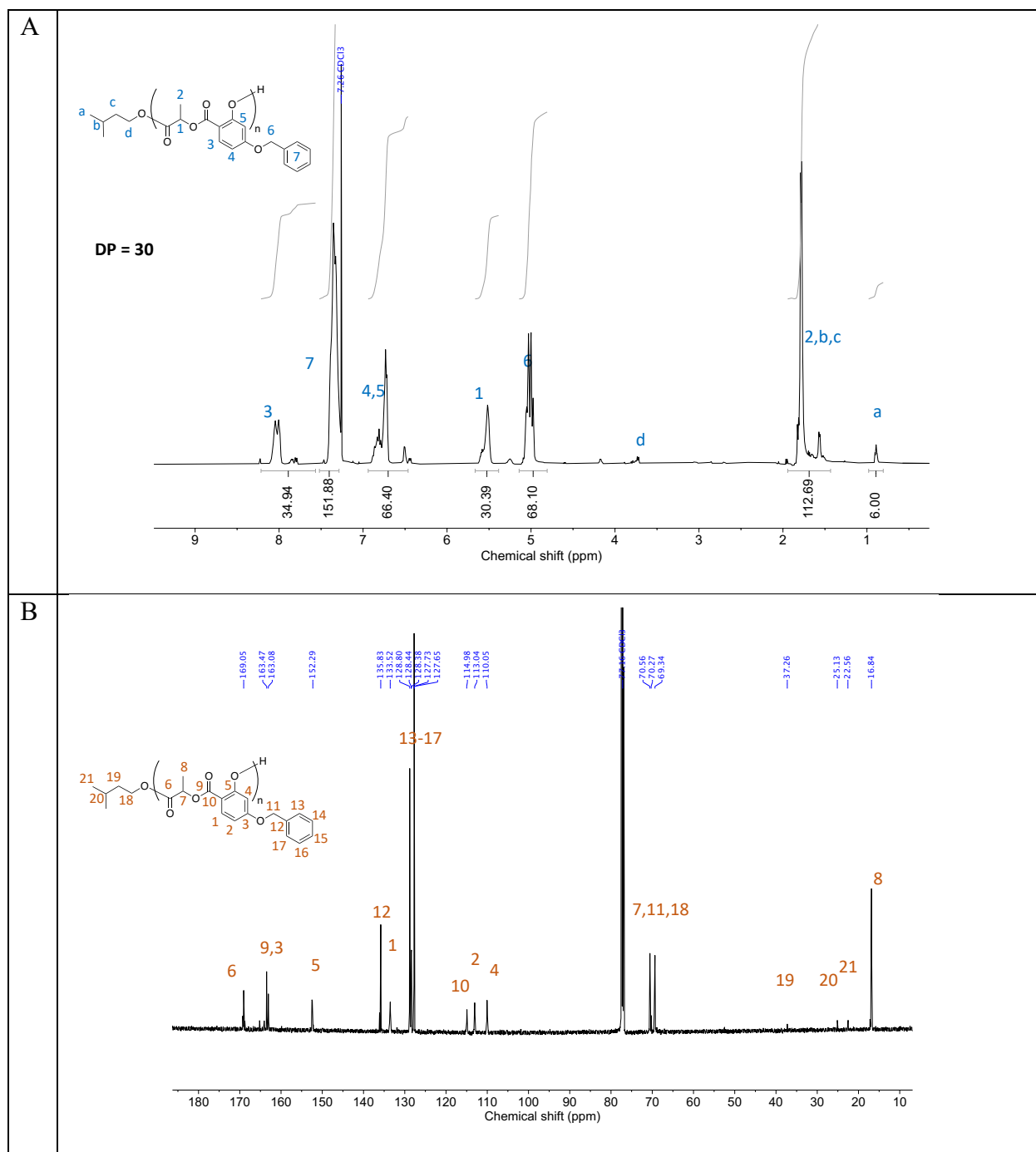


Figure S3.71. A) 1H -NMR and B) ^{13}C -NMR of 24 in CDCl₃.

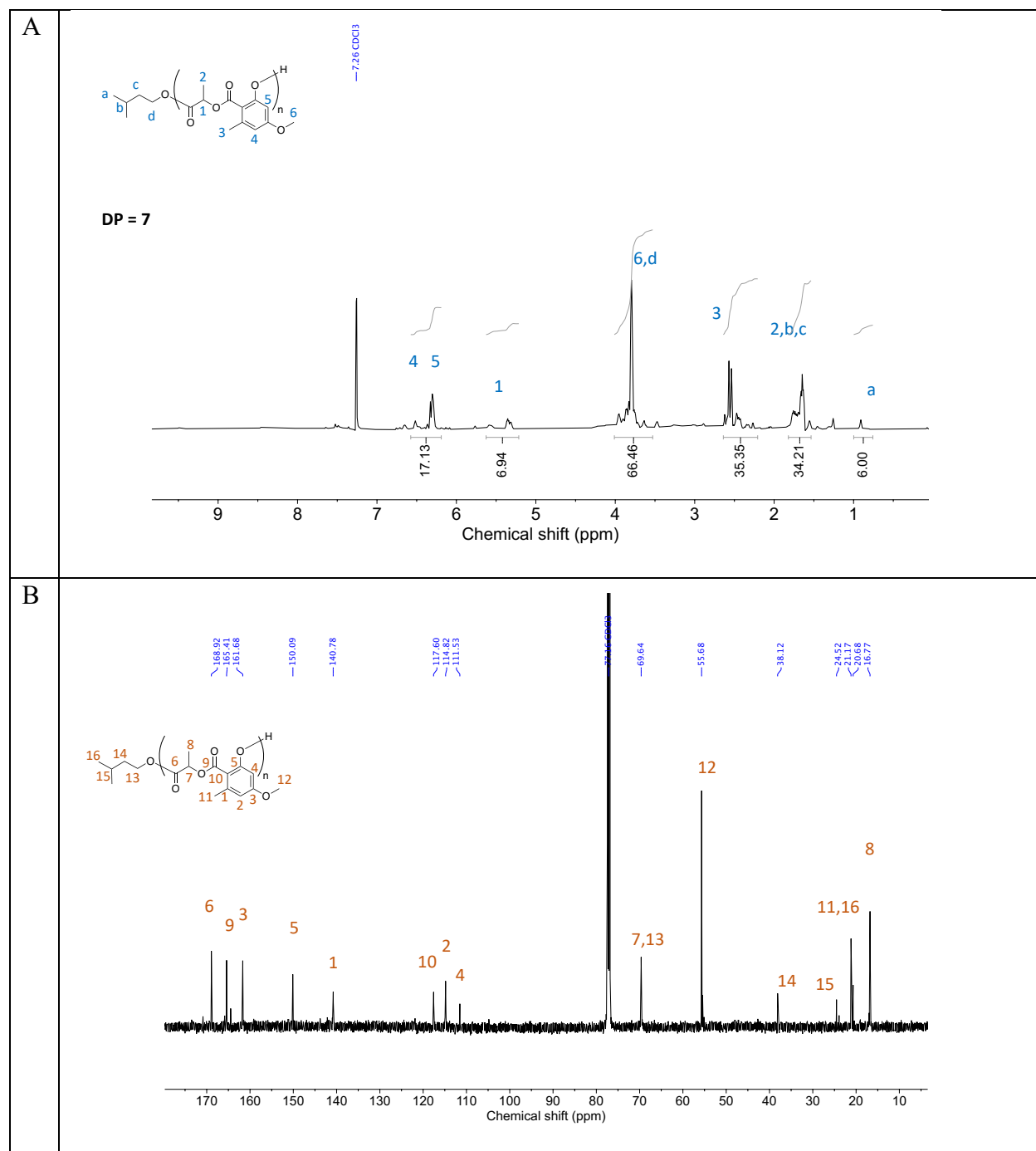


Figure S3.72. A) ¹H-NMR and B) ¹³C-NMR of 25 in CDCl₃.

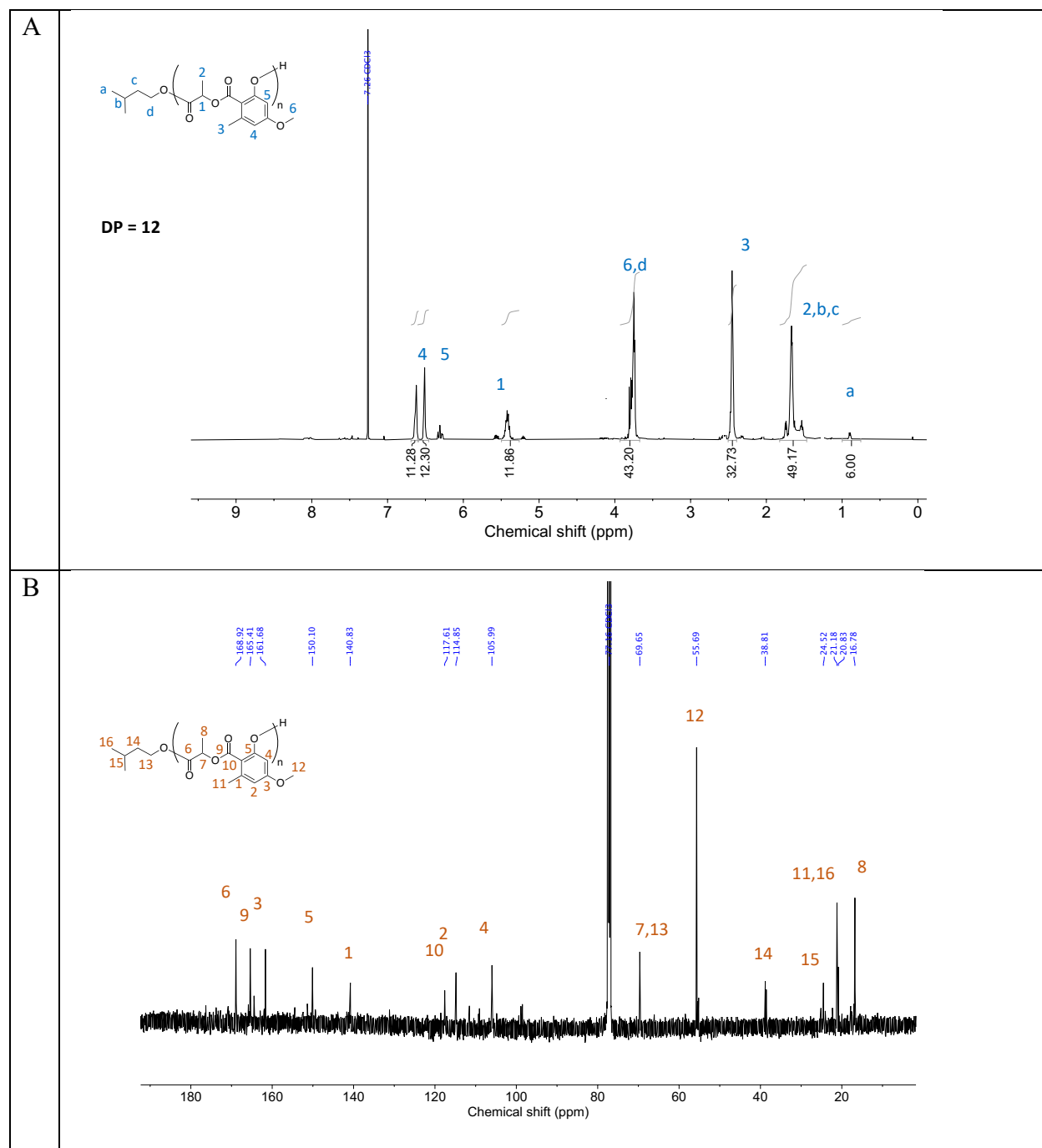


Figure S3.73. A) ¹H-NMR and B) ¹³C-NMR of 26 in CDCl₃.

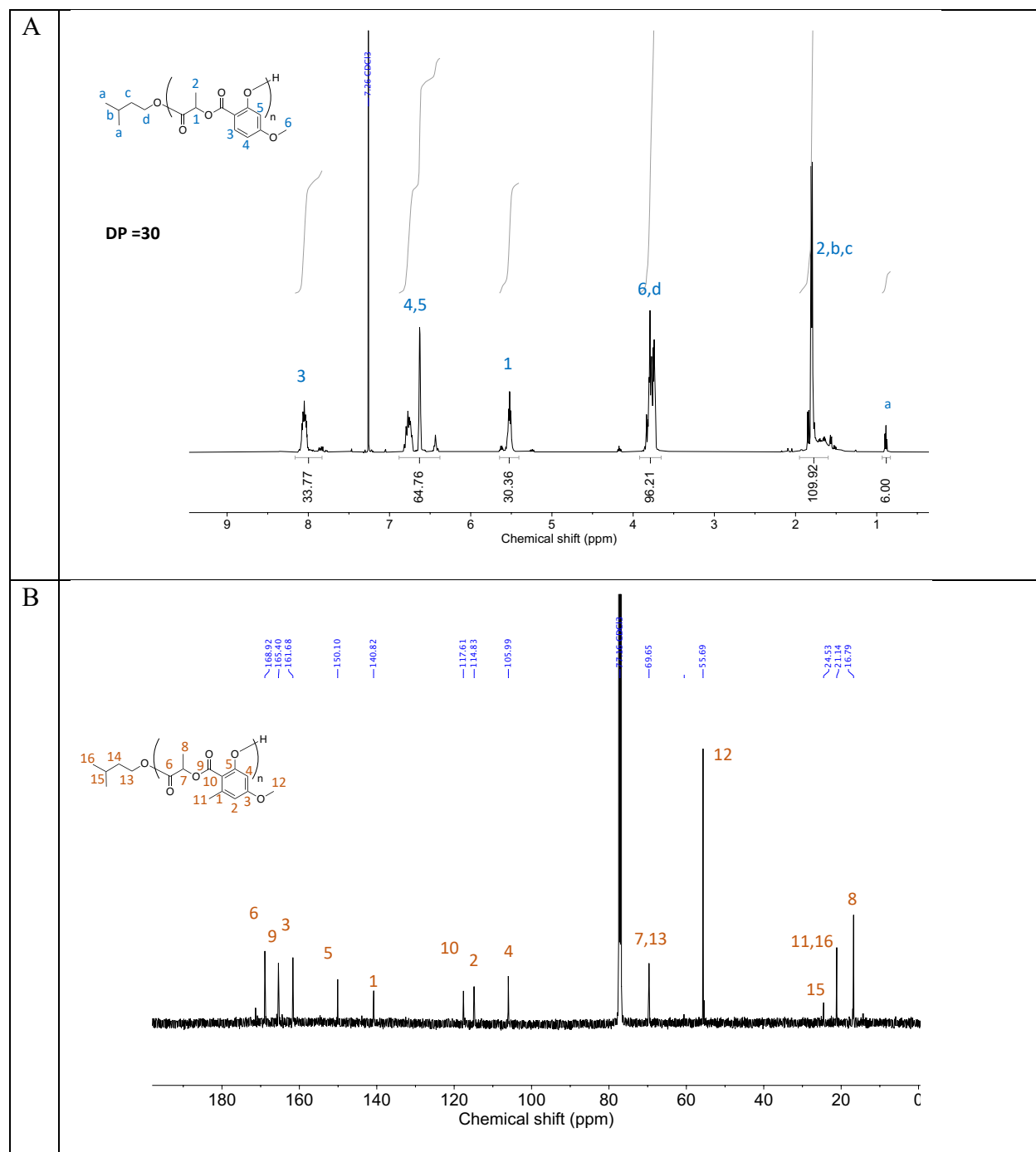


Figure S3.74. A) ¹H-NMR and B) ¹³C-NMR of 27 in CDCl₃.

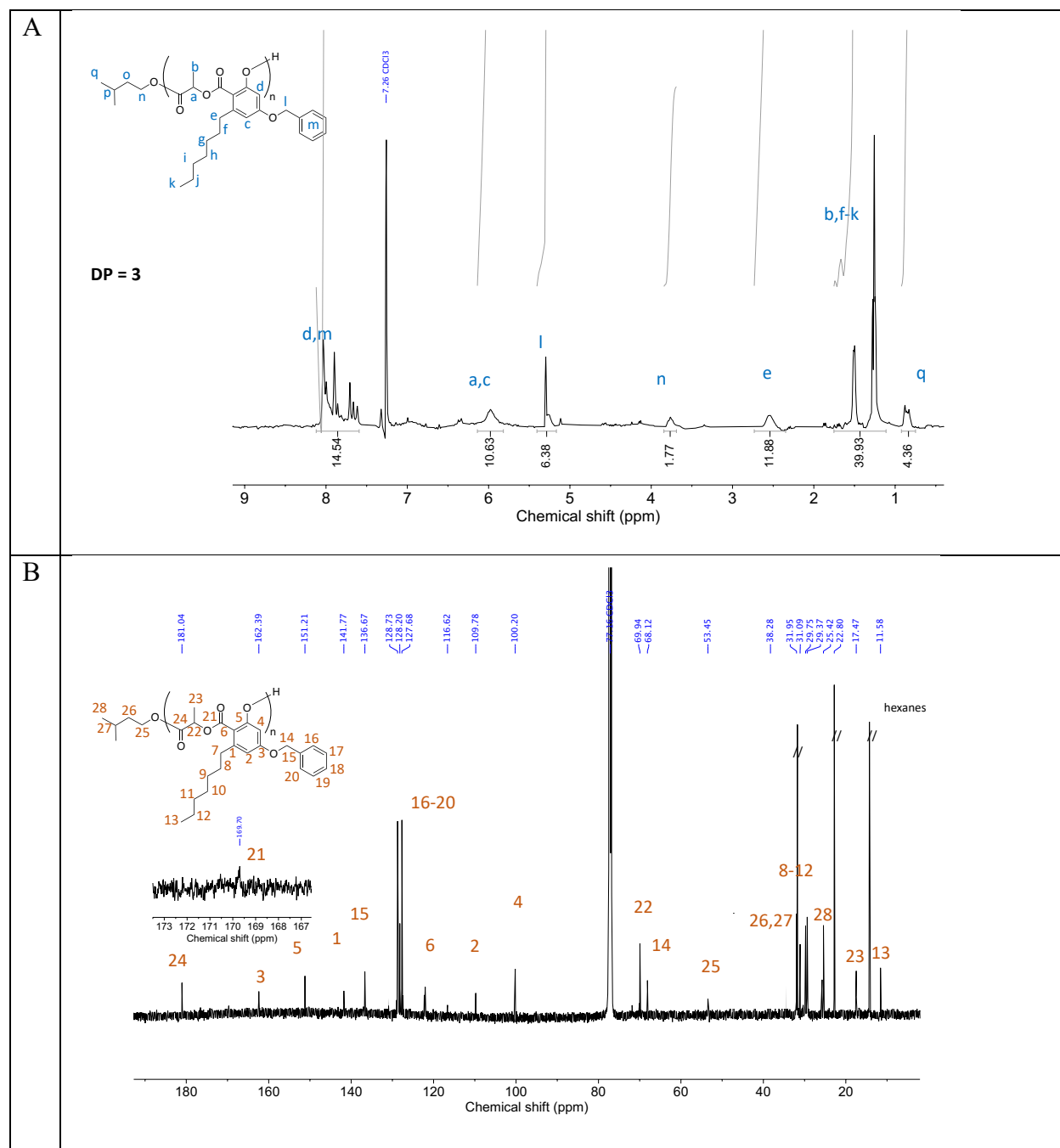


Figure S3.75. A) ^1H -NMR and B) ^{13}C -NMR of 28 in CDCl₃.

3.4.3. Polylysine

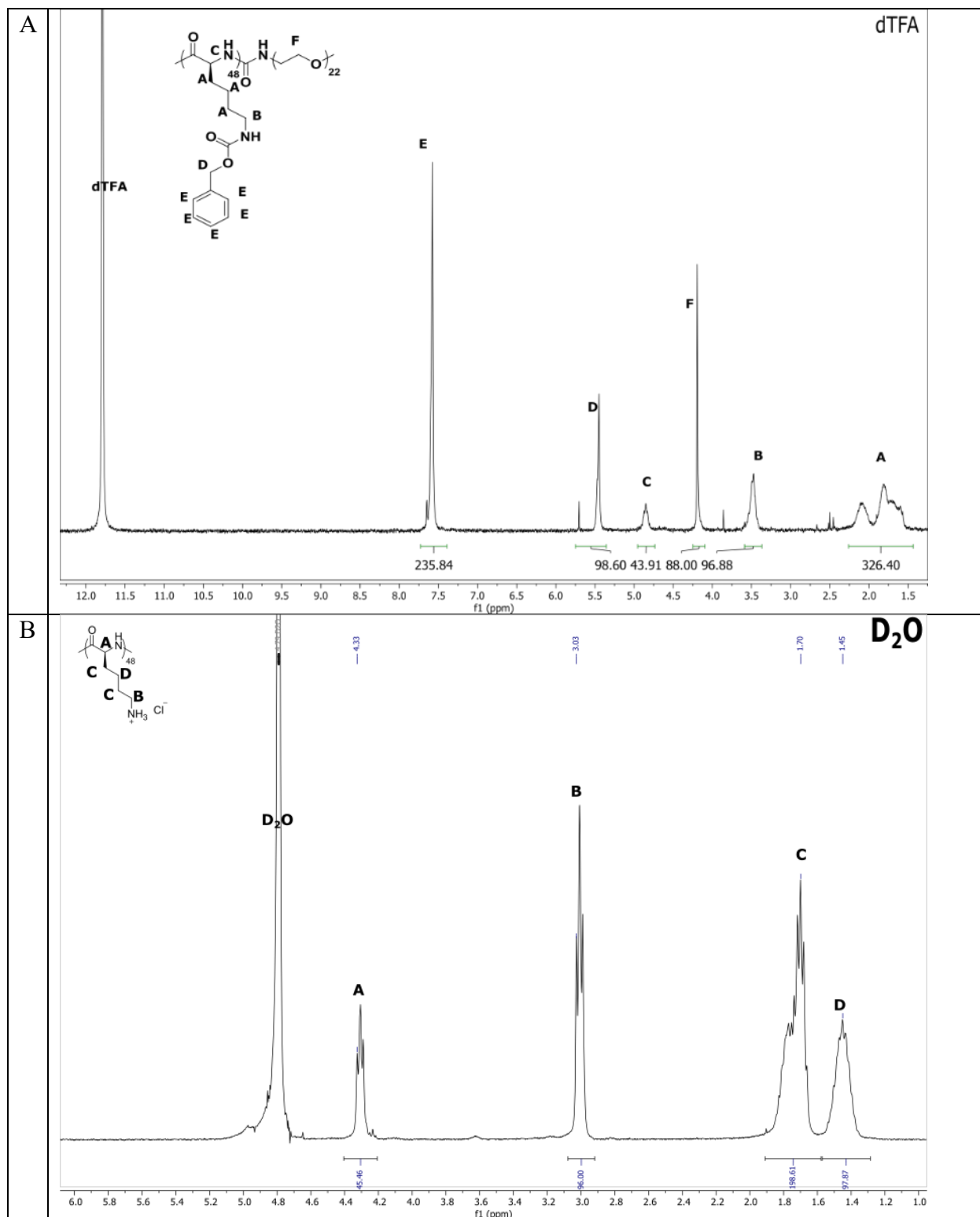


Figure S3.76. ^1H NMR of A) polyZK₄₈ after reaction with mPEG-NCO for end-group analysis and molecular weight determination, and B) polyK₄₈.

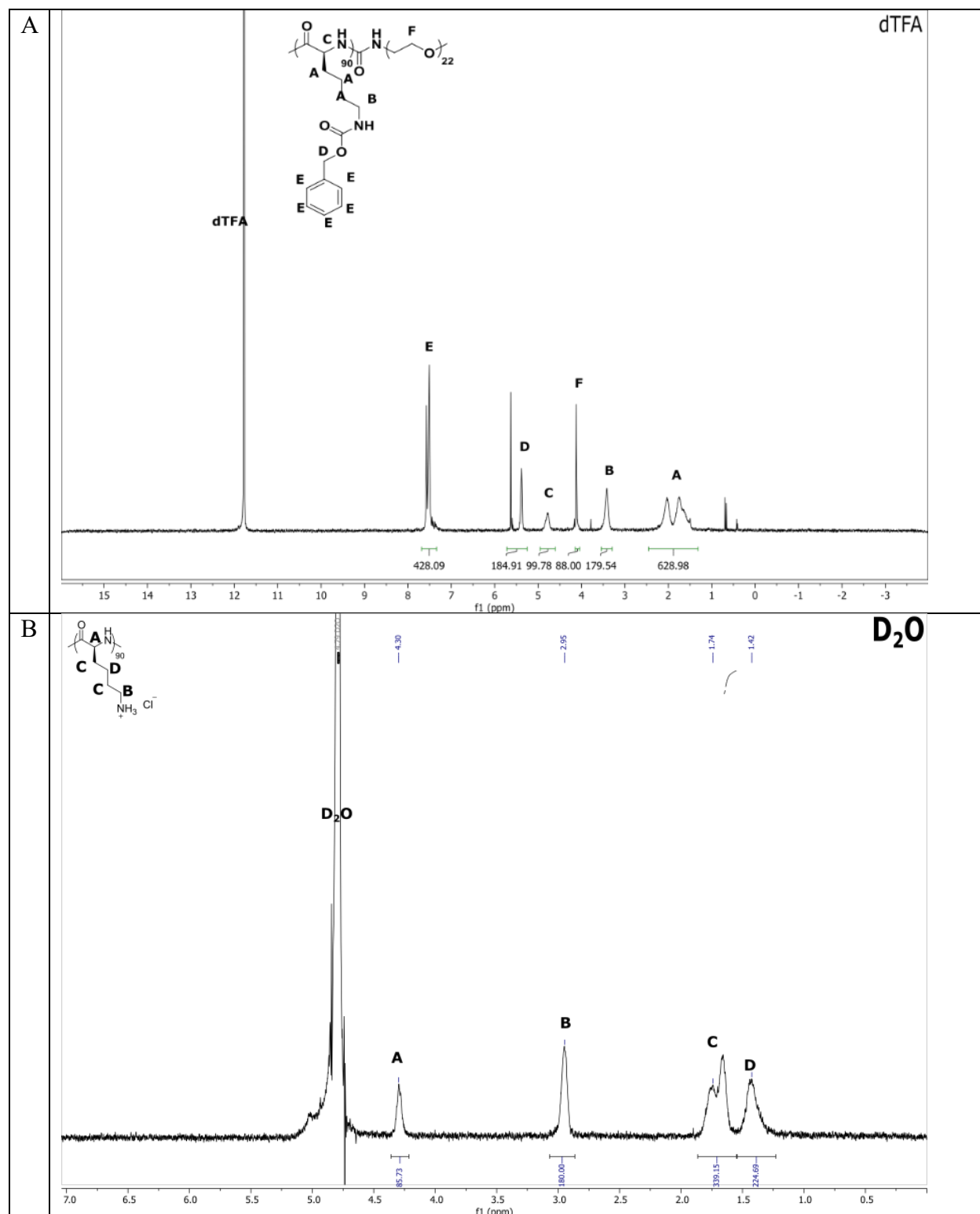


Figure S3.77. ¹H NMR of A) polyZK₉₀ after reaction with mPEG-NCO for end-group analysis and molecular weight determination, and B) polyK₉₀.

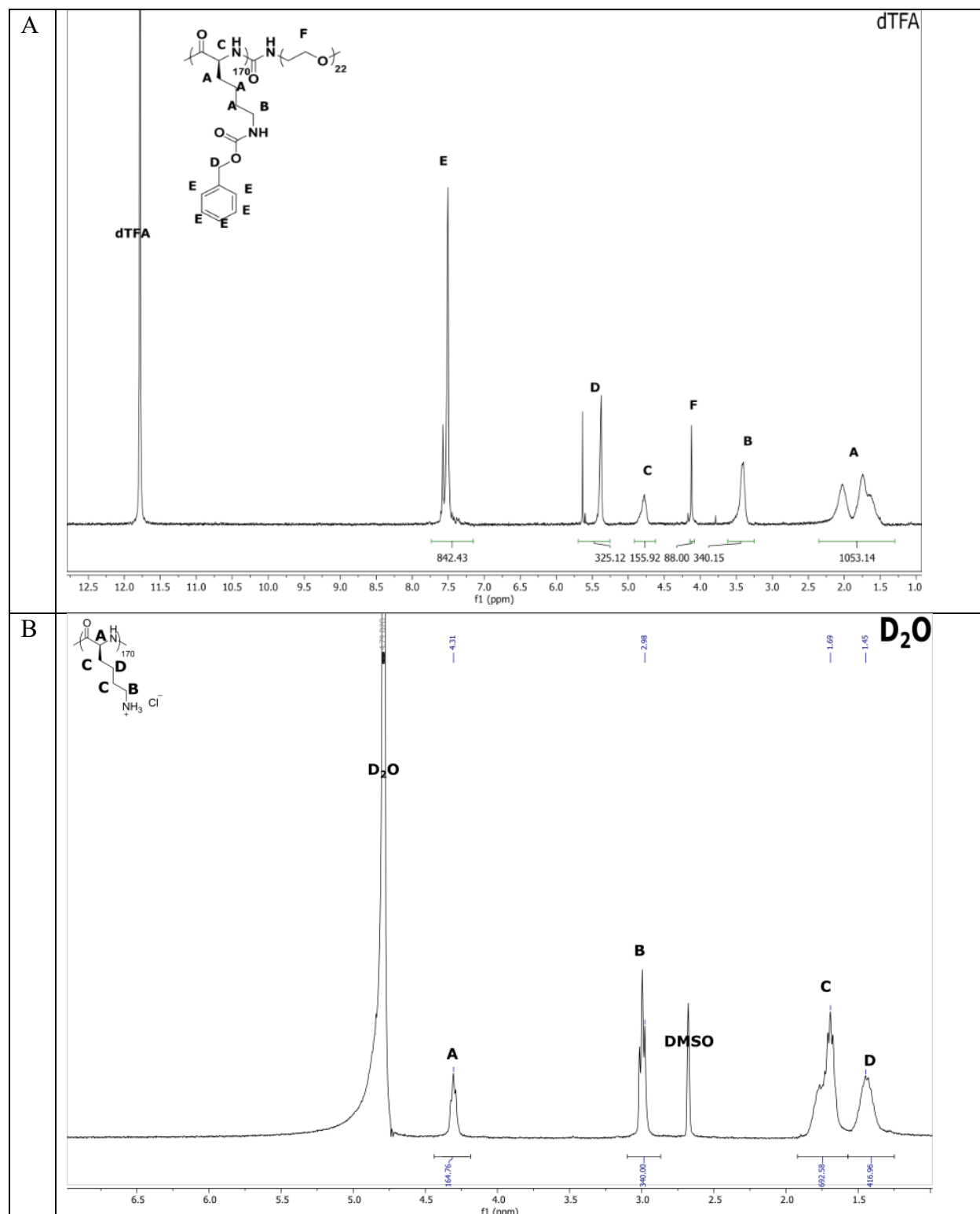


Figure S3.78. ¹H NMR of A) polyZK₁₇₀ after reaction with mPEG-NCO for end-group analysis and molecular weight determination, and B) polyK₁₇₀.

3.5 Supplementary tables.

Polymer	DP theoretical	DP from NMR	Caffeine content theoretical (%)	Caffeine content from ¹ H-NMR (%)	M _n (kg/mol)	Đ	Yield (%)
P-(t-Bu-DMEA-Caf30%)MA DP30	30	34	30	35	6.4	1.16	70
P-(t-Bu-DMEA-Caf10%)MA DP 30	30	29	10	14	6.3	1.06	71
P-(t-Bu-DMEA)MA DP30	30	30	0	0	4.7	1.03	76
P-(DMEA-Caf30%)MA DP30	30	36	30	28	N.A.	N.A.	72
P-(DMEA-Caf10%)MA DP30	30	31	10	9	4.2	1.16	73
P-(DMEA)MA DP30		32	0	0	5.1	1.18	74

Table S3.1. Characterization of the copolymers bearing caffeine and cationic or zwitterionic side chains and DP = 30 from RAFT polymerization by ¹H-NMR, SEC and yields. N.A. = not analyzed due to poor solubility under analytical conditions.

Entry	Catalyst	Base	Ratio (monomer/ catalyst/ base/ initiator)	Solvent	T (°C)	Conversion ¹ H-NMR	Time (h)	M _n (kg/ mol)	Đ
1	-	-	-	THF	23	0	1		
2	DMAP	-	50:2:1	Tol	110	98%	15	2.1	1.90
3	3-O urea	MT BD	50:2:1:1	THF	23	99%	1	12.6	1.96
4	3-O urea	MT BD	50:1:1:1	THF	23	99%	1	16.6	1.14
5	-	MT BD	50:1:1	THF	23	99%	1	14.6	2.37
6	3-O urea		50:1:1	THF	23	99%	1	21.4	1.59

Table S3.2. Evaluation of the conditions during ring opening polymerization of monomer 13 using 3-methylbutan-1-ol as initiator and different catalysts. Entry 4 is the conditions for the polymerization in this work.

DP _{theo}	DP _{NMR}	M _n (kg/mol)	Đ	Yield (%)
30	28	6.8	1.11	78
50	66	16.6	1.14	75
70	76	25.4	1.20	84
90	94	20.2	1.24	99

Table S3.3. Characterization of polymers from ROP of monomer 13 using the system monomer/3-O urea/MTBD/initiator. DP from ¹H-NMR, SEC in THF and yields. The 3-O urea and MTBD were varied according to the initiator.

Polymer	R ₁	R ₂	M/I _{NMR}	Size (nm)	PDI
16	Me	H	5	193 ± 81	0.167
17	Me	H	13	282 ± 54	0.513
18	Me	H	30	315 ± 183	0.358
19	Allyl	H	5	117 ± 17	0.702
20	Allyl	H	11	198 ± 40	0.439
21	Allyl	H	22	149 ± 53	0.103
22	Bn	H	6	205 ± 69	0.102
23	Bn	H	10	182 ± 85	0.173
24	Bn	H	30	194 ± 72	0.351
25	Me	Me	7	178 ± 84	0.167
26	Me	Me	12	123 ± 29	0.105
27	Me	Me	28	686 ± 137	0.454
28	Bn	Hep	3	594 ± 118	0.484

Table S3.4. Dynamic light scattering (DLS) results of the polymers at 1 mg/mL in PBS.

DP	<i>S. aureus</i> (ATCC 25904)		<i>E. coli</i> (ATCC 25922)		<i>P. aeruginosa</i> (ATCC 25102)	
	μM	μg/mL	μM	μg/mL	μM	μg/mL
40	5.00	33	10.00	67	20.00	130
48	5.00	40	5.00	40	5.00	40
67	5.00	55	2.50	28	5.00	55
75	5.00	62	2.50	31	5.00	62
90	2.50	37	2.50	37	5.00	74
110	2.50	45	1.25	23	5.00	91
128	2.50	53	1.25	26	5.00	110
148	2.50	61	0.625	15	5.00	120
155	2.50	64	0.625	16	2.50	64
160	1.25	33	0.625	16	2.50	66
170	1.25	35	0.625	18	2.50	70
200	1.25	41	0.625	21	2.50	82

Table S3.5. Minimum Bactericidal Concentration (MBC) of polyK of various degrees of polymerization (DP).

Chapter 4. Characterization of polylysine-phage bioconjugates and complexes

Adapted from:

- Peng, H.; **Vexler, S.**; Xu, S.; Chen, I. A. Real-time in vivo bacterial imaging by CT and fluorescence using phage-gold nanorod bioconjugates as contrast agents. *ACS Biomaterials Science & Engineering* **2025**.
<https://doi.org/10.1021/acsbiomaterials.4c02190>
- **Vexler, S.**; Morrison, C.; Jain, S.; Yang, Y.; Tsunemoto, H.; Zhang, N.; McCarthy, K.; Mihalache, B.; Kutach, M.; Yang, S.; Pogliano, J.; Annabi, N.; Deming, T.J.; Chen, I.A. Phage-Polymer conjugates inhibit *S. aureus* with high cytocompatibility and low bacterial resistance. (under review)

Many cationic polymers are known to be antimicrobial due to disruptive interactions with negatively charged bacterial cell membranes. However, this robust biophysical mechanism can also cause nonspecific disruption of mammalian cell membranes, leading to efforts to synthesize polymers with improved biocompatibility.^{177,189,190} Alternatively, in the approach described here, we propose that phage specificity could compensate for the polymer's lack of specificity. Additionally, if bactericidal effect of phage infection is replaced by the lytic activity of a synthetic polymer, the host range can instead be expanded by engineering the phage's binding specificity.

Previously, we engineered the receptor binding protein of M13 bacteriophage to bind to the common *Staphylococcus aureus* surface polysaccharide poly-*N*-acetylglucosamine (PNAG). The major coat protein that comprises the bulk of the phage is present in thousands of copies per

virion and provides solvent-accessible functional groups for potential bioconjugation of antimicrobial cargo, like synthetic polymers.^{45,81} α -poly(L-lysine) was chosen as a representative cationic polymer due to its well-documented antimicrobial properties^{49,186–188} combined with known biocompatibility problems.^{191,192} Chemical synthesis of α -poly(L-lysine) can be performed on the gram scale with control over the degree of polymerization (DP).^{154–156} Here, α -poly(L-lysine) with varying DP were separately attached either covalently or electrostatically to the modified phage, and antimicrobial activity and biocompatibility of the resulting bioconjugates and electrostatic complexes were studied.

4.1 Methodology

Phage loading with polyK. 1×10^{13} virions in 1 mL of PBS were added to 2.6 mg of sulfo-NHS-acetate and incubated overnight at room temperature under rotation to protect primary amines. The next day, 100 μ L of 1 M Tris HCl, pH 7.5, was added to quench the reaction. The protected phage was dialyzed using a Slide-A-Lyzer™ MINI Dialysis Devices, 20K MWCO (ThermoFisher Scientific) with 0.1 M MES buffer, pH 4.5 – 5. To 1×10^{13} protected virions, 546 μ L of polyK (prepared at a stock concentration of 200 μ M in MES buffer) and 100 μ L of either 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) at a concentration of 10 mg/mL in H₂O (covalent loading) or H₂O (no EDC for electrostatic loading) were added. The final volume was adjusted to 2 mL with MES buffer, and the reactions were incubated overnight at room temperature under rotation. The following day, the polyK-phage product was dialyzed into PBS using Pur-A-Lyzer™ Maxi Dialysis tubes, 50 kDa MWCO (Sigma-Aldrich). A speed vacuum was used to reduce the volume to approximately 0.5 mL. Concentrations of products were estimated by UV-Vis absorbance as described previously. Conjugates or electrostatic complexes of polyK and the anti-PNAG phages are called ‘PKAP phages’; the degree of polymerization of

polyK and method of attachment (C for covalent, E for electrostatic) are specified in the subscript.

PKAP phage Bradford assay. PolyK was diluted in PBS to prepare a standard curve ranging from 25 to 800 nM. PKAP phages were first diluted 20-fold (covalent constructs) or 50-fold (electrostatic constructs) in PBS before preparing a series of two-fold dilutions. Protected phage, prepared as described above, was diluted at concentrations of 10^{12} , 5×10^{12} , and 2.5×10^{12} virions/mL in PBS. 250 μ L of Pierce™ Bradford Plus Protein Assay Reagent (ThermoFisher Scientific) was added to 50 μ L of sample in a clear flat bottom 96-well plate. The plate was incubated at room temperature for 10 minutes before reading the absorbance at 595 nm with a Tecan Infinite 200 Pro microplate reader. The average absorbance of three blank replicates (PBS) was subtracted from the average of two replicates for each polyK concentration, then plotted against concentration. Points within the linear range were used to generate a standard curve to determine the polyK concentration of PKAP phage dilutions within the same range.

Virion quantitation in PKAP phage by primary amine content. Protected M13 ^{α} PNAG was diluted in a 0.1 M sodium bicarbonate buffer, pH 10.5, to prepare a standard curve ranging from 5×10^9 to 10^{12} virions/mL. PKAP phages were diluted in the same buffer to an approximate concentration between 2×10^{10} and 10^{11} virions/mL as estimated by absorbance, such that the concentration of polyK present on the PKAP phage was between the linear range of 0.25 and 5 μ M; a dilution of polyK at the same concentration as the corresponding PKAP phage was also prepared in sodium bicarbonate buffer. 40 μ L of each sample in triplicate was added to a 96-well, solid black, flat bottom polystyrene plate, in addition to a blank consisting of only sodium

bicarbonate buffer. 200 μL of Fluoraldehyde™ o-Phthaldialdehyde Reagent Solution (OPA) was added to each well and the plate was incubated for 3 minutes in the dark before measuring the fluorescence on a Tecan Infinite 200 Pro microplate reader with an excitation wavelength of 340 nm and emission wavelength of 455 nm. The blank-adjusted value of the polyK was subtracted from the blank-adjusted value of the PKAP phage, and the resulting value was fitted to the protected M13 $^{\alpha\text{PNAG}}$ phage standard curve to determine the virion concentration of each PKAP.

Virions quantification by Qubit Fluorometer. A modified version of a previously published protocol to determine the ssDNA concentration of phage bioconjugates was used.¹⁹³ A standard curve of phage was prepared in PBS from 10^{12} virions/mL to 3.13×10^{10} virions/mL. To 250 μL of PKAP phage, phage standard, or a blank (PBS), 250 μL of ZymoPURE P2 (Blue) was added. Samples were mixed by inverting the tube ten times and incubated for 3 minutes at room temperature. After incubating, 250 μL of ZymoPURE P3 (Yellow) was added and mixed by inversion until the solution was completely yellow. The samples were centrifuges for 10 minutes at 16,000 rcf. Then, 600 μL was transferred to a clean 1.5 mL microcentrifuge tube. With the drying rate set to high, a vacuum concentrator was used to reduce the sample volume to approximately 50 μL . Using 20 μL of each sample and following the manufacturer's instructions, a Qubit Fluorometer was used to quantify the amount of ssDNA present. The value of the blank was subtracted from each sample to account for interference from the ZymoPURE buffers.

Amino acid analysis of PKAP phage. M13 $^{\alpha\text{PNAG}}$ phage, polyK₉₀, PKAP_{90C}, and PKAP_{90E} were sent to the Molecular Structure Facility, UC Davis (Davis, CA, USA). Samples were transferred

using two 5/50 formic acid/acetonitrile washes and dried. Liquid phase hydrolysis (6 N HCl and 1% phenol) was performed at 110 °C for 24 hours, after which samples were dried again and dissolved in norleucine dilution buffer to the final volume. 50 µL of sample (2.0 nmol equivalent of norleucine) was injected onto a Hitachi L-8800 amino acid analyzer.

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration

(MBC). A standard broth-microdilution protocol was used to determine the minimum inhibitory concentration based on Clinical and Laboratory Standards Institute (CLSI) recommendations.¹⁵⁹ A series of two-fold dilutions of PKAP phage in PBS were prepared, then diluted ten-fold with MHB. 50 µL of each dilution was added to a 96-well round bottom plate. Assays were performed in duplicate.

Single colonies were picked and resuspended in MHB from cultures grown overnight on Mueller Hinton agar at 37 °C. The OD₆₂₅ was measured and adjusted between 0.08 and 0.13, corresponding to the optical density of McFarland standard 0.5, then diluted 100-fold. 50 µL of bacterial suspension was added to each well containing diluted PKAP phage, resulting in approximately 5×10^5 CFU/mL per well.¹⁵⁹ A growth control, consisting of 50 µL of bacterial suspension and 50 µL of broth, and sterility control, consisting of 100 µL broth only, were also prepared. Covered plates were incubated at 37°C without shaking. After 24 hours, the MIC was determined as the lowest concentration with no visible growth.

To determine the MBC, 50 µL from each well with no visible growth was plated on Mueller Hinton agar and incubated overnight at 37 °C. The MBC was determined as the lowest concentration of treatment yielding fewer than 25 colonies per plate, corresponding to survival of $\leq 0.1\%$ of the original inoculum.

Fluorescence microscopy of *S. aureus*. Single colonies *S. aureus* ATCC 29213 were picked from LB agar plates, inoculated in cation-adjusted Mueller Hinton Broth (CA-MHB), and incubated overnight at 37 °C, with rolling. Overnight cultures were diluted into fresh CA-MHB, and incubated at 37 °C, with shaking, until they reached OD600 $\approx$ 0.10-0.15 (equivalent to $\sim 1 \times 10^8$ CFU/mL). Bacteria were then treated at the MIC with polyK₉₀, PKAP_{90C}, or PKAP_{90E}, and incubated at 37 °C, with rolling. After 120 minutes of treatment, cells were collected and stained with a dye mix containing FM4-64, DAPI, and SYTOX-Green, before being transferred to an agarose pad for imaging.

Mammalian cell metabolism and viability. The impact of polyK or PKAP phage on human embryonic kidney cell (HEK 293; ATCC CRL-1573) metabolism was measured according to previously reported protocols.^{83,88,177} HEK 293 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics (penicillin/streptomycin) using Falcon[®] polystyrene tissue culture flasks with ventilated caps (Corning). The cells were incubated at 37 °C with 5% CO₂ until confluency. Then, cells were detached from the culture flask and seeded at a density of 1×10^4 cells/well in a 96-well plate (Celltreat). After culturing the cells overnight, the media was replaced with 100 μ L media supplemented with polyK or PKAP phage at various concentrations, or media with no supplements as the negative control, with triplicate samples for each condition. After a further 24 hours of incubation, metabolic activity was quantified using a PrestoBlue[™] assay according to the manufacturer's instructions, allowing 4 hours of incubation with the reagent.

For the five-day study, cells were cultured to confluency as described above, seeded at a density of 1×10^4 cells/well, and incubated with DMEM in each well of 24-well plates

(Corning). Five hours after seeding, the media was changed to DMEM containing 10% FBS and 1% antibiotics (penicillin/streptomycin) supplemented with polyK or PKAP phage at the same concentration as the *S. aureus* MIC, or to media with no supplements as the negative control. Triplicate samples were prepared for each condition, and media supplemented with polyK or PKAP phage for experimental groups was refreshed every 2 days. HEK 293 cell metabolic activity was quantified using a PrestoBlue assay according to the manufacturer's protocol on days 1, 3, and 5 of culture, replacing media with fresh supplemented media after the assay. Cell viability was assessed with calcein AM and ethidium homodimer-1 staining. Cells were imaged with an AxioObserver Z1 inverted microscope (Zeiss).

Hemolysis assay. Hemolysis assays were performed according to previously reported protocols.^{88,177,194} Three mL of 10% sheep red blood cells (MP Biomedicals) were added to 14 mL of PBS (pH ~7), gently mixed, and centrifuged for 10 minutes at $800 \times g$, 4 °C. Pelleted cells were washed four times by removing the supernatant, adding 14 mL of fresh PBS, and centrifuging again under the same conditions. After the final wash step, 200 μ L of cells were removed from the bottom of the vial and added to 9.8 mL of PBS to prepare the blood cell solution.

Two-fold serial dilutions of polyK or PKAP phage in PBS were prepared. 100 μ L of test reagent, detergent control (20% Triton X-100 in PBS), or negative control (PBS) were added to 100 μ L of blood solution in a 96-well round bottom plate, then incubated at 37 °C for one hour. After incubation, the plate was centrifuged at $1,700 \times g$ for 5 minutes. 100 μ L of supernatant was carefully collected without disrupting the pellet and transferred to a 96-well flat bottom plate.

Absorbance at 405 nm was measured and percent hemolysis was calculated according to the following equation: % *hemolysis* = $100 \times (A_{\text{sample}} - A_{\text{PBS}}) / (A_{\text{TritonX}} - A_{\text{PBS}})$.

Release of electrostatically loaded polyK. Electrostatically loaded PKAP phage was diluted to a concentration of 35 μM polyK in MilliQ water and 1 mL was added to a Slide-A-Lyzer™ MINI Dialysis Device, 20K MWCO (ThermoFisher Scientific). 45 mL of 1 $\times$ PBS buffer with 0, 0.1, 1, or 10 mg/mL Dextran sulfate sodium salt from *Leuconostoc* spp., average mol wt >500,000 (Sigma-Aldrich), was added to the conical tube of the dialysis device. A stir bar was added to the conical tube, and dialysis devices were incubated at 4 °C on a stir plate. After 1, 2, 4, 6, 24, and 48 hours, 50 μL of sample was removed and the buffer was replaced. Each condition was assessed in duplicate, the 50 μL aliquots were diluted ten-fold with PBS, and the amount of polyK remaining on the phage was assessed by the Bradford assay in triplicate as described above.

Resistance development. The broth microdilution assay described above was used to assess the development of resistance in *S. aureus* (ATCC 29213) against oxacillin, polyK, and PKAP phage. After the first assay, bacteria growing at sub-MIC concentrations for each treatment or without treatment were pooled and adjusted to a final volume of 2 mL in Mueller Hinton Broth. The OD₆₂₅ of the pool was measured and adjusted to be between 0.08 and 0.13, corresponding to the optical density of McFarland standard 0.5, and then diluted 100-fold to be used as the bacterial suspension to inoculate the next assay. 10 μL of this bacterial suspension was plated on Mueller Hinton agar to check for contamination. Additionally, for each round of selection, a glycerol stock was prepared for each of the four conditions (no treatment, oxacillin, polyK, or

PKAP phage) by adding 500 μ L bacterial suspension at McFarland standard 0.5 to 500 μ L of 50% glycerol. After thirteen rounds of selection, the MIC was re-evaluated for all treatments, along with vancomycin as a positive control.

Glycerol stocks from the thirteenth round of each selection condition, along with the initial *S. aureus* strain (ATCC 29213), were streaked on Mueller Hinton agar. 5 mL of LB broth was inoculated with a single colony and grown overnight. The following day, genomic DNA was extracted with a ZymoBIOMICS DNA Kit and sent for whole-genome sequencing. Snippy (version 4.6.0) was used to align mutant sequences to the original strain and determine variants.¹⁹⁵

4.2 Results

Covalent or electrostatic loading of polyK onto M13 ^{α PNAG} to produce polyK-anti-PNAG (PKAP) phages. Unmodified M13 ^{α PNAG} was characterized by zeta potential using previously described settings¹⁹⁶ and showed a negative zeta potential ($\zeta = -15.6 \pm 1.5$ mV), as expected from the overall charge of g8p. An initial attempt to electrostatically load polyK₉₀ by mixing and incubation with M13 ^{α PNAG} resulted in visible aggregation and ζ near zero (-0.5 ± 1.2 mV). To avoid aggregation, M13 ^{α PNAG} was pre-treated with sulfo-NHS-acetate to acylate primary amines. The protected M13 ^{α PNAG} showed a more negative zeta potential ($\zeta = -20.6 \pm 2.8$ mV), consistent with acylation of amines. After protection, the phage intermediate was loaded with polyK₄₈, polyK₉₀, or polyK₁₇₀. The polyK polymers alone exhibited positive ζ , with greater ζ at higher DP (**Table 4.1**), as expected. In one method, loading was performed through overnight incubation in appropriate buffer, allowing formation of noncovalent, presumed electrostatic, complexes (designated by an “E”) between virions and polymers. In the second method, virions and polymers were cross-linked with EDC chemistry (designated by a “C”) to form covalent

bioconjugates (**Table 4.1**). Both types of polymer-phage products were purified by dialysis and designated as PKAP (polyK-anti-PNAG) phages. Six variations of PKAP phages were synthesized by combining the three polyK variants and two loading methods. All of the PKAP phages displayed positive ζ (**Table 4.1**), indicating successful conjugate or complex formation, as the positive ζ is consistent with a predominance of amine groups at the virion surface from loading the polymer onto the negatively charged virions. Higher ζ was observed with greater DP, mirroring the trend in the polymers. Covalent loading and electrostatic loading resulted in similar ζ of the resulting PKAP phages.

		Degrees of Polymerization (<i>n</i>)		
		<i>n</i> = 48	<i>n</i> = 90	<i>n</i> = 170
polyK		polyK ₄₈ 11.1 ± 4.0	polyK ₉₀ 19.3 ± 1.1	polyK ₁₇₀ 21.8 ± 3.0
Method of Loading	Covalent	PKAP _{48C} 9.1 ± 0.2	PKAP _{90C} 12.8 ± 1.0	PKAP _{170C} 14.7 ± 2.0
	Electrostatic	PKAP _{48E} 8.3 ± 0.8	PKAP _{90E} 10.4 ± 1.2	PKAP _{170E} 11.9 ± 1.1

Table 4.1. Synthetic PKAP phage conjugates and complexes prepared from M13^αPNAG and three polyK polymers with zeta potentials (ζ). Bioconjugates (designated ‘C’) were synthesized using carbodiimide chemistry. Noncovalent complexes (designated ‘E’) were formed by electrostatic interactions of positively charged polymers with negatively charged virions. ζ is given for each polymer, conjugate or complex. PKAP phages exhibit positive ζ in all cases.

Characterization of PKAP compositions. Loading of polyK was observed to affect the UV-Vis absorption spectrum, altering the peak position and shape for PKAP phages compared to M13^αPNAG (**Figure S4.1**), indicating effects of polymer loading on electronic transitions. The equation typically used to quantify unmodified phage concentration is based on absorbance at

269 and 320 nm. For an orthogonal method to characterize PKAP phages, we quantified the polyK concentration using the Bradford reagent, which was insensitive to the virions. Then, the virion concentration was measured using the o-phthaldialdehyde reagent after accounting for the known polyK concentration.

The amount of polyK loaded in each sample was determined by Bradford assay, which is primarily based on binding of the Bradford reagent to basic moieties and to aromatic moieties to a lesser extent. Standard curves were generated for each polyK (**Figure S4.2**). The protected M13^{αPNAG} phage produced a negligible signal with the Bradford reagent for concentrations $\leq 10^{12}$ virions/mL (**Table S4.1**). The phage concentration in all PKAP dilutions, approximated by the UV-Vis absorbance, was kept under this range, such that Bradford reagent signal could be attributed solely to the polyK. Comparison to the standard curves thus gave the concentration of polyK in each PKAP sample (**Table S4.2**).

The concentration of phage virions was determined by a fluorescence assay using o-phthaldialdehyde (OPA). The protected M13^{αPNAG} phage was observed to produce a linear standard curve between 10^{10} and 10^{12} virions/mL (**Figure S4.3A**). At the same time, polyK also produced a linear standard curve between 0.125 and 5 μ M (**Figure S4.3B**). Since the concentration of polyK was measured independently by the Bradford assay, the OPA fluorescence of the known concentration of polyK was subtracted from the PKAP phage fluorescence, and the remaining fluorescence could be attributed to the phage virions. In general, the virions accounted for 30-60% of the total OPA fluorescence. The virion fluorescence was then compared to the M13^{αPNAG} standard curve to determine the phage virion concentration within the PKAP phage sample (**Table S4.3**).

Additionally, we attempted to quantify the concentration of phage virions using a previously developed method for phage-gold nanorod bioconjugates.¹⁹³ Briefly, buffers from commercially available MiniPrep kits are used to chemically denature the phage, and DNA is isolated. Centrifugation pellets the gold nanorods, separating it from the ssDNA that remains in the supernatant. To minimize the loss of ssDNA, the phage DNA is concentrated by speed vacuum centrifugation rather than MiniPrep columns. Using a Qubit Fluorometer, amounts as small as 10 pg/ μ L phage DNA are detected, and compared to a standard curve of virions for quantification (**Figure S4.4**). However, quantification by this method resulted in substantially lower phage concentrations than measured by OPA, suggesting the cationic polyK may be interacting with the negatively charged DNA. Further attempts to isolate and quantify the phage DNA by chemical or enzymatic digestion also failed.

The results of the Bradford and OPA assay were combined to determine the amount of polyK per virion in each PKAP phage sample (**Table 4.2**). At each DP of polyK, electrostatic interactions loaded 1.7 to 2.5 times more polyK than the covalent counterpart. The g8p protein has three solvent exposed carboxyl groups (Glu2, Asp4, and Asp5) available for EDC conjugation, limiting the number of potential covalent bonds that can form.^{45,82} These three residues are also likely participants in electrostatic interactions, along with the partially buried Glu20 that is normally inaccessible to EDC chemistry. Across both methods of loading, a smaller DP corresponded to more polyK molecules loaded per virion, suggesting that the surface area of the virions may limit the amount of loading.

An independent measurement of the ratio of polyK to g8p could be obtained by amino acid composition analysis by LCMS after intensive hydrolysis (**Figure S4.5**). M13 ^{α PNAG}, PKAP_{90C} and PKAP_{90E} were subjected to amino acid analysis (**Table S4.4**). The amount of

polyK₉₀ lysine in PKAP phage samples was calculated by subtracting the amount of phage lysine (detected in M13^{αPNAG}), after normalizing for virion concentration. The amount of polyK₉₀ lysine was normalized by DP and compared to the detectable virion concentration to obtain the number of polyK₉₀ molecules per virion. The concentration of polyK₉₀ was found to be 8.9 and 14 μM per 10¹² virions/mL in PKAP_{90C} and PKAP_{90E}, respectively. These values correspond to 2.5 polyK₉₀ molecules per g8p for PKAP_{90C} and 3.8 polyK₉₀ molecules per g8p for PKAP_{90E}. Amino acid composition analysis thus agreed with the Bradford and OPA analysis and confirmed higher polyK₉₀ loading for electrostatic compared to covalent attachment. Overall, these analyses showed that approximately 1 to 4 molecules of polyK were attached to PKAP phages per copy of g8p protein, with more polyK molecules per g8p found with electrostatic complexes and lower polymer length.

PKAP	Conc. polyK (μM) per 10 ¹² virions/mL	polyK molecules per phage	polyK molecules per g8p
48C	8.50 ± 0.22	5120 ± 130	2.34 ± 0.06
48E	14.70 ± 0.55	8850 ± 330	4.04 ± 0.15
90C	6.36 ± 0.36	3830 ± 220	1.75 ± 0.10
90E	11.6 ± 1.3	7000 ± 750	3.20 ± 0.34
170C	3.35 ± 0.28	2020 ± 170	0.92 ± 0.08
170E	8.24 ± 1.20	4960 ± 720	2.27 ± 0.33

Table 4.2. PKAP phage composition. The concentration of polyK was determined by the Bradford assay, allowing determination of the phage virion concentration by the OPA assay and calculation of the polyK molecules per phage. Given 2190 copies of g8p per virion, the ratio of polyK molecules per g8p copy was calculated.

Antibacterial activity of PKAP phages. The six PKAP phages were tested against *S. aureus*, a prolific PNAG producer, as well as *Escherichia coli* and *Pseudomonas aeruginosa*. A reduction

in MIC was observed for the PKAP phages against *S. aureus* compared to the corresponding polyK solution (**Table 4.3**). Greater reduction was observed for shorter polyK length; MIC decreases of 5 to 7-fold were observed for PKAP₄₈ and PKAP₉₀ constructs, while only a 2-fold decrease was observed for PKAP₁₇₀ constructs. In contrast to *S. aureus*, PKAP phages did not decrease MICs for *E. coli* or *P. aeruginosa* (**Table S4.5**), indicating specificity towards *S. aureus*. The minimum bactericidal concentration (MBC) was found to be identical to the MIC for *S. aureus* across all constructs (**Table S4.6**), indicating bactericidal activity as also observed for the polymers alone. M13^{αPNAG} (no polymer) demonstrated no inhibition of bacterial growth for any of the three bacterial species for up to a concentration of 10¹² virions/mL, as expected, confirming that the antibacterial activity of PKAP phages is derived from the polyK cargo.

PKAP_{90C} and PKAP_{90E} were further tested against additional *S. aureus* strains, including methicillin-resistant clinical isolates (MRSA), and *Staphylococcus epidermidis*, which also presents PNAG in its polysaccharide capsule. PolyK₉₀ and the corresponding PKAP phages exhibited the same MIC of *S. aureus* Newman towards all methicillin-resistant clinical isolates as well as quality control strain ATCC 29213 (**Table 4.3**). Both oxacillin-sensitive and oxacillin-resistant strains of *S. epidermidis* were sensitive to polyK₉₀, and PKAP_{90C} and PKAP_{90E} reduced the MIC by 2.4-fold. These data confirmed that drug-resistant staphylococci are still susceptible to the membrane-disruptive mechanism of polyK, as expected. Additionally, to test stability of PKAP_{90C}, its MIC against *S. aureus* ATCC 29213 was measured again after storage at 4 °C for 13 weeks. No change in MIC was observed, confirming that PKAP_{90C} retained activity over the storage period.

Species	Strain	DP	PolyK (μ M)	PKAP-C (μ M polyK)	PKAP-E (μ M polyK)
<i>S. aureus</i>	Newman (ATCC 25904)	48	5.00	0.83	0.76
		90	2.50	0.53	0.54
		170	1.25	0.59	0.61
	ATCC 29213	90	2.50	0.53*	0.54
	ATCC 43300		2.50	0.53	0.54
	Clinical isolate A, methicillin resistant		2.50	0.53	0.54
	Clinical isolate B, methicillin resistant		2.50	0.53	0.54
	Clinical isolate C, methicillin sensitive		2.50	0.53	0.54
	Clinical isolate A, oxacillin resistant		0.625	0.26	0.27
<i>S. epidermidis</i>	Clinical isolate B, oxacillin sensitive		0.625	0.26	0.27

Table 4.3. Minimum inhibitory concentration (MIC) of polyK and PKAP phages. Broth microdilution was used to determine the MIC for different strains of *S. aureus* and *S. epidermidis*. An asterisk indicates no change in MIC after the PKAP phage was stored at 4 °C for 13 weeks.

Mechanism of cell-killing by PKAP phages. PolyK (α -poly(L-lysine)-NH₂) is known to cause rapid cell death by disrupting bacterial membranes.¹⁸⁶ The kinetics of cell death induced by polyK₉₀ was compared to that of PKAP_{90C} and PKAP_{90E}. *S. aureus* ATCC 29213 was incubated with each treatment at the MIC, and aliquots were diluted and spread on agar plates at a series of time points for colony counting after overnight incubation at 37 °C. PolyK₉₀ and PKAP_{90E} showed similar kinetics. Interestingly, PKAP_{90C} exhibited significantly faster cell-killing (**Figure 4.1**). At 30 minutes, PKAP_{90C} treatment produced a 1.8-log reduction in colony-forming units

(CFU), compared to a 0.5-log and 0.3-log reduction for polyK₉₀ and PKAP_{90E}, demonstrating that the bioconjugate improves killing rate. After four hours, all constructs induced a > 2.5-log reduction, with PKAP_{90C} inducing the greatest reduction (3.0-log reduction). Time-kill curves were fit to a single-exponential decay model, finding a higher apparent rate constant ($k = 0.12 \text{ min}^{-1}$) for PKAP_{90C} compared to polyK₉₀ ($k = 0.034 \text{ min}^{-1}$) or PKAP_{90E} ($k = 0.028 \text{ min}^{-1}$) (Table S4.7). The faster killing rate of the bioconjugate indicates that concerted presentation of the polymer on the phage may accelerate cell-killing, while the equal rate of polyK₉₀ and PKAP_{90E} indicates that the electrostatic complex acts through the same mechanism as the free polymer.

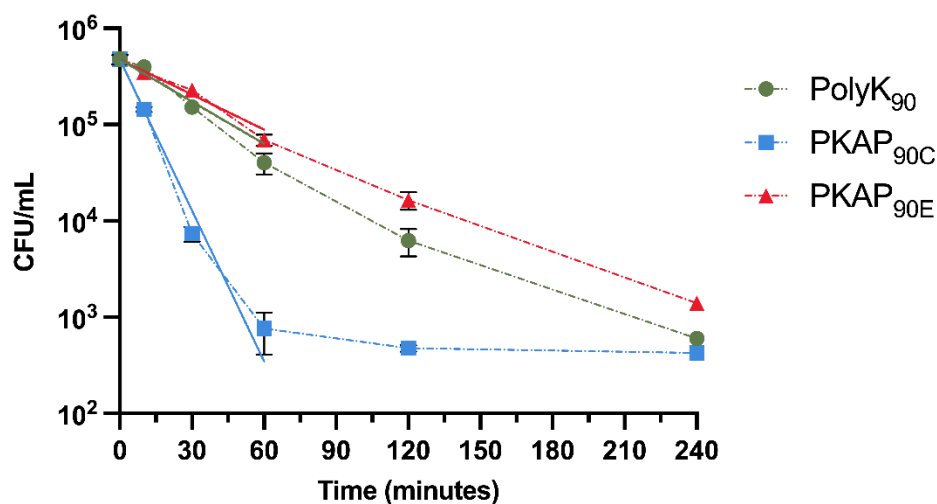


Figure 4.1. Time-kill kinetics of polyK₉₀, PKAP_{90E} and PKAP_{90C}. Decrease in viable *S. aureus* (ATCC 29213) cells over time when incubated with polyK₉₀, PKAP_{90C}, or PKAP_{90E} at their respective MICs. Error bars represent standard deviation between biological duplicates. Straight colored lines correspond to single-exponential curve fits of the first 60 minutes (Table S9).

The effects of PKAP treatment on *S. aureus* cells were characterized by fluorescence microscopy. Cells were incubated with polyK₉₀, PKAP_{90C}, or PKAP_{90E} at their respective MIC for 120 minutes before staining and imaging. Staining of cells incubated with M13^{αPNAG} was indistinguishable from untreated cells. In contrast, all three treatments yielded cells that were

permeabilized to SYTOX Green, typically excluded from intact cells, and showed clearance of cell bodies under phase contrast, supporting the mechanism of membrane disruption (**Figure 4.2**).

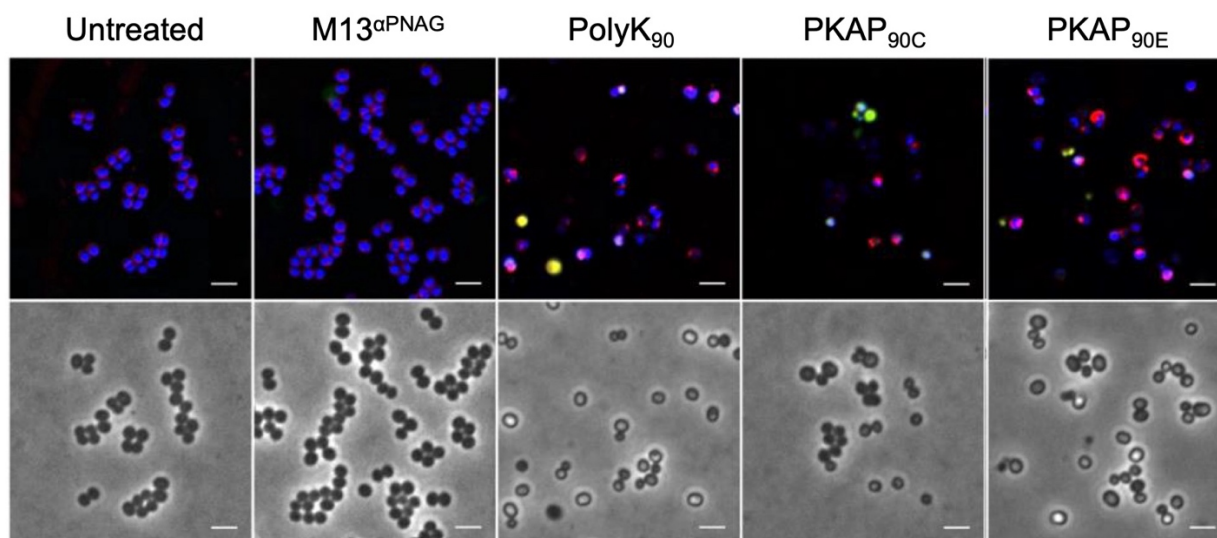


Figure 4.2. Fluorescence (top row) and phase contrast (bottom row) microscopy of *S. aureus* treated with polyK₉₀ and PKAP₉₀ phages. After 120 minutes of treatment at the MIC, cells were stained with FM4-64 (red, membrane dye), DAPI (blue, membrane-permeable DNA dye), and SYTOX Green (green, membrane-impermeable DNA dye). Scale bar = 2 μ m.

The interaction between PKAP phages and *S. aureus* was further evaluated by TEM. M13 ^{α PNAG} or PKAP_{90E} phages were incubated with *S. aureus* (Newman) and then applied to TEM grids. To visualize M13-derived phages by negative stain TEM, a previously developed protocol was used to immunolabel g8p with gold nanoparticles, facilitating their identification (**Figure 4.3**).¹⁹⁷ Both M13 ^{α PNAG} and PKAP_{90E} phages were observed bound to *S. aureus* cells, and PKAP_{90E} induced significant bacterial disruption compared to normal morphology (**Figure 4.3E**). Bacterial membrane disruption after incubation with PKAP_{90C} was also apparent (**Figure 4.3F**). However, PKAP_{90C} itself could not be visualized by immunolabeling, likely because the

covalent attachment of polyK₉₀ interfered with the binding of the primary antibody to g8p. Overall, the TEM data was consistent with bacterial lysis induced by the PKAP phages.

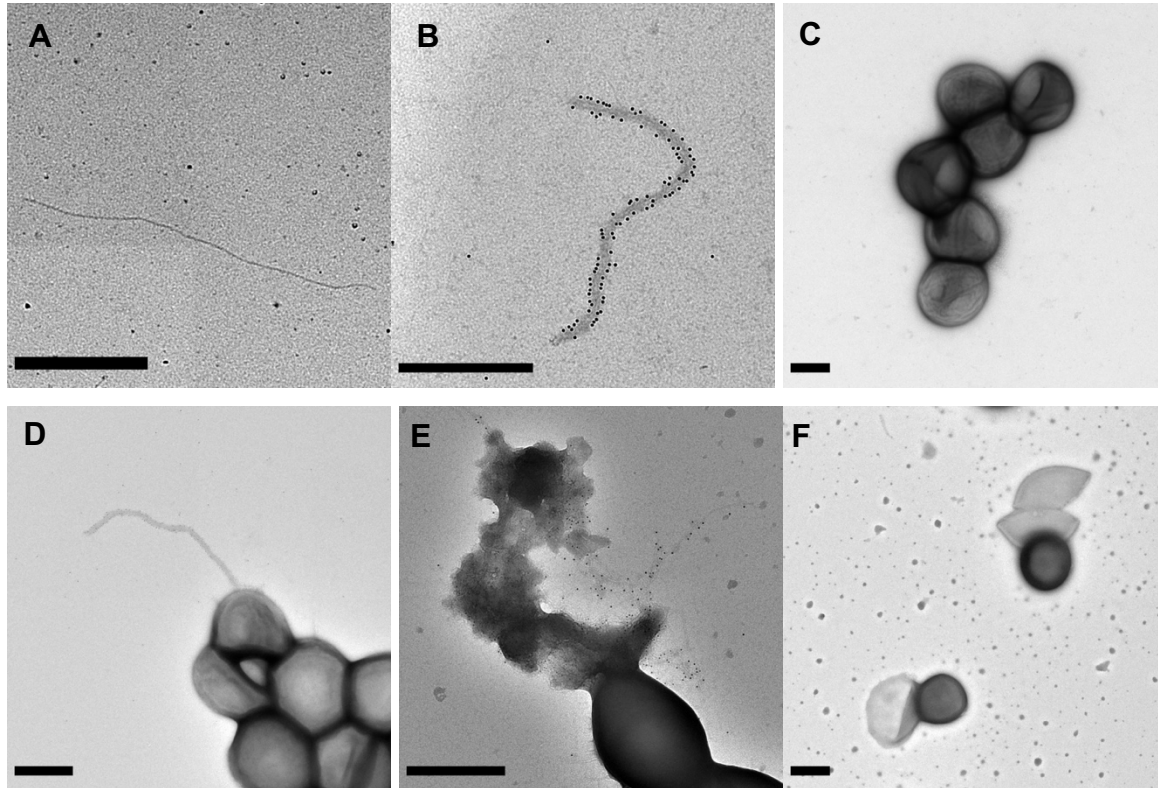


Figure 4.3. Transmission electron microscopy (TEM) visualizes interactions between M13^{αPNAG} and *S. aureus*. A) Unlabeled M13^{αPNAG} phage, B) M13^{αPNAG} phage labeled with gold nanoparticles, C) *S. aureus* Newman, D) *S. aureus* incubated with immunolabeled M13^{αPNAG} phage, E) *S. aureus* incubated with immunolabeled PKAP_{90E} phage and F) *S. aureus* incubated with PKAP_{90C} phage. Scale bar = 500 nm.

Cytotoxicity of PKAP phages. For an initial assessment of cytotoxicity, HEK 293 cells were incubated with PKAP phages or the corresponding polyKs for twenty-four hours. Metabolic activity was assessed with a PrestoBlue assay and normalized by the activity of untreated cells. Exposure to M13^{αPNAG} showed little effect compared to control ($114\% \pm 5\%$), as expected. 24-h treatment at the *S. aureus* MIC for polyK₉₀ and polyK₁₇₀ significantly decreased HEK 293 cell metabolic activity to $51\% \pm 8.5\%$ and $24\% \pm 7.8\%$, respectively, of the control (**Figure 4.4B, C**),

indicating substantial cytotoxicity as expected for these cationic polymers,¹⁸⁶ although 24-h treatment with polyK₄₈ was better tolerated (**Figure 4.4A**). Higher polyK₉₀ concentration resulted in decreased HEK 293 metabolism (**Figure S4.6**), with metabolic activity dropping to $16\% \pm 11.6\%$ at 2X MIC.

In contrast to polyK₉₀ and polyK₁₇₀, 24-h treatment at the MIC for PKAP_{90C}, PKAP_{90E}, PKAP_{170C} and PKAP_{170E} all resulted in significantly greater HEK 293 cell metabolic activity ($80\% \pm 10\%$, $109\% \pm 9\%$, $65\% \pm 8\%$, and $108\% \pm 13\%$, respectively) compared to the corresponding polyK treatment (**Figure 4.4**). Since $>80\%$ cell activity is considered non-toxic, exposure to the MIC of PKAP_{90C} or PKAP_{90E} would be considered non-toxic.¹⁹⁸ However, higher concentrations ($>7X$ MIC) of PKAP_{90C} or PKAP_{90E} did result in cytotoxicity (**Figure S4.6**). Nevertheless, increasing the concentration of PKAP_{90C} to 7.7X MIC still gave $66\% \pm 6\%$ metabolic activity, showing that even 7.7X MIC of PKAP_{90C} was better tolerated compared to 2X MIC of polyK₉₀ ($p = 0.0036$).

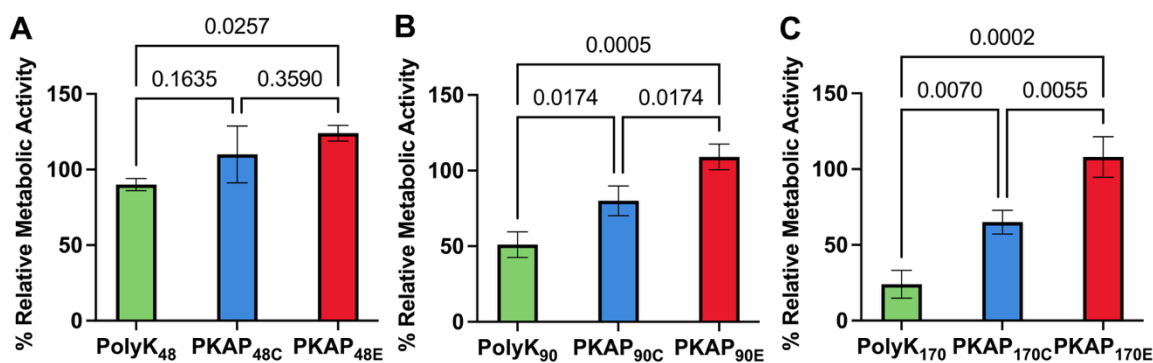


Figure 4.4. Relative metabolic activity of polyK and PKAP treated HEK 293 cells. HEK 293 cells were treated for 24 h with concentrations equivalent to *S. aureus* MIC of polyK, PKAP_C or PKAP_E with DP = A) 48, B) 90, or C) 170. Metabolic activity was measured with PrestoBlue and expressed as a percentage of untreated cells. Significance was assessed by a one-way ANOVA and Tukey's post-hoc multiple comparisons test.

Since antimicrobial regimens typically cover multiple days, biocompatibility was also assessed with continuing exposure to the agents over a time course of 5 days, to capture long-term inhibition or recovery of metabolic activity. HEK 293 cells were incubated with PKAP phages or the corresponding polyKs at a concentration equal to the *S. aureus* MIC. Cell metabolic activity was assessed on days 1, 3, and 5, and after each time point, spent media was replaced with fresh media containing the treatment concentrations (**Figure 4.5**). Without treatment, HEK 293 cells show consistent increase in metabolic activity, indicating growth over the five-day period. However, HEK 293 cells treated with any of the polyKs showed no significant increase in metabolism, indicating inhibition of cell growth and thus poor biocompatibility, even for polyK₄₈. That is, although polyK₄₈ did not appear to affect metabolic activity at 24 h, it did inhibit long-term growth over 5 days. Like the polyKs, both PKAP_{170C} and PKAP_{170E} also showed a lack of cell growth over 5 days (**Figure 4.5C**), indicating that the polymer with DP=170 is not biocompatible for longer exposures, even in the PKAP context.

In contrast, treatment with PKAP_{48C} resulted in metabolic activity over 5 days similar to the control. PKAP_{48E} also gave increased metabolism over time, indicating growing cells, but at roughly half the rate of the control (**Figure 4.5A**), indicating that the electrostatic complex was more cytotoxic than the bioconjugate ($p < 0.0001$). This trend was mirrored for PKAP_{90C} and PKAP_{90E}, although both showed less metabolic activity compared to the corresponding DP = 48 agents (**Figure 4.5B**). Nevertheless, the metabolic activity with PKAP_{90C} treatment was still significantly greater than with PKAP_{48E} treatment ($p < 0.0001$). Thus, the most biocompatible treatment at the MIC was PKAP_{48C}, closely followed by PKAP_{90C}.

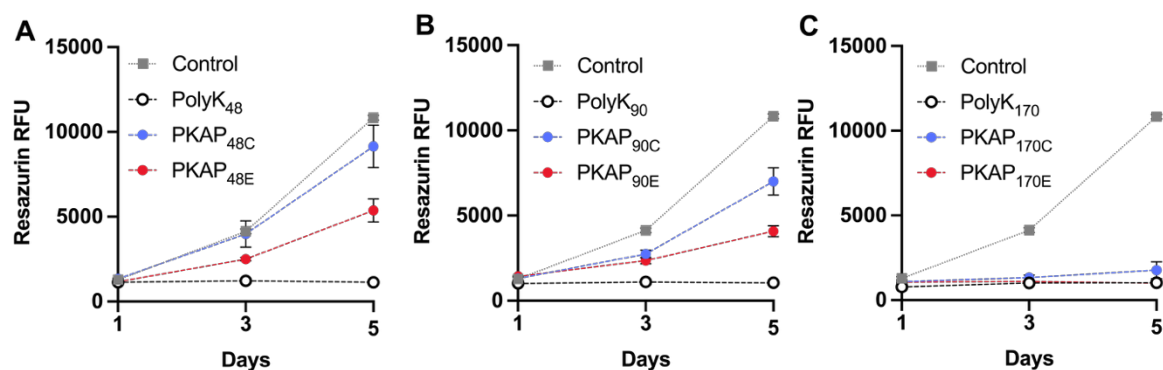


Figure 4.5. Metabolic activity of polyK and PKAP treated HEK 293 cells over a five-day period. A PrestoBlue assay measured metabolic activity of HEK 293 cells treated at the *S. aureus* MIC of A) polyK₄₈, PKAP_{48C}, or PKAP_{48E}, B) polyK₉₀, PKAP_{90C}, or PKAP_{90E}, and C) polyK₁₇₀, PKAP_{170C}, or PKAP_{170E}. No treatment was given for the control. Significance was assessed with a two-way ANOVA followed by Tukey's test.

To visualize cell density and viability, cells incubated with polyK₉₀, PKAP_{90C}, or PKAP_{90E} at the MIC were stained with live/dead stain (calcein AM and ethidium homodimer-1) on days 1 (**Figure S4.7**) and 5 (**Figure 4.6**). The total number of live and dead cells from five images per condition were used to calculate the percentage of live cells (**Figure S4.8**). After one day of treatment, cell viability was consistent with the levels of metabolism measured during the initial twenty-four-hour assay by PrestoBlue; namely, PKAP_{90C}- or PKAP_{90E}-treated cells exceeded 80% viability, while cells treated with polyK₉₀ exhibited reduced (42%) viability. After five days, PKAP_{90C}- or PKAP_{90E}-treated cells exhibited substantial microscopic growth, comparable to the untreated cells, with viability above 98%. In contrast, viability of polyK₉₀-treated cells dropped to 29% and displayed minimal microscopic growth. Together, these results confirm that PKAP modification substantially increases the cytocompatibility of polyK.

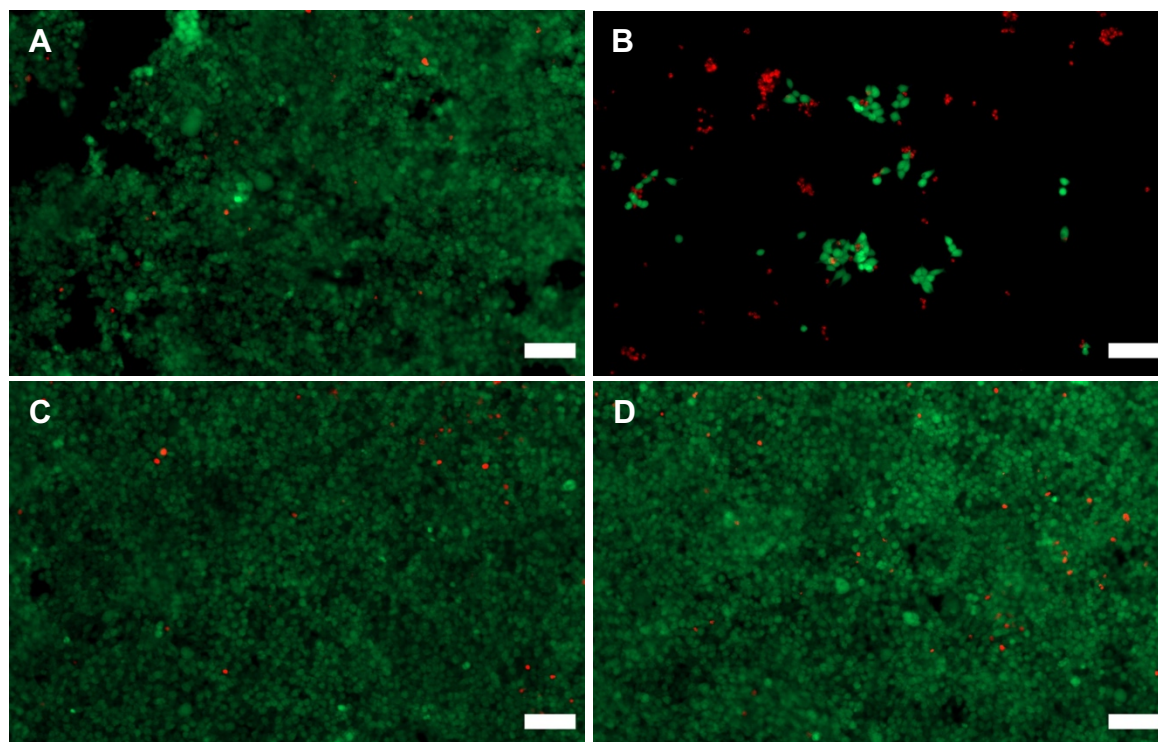


Figure 4.6. Representative images of live-dead staining on day 5. HEK 293 cells were imaged after five days of continued exposure to: A) control (no treatment), B) polyK₉₀, C) PKAP_{90C}, or D) PKAP_{90E} at the *S. aureus* MIC. Cells were stained with calcein AM and ethidium homodimer-1 to determine viability. Scale bar = 100 μ m.

Hemolytic activity of PKAP phages. The hemolytic effects of polyK and PKAP phages on sheep red blood cells were evaluated at varying concentrations. At the *S. aureus* MIC, all polyKs gave less than 5% hemolysis (**Figure 4.7A**), but with a concentration of 40 μ M (8 $\times$ MIC for polyK₄₈), all three polyKs induced $\geq 5\%$ hemolysis. All six PKAP phages were tested at 0.5X, 1X and 8X MIC. Hemolysis remained at $< 5\%$ in all cases, with the exception of PKAP_{170C} at 8 $\times$ MIC (**Figure 4.7D**). These results confirmed the greater biocompatibility of the PKAP phages (DP = 48 and 90) compared to the corresponding polyKs.

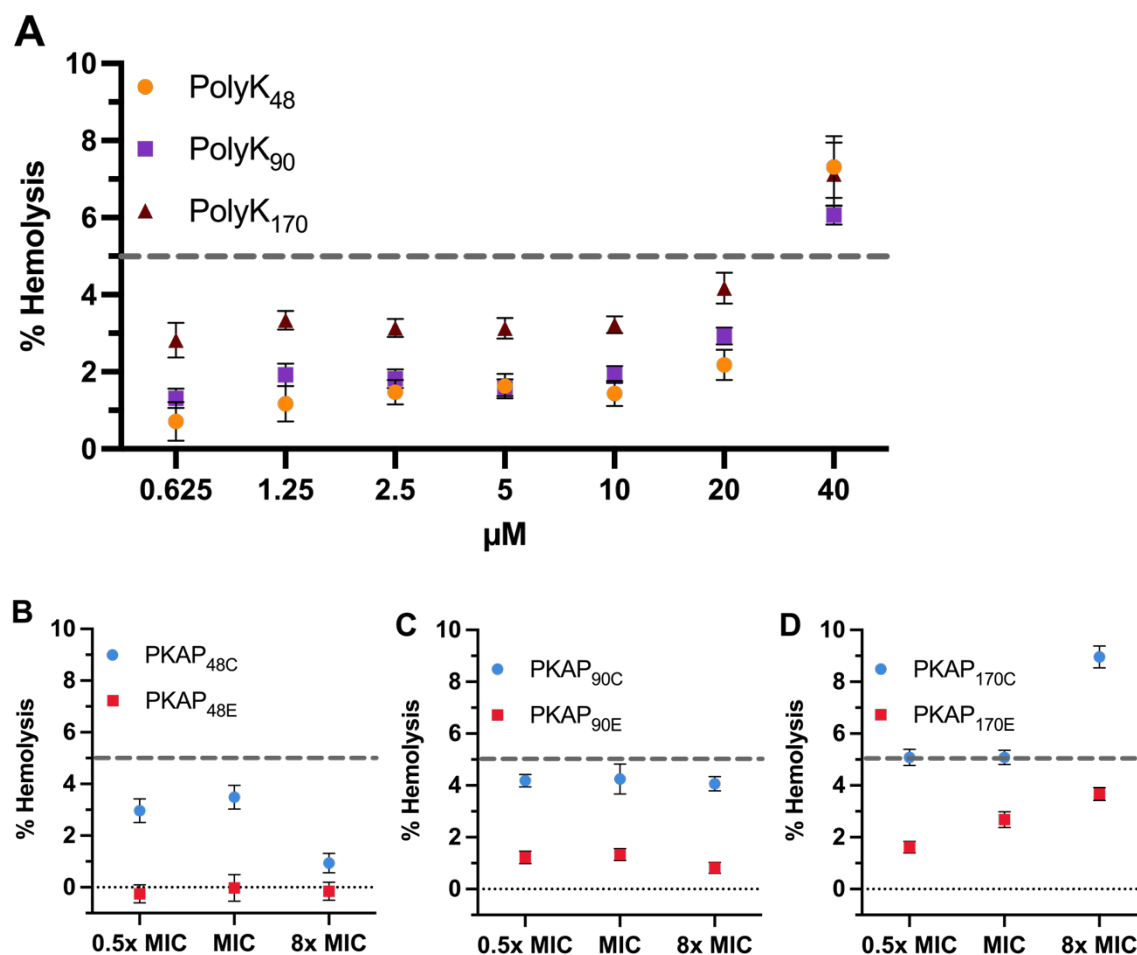


Figure 4.7. Hemolysis in the presence of polyK and PKAP phages. A) PolyK DP = 48, 90, or 170 were evaluated between concentrations of 0.625 and 40 μ M, presenting < 5% hemolysis at MIC (5, 2.5, and 5 μ M respectively. B) PKAP_{48C}, PKAP_{48E}, C) PKAP_{90C}, PKAP_{90E} and D) PKAP_{170C}, and PKAP_{170E} were evaluated at *S. aureus* MIC (0.83, 0.76, 0.53, 0.54, 0.59, and 0.61 μ M polyK, respectively), along with 0.5 $\times$ and 8 $\times$ MIC.

Release of polyK from the electrostatic PKAP complex. Prolonged 5-day exposure to the electrostatic PKAP complexes resulted in less cell growth compared to the PKAP bioconjugates. Given the high cytotoxicity of the free polymers, a possible explanation for this observation is that polyK may dissociate from the electrostatic complex with phages over longer time periods. To test this hypothesis, the release of polyK₉₀ from PKAP_{90E} was evaluated by dialysis into a buffer containing varying amounts of the anionic polysaccharide dextran sulfate as a negatively

charged ‘sink’ for any dissociated polyK. The amount of polyK₉₀ remaining in the dialysis cassette over time was assessed with the Bradford assay (**Figure 4.8**). Without the dextran sulfate sink, 80% of the initial amount of polyK₉₀ remained in the cassette at 48 h, constituting a significant loss of polymer from the complex ($p = 0.0014$, two-way ANOVA, Tukey’s test). Increasing concentrations of dextran sulfate outside the cassette resulted in faster release over time. Release curves of polyK₉₀ from PKAP_{90E} with varying concentrations of dextran sulfate were fit to a pseudo-first-order exponential decay model (**Table S4.8**). The apparent rate constant for polyK₉₀ release increased with concentration of dextran sulfate (**Figure S4.9**), indicating a dynamic process with rapid release and rebinding. These results confirm that polyK₉₀ was indeed released from the electrostatic complex, on a time scale that could affect cell growth due to the cytotoxicity of the free polymer. Rapid, reversible polymer release is also consistent with the time-kill assay showing similar kinetics of bacterial cell death for polyK₉₀ and PKAP_{90E}.

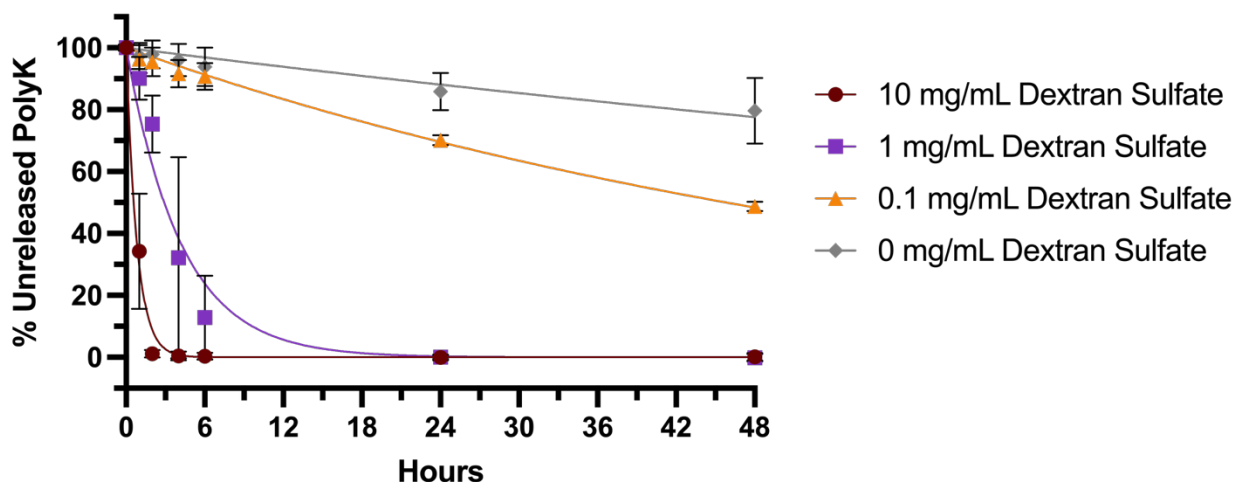


Figure 4.8. Release of electrostatically loaded polyK₉₀ from PKAP_{90E}. The amount of polyK₉₀ remaining after dialysis with PBS with 0, 0.1, 1, or 10 mg/mL dextran sulfate was evaluated by Bradford assay. As the dextran sulfate concentration in the dialysis buffer increased, the rate of polyK₉₀ release increased.

Lack of resistance development. Antimicrobial polymers are believed to be “resistance resistant,” i.e., mutations conferring resistance are rare, which would be a key advantage over other antibiotic classes.^{199,200} The ability of *S. aureus* (ATCC 29213) to develop resistance to PKAP_{90C} phage compared to oxacillin and polyK₉₀ was assessed by serial passaging at sublethal conditions. This experimental evolution was conducted following the broth microdilution procedure, in which the next passage was inoculated using the pooled bacterial cultures from sub-MIC concentrations. After five rounds of serial passaging, the oxacillin MIC increased from 0.5 to 4 µg/mL, reaching the breakpoint for resistance as defined by the CLSI Performance Standards for Antimicrobial Susceptibility Testing.²⁰¹ By the 13th round, the oxacillin MIC had reached 16 µg/mL, a 32-fold increase from its original value. In contrast, the MIC for both polyK₉₀ and PKAP_{90C} stayed within 2-fold (i.e., within measurement error) of the initial MIC despite serial passaging in sublethal concentrations of the respective treatment (**Figure 4.9**).²⁰²

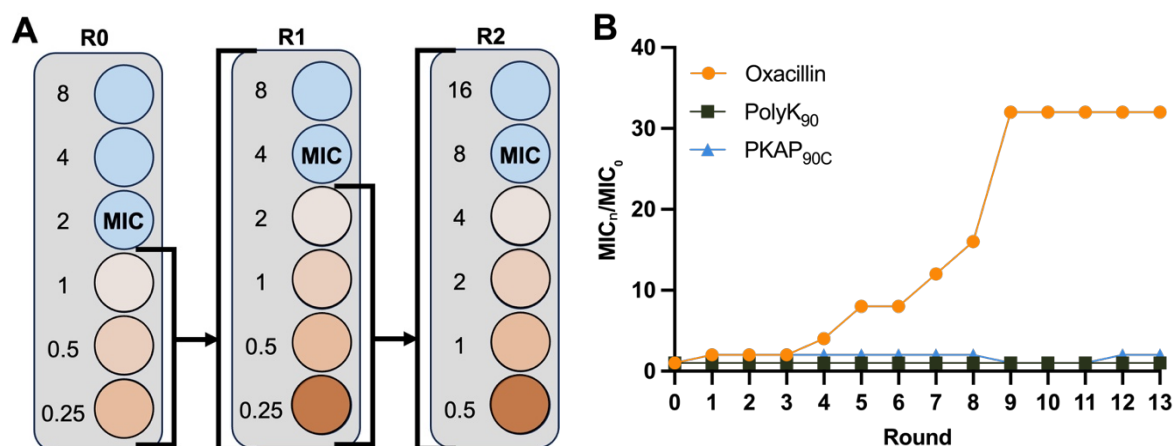


Figure 4.9. PolyK₉₀ and PKAP_{90C} do not induce resistance in adaptive laboratory evolution. A) Schematic for adaptive laboratory evolution using serial broth microdilution to generate resistance. B) The MICs for each round for oxacillin, polyK₉₀, and PKAP_{90C}, normalized by the initial MIC concentration (round 0).

After the final (13th) round of selection, the MIC of the evolved culture from each selection-pressure group (oxacillin, polyK₉₀ and PKAP_{90C}) was measured for all three antimicrobials, to assess whether cross-resistance had developed. No significant cross-resistance was observed (**Table S4.9**). In addition, to assess spontaneous emergence of resistance without selection pressure, *S. aureus* was serially passaged without treatment (i.e., in media alone) for 13 rounds, and the MIC of the culture for oxacillin, polyK₉₀ and PKAP_{90C} was measured over rounds. Resistance to oxacillin gradually increased by 4-fold by the end of the experiment, but no change was observed for the MICs of polyK₉₀ and PKAP_{90C} (**Figure S4.10**). These observations support the concept that resistance to antimicrobial polymers, and now polymer conjugates, is slow to emerge.

To identify genetic changes in the experimental evolution, bacteria from the thirteenth round were grown on Mueller Hinton agar and single colonies were selected for whole-genome sequencing, along with the initial *S. aureus* strain (ATCC 29213). Genetic differences between the isolates and the reference strain were identified with snippy (**Table S4.10**).¹⁹⁵ Nine point mutations (four synonymous, five non-synonymous) were identified in the control culture without treatment, and none of these were found in other selection lines, indicating genetic drift during the experiment. Six mutations (all non-synonymous) were identified in the oxacillin isolate, including a mutation in *vraS*, a histidine kinase that senses cell wall stress and regulates resistance to β -lactams and vancomycin.²⁰³ None of the mutations were present in the Comprehensive Antibiotic Resistance Database,²⁰⁴ indicating the de novo development of oxacillin resistance rather than horizontal gene transfer of the *mecA* gene.²⁰⁵

In the polyK₉₀ selection line, two mutations (both non-synonymous) were found, including one mutation creating a premature stop codon (nonsense mutation) in *graR*, which

regulates the response to cationic antimicrobial peptides.²⁰⁶ In the PKAP_{90C} selection line, five mutations (all non-synonymous) were found, including a frameshift in *agrC*, a membrane protein that binds autoinducing peptides and activates downstream responses.²⁰⁷ Despite these mutations, no change in the MIC was observed for polyK₉₀ or PKAP_{90C}. Since *vraS* and *graR* are associated with vancomycin resistance,^{208–211} we measured the MIC for vancomycin in these isolates; however, no vancomycin resistance was observed (**Table S4.10**). Overall, while individual mutations suggest genetic potential for a response to polyK₉₀ and PKAP_{90C}, no phenotypic change in the MIC was observed during the experiment.

4.3 Discussion

The prevalence of AMR among bacterial pathogens motivates the development of ‘resistance-proof’ treatments that work through robust biophysical mechanisms, such as membrane disruption by cationic polymers. However, such mechanisms are often non-selective and result in damage to mammalian cells. On the other hand, phages are often overly specific, but we hypothesized that the shortcomings of polymers and phages could compensate for one another in a hybrid agent. A prior study noted that incubation of bacterial cells with both naturally derived ϵ -poly(L-lysine) and environmentally isolated wild-type phages gave added cell-killing over either agent alone,²¹² as expected given their different modes of action, but did not synthesize conjugates or test for selectivity. Interestingly, α -poly(L-lysine) has been observed to inactivate the lytic coliphage T2,²¹³ presumably through non-specific electrostatic interactions, but oligolysine has also been used as a chemical “wrapper” to reduce non-specific binding of M13 during phage display.²¹⁴ These prior observations indicate that interactions between phage and cationic polymers can cause different effects depending on the system. Therefore, we loaded

M13 with the nonspecific lytic polymer α -poly(L-lysine) (also called polyK) either covalently or electrostatically and engineered the phage to selectively bind the *S. aureus* surface polysaccharide PNAG. We characterized and evaluated efficacy and selectivity for both methods of loading along with various degrees of polymerization of polyK.

High loading efficiency was achieved through both loading methods, with one to four polyK molecules loaded per copy of g8p, such that polyK outweighed phage proteins approximately five or nine times for PKAP_{90C} and PKAP_{90E}, respectively. While electrostatic interactions loaded more polymer per phage than covalent crosslinking, both methods resulted in nearly identical reductions in MIC towards the target species *S. aureus*. However, increasing DP led to fewer polymer molecules loaded per phage and a smaller reduction in MIC regardless of attachment strategy. Both methods of loading preserved the intrinsic lytic and bactericidal activity of the polymer. Poly(L-lysine) is understood to disrupt membranes through the “carpet” mechanism, where cationic polymer molecules aggregate on the negatively charged phospholipid membrane, and upon achieving a critical local concentration, induce membrane distortion leading to cell death.^{143,144} Notably, electrostatically loaded polyK in PKAP_{90E} and free polyK₉₀ killed cells at the same rate, and electrostatically loaded polyK₉₀ was found to dissociate rapidly from PKAP_{90E} from the phage in dialysis experiments. These observations are consistent with a mechanism for electrostatic PKAP constructs in which polyK is released and then interacts with the bacterial cell membrane. In contrast, the covalently loaded PKAP_{90C} gave significantly faster kill rates than free polyK₉₀ or PKAP_{90E}, with a death rate constant 3.6 times higher than free polyK₉₀. This comparison is consistent with PKAP_{90C} acting through a different mechanism, perhaps by pre-assembling the polymer “carpet” and thus bypassing the need to accumulate polyK to the critical concentration within the membrane.

Selectivity of the phage constructs was engineered using an scFv sequence derived from a known anti-PNAG antibody,^{92,94,95} and verified by ELISA and glycan-binding arrays. Although free polyK treatment at the MIC completely suppressed HEK 293 cell metabolism over 5 days, PKAP phage treatments at the respective MICs were tolerated by mammalian cells, with PKAP_{48C}, PKAP_{48E} and PKAP_{90C} allowing >50% of normal growth. Interestingly, the covalent constructs were significantly less cytotoxic than the corresponding electrostatic constructs. This difference is consistent with the mechanistic observations, in that electrostatic constructs release free polyK molecules that may interact with mammalian cells, while covalent constructs do not release polyK. In general, lower degrees of polymerization led to higher biocompatibility. Mammalian cells also tolerated higher concentrations of PKAP_{90C} and PKAP_{90E} than free polyK₉₀. To calculate the selectivity index (SI, or the 50% cytotoxic concentration CC₅₀ for mammalian cells divided by the minimum inhibitory concentration on *S. aureus*), metabolic activity of HEK 293 cells was measured after 24 hour treatment by different concentrations of polyK₉₀, PKAP_{90C}, and PKAP_{90E} to estimate the CC₅₀ for each treatment (**Figure S4.6**). Treatments of polyK₉₀ at 2.5 μ M, PKAP_{90C} at 3.8 μ M, and PKAP_{90E} at 6.3 μ M, gave 44-66% metabolic activity with no significant difference between the values; these concentrations were therefore used as the CC₅₀ values.²¹⁵ Note that use of CC₅₀ from the HEK 293 metabolic activity assay would give substantially lower absolute values for SI compared to using the hemolytic concentration (HC₅₀); nevertheless, relative trends can be observed.²¹⁶ The MIC and CC₅₀ of polyK₉₀ are equal, so its selectivity index ($SI = CC_{50}/MIC$) is 1, consistent with a poor therapeutic candidate. In contrast, the selectivity index was 7 for PKAP_{90C} and 12 for PKAP_{90E}, indicating an order of magnitude improvement by using the PKAP constructs. Despite a lower selectivity index, PKAP_{90C} phage demonstrated superior biocompatibility over five days

compared to PKAP_{90E} and PKAP_{48E}, consistent with polyK release over time from the electrostatic complexes and subsequent interference with mitochondrial function.¹⁹² Given these considerations, PKAP_{90C} was chosen to test whether the phage-polymer conjugate was ‘resistance-proof’, as intended.

In order to evolve resistant organisms, serial passaging of *S. aureus* was performed in the presence of sub-inhibitory concentrations of oxacillin, polyK₉₀, or PKAP_{90C}. Oxacillin resistance emerged and increased steadily, with the evolved culture able to tolerate a 32-fold increased concentration of oxacillin by the end of the experiment. Remarkably, both polyK₉₀ and PKAP_{90C} treatments gave no increase in resistance during the same duration. Therefore, PKAP_{90C} combines the resistance-proof property of the cationic polymer with phage-based selectivity that overcomes the polymer’s cytotoxicity.

4.4 Supplementary figures

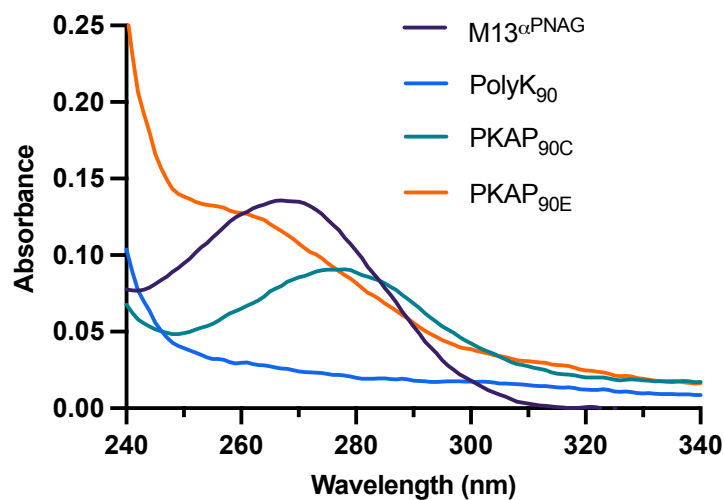


Figure S4.1. UV-Vis absorbance spectrum of M13^αPNAG, polyK₉₀, PKAP_{90C}, and PKAP_{90E}. M13 phages exhibit a broad peak at 269 nm due to the presence of ssDNA and virion coat proteins. α -poly(L-lysine)-NH₂ exhibits little absorption at 269 nm. Loading of α -poly(L-lysine)-NH₂ altered the absorbance spectrum, with PKAP_{90C} phage presenting a red shift and PKAP_{90E} showing peak broadening.

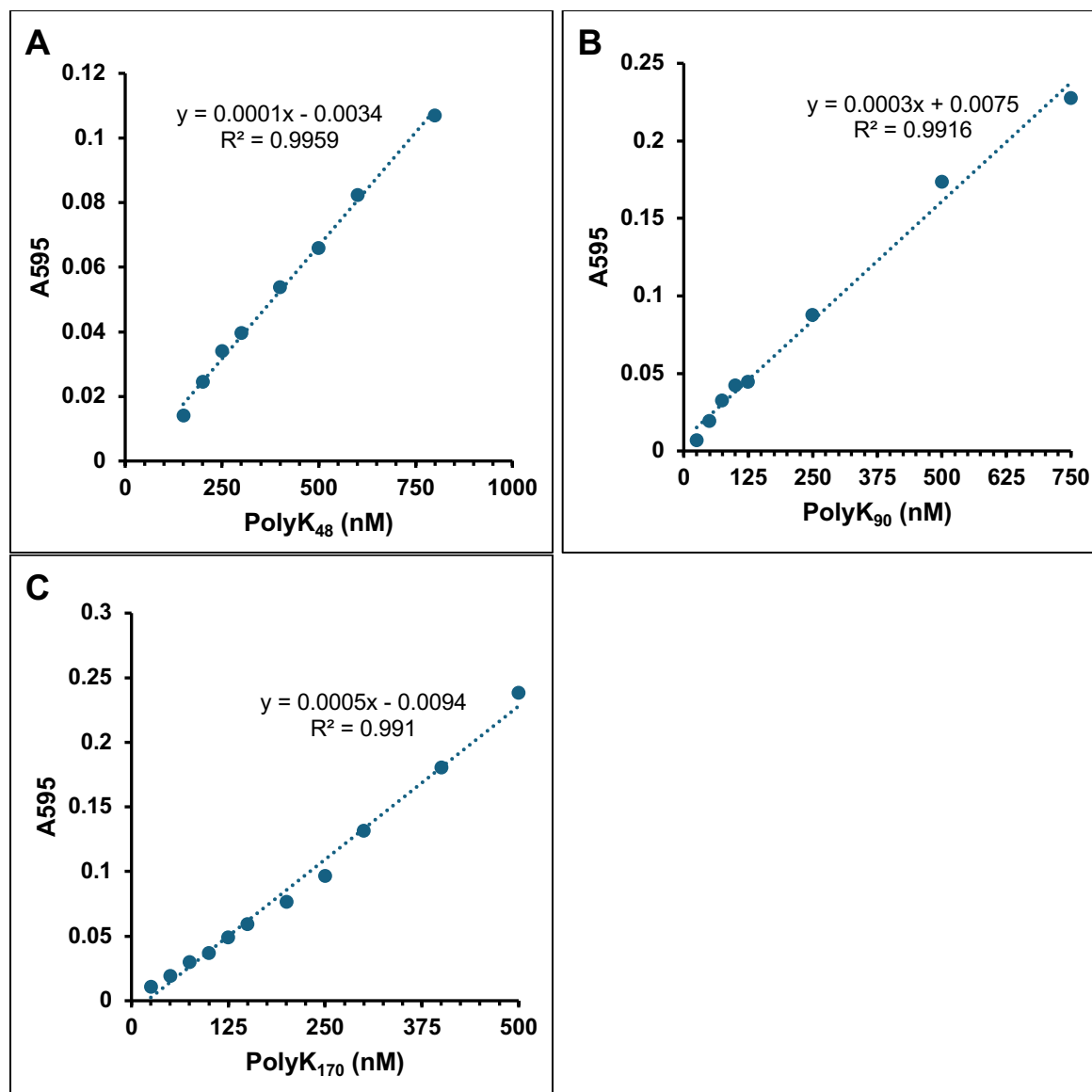


Figure S4.2. Bradford assay standard curves for A) polyK₄₈, B) polyK₉₀, and C) polyK₁₇₀.

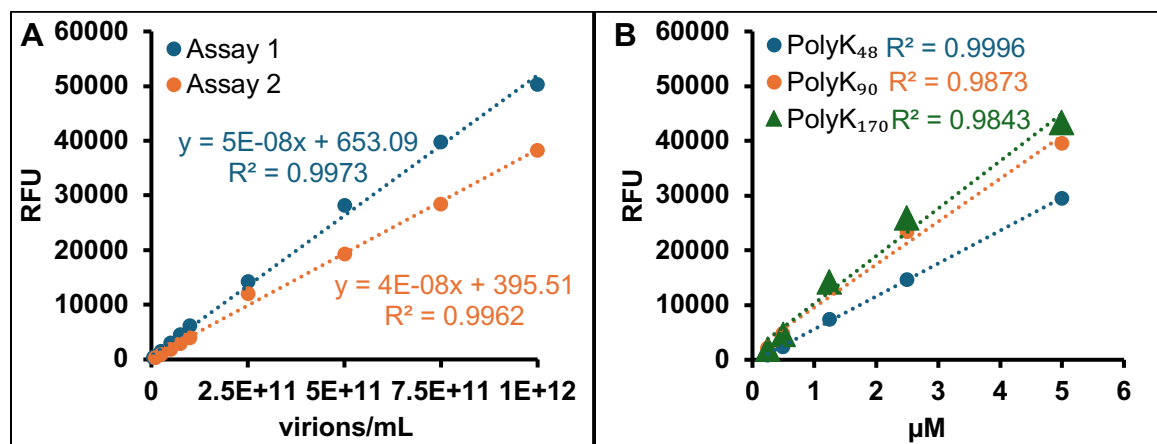


Figure S4.3. OPA standard curves. A) Protected M13^{αPNAG} phage was reacted with OPA to generate standard curves for quantifying the virions present in PKAP phages. PKAP_{48C}, PKAP_{48E}, and PKAP_{90C} were quantified on one plate (assay 1), and PKAP_{90E}, PKAP_{170C}, and PKAP_{170E} were quantified on another plate (assay 2). B) PolyK₄₈, 90, and 170 all produce linear signal when reacted with OPA between 0.125 and 5 μM .

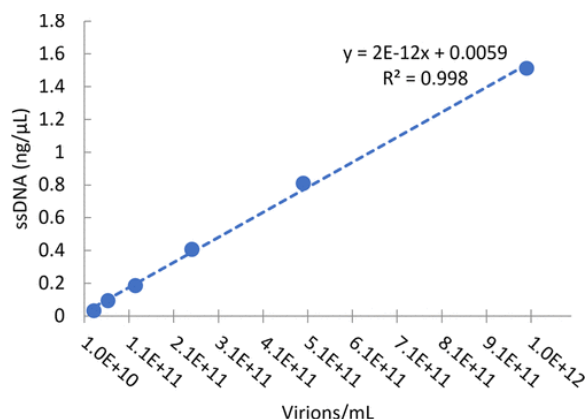
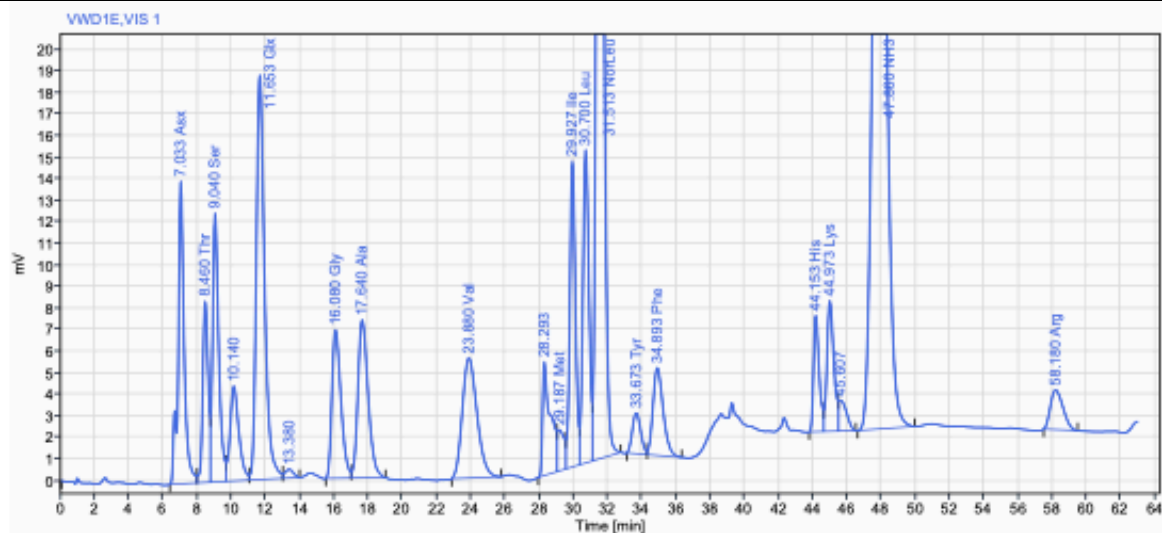


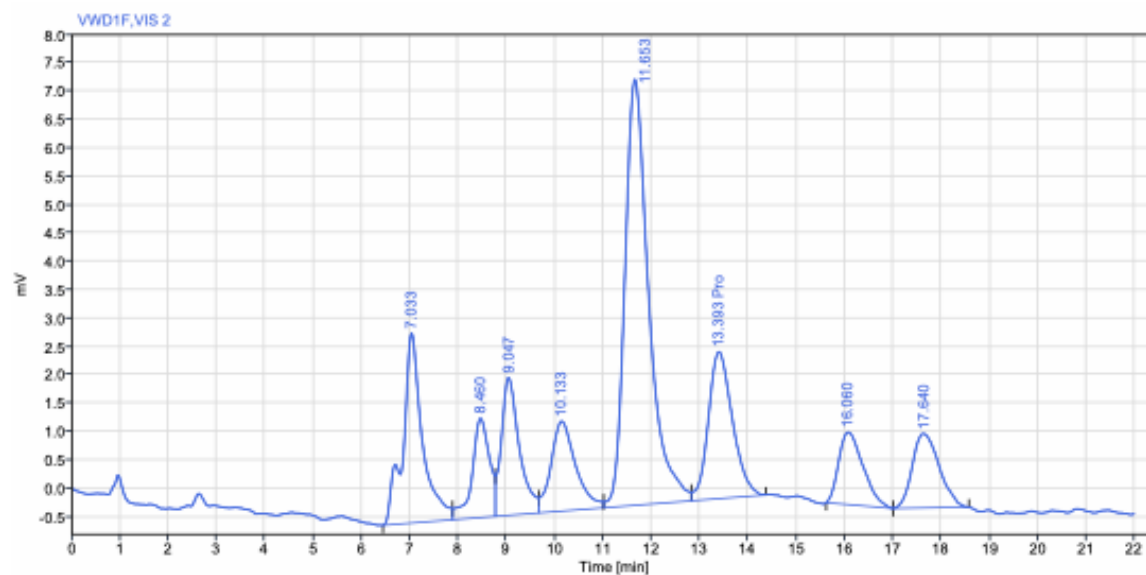
Figure S4.4. Standard curve for virion quantification by Qubit Fluorometer. Phage was prepared at known concentrations of 10^{12} , 5×10^{11} , 2.5×10^{11} , 1.25×10^{11} , 6.25×10^{10} , and 3.13×10^{10} virions/mL. ssDNA concentrations (ng/ μL) were determined by a Qubit assay. The resulting linear trendline has the equation $y = 2 \times 10^{-12}x + 0.0059$, where x is phage concentration and y is ssDNA concentration.

A



Signal: VWD1E,VIS 1

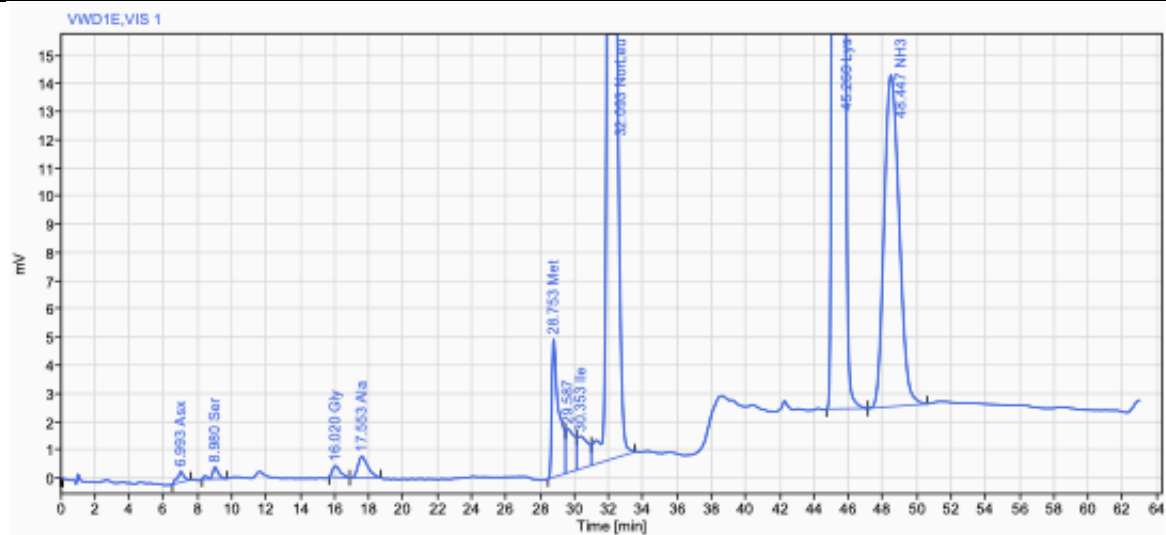
Name	RT [min]	Area	Concentration [nmol]
Asx	7.033	346.122	0.521
Thr	8.460	185.447	0.290
Ser	9.040	308.592	0.389
Glx	11.653	631.081	0.908
Gly	16.080	250.898	0.362
Ala	17.640	303.574	0.415
Val	23.880	332.144	0.465
Met	29.187	52.115	0.070
Ile	29.927	325.053	0.441
Leu	30.700	354.067	0.476
NorLeu	31.513	1495.286	1.955
Tyr	33.673	63.866	0.094
Phe	34.893	168.385	0.239
His	44.153	122.932	0.171
Lys	44.973	170.754	0.215
NH3	47.860	2070.972	3.069
Arg	58.180	93.665	0.137



Signal: VWD1F,VIS 2

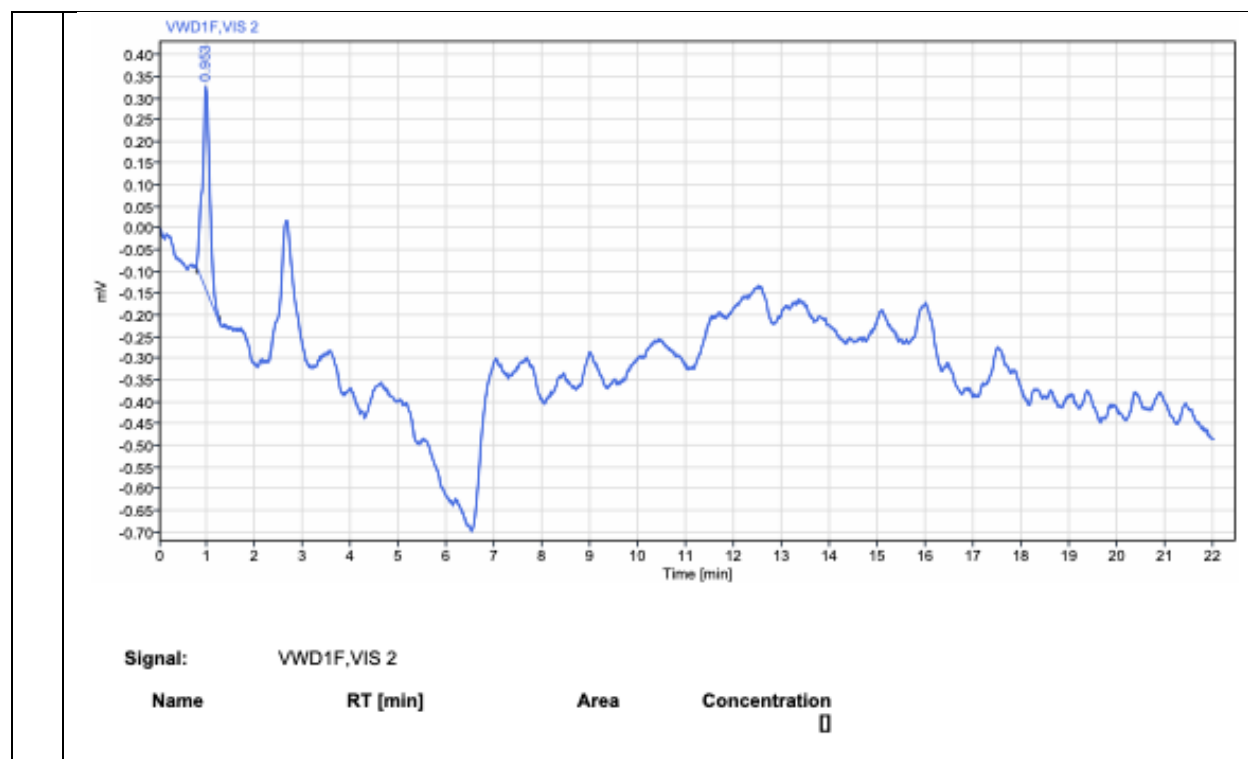
Name	RT [min]	Area	Concentration [nmol]
Pro	13.393	89.977	0.561

B

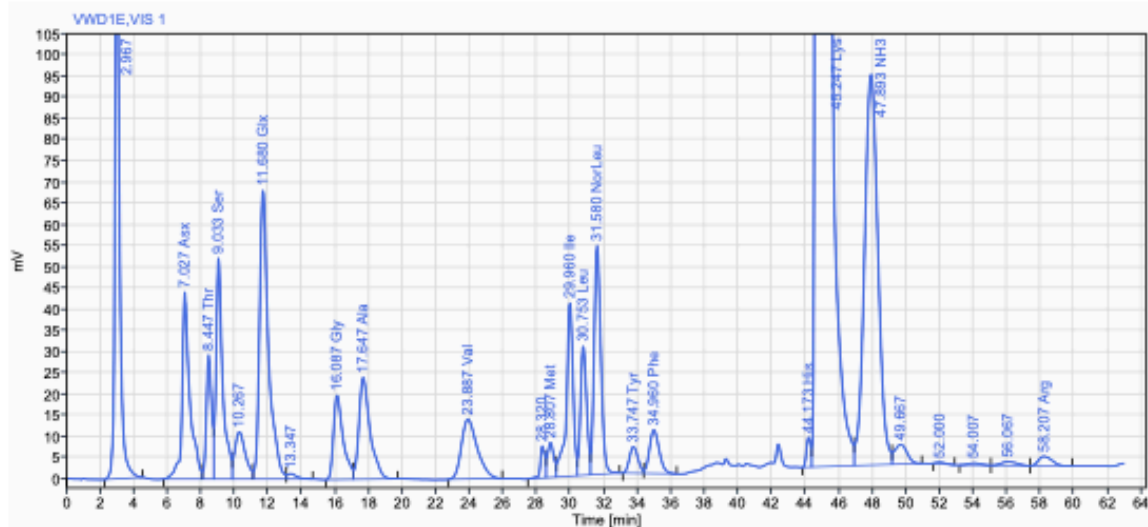


Signal: VWD1E,VIS 1

Name	RT [min]	Area	Concentration [nmol]
Asx	6.993	9.897	0.015
Ser	8.980	10.942	0.015
Gly	16.020	12.718	0.018
Ala	17.553	30.150	0.042
Met	28.753	140.037	3.099
Ile	30.353	51.060	0.070
NorLeu	32.093	1523.988	2.089
Lys	45.260	4555.501	5.773
NH3	48.447	721.214	1.093

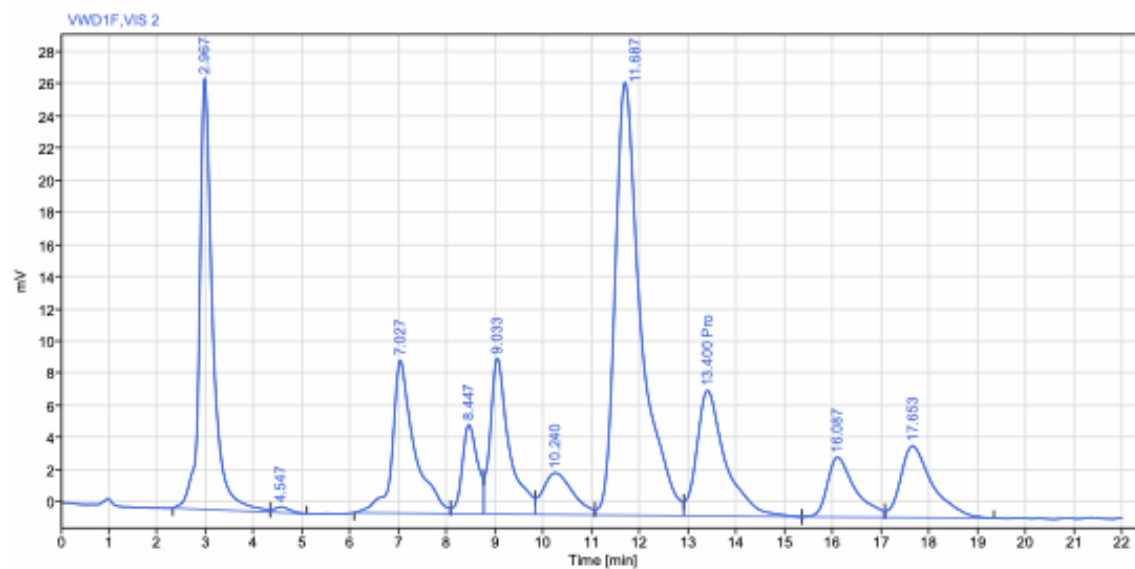


C



Signal: VWD1E,VIS 1

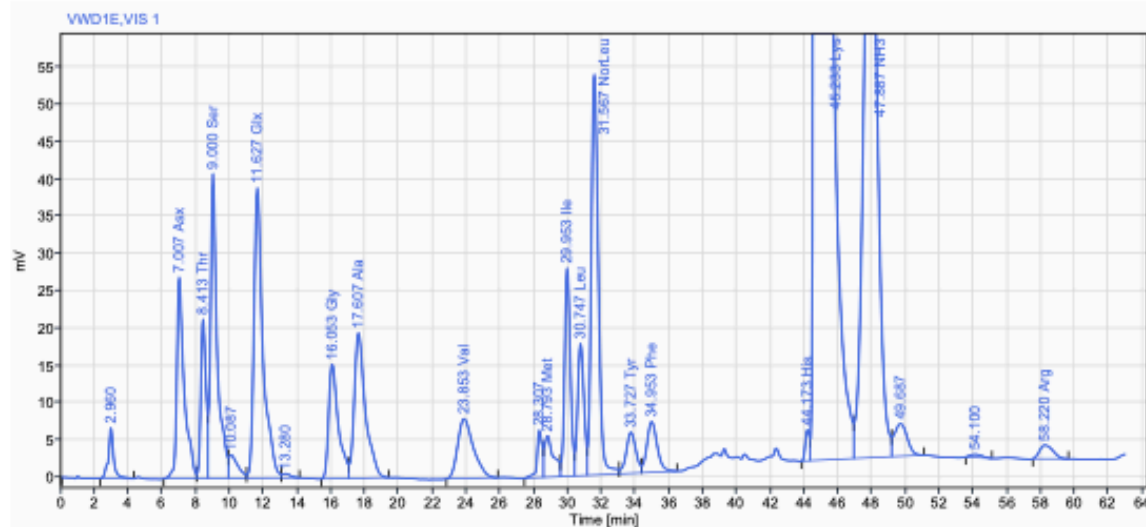
Name	RT [min]	Area	Concentration [nmol]
Asx	7.027	1416.248	2.130
Thr	8.447	641.534	1.004
Ser	9.033	1429.409	1.803
Glx	11.680	2544.594	3.663
Gly	16.087	801.176	1.154
Ala	17.647	1108.575	1.514
Val	23.887	924.554	1.294
Met	28.807	215.913	0.288
Ile	29.960	1144.490	1.552
Leu	30.753	777.428	1.045
NorLeu	31.580	1508.517	1.973
Tyr	33.747	220.580	0.323
Phe	34.960	415.313	0.588
His	44.173	126.312	0.176
Lys	45.247	59666.864	75.266
NH3	47.893	5156.453	7.640
Arg	58.207	124.248	0.182



Signal: VWD1F,VIS 2

Name	RT [min]	Area	Concentration [nmol]
Pro	13.400	321.350	2.005

D



Signal: VWD1E,VIS 1

Name	RT [min]	Area	Concentration [nmol]
Asx	7.007	819.117	1.232
Thr	8.413	449.099	0.703
Ser	9.000	1127.350	1.422
Glx	11.627	1450.909	2.089
Gly	16.053	615.163	0.886
Ala	17.607	895.625	1.223
Val	23.853	518.788	0.726
Met	28.793	209.406	0.279
Ile	29.953	663.508	0.900
Leu	30.747	462.014	0.621
NorLeu	31.567	1556.604	2.036
Tyr	33.727	219.213	0.321
Phe	34.953	290.133	0.411
His	44.173	72.152	0.100
Lys	45.233	59245.983	74.736
NH3	47.887	4838.750	7.170
Arg	58.220	99.985	0.146

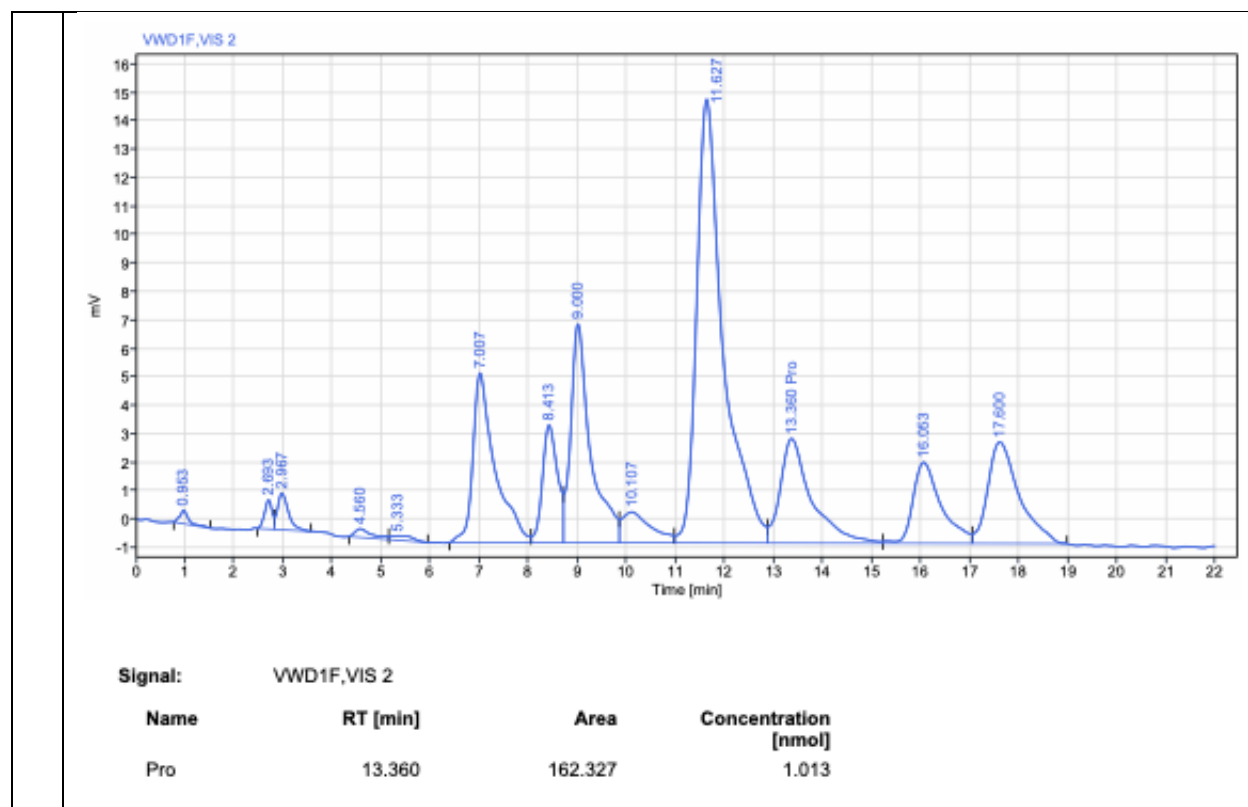


Figure S4.5. Chromatograms of amino acid analysis for A) M13^{αPNAG}, B) polyK₉₀, C) PKAP_{90C}, and D) PKAP_{90E}.

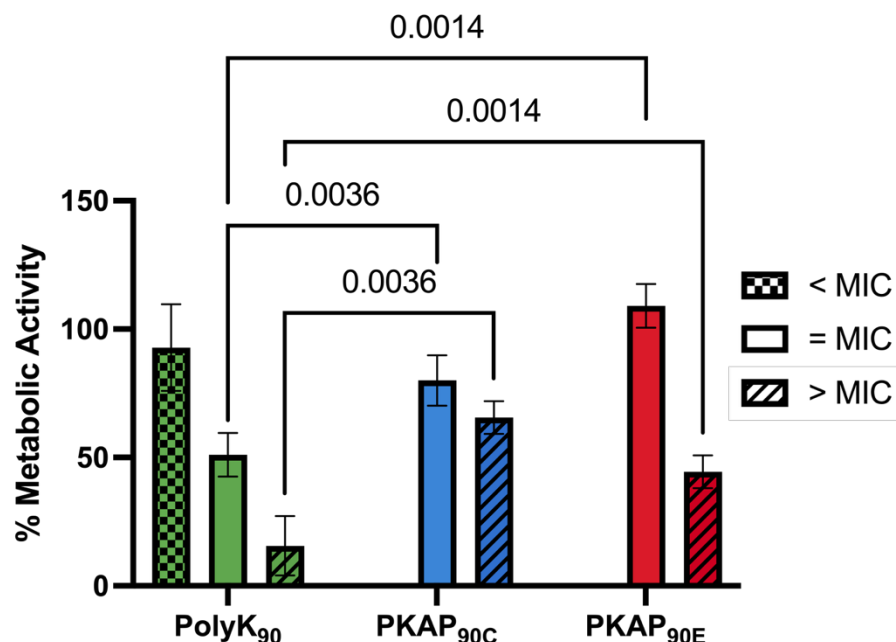


Figure S4.6. HEK293 cell metabolic activity at additional polyK and PKAP₉₀ concentrations. HEK 293 cells were treated for twenty-four hours with polyK90 at 0.5 μ M, 2.5 μ M (MIC), or 5 μ M, PKAP90C at 0.53 μ M (MIC) or 3.83 ± 0.55 ($7.7\times$ MIC), PKAP90E at 0.54 μ M (MIC) or 6.31 ± 1.77 μ M ($12.5\times$ MIC). Metabolic activity was measured with PrestoBlue and expressed as a percentage of untreated cells, and significance was assessed by a two-way ANOVA and Tukey's test. Cells treated with PKAP90C at $7.7\times$ MIC or PKAP90E at $12.5\times$ MIC presented $66\% \pm 6\%$ and 44% metabolic activity of the control; there was no significant difference between these two treatments or cells treated with 2.5 μ M polyK.

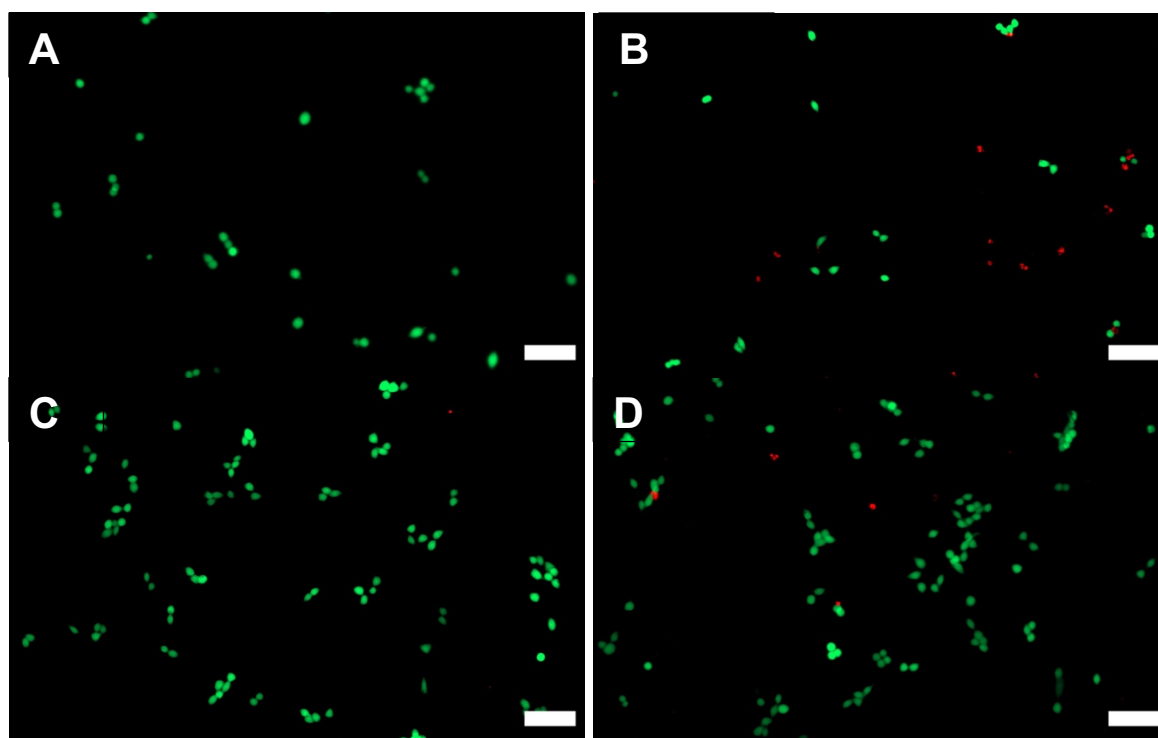


Figure S4.7. Fluorescence microscopy visualizes mammalian cell viability after twenty-four hours of treatment. After twenty-four hours of treatment with A) no supplements, B) polyK₉₀, C) PKAP_{90C}, or D) PKAP_{90E} at the *S. aureus* MIC, HEK 293 cells were stained with calcein AM and ethidium homodimer-1 to determine viability. Scale bar = 100 μ m.

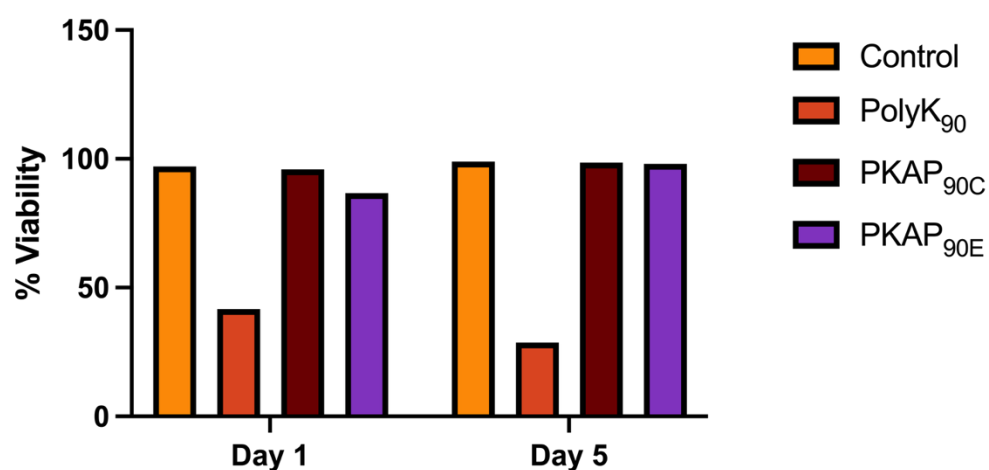


Figure S4.8. Mammalian cell percent viability on days 1 and 5. Percent viability was calculated as number of live cells (stained with calcein AM) over total number of cells (stained with either calcein AM or ethidium homodimer-1) from five images.

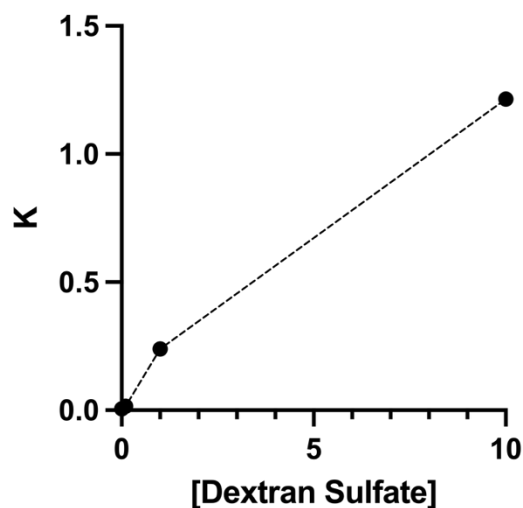


Figure S4.9. Dextran sulfate concentration (mg/mL) vs rate constant, k (h^{-1}), of polyK₉₀ release from PKAP_{90E} (Table S9).

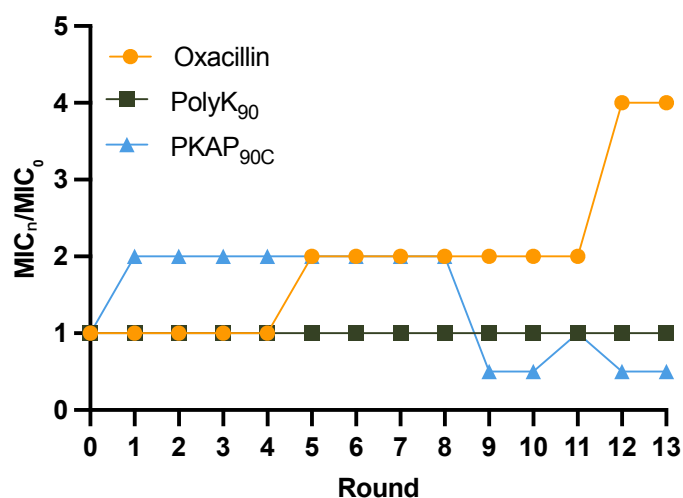


Figure S4.10. MIC of control group after serial passaging. A control culture was serially passaged with no treatment (i.e., sub-cultured following the same protocol as the treatment groups, but without any treatment). At each round, the MIC of this control culture was measured for oxacillin, polyK₉₀, and PKAP_{90C}. The MIC measured at each round was normalized by the corresponding initial MIC and plotted over rounds. Only increased resistance to oxacillin was observed.

4.5 Supplementary tables

Phage Conc. (virions/mL)	A595 - Blank
10^{13}	0.04615
	0.05115
	0.03885
10^{12}	-0.00045
	0.00165
	-0.00725
5×10^{11}	0.01065
	-0.01115
	-0.00815
2.5×10^{11}	0.00665
	-0.00135
	0.00815

Table S4.1. Reaction of phage with Bradford reagent. Protected phage at varying concentrations (column 1) was incubated with Bradford reagent. The absorbance at 595 nm was measured and the absorbance value of a blank consisting of PBS was subtracted (column 2), producing values too low to be quantified with a standard curve for polyK₉₀ for concentrations below 10^{12} virions/mL.

PKAP	Dilution Factor	A595	A595 - Blank	PolyK Conc. of Dilution (nM)	PolyK Conc. Of Stock (μM)	Avg. PolyK Conc. Of Stock (μM)
48C	0.00625	0.44260001	0.07030001	737.0	117.9	113.3 ± 10.3
		0.44119999	0.06889999	724.0	115.7	
		0.43509999	0.06279999	662.0	105.9	
	0.003125	0.40349999	0.03119999	346.0	110.7	
		0.40939999	0.03709999	405.0	129.6	
		0.40020001	0.02790001	313.0	100.2	
48E	0.003125	0.426	0.0537	571.0	182.7	193.5 ± 24.8
		0.42129999	0.04899999	524.0	167.7	
		0.42539999	0.05309999	565.0	180.8	
	0.001563	0.40259999	0.03029999	337.0	215.7	
		0.3973	0.02500001	284.0	181.8	
		0.4052	0.03290001	363.0	232.3	
90C	0.005	0.56629997	0.22024997	709.2	141.8	136.0 ± 19.6
		0.57050002	0.22445001	723.2	144.6	
		0.5679	0.22184999	714.5	142.9	
	0.0025	0.46849999	0.12244998	383.2	153.3	
		0.47139999	0.12534998	392.8	157.1	
		0.47330001	0.12725	399.2	159.7	
	0.00125	0.40349999	0.05744998	166.5	133.2	
		0.40279999	0.05674998	164.2	131.3	
		0.39829999	0.05224998	149.2	119.3	
	0.000625	0.37529999	0.02924998	72.5	116.0	
		0.38010001	0.03405	88.5	141.6	
		0.37059999	0.02454998	56.8	90.9	
90E	0.00125	0.50120002	0.15515001	492.2	393.7	362.4 ± 101.6
		0.48879999	0.14274998	450.8	360.7	
		0.49700001	0.15095	478.2	382.5	
	0.000625	0.4314	0.08534999	259.5	415.2	
		0.42539999	0.07934998	239.5	383.2	
		0.42969999	0.08364998	253.8	406.1	
	0.0003125	0.39480001	0.04875	137.5	440.0	
		0.38789999	0.04184999	114.5	366.4	
		0.396	0.04994999	141.5	452.8	
	0.00015625	0.36759999	0.02154998	46.8	299.7	
		0.35659999	0.01054998	10.2	65.1	
		0.37149999	0.02544998	59.8	382.9	
170C	0.0015625	0.51160002	0.13295002	284.7	182.2	169.6 ± 19.3
		0.51050001	0.13185002	282.5	180.8	
		0.50569999	0.12705	272.9	174.7	
		0.4219	0.04325001	105.3	134.8	

	0.000781 25	0.43810001	0.05945002	137.7	176.3	
		0.43489999	0.05624999	131.3	168.1	
	0.000390 63	0.39719999	0.01854999	55.9	143.1	
		0.40759999	0.02894999	76.7	196.4	
		0.40239999	0.02374999	66.3	169.7	
170E	0.001562 5	0.5043	0.12565	270.1	172.9	175.1 ± 19.9
		0.51670003	0.13805003	294.9	188.7	
		0.51190001	0.13325001	285.3	182.6	
	0.000781 25	0.4339	0.05525	129.3	165.5	
		0.44299999	0.06434999	147.5	188.8	
		0.42559999	0.04695	112.7	144.3	
	0.000390 63	0.3976	0.01895	56.7	145.2	
		0.4084	0.02975	78.3	200.4	
		0.40580001	0.02715002	73.1	187.1	

Table S4.2. PolyK concentration on PKAP phages measured by Bradford reagent. For each PKAP phage construct (column 1), a series of dilutions were prepared (column 2); the absorbance at 595 nm was measured after incubating with Bradford reagent (column 3) and the absorbance value of a blank (PBS incubated with Bradford) was subtracted (column 4). Standard curves of polyK of the same DP were used to determine the polyK concentration of the blank-corrected values (column 5), then divided by the dilution factor to determine the stock polyK concentration (column 6).

PKAP	RFU PKAP	Avg. RFU PKAP - Blank	RFU PolyK	Avg. RFU PolyK - Blank	Avg. RFU Phage	Virions/mL
48C	23581	23296 ± 165	11457	10813 ± 269	12484 ± 316	2.37E+11 ± 6.31E+09
	23514		11077			
	23827		10937			
48E	9745	9523 ± 113	6091	5760 ± 43	3763 ± 121	6.22E+10 ± 2.42E+09
	9891		6070			
	9967		6153			
90C	26471	26070 ± 439	16509	15618 ± 427	10451 ± 612	2.51E+11 ± 1.53E+10
	27074		16232			
	26221		15671			
90E	14238	13604 ± 321	8703	8103 ± 485	5501 ± 582	9.70E+10 ± 1.16E+10
	14003		7888			
	13604		8752			
170C	17005	16720 ± 671	9116	8437 ± 271	8283 ± 724	1.97E+11 ± 1.81E+10
	16717		9110			
	17996		8643			
170E	18795	18512 ± 659	14164	13259 ± 503	5253 ± 828	1.42E+11 ± 2.07E+10
	19775		13961			
	18523		13210			

Table S4.3. Reaction of PKAP phages with OPA reagent. PKAP phages (column 1) were reacted with OPA reagent and fluorescence (excitation at 340, emission at 455 nm) was measured (column 2). Triplicates were averaged and the fluorescence of a blank (PBS incubated with OPA reagent) was subtracted (column 3). The fluorescence of an aliquot of polyK at the same concentration present on the PKAP phage (as previously determined by the Bradford assay) after reacting with OPA was measured (column 4); triplicates were averaged and the fluorescence of a blank consisting of PBS was subtracted (column 5). The RFU of the polyK was subtracted from the RFU of the PKAP phage (column 6) and the resulting value was fit to the standard curve of blocked phage (Figure S10) to determine the phage virion concentration in virions/mL (column 7). Attempts to quantify PKAP phage DNA by extraction or isolation failed due to essentially irreversible binding of the polyK to phage DNA upon its release.

	M13^αPNAG	PolyK₉₀	PKAP_{90C}	PKAP_{90E}
Initial Volume (μL)	109	130	65	140
Final Volume (μL)	2000	1000	2000	2000
Amino Acid	Nanomoles/50 μL			
Asx	0.533	0	2.159	1.21
Thr	0.297	0	1.018	0.691
Ser	0.398	0	1.828	1.397
Glx	0.929	0	3.713	2.052
Pro	0.574	0	2.032	0.995
Gly	0.37	0	1.17	0.87
Ala	0.425	0	1.535	1.201
Val	0.476	0	1.312	0.713
Ile	0.451	0	1.573	0.884
Leu	0.487	0	1.059	0.61
Tyr	0.096	0	0.327	0.315
Phe	0.245	0	0.596	0.404
His	0.175	0	0.178	0.098
Lys	0.22	5.527	76.296	73.415
Arg	0.14	0	0.184	0.143
Asx	0.533	0	2.159	1.21
Total	5.81	5.53	94.98	85.00

Table S4.4. Phage amino acid analysis. The initial volume, final volume, and nanomoles of each amino acid per 50 μL injection volume for all samples subjected to amino acid analysis. Phage and polyK concentrations were determined as follows: The concentration of each amino acid was divided by 50 μL, multiplied by the final volume, and then divided by the initial volume to determine the original sample concentration. The total concentration of phage amino acids (2.13 nanomoles/μL) was divided by the original phage concentration (10^{13} virions/mL) to find 2.13×10^{-10} nanomoles of amino acids detected per virion. The ratio of lysine to all other amino acids was observed to be 0.04 for M13^αPNAG. The amount of all non-lysine amino acids in PKAP_{90C} and PKAP_{90E} (11.50 and 3.31 nanomoles/μL, respectively) were multiplied by 0.04 to calculate the amount of lysine contributed by phage virions, rather than polyK. The total amino acid amount contributed by phage virions for PKAP_{90C} and PKAP_{90E} (11.95 and 3.44 nanomoles/μL respectively) were divided by 2.13×10^{-10} nanomoles/virion and multiplied by 1000 to determine the concentration in virions/mL, finding PKAP_{90C} at 5.60×10^{13} virions/mL and PKAP_{90E} at 1.61×10^{13} virions/mL. For each PKAP, the concentration of non-phage lysine in nanomoles/μL was converted to μM of polyK based on average degree of polymerization, then divided by the phage concentration (virions/mL) to determine molecules of polyK per virion.

Species	DP	PolyK (μM)	PKAP-C ($\mu\text{M polyK}$)	PKAP-E ($\mu\text{M polyK}$)
<i>E. coli</i> (ATCC 25922)	48	5	13	12
	90	2.5	2.1	2.2
	170	0.63	1.2	1.2
<i>P. aeruginosa</i> (ATCC 25102)	48	5	> 13	12
	90	5	8.5	8.7
	170	2.5	2.4	4.9

Table S4.5. Minimum inhibitory concentration (MIC) of polyK and PKAP phages for *E. coli* (ATCC 25922) and *P. aeruginosa* (ATCC 25102) determined by broth microdilution.

Species	Strain	DP	PolyK (μM)	PKAP-C ($\mu\text{M polyK}$)	PKAP-E ($\mu\text{M polyK}$)
<i>S. aureus</i>	Newman (ATCC 25904)	48	5	0.83	0.76
		90	2.5	0.53	0.54
		170	1.25	0.59	0.61
	ATCC 29213	90	2.5	0.53	0.54
	ATCC 43300		2.5	0.53	0.54
	Clinical isolate A, methicillin resistant		2.5	0.53	0.54
	Clinical isolate B, methicillin resistant		2.5	0.53	0.54
	Clinical isolate C, methicillin sensitive		2.5	0.53	0.54
<i>S. epidermidis</i>	Clinical isolate A, oxacillin resistant		0.63	0.26	0.27
	Clinical isolate A, oxacillin sensitive		0.63	0.26	0.27
<i>E. coli</i>	ATCC 25922	48	5	13	12
		90	2.5	2.1	2.2
		170	0.625	1.2	1.2
<i>P. aeruginosa</i>	ATCC 25102	48	5	> 13	48
		90	5	> 8.5	17
		170	2.5	4.7	9.7

Table S4.6. Minimum bactericidal concentration (MBC) of polyK and PKAP phages. After determining the MIC by a broth microdilution method, 50 μL aliquots were plated on Mueller Hinton agar and grown overnight at 37 °C. Concentrations growing only 0.1% or less than the number of colonies as the original inoculum were considered to be the MBC.

Construct	k (min ⁻¹)	R ²
PKAP _{90C}	0.12 (0.114 to 0.128)	0.9998
PolyK ₉₀	0.034 (0.021 to 0.054)	0.9653
PKAP _{90E}	0.028 (0.022 to 0.035)	0.9894

Table S4.7. Time-kill curves of polyK₉₀, PKAP_{90C}, and PKAP_{90E} were fit to an exponential death model, $[Y(t) = (Y_0) \cdot e^{-k \cdot t}]$, where Y_0 is the initial number of colony forming units, k is the death rate constant, and t is time. Data from zero to 60 minutes were fit. 95% confidence intervals are listed in parentheses.

Dextran Sulfate (mg/mL)	k (h ⁻¹)	Half-life (h)	R ²
10	1.21 (1.06 to 1.40)	0.571 (0.495 to 0.653)	0.957
1	0.239 (0.196 to 0.290)	2.90 (2.39 to 3.53)	0.870
0.1	0.0151 (0.0141 to 0.0161)	45.8 (43.0 to 49.0)	0.960
0	0.00528 (0.00417 to 0.00644)	131 (108 to 166)	0.556

Table S4.8. Release curves of polyK₉₀ from PKAP_{90E} with varying concentrations of dextran sulfate were fit to a pseudo-first order exponential decay model, $[Y(\%) = (100\%) \cdot e^{-k \cdot t}]$, where Y is percent retained, k is the apparent rate constant, and t is time. 95% confidence intervals are listed in parentheses.

	Oxacillin		PolyK₉₀		PKAP_{90C}		Vancomycin
	Final MIC (µg/mL)	Fold-Change	Final MIC (µM)	Fold-Change	Final MIC (µM)	Fold-Change (µg/mL)	Final MIC (µg/mL)
No Selection	2	4	2.5	1	0.26	0.5	1
Oxacillin Selection	16	32	2.5	1	≤ 0.13	≤ 0.25	1
PolyK₉₀ Selection	0.5	1	2.5	1	0.52	1	1
PKAP_{90C} Selection	0.5	1	2.5	1	0.52	1	1

Table S4.9. Cross-evaluation of MIC after thirteen rounds of selection. After thirteen rounds of selection, the MICs for the evolved cultures under each of the three selection pressures, in addition to a control that had been subcultured thirteen times with no selection pressure, were evaluated against all of the treatments, in addition to vancomycin. Fold-change is given in comparison to the MIC of the initial culture. Only MICs to oxacillin were altered by serial passaging under any conditions.

Mutant	Type	Gene	Product	Effect
Oxacillin	del	vraF	ABC transporter ATP-binding protein VraF	frameshift_variant c.210delA p.Lys70fs
	ins	relA	GTP pyrophosphokinase	frameshift_variant c.1199dupC p.Ala401fs
	snp	cinA	Putative competence-damage inducible protein	missense_variant c.736G>C p.Glu246Gln
	snp	agrA	quorum-sensing response regulator AgrA	missense_variant c.330A>T p.Lys110Asn
	snp	rfe	Glycosyl transferase family protein	missense_variant c.750C>A p.Phe250Leu
	snp	vraS	Sensor protein VraS	missense_variant c.865C>A p.Gln289Lys
PolyK₉₀	snp	graR	response regulator transcription factor GraR/ApsR	stop_gained c.185G>A p.Trp62*
	snp	hsdR	type I restriction endonuclease subunit R	missense_variant c.1378G>A p.Glu460Lys
PKAP_{90C}	snp	bglG	Activator of the mannose operon (Transcriptional antiterminator), BglG family	missense_variant c.1864T>A p.Leu622Ile
	snp	mprF	bifunctional lysylphosphatidylglycerol flippase/synthetase MprF	missense_variant c.1021T>A p.Leu341Ile
	snp	cps2a	polyisoprenyl-teichoic acid--peptidoglycan teichoic acid transferase	missense_variant c.604G>C p.Ala202Pro
	del	agrC	quorum-sensing sensor histidine kinase AgrC	frameshift_variant c.1116delA p.Pro373fs
	snp		major facilitator superfamily protein superfamily	missense_variant c.266G>A p.Gly89Asp
No selection	snp	metE	5-methyltetrahydropteroyltryglutamate- - homocysteine S-methyltransferase	synonymous_variant c.681T>A p.Ala227Ala
	snp	qoxA	cytochrome aa3 quinol oxidase subunit II	missense_variant c.799G>A p.Ala267Thr

	snp		E domain-containing protein	missense_variant c.2965A>T p.Ile989Phe
	snp		excalibur calcium-binding domain-containing protein	missense_variant c.352G>A p.Asp118Asn
	snp	harA	haptoglobin-binding heme uptake protein HarA	missense_variant c.2435C>A p.Thr812Lys
	snp		hemolysin family protein	synonymous_variant c.309G>A p.Thr103Thr
	snp	uppS	isoprenyl transferase	synonymous_variant c.501C>T p.Asp167Asp
	snp		ribulokinase	synonymous_variant c.156T>C p.Tyr52Tyr
	snp	fmtA	teichoic acid D-Ala esterase FmtA	missense_variant c.643A>T p.Asn215Tyr

Table S4.10. Mutations identified by snippy (v4.6.0) arising from thirteen rounds of selection pressure or unselected subculturing compared to the original *S. aureus* strain, ATCC 29213. Del = deletion, ins = insertion, snp = single nucleotide polymorphism.

Chapter 5. Conclusions

Antimicrobial resistance (AMR) poses a dire threat to public health, with resulting deaths expected to surpass those caused by cancer by 2050.¹¹ Additionally, the 2020 COVID-19 pandemic led to hospital overcrowding and a diminished focus on antibiotic stewardship, increasing the spread of resistant healthcare-associated infections (HAIs) like MRSA.^{217,218} To combat the rise of AMR, we urgently need to develop treatments with a lower potential for inducing resistance. Two promising therapies are antimicrobial polymers and bacteriophages, but they face opposite drawbacks as systemic agents; a lack of polymer specificity leads to cytotoxicity, while the overly specific host range of phages hinders timely and widespread administration. In this dissertation, we hypothesized that a synergistic agent could overcome these drawbacks, and engineered the well-studied M13 bacteriophage into an antimicrobial polymer delivery platform.

In Chapter 2, the receptor binding protein of M13 was modified to express an antibody fragment specific to poly-*N*-acetylglucosamine (PNAG), a common *S. aureus* surface polysaccharide. The apparent dissociation constant of the anti-PNAG phage, designated M13^{aPNAG}, to *S. aureus* biofilm polysaccharides was comparable to the monoclonal antibody.⁹⁵ Additionally, that phage exhibited strong binding to whole *S. aureus* cells and no binding to mammalian cells, highlighting its utility as a targeted delivery vehicle.

In Chapter 3, we assessed the antimicrobial activity of three separate libraries of bio-inspired polymers with either methacrylate, hydroxybenzoate-co-lactide, or peptide backbones. Each library exhibited different trends with respect to degrees of polymerization, hydrophobicity, and charge, emphasizing the diversity of antimicrobial polymers. This highlights the challenge of rationally designing new antimicrobial polymers, though advances in machine learning may

facilitate de novo creation while minimizing experimental burden.^{219,220} Considering factors such as degradability, solubility, and yield, α -poly(L-lysine), referred to as “polyK” was ultimately selected for phage conjugation.

In Chapter 4, polyK with degrees of polymerization equal to 48, 90, or 170 was loaded onto M13^{aPNAG} phage by covalent crosslinking or electrostatic attractions, creating polyK-anti-PNAG (PKAP) phages. The polymer-phage hybrids exhibited improved antimicrobial activity, reduced cytotoxicity, and no emergence of resistance in *S. aureus* during serial passaging. Phage-based delivery is thus an effective strategy for increasing the specificity of antimicrobial polymers while retaining their intrinsic resistance-proof nature.

While successful in reducing in vitro cytotoxicity, it remains to be seen if PKAP phages can treat wound or systemic infections in a mouse model. Previous works demonstrate the efficacy of poly(L-lysine) and poly(L-leucine) block copolymer hydrogels in treating infected wounds,¹⁸⁸ but treatment of bacteremia is less explored due to documented systemic toxicity.²²¹ Future studies are necessary to determine if phage delivery is sufficient to overcome barriers to systemic administration.

Building on the findings of this dissertation, M13-based polymer delivery represents a generalizable platform that can be expanded to treating other infections. Further modifications to the receptor binding protein could enable targeting of gram-negative organisms, as polyK exhibited broad spectrum antimicrobial activity. The efficiency in loading other polymers remains unexplored. As the hydrophilic and cationic nature of polyK facilitated both covalent and electrostatic attachment to M13, it can be anticipated more hydrophobic or neutral polymers would see lower drug-to-phage ratios. However, conjugating different polymers could yield greater potential for clinical applications, if they possess an initially higher selectivity index than

polyK, or enable targeting of species with limited susceptibility to polyK, such as fungi.

Polymer-phage delivery could be adapted for cancer treatment; there is substantial effort to document cancer antigens as targets for antibody drug conjugates,²²² and many cancer cells exhibit a stronger negative surface charge than healthy cells, making them more susceptible to cationic polymers.²²³ Looking forward, we are optimistic that future researchers will build on the findings of this work in the fight to combat antimicrobial resistance.

References

- (1) Centers for Disease Control and Prevention. Achievements in Public Health, 1900-1999: Changes in the Public Health System. *Morbidity and Mortality Weekly Report* **1999**, 48 (29).
- (2) Fleming, A. On the Antibacterial Action of Cultures of a *Penicillium*, with Special Reference to Their Use in the Isolation of *B. Influenzæ*. *Br J Exp Pathol* **1929**, 10 (3), 226–236.
- (3) Chain, E.; Florey, H. W.; Gardner, A. D.; Heatley, N. G.; Jennings, M. A.; Orr-Ewing, J.; Sanders, A. G. PENICILLIN AS A CHEMOTHERAPEUTIC AGENT. *The Lancet* **1940**, 236 (6104), 226–228. <https://doi.org/10.5555/uri:pii:S0140673601087281>.
- (4) Hutchings, M. I.; Truman, A. W.; Wilkinson, B. Antibiotics: Past, Present and Future. *Current Opinion in Microbiology* **2019**, 51, 72–80. <https://doi.org/10.1016/j.mib.2019.10.008>.
- (5) *Nobel Prize in Physiology or Medicine 1945*. NobelPrize.org. <https://www.nobelprize.org/prizes/medicine/1945/summary/> (accessed 2026-04-26).
- (6) Neushul, P. Science, Government and the Mass Production of Penicillin. *J Hist Med Allied Sci* **1993**, 48 (4), 371–395. <https://doi.org/10.1093/jhmas/48.4.371>.
- (7) Davies, J. Where Have All the Antibiotics Gone? *Can J Infect Dis Med Microbiol* **2006**, 17 (5), 287–290. <https://doi.org/10.1155/2006/707296>.
- (8) Rammelkamp, C. H.; Maxon, T. Resistance of *Staphylococcus Aureus* to the Action of Penicillin. *Experimental Biology and Medicine* **1942**, 51 (3), 386–389. <https://doi.org/10.3181/00379727-51-13986>.
- (9) World Health Organization. *Global Antibiotic Resistance Surveillance Report 2025: WHO Global Antimicrobial Resistance and Use Surveillance System (GLASS) – Summary*; World Health Organization, 2025. <https://doi.org/10.2471/B09585>.
- (10) Naghavi, M.; Vollset, S. E.; Ikuta, K. S.; Swetschinski, L. R.; Gray, A. P.; Wool, E. E.; Robles Aguilar, G.; Mestrovic, T.; Smith, G.; Han, C.; Hsu, R. L.; Chalek, J.; Araki, D. T.; Chung, E.; Raggi, C.; Gershberg Hayoon, A.; Davis Weaver, N.; Lindstedt, P. A.; Smith, A. E.; Altay, U.; Bhattacharjee, N. V.; Giannakis, K.; Fell, F.; McManigal, B.; Ekapirat, N.; Mendes, J. A.; Rungchien, T.; Srimokla, O.; Abdelkader, A.; Abd-Elsalam, S.; Aboagye, R. G.; Abolhassani, H.; Abualruz, H.; Abubakar, U.; Abukhadajah, H. J.; Aburuz, S.; Abu-Zaid, A.; Achalapong, S.; Addo, I. Y.; Adekanmbi, V.; Adeyeoluwa, T. E.; Adnani, Q. E. S.; Adzigbli, L. A.; Afzal, M. S.; Afzal, S.; Agodi, A.; Ahlstrom, A. J.; Ahmad, A.; Ahmad, S.; Ahmad, T.; Ahmadi, A.; Ahmed, A.; Ahmed, H.; Ahmed, I.; Ahmed, M.; Ahmed, S.; Ahmed, S. A.; Akkaif, M. A.; Al Awaidey, S.; Al Thaher, Y.; Alalalmeh, S. O.; AlBataineh, M. T.; Aldhaleei, W. A.; Al-Gheethi, A. A. S.; Alhaji, N. B.; Ali, A.; Ali, L.; Ali, S. S.; Ali, W.; Allel, K.; Al-Marwani, S.; Alrawashdeh, A.; Altaf, A.; Al-Tammemi, A. B.; Al-Tawfiq, J. A.; Alzoubi, K. H.; Al-Zyoud, W. A.; Amos, B.; Amuasi, J. H.; Ancuceanu, R.; Andrews, J. R.; Anil, A.; Anuoluwa, I. A.; Anvari, S.; Anyasodor, A. E.; Apostol, G. L. C.; Arabloo, J.; Arafat, M.; Aravkin, A. Y.; Areda, D.; Aremu, A.; Artamonov, A. A.; Ashley, E. A.; Asika, M. O.; Athari, S. S.; Atout, M. M. W.; Awoke, T.; Azadnajafabad, S.; Azam, J. M.; Aziz, S.; Azzam, A. Y.; Babaei, M.; Babin, F.-X.; Badar, M.; Baig, A. A.; Bajcetic, M.; Baker, S.; Bardhan, M.; Barqawi, H. J.; Basharat, Z.; Basiru, A.; Bastard, M.; Basu, S.;

Bayleyegn, N. S.; Belete, M. A.; Bello, O. O.; Beloukas, A.; Berkley, J. A.; Bhagavathula, A. S.; Bhaskar, S.; Bhuyan, S. S.; Bielicki, J. A.; Briko, N. I.; Brown, C. S.; Browne, A. J.; Buonsenso, D.; Bustanji, Y.; Carvalheiro, C. G.; Castañeda-Orjuela, C. A.; Cenderadewi, M.; Chadwick, J.; Chakraborty, S.; Chandika, R. M.; Chandy, S.; Chansamouth, V.; Chattu, V. K.; Chaudhary, A. A.; Ching, P. R.; Chopra, H.; Chowdhury, F. R.; Chu, D.-T.; Chutiyami, M.; Cruz-Martins, N.; Da Silva, A. G.; Dadras, O.; Dai, X.; Darcho, S. D.; Das, S.; De La Hoz, F. P.; Dekker, D. M.; Dhama, K.; Diaz, D.; Dickson, B. F. R.; Djorie, S. G.; Dodangeh, M.; Dohare, S.; Dokova, K. G.; Doshi, O. P.; Dowou, R. K.; Dsouza, H. L.; Dunachie, S. J.; Dziedzic, A. M.; Eckmanns, T.; Ed-Dra, A.; Eftekharimehrabad, A.; Ekundayo, T. C.; El Sayed, I.; Elhadi, M.; El-Huneidi, W.; Elias, C.; Ellis, S. J.; Elsheikh, R.; Elsohaby, I.; Eltaha, C.; Eshrati, B.; Eslami, M.; Eyre, D. W.; Fadaka, A. O.; Fagbamigbe, A. F.; Fahim, A.; Fakhri-Demeshghieh, A.; Fasina, F. O.; Fasina, M. M.; Fatehizadeh, A.; Feasey, N. A.; Feizkhah, A.; Fekadu, G.; Fischer, F.; Fitriana, I.; Forrest, K. M.; Fortuna Rodrigues, C.; Fuller, J. E.; Gadanya, M. A.; Gajdacs, M.; Gandhi, A. P.; Garcia-Gallo, E. E.; Garrett, D. O.; Gautam, R. K.; Gebregergis, M. W.; Gebrehiwot, M.; Gebremeskel, T. G.; Geffers, C.; Georgalis, L.; Ghazy, R. M.; Golechha, M.; Golinelli, D.; Gordon, M.; Gulati, S.; Gupta, R. D.; Gupta, S.; Gupta, V. K.; Habteyohannes, A. D.; Haller, S.; Harapan, H.; Harrison, M. L.; Hasaballah, A. I.; Hasan, I.; Hasan, R. S.; Hasani, H.; Haselbeck, A. H.; Hasnain, M. S.; Hassan, I. I.; Hassan, S.; Hassan Zadeh Tabatabaei, M. S.; Hayat, K.; He, J.; Hegazi, O. E.; Heidari, M.; Hezam, K.; Holla, R.; Holm, M.; Hopkins, H.; Hossain, M. M.; Hosseinzadeh, M.; Hostiuc, S.; Hussein, N. R.; Huy, L. D.; Ibáñez-Prada, E. D.; Ikiroma, A.; Ilic, I. M.; Islam, S. M. S.; Ismail, F.; Ismail, N. E.; Iwu, C. D.; Iwu-Jaja, C. J.; Jafarzadeh, A.; Jaiteh, F.; Jalilzadeh Yengejeh, R.; Jamora, R. D. G.; Javidnia, J.; Jawaaid, T.; Jenney, A. W. J.; Jeon, H. J.; Jokar, M.; Jomehzadeh, N.; Joo, T.; Joseph, N.; Kamal, Z.; Kanmodi, K. K.; Kantar, R. S.; Kapisi, J. A.; Karaye, I. M.; Khader, Y. S.; Khajuria, H.; Khalid, N.; Khamesipour, F.; Khan, A.; Khan, M. J.; Khan, M. T.; Khanal, V.; Khidri, F. F.; Khubchandani, J.; Khusuwan, S.; Kim, M. S.; Kisa, A.; Korshunov, V. A.; Krapp, F.; Krumkamp, R.; Kuddus, M.; Kulimbet, M.; Kumar, D.; Kumaran, E. A. P.; Kuttikkattu, A.; Kyu, H. H.; Landires, I.; Lawal, B. K.; Le, T. T. T.; Lederer, I. M.; Lee, M.; Lee, S. W.; Lepape, A.; Lerango, T. L.; Ligade, V. S.; Lim, C.; Lim, S. S.; Limenh, L. W.; Liu, C.; Liu, X.; Liu, X.; Loftus, M. J.; M Amin, H. I.; Maass, K. L.; Maharaj, S. B.; Mahmoud, M. A.; Maikanti-Charalampous, P.; Makram, O. M.; Malhotra, K.; Malik, A. A.; Mandilara, G. D.; Marks, F.; Martinez-Guerra, B. A.; Martorell, M.; Masoumi-Asl, H.; Mathioudakis, A. G.; May, J.; McHugh, T. A.; Meiring, J.; Meles, H. N.; Melese, A.; Melese, E. B.; Minervini, G.; Mohamed, N. S.; Mohammed, S.; Mohan, S.; Mokdad, A. H.; Monasta, L.; Moodi Ghalibaf, A.; Moore, C. E.; Moradi, Y.; Mossialos, E.; Mougin, V.; Mukoro, G. D.; Mulita, F.; Muller-Pebody, B.; Murillo-Zamora, E.; Musa, S.; Musicha, P.; Musila, L. A.; Muthupandian, S.; Nagarajan, A. J.; Naghavi, P.; Nainu, F.; Nair, T. S.; Najmuldeen, H. H. R.; Natto, Z. S.; Nauman, J.; Nayak, B. P.; Nchanji, G. T.; Ndishimye, P.; Negoï, I.; Negoï, R. I.; Nejadghaderi, S. A.; Nguyen, Q. P.; Noman, E. A.; Nwakanma, D. C.; O'Brien, S.; Ochoa, T. J.; Odetokun, I. A.; Ogundijo, O. A.; Ojo-Akosile, T. R.; Okeke, S. R.; Okonji, O. C.; Olagunju, A. T.; Olivas-Martinez, A.; Olorukooba, A. A.; Olwoch, P.; Onyedibe, K. I.; Ortiz-Brizuela, E.; Osuolale, O.; Ounchanum, P.; Oyeyemi, O. T.; P A, M. P.; Paredes, J. L.; Parikh, R. R.; Patel, J.; Patil, S.; Pawar, S.; Peleg, A. Y.; Peprah, P.; Perdigão, J.; Perrone, C.; Petcu, I.-R.; Phommasone, K.; Piracha, Z. Z.; Poddighe, D.; Pollard, A. J.; Poluru, R.; Ponce-De-

- Leon, A.; Puvvula, J.; Qamar, F. N.; Qasim, N. H.; Rafai, C. D.; Raghav, P.; Rahbarnia, L.; Rahim, F.; Rahimi-Movaghar, V.; Rahman, M.; Rahman, M. A.; Ramadan, H.; Ramasamy, S. K.; Ramesh, P. S.; Ramteke, P. W.; Rana, R. K.; Rani, U.; Rashidi, M.-M.; Rathish, D.; Rattanaovong, S.; Rawaf, S.; Redwan, E. M. M.; Reyes, L. F.; Roberts, T.; Robotham, J. V.; Rosenthal, V. D.; Ross, A. G.; Roy, N.; Rudd, K. E.; Sabet, C. J.; Saddik, B. A.; Saeb, M. R.; Saeed, U.; Saeedi Moghaddam, S.; Saengchan, W.; Safaei, M.; Saghazadeh, A.; Saheb Sharif-Askari, N.; Sahebkar, A.; Sahoo, S. S.; Sahu, M.; Saki, M.; Salam, N.; Saleem, Z.; Saleh, M. A.; Samodra, Y. L.; Samy, A. M.; Saravanan, A.; Satpathy, M.; Schumacher, A. E.; Sedighi, M.; Seekaew, S.; Shafie, M.; Shah, P. A.; Shahid, S.; Shahwan, M. J.; Shakoor, S.; Shalev, N.; Shamim, M. A.; Shamshirgaran, M. A.; Shamsi, A.; Sharifan, A.; Shastri, R. P.; Shetty, M.; Shittu, A.; Shrestha, S.; Siddig, E. E.; Sideroglou, T.; Sifuentes-Osornio, J.; Silva, L. M. L. R.; Simões, E. A. F.; Simpson, A. J. H.; Singh, A.; Singh, S.; Sinto, R.; Soliman, S. S. M.; Sorane, S.; Stoesser, N.; Stoeva, T. Z.; Swain, C. K.; Szarpak, L.; T Y, S. S.; Tabatabai, S.; Tabche, C.; Taha, Z. M.-A.; Tan, K.-K.; Tasak, N.; Tat, N. Y.; Thairakong, A.; Thangaraju, P.; Tigoi, C. C.; Tiwari, K.; Tovani-Palone, M. R.; Tran, T. H.; Tumurkhuu, M.; Turner, P.; Udoakang, A. J.; Udoh, A.; Ullah, N.; Ullah, S.; Vaithinathan, A. G.; Valenti, M.; Vos, T.; Vu, H. T. L.; Waheed, Y.; Walker, A. S.; Walson, J. L.; Wangrangsimakul, T.; Weerakoon, K. G.; Wertheim, H. F. L.; Williams, P. C. M.; Wolde, A. A.; Wozniak, T. M.; Wu, F.; Wu, Z.; Yadav, M. K. K.; Yaghoubi, S.; Yahaya, Z. S.; Yarahmadi, A.; Yezli, S.; Yismaw, Y. E.; Yon, D. K.; Yuan, C.-W.; Yusuf, H.; Zakham, F.; Zamagni, G.; Zhang, H.; Zhang, Z.-J.; Zielińska, M.; Zumla, A.; Zyoud, S. H. H.; Zyoud, S. H.; Hay, S. I.; Stergachis, A.; Sartorius, B.; Cooper, B. S.; Dolecek, C.; Murray, C. J. L. Global Burden of Bacterial Antimicrobial Resistance 1990–2021: A Systematic Analysis with Forecasts to 2050. *The Lancet* **2024**, *404* (10459), 1199–1226. [https://doi.org/10.1016/S0140-6736\(24\)01867-1](https://doi.org/10.1016/S0140-6736(24)01867-1).
- (11) United Nations Environment Programme. *Bracing for Superbugs: Strengthening Environmental Action in the One Health Response to Antimicrobial Resistance*; Geneva, 2023. <https://www.unep.org/resources/superbugs/environmental-action>.
 - (12) Turner, N. A.; Sharma-Kuinkel, B. K.; Maskarinec, S. A.; Eichenberger, E. M.; Shah, P. P.; Carugati, M.; Holland, T. L.; Fowler, V. G. Methicillin-Resistant *Staphylococcus Aureus*: An Overview of Basic and Clinical Research. *Nat Rev Microbiol* **2019**, *17* (4), 203–218. <https://doi.org/10.1038/s41579-018-0147-4>.
 - (13) 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>.
 - (14) Centers for Disease Control and Prevention. *HAI Pathogens and Antimicrobial Resistance Report, 2018 - 2021*; U.S. Department of Health and Human Services, CDC: Atlanta, GA, 2023. <https://www.cdc.gov/nhsn/hai-report/index.html>.
 - (15) Lakhundi, S.; Zhang, K. Methicillin-Resistant *Staphylococcus Aureus*: Molecular Characterization, Evolution, and Epidemiology. *Clin Microbiol Rev* **2018**, *31* (4), e00020-18. <https://doi.org/10.1128/CMR.00020-18>.
 - (16) Taylor, T. A.; Tobin, E. H.; Unakal, C. G. *Staphylococcus Aureus Infection*. In *StatPearls*; StatPearls Publishing: Treasure Island (FL), 2026.

- (17) Dobrindt, U.; Hacker, J. Whole Genome Plasticity in Pathogenic Bacteria. *Current Opinion in Microbiology* **2001**, *4* (5), 550–557. [https://doi.org/10.1016/S1369-5274\(00\)00250-2](https://doi.org/10.1016/S1369-5274(00)00250-2).
- (18) Goerke, C.; Wolz, C. Regulatory and Genomic Plasticity of *Staphylococcus Aureus* during Persistent Colonization and Infection. *International Journal of Medical Microbiology* **2004**, *294* (2), 195–202. <https://doi.org/10.1016/j.ijmm.2004.06.013>.
- (19) Miller, W. R.; Arias, C. A. ESKAPE Pathogens: Antimicrobial Resistance, Epidemiology, Clinical Impact and Therapeutics. *Nat Rev Microbiol* **2024**, *22* (10), 598–616. <https://doi.org/10.1038/s41579-024-01054-w>.
- (20) Cheung, G. Y. C.; Bae, J. S.; Otto, M. Pathogenicity and Virulence of *Staphylococcus Aureus*. *Virulence* **2021**, *12* (1), 547–569. <https://doi.org/10.1080/21505594.2021.1878688>.
- (21) Archer, N. K.; Mazaitis, M. J.; Costerton, J. W.; Leid, J. G.; Powers, M. E.; Shirtliff, M. E. *Staphylococcus Aureus* Biofilms. *Virulence* **2011**, *2* (5), 445–459. <https://doi.org/10.4161/viru.2.5.17724>.
- (22) Kahl, B. C.; Becker, K.; Löffler, B. Clinical Significance and Pathogenesis of Staphylococcal Small Colony Variants in Persistent Infections. *Clinical Microbiology Reviews* **2016**, *29* (2), 401–427. <https://doi.org/10.1128/cmr.00069-15>.
- (23) Tobin, E. H.; Jogu, P.; Koirala, J. Methicillin-Resistant *Staphylococcus Aureus*. In *StatPearls*; StatPearls Publishing: Treasure Island (FL), 2026.
- (24) MacFarquhar, J. K.; Bajpai, A.; Fisher, T.; Barr, C.; Kent, A. G.; McKay, S. L.; Campbell, D.; Gargis, A. S.; Balbuena, R.; Lonsway, D.; Karlsson, M.; Walters, M. S.; Ham, D. C.; Glover, W. A. Identification and Characterization of Vancomycin-Resistant *Staphylococcus Aureus* CC45/USA600, North Carolina, USA, 2021 - Volume 31, Number 1—January 2025 - Emerging Infectious Diseases Journal - CDC. <https://doi.org/10.3201/eid3101.241573>.
- (25) Srinivasan, A.; Dick, J. D.; Perl, T. M. Vancomycin Resistance in Staphylococci. *Clin Microbiol Rev* **2002**, *15* (3), 430–438. <https://doi.org/10.1128/CMR.15.3.430-438.2002>.
- (26) Creech, C. B.; Al-Zubeidi, D. N.; Fritz, S. A. Prevention of Recurrent Staphylococcal Skin Infections. *Infect Dis Clin North Am* **2015**, *29* (3), 429–464. <https://doi.org/10.1016/j.idc.2015.05.007>.
- (27) Liu, M.; Sun, F.; Qi, Y.; Qi, W.; Li, J. Investigation into Antibiotic Resistance Profiles of *Staphylococcus Aureus* Among Chronic Rhinosinusitis Patients and Multifaceted Analysis of Factors Influencing Methicillin-Resistant *Staphylococcus Aureus* Infection. *Infect Drug Resist* **2025**, *18*, 4363–4371. <https://doi.org/10.2147/IDR.S546894>.
- (28) Keen, E. C. A Century of Phage Research: Bacteriophages and the Shaping of Modern Biology. *Bioessays* **2015**, *37* (1), 6–9. <https://doi.org/10.1002/bies.201400152>.
- (29) Sanz-Gaitero, M.; Seoane-Blanco, M.; van Raaij, M. J. Structure and Function of Bacteriophages. In *Bacteriophages*; Springer, Cham, 2021; pp 19–91. https://doi.org/10.1007/978-3-319-41986-2_1.
- (30) Dion, M. B.; Oechslein, F.; Moineau, S. Phage Diversity, Genomics and Phylogeny. *Nat Rev Microbiol* **2020**, *18* (3), 125–138. <https://doi.org/10.1038/s41579-019-0311-5>.

- (31) Klumpp, J.; Dunne, M.; Loessner, M. J. A Perfect Fit: Bacteriophage Receptor-Binding Proteins for Diagnostic and Therapeutic Applications. *Current Opinion in Microbiology* **2023**, *71*, 102240. <https://doi.org/10.1016/j.mib.2022.102240>.
- (32) Kasman, L. M.; Porter, L. D. Bacteriophages. In *StatPearls*; StatPearls Publishing: Treasure Island (FL), 2026.
- (33) Kurilovich, E.; Geva-Zatorsky, N. Effects of Bacteriophages on Gut Microbiome Functionality. *Gut Microbes* **17** (1), 2481178. <https://doi.org/10.1080/19490976.2025.2481178>.
- (34) Publications Service. On an Invisible Microbe Antagonistic toward Dysenteric Bacilli: Brief Note by Mr. F. D'Herelle, Presented by Mr. Roux. *Research in Microbiology* **2007**, *158* (7), 553–554. <https://doi.org/10.1016/j.resmic.2007.07.005>.
- (35) Phage Therapy—History from Twort and d'Herelle Through Soviet Experience to Current Approaches. In *Advances in Virus Research*; Academic Press, 2012; Vol. 83, pp 3–40. <https://doi.org/10.1016/B978-0-12-394438-2.00001-3>.
- (36) Aswani, V. H.; Shukla, S. K. An Early History of Phage Therapy in the United States: Is It Time to Reconsider? *Clin Med Res* **2021**, *19* (2), 82–89. <https://doi.org/10.3121/cmr.2021.1605>.
- (37) Schooley, R. T.; Biswas, B.; Gill, J. J.; Hernandez-Morales, A.; Lancaster, J.; Lessor, L.; Barr, J. J.; Reed, S. L.; Rohwer, F.; Benler, S.; Segall, A. M.; Taplitz, R.; Smith, D. M.; Kerr, K.; Kumaraswamy, M.; Nizet, V.; Lin, L.; McCauley, M. D.; Strathdee, S. A.; Benson, C. A.; Pope, R. K.; Leroux, B. M.; Picel, A. C.; Mateczun, A. J.; Cilwa, K. E.; Regeimbal, J. M.; Estrella, L. A.; Wolfe, D. M.; Henry, M. S.; Quinones, J.; Salka, S.; Bishop-Lilly, K. A.; Young, R.; Hamilton, T. Development and Use of Personalized Bacteriophage-Based Therapeutic Cocktails To Treat a Patient with a Disseminated Resistant *Acinetobacter Baumannii* Infection. *Antimicrobial Agents and Chemotherapy* **2017**, *61* (10), 10.1128/aac.00954-17. <https://doi.org/10.1128/aac.00954-17>.
- (38) Mertz, L. Battling Superbugs: How Phage Therapy Went From Obscure to Promising. *IEEE Pulse* **2019**, *10* (1), 3–9. <https://doi.org/10.1109/MPULS.2018.2885833>.
- (39) Aslam, S.; Lampley, E.; Wooten, D.; Karris, M.; Benson, C.; Strathdee, S.; Schooley, R. T. Lessons Learned From the First 10 Consecutive Cases of Intravenous Bacteriophage Therapy to Treat Multidrug-Resistant Bacterial Infections at a Single Center in the United States. *Open Forum Infect Dis* **2020**, *7* (9), ofaa389. <https://doi.org/10.1093/ofid/ofaa389>.
- (40) Sawa, T.; Moriyama, K.; Kinoshita, M. Current Status of Bacteriophage Therapy for Severe Bacterial Infections. *J intensive care* **2024**, *12* (1), 44. <https://doi.org/10.1186/s40560-024-00759-7>.
- (41) Pires, D. P.; Costa, A. R.; Pinto, G.; Meneses, L.; Azeredo, J. Current Challenges and Future Opportunities of Phage Therapy. *FEMS Microbiol Rev* **2020**, *44* (6), 684–700. <https://doi.org/10.1093/femsre/fuaa017>.
- (42) Torriero-Smith, N.; Rogers, B. A.; McDonald, M. J.; Barr, J. J. Harnessing ‘Phage Training’ to Bolster the Therapeutic Potential of Bacteriophages. *Current Opinion in Microbiology* **2026**, *89*, 102696. <https://doi.org/10.1016/j.mib.2025.102696>.

- (43) Vu, T. N.; Clark, J. R.; Jang, E.; D'Souza, R.; Nguyen, L. P.; Pinto, N. A.; Yoo, S.; Abadie, R.; Maresso, A. W.; Yong, D. Appelmans Protocol – A Directed *in Vitro* Evolution Enables Induction and Recombination of Prophages with Expanded Host Range. *Virus Research* **2024**, 339, 199272. <https://doi.org/10.1016/j.virusres.2023.199272>.
- (44) Pires, D. P.; Cleto, S.; Sillankorva, S.; Azeredo, J.; Lu, T. K. Genetically Engineered Phages: A Review of Advances over the Last Decade. *Microbiol Mol Biol Rev* **2016**, 80 (3), 523–543. <https://doi.org/10.1128/MMBR.00069-15>.
- (45) Peng, H.; Chen, I. A.; Qimron, U. Engineering Phages to Fight Multidrug-Resistant Bacteria. *Chem Rev* **2024**, 125 (2), 933–971. <https://doi.org/10.1021/acs.chemrev.4c00681>.
- (46) Łobocka, M.; Dąbrowska, K.; Górski, A. Engineered Bacteriophage Therapeutics: Rationale, Challenges and Future. *BioDrugs* **2021**, 35 (3), 255–280. <https://doi.org/10.1007/s40259-021-00480-z>.
- (47) Cornell, R. J.; Donaruma, L. G. 2-Methacryloxytropone. Intermediates for the Synthesis of Biologically Active Polymers. *J. Med. Chem.* **1965**, 8 (3), 388–390. <https://doi.org/10.1021/jm00327a025>.
- (48) Allan, C. R.; Hadwiger, L. A. The Fungicidal Effect of Chitosan on Fungi of Varying Cell Wall Composition. *Experimental Mycology* **1979**, 3 (3), 285–287. [https://doi.org/10.1016/S0147-5975\(79\)80054-7](https://doi.org/10.1016/S0147-5975(79)80054-7).
- (49) Shima, S.; Matsuoka, H.; Iwamoto, T.; Sakai, H. Antimicrobial Action of Epsilon-Poly-L-Lysine. *J Antibiot (Tokyo)* **1984**, 37 (11), 1449–1455. <https://doi.org/10.7164/antibiotics.37.1449>.
- (50) Ul Haq, I.; Pinto Vieira, R.; Lima, W. G.; de Lima, M. E.; Krukiewicz, K. Antimicrobial Polymers: Elucidating the Role of Functional Groups on Antimicrobial Activity. *Arab Journal of Basic and Applied Sciences* **2024**, 31 (1), 325–344. <https://doi.org/10.1080/25765299.2024.2366543>.
- (51) 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>.
- (52) 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>.
- (53) Siedenbiedel, F.; Tiller, J. C. Antimicrobial Polymers in Solution and on Surfaces: Overview and Functional Principles. *Polymers* **2012**, 4 (1), 46–71. <https://doi.org/10.3390/polym4010046>.
- (54) Nathan, A.; Zalipsky, S.; Ertel, S. I.; Agathos, S. N.; Yarmush, M. L.; Kohn, J. Copolymers of Lysine and Polyethylene Glycol: A New Family of Functionalized Drug Carriers. *Bioconjugate Chem.* **1993**, 4 (1), 54–62. <https://doi.org/10.1021/bc00019a008>.
- (55) Sahariah, P.; Másson, M. Antimicrobial Chitosan and Chitosan Derivatives: A Review of the Structure–Activity Relationship. *Biomacromolecules* **2017**, 18 (11), 3846–3868. <https://doi.org/10.1021/acs.biomac.7b01058>.

- (56) Wang, L.; Zhang, C.; Zhang, J.; Rao, Z.; Xu, X.; Mao, Z.; Chen, X. Epsilon-Poly-L-Lysine: Recent Advances in Biomanufacturing and Applications. *Front. Bioeng. Biotechnol.* **2021**, *9*. <https://doi.org/10.3389/fbioe.2021.748976>.
- (57) Oliveira Júnior, N. G.; Souza, C. M.; Buccini, D. F.; Cardoso, M. H.; Franco, O. L. Antimicrobial Peptides: Structure, Functions and Translational Applications. *Nat Rev Microbiol* **2025**, *23* (11), 687–700. <https://doi.org/10.1038/s41579-025-01200-y>.
- (58) Huan, Y.; Kong, Q.; Mou, H.; Yi, H. Antimicrobial Peptides: Classification, Design, Application and Research Progress in Multiple Fields. *Front. Microbiol.* **2020**, *11*. <https://doi.org/10.3389/fmicb.2020.582779>.
- (59) 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>.
- (60) Si, Z.; Chan-Park, M. B. Recent Advances in Designing Antimicrobial Polymers with Diverse Uptake and Killing Mechanisms. *Progress in Polymer Science* **2026**, *174*, 102080. <https://doi.org/10.1016/j.progpolymsci.2026.102080>.
- (61) Chindera, K.; Mahato, M.; Kumar Sharma, A.; Horsley, H.; Kloc-Muniak, K.; Kamaruzzaman, N. F.; Kumar, S.; McFarlane, A.; Stach, J.; Bentin, T.; Good, L. The Antimicrobial Polymer PHMB Enters Cells and Selectively Condenses Bacterial Chromosomes. *Sci Rep* **2016**, *6* (1), 23121. <https://doi.org/10.1038/srep23121>.
- (62) Zhou, M.; Qian, Y.; Xie, J.; Zhang, W.; Jiang, W.; Xiao, X.; Chen, S.; Dai, C.; Cong, Z.; Ji, Z.; Shao, N.; Liu, L.; Wu, Y.; Liu, R. Poly(2-Oxazoline)-Based Functional Peptide Mimics: Eradicating MRSA Infections and Persists While Alleviating Antimicrobial Resistance. *Angewandte Chemie International Edition* **2020**, *59* (16), 6412–6419. <https://doi.org/10.1002/anie.202000505>.
- (63) Bai, S.; Wang, J.; Yang, K.; Zhou, C.; Xu, Y.; Song, J.; Gu, Y.; Chen, Z.; Wang, M.; Shoen, C.; Andrade, B.; Cynamon, M.; Zhou, K.; Wang, H.; Cai, Q.; Oldfield, E.; Zimmerman, S. C.; Bai, Y.; Feng, X. A Polymeric Approach toward Resistance-Resistant Antimicrobial Agent with Dual-Selective Mechanisms of Action. *Science Advances* **2021**, *7* (5), eabc9917. <https://doi.org/10.1126/sciadv.abc9917>.
- (64) Miranda-Velez, M.; Sarker, G. S.; Ramisetty, P.; Geden, S.; Bartolomeu Halicki, P. C.; Annamalai, T.; Tse-Dinh, Y.-C.; Rohde, K. H.; Moon, J. H. Proton Motive Force-Disrupting Antimycobacterial Guanyurea Polymer. *Biomacromolecules* **2022**, *23* (11), 4668–4677. <https://doi.org/10.1021/acs.biomac.2c00902>.
- (65) Lam, S. J.; O'Brien-Simpson, N. M.; Pantarat, N.; Sulistio, A.; Wong, E. H. H.; Chen, Y.-Y.; Lenzo, J. C.; Holden, J. A.; Blencowe, A.; Reynolds, E. C.; Qiao, G. G. Combating Multidrug-Resistant Gram-Negative Bacteria with Structurally Nanoengineered Antimicrobial Peptide Polymers. *Nat Microbiol* **2016**, *1* (11), 16162. <https://doi.org/10.1038/nmicrobiol.2016.162>.
- (66) Mousavi, S. M.; Hashemi, S. A.; Yari Kalashgrani, M.; Zare, I.; Rahmanian, V.; Chiang, W.-H.; Mostafavi, E. Synthetic Polymers as Antibacterial and Antiviral Agents. In *Antibacterial and Antiviral Functional Materials, Volume 2*; ACS Symposium Series;

- American Chemical Society, 2024; Vol. 1472, pp 1–46. <https://doi.org/10.1021/bk-2024-1472.ch001>.
- (67) Asif, F.; Zaman, S. U.; Arnab, Md. K. H.; Hasan, M.; Islam, Md. M. Antimicrobial Peptides as Therapeutics: Confronting Delivery Challenges to Optimize Efficacy. *The Microbe* **2024**, 2, 100051. <https://doi.org/10.1016/j.microb.2024.100051>.
 - (68) Smola-Dmochowska, A.; Lewicka, K.; Macyk, A.; Rychter, P.; Pamuła, E.; Dobrzyński, P. Biodegradable Polymers and Polymer Composites with Antibacterial Properties. *Int J Mol Sci* **2023**, 24 (8), 7473. <https://doi.org/10.3390/ijms24087473>.
 - (69) Lin, M.; Sun, J. Antimicrobial Peptide-Inspired Antibacterial Polymeric Materials for Biosafety. *Biosafety and Health* **2022**, 4 (4), 269–279. <https://doi.org/10.1016/j.bsheal.2022.03.009>.
 - (70) Abedon, S. T.; Kuhl, S. J.; Blasdel, B. G.; Kutter, E. M. Phage Treatment of Human Infections. *Bacteriophage* **2011**, 1 (2), 66–85. <https://doi.org/10.4161/bact.1.2.15845>.
 - (71) Alkarri, S.; Bin Saad, H.; Soliman, M. On Antimicrobial Polymers: Development, Mechanism of Action, International Testing Procedures, and Applications. *Polymers* **2024**, 16 (6), 771. <https://doi.org/10.3390/polym16060771>.
 - (72) Cook, B. W. M.; Hynes, A. P. Re-Evaluating What Makes a Phage Unsuitable for Therapy. *npj Antimicrob Resist* **2025**, 3 (1), 45. <https://doi.org/10.1038/s44259-025-00117-z>.
 - (73) 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? *International Journal of Molecular Sciences* **2019**, 20 (11), 2747. <https://doi.org/10.3390/ijms20112747>.
 - (74) Ning, H.-Q.; Lin, H.; Wang, J.-X. Synergistic Effects of Endolysin Lysqdv001 and ϵ -Poly-Lysine in Controlling *Vibrio Parahaemolyticus* and Its Biofilms. *International Journal of Food Microbiology* **2021**, 343, 109112. <https://doi.org/10.1016/j.ijfoodmicro.2021.109112>.
 - (75) Park, J.; Hassan, M. A.; Nabawy, A.; Jiang, M.; Parmar, K.; Reddivari, A.; Goswami, R.; Jeon, T.; Patel, R.; Rotello, V. M. Engineered Bacteriophage-Polymer Nanoassemblies for Treatment of Wound Biofilm Infections. *ACS Nano* **2024**, 18 (39), 26928–26936. <https://doi.org/10.1021/acsnano.4c08671>.
 - (76) Meng, L.; Yang, F.; Pang, Y.; Cao, Z.; Wu, F.; Yan, D.; Liu, J. Nanocapping-Enabled Charge Reversal Generates Cell-Enterable Endosomal-Escapable Bacteriophages for Intracellular Pathogen Inhibition. *Science Advances* **2022**, 8 (28), eabq2005. <https://doi.org/10.1126/sciadv.abq2005>.
 - (77) Jia, Q.; Xiang, Y. Cryo-EM Structure of a Bacteriophage M13 Mini Variant. *Nat Commun* **2023**, 14 (1), 5421. <https://doi.org/10.1038/s41467-023-41151-7>.
 - (78) Smeal, S. W.; Schmitt, M. A.; Pereira, R. R.; Prasad, A.; Fisk, J. D. Simulation of the M13 Life Cycle I: Assembly of a Genetically-Structured Deterministic Chemical Kinetic Simulation. *Virology* **2017**, 500, 259–274. <https://doi.org/10.1016/j.virol.2016.08.017>.
 - (79) Barderas, R.; Benito-Peña, E. The 2018 Nobel Prize in Chemistry: Phage Display of Peptides and Antibodies. *Anal Bioanal Chem* **2019**, 411 (12), 2475–2479. <https://doi.org/10.1007/s00216-019-01714-4>.

- (80) Ledsgaard, L.; Kilstrup, M.; Karatt-Vellatt, A.; McCafferty, J.; Laustsen, A. H. Basics of Antibody Phage Display Technology. *Toxins (Basel)* **2018**, *10* (6), 236. <https://doi.org/10.3390/toxins10060236>.
- (81) Wang, R.; Li, H.-D.; Cao, Y.; Wang, Z.-Y.; Yang, T.; Wang, J.-H. M13 Phage: A Versatile Building Block for a Highly Specific Analysis Platform. *Anal Bioanal Chem* **2023**, 1–18. <https://doi.org/10.1007/s00216-023-04606-w>.
- (82) Chung, W.-J.; Lee, D.-Y.; Yoo, S. Y. Chemical Modulation of M13 Bacteriophage And Its Functional Opportunities for Nanomedicine. *IJN* **2014**, *9* (1), 5825–5836. <https://doi.org/10.2147/IJN.S73883>.
- (83) Peng, H.; Borg, R. E.; Dow, L. P.; Pruitt, B. L.; Chen, I. A. Controlled Phage Therapy by Photothermal Ablation of Specific Bacterial Species Using Gold Nanorods Targeted by Chimeric Phages. *Proceedings of the National Academy of Sciences* **2020**, *117* (4), 1951–1961. <https://doi.org/10.1073/pnas.1913234117>.
- (84) Peng, H.; Rossetto, D.; Mansy, S. S.; Jordan, M. C.; Roos, K. P.; Chen, I. A. Treatment of Wound Infections in a Mouse Model Using Zn²⁺-Releasing Phage Bound to Gold Nanorods. *ACS Nano* **2022**, *16* (3), 4756–4774. <https://doi.org/10.1021/acsnano.2c00048>.
- (85) Yang, Y.; Kang, D.; Mihalache, B.; Vexler, S.; Jain, S.; Peng, H.; Annabi, N.; Yang, S.; Chen, I. A. Metagenome-Inspired Libraries to Engineer Phage M13 for Targeted Killing of Gram-Negative Bacterial Species. *Nucleic Acids Res* **2025**, *53* (18), gkaf984. <https://doi.org/10.1093/nar/gkaf984>.
- (86) Lin, A.; Jimenez, J.; Derr, J.; Vera, P.; Manapat, M. L.; Esvelt, K. M.; Villanueva, L.; Liu, D. R.; Chen, I. A. Inhibition of Bacterial Conjugation by Phage M13 and Its Protein G3p: Quantitative Analysis and Model. *PLOS ONE* **2011**, *6* (5), e19991. <https://doi.org/10.1371/journal.pone.0019991>.
- (87) Freese, P. D.; Korolev, K. S.; Jiménez, J. I.; Chen, I. A. Genetic Drift Suppresses Bacterial Conjugation in Spatially Structured Populations. *Biophysical Journal* **2014**, *106* (4), 944–954. <https://doi.org/10.1016/j.bpj.2014.01.012>.
- (88) Yang, Y.; Vexler, S.; Jordan, M. C.; Abbondante, S.; Kang, D.; Peng, H.; Marshall, M.; Naini, B. V.; Jain, S.; Lai, Y.-C.; Annabi, N.; Roos, K. P.; Pearlman, E.; Chen, I. A. A Synthetic Phage-Peptide Conjugate as a Potent Antibacterial Agent for Pseudomonas Aeruginosa Infections. *ACS Cent. Sci.* **2025**. <https://doi.org/10.1021/acscentsci.5c00562>.
- (89) Peng, H.; Borg, R. E.; Nguyen, A. B. N.; Chen, I. A. Chimeric Phage Nanoparticles for Rapid Characterization of Bacterial Pathogens: Detection in Complex Biological Samples and Determination of Antibiotic Sensitivity. *ACS Sens.* **2020**, *5* (5), 1491–1499. <https://doi.org/10.1021/acssensors.0c00654>.
- (90) Click, E. M.; Webster, R. E. The TolQRA Proteins Are Required for Membrane Insertion of the Major Capsid Protein of the Filamentous Phage F1 during Infection. *Journal of Bacteriology* **1998**, *180* (7), 1723–1728. <https://doi.org/10.1128/jb.180.7.1723-1728.1998>.
- (91) Sturgis, J. N. Organisation and Evolution of the Tol-Pal Gene Cluster. *J Mol Microbiol Biotechnol* **2001**, *3* (1), 113–122.

- (92) Skurnik, D.; Cywes-Bentley, C.; Pier, G. B. The Exceptionally Broad-Based Potential of Active and Passive Vaccination Targeting the Conserved Microbial Surface Polysaccharide PNAG. *cdc* **2016**, *15* (8), 1041–1053. <https://doi.org/10.1586/14760584.2016.1159135>.
- (93) Kelly-Quintos, C.; Cavacini, L. A.; Posner, M. R.; Goldmann, D.; Pier, G. B. Characterization of the Opsonic and Protective Activity against *Staphylococcus Aureus* of Fully Human Monoclonal Antibodies Specific for the Bacterial Surface Polysaccharide Poly-N-Acetylglucosamine. *Infection and Immunity* **2006**, *74* (5), 2742–2750. <https://doi.org/10.1128/IAI.74.5.2742-2750.2006>.
- (94) Cywes-Bentley, C.; Skurnik, D.; Zaidi, T.; Roux, D.; DeOliveira, R. B.; Garrett, W. S.; Lu, X.; O'Malley, J.; Kinzel, K.; Zaidi, T.; Rey, A.; Perrin, C.; Fichorova, R. N.; Kayatani, A. K. K.; Maira-Litrán, T.; Gening, M. L.; Tsvetkov, Y. E.; Nifantiev, N. E.; Bakaletz, L. O.; Pelton, S. I.; Golenbock, D. T.; Pier, G. B. Antibody to a Conserved Antigenic Target Is Protective against Diverse Prokaryotic and Eukaryotic Pathogens. *PNAS* **2013**, *110* (24), E2209–E2218. <https://doi.org/10.1073/pnas.1303573110>.
- (95) Soliman, C.; Walduck, A. K.; Yuriev, E.; Richards, J. S.; Cywes-Bentley, C.; Pier, G. B.; Ramsland, P. A. Structural Basis for Antibody Targeting of the Broadly Expressed Microbial Polysaccharide Poly-N-Acetylglucosamine. *Journal of Biological Chemistry* **2018**, *293* (14), 5079–5089. <https://doi.org/10.1074/jbc.RA117.001170>.
- (96) Day, L. A.; Wiseman, R. L. A Comparison of DNA Packaging in the Virions of Fd, Xf, and Pfl. In *The Single-stranded DNA phages*; Denhardt, D. T., Dressler, D., Ray, D. S., Eds.; Cold Spring Harbor monograph series; Cold Spring Harbor Laboratory: Cold Spring Harbor, N.Y, 1978; pp 605–625.
- (97) Bales, P. M.; Renke, E. M.; May, S. L.; Shen, Y.; Nelson, D. C. Purification and Characterization of Biofilm-Associated EPS Exopolysaccharides from ESKAPE Organisms and Other Pathogens. *PLOS ONE* **2013**, *8* (6), e67950. <https://doi.org/10.1371/journal.pone.0067950>.
- (98) DuBois, Michel.; Gilles, K. A.; Hamilton, J. K.; Rebers, P. A.; Smith, Fred. Colorimetric Method for Determination of Sugars and Related Substances. *Anal. Chem.* **1956**, *28* (3), 350–356. <https://doi.org/10.1021/ac60111a017>.
- (99) Stowell, S. R.; Arthur, C. M.; McBride, R.; Berger, O.; Razi, N.; Heimbürg-Molinaro, J.; Rodrigues, L. C.; Gourdine, J.-P.; Noll, A. J.; von Gunten, S.; Smith, D. F.; Knirel, Y. A.; Paulson, J. C.; Cummings, R. D. Microbial Glycan Microarrays Define Key Features of Host-Microbial Interactions. *Nat Chem Biol* **2014**, *10* (6), 470–476. <https://doi.org/10.1038/nchembio.1525>.
- (100) Cerca, N.; Jefferson, K. K.; Maira-Litrán, T.; Pier, D. B.; Kelly-Quintos, C.; Goldmann, D. A.; Azeredo, J.; Pier, G. B. Molecular Basis for Preferential Protective Efficacy of Antibodies Directed to the Poorly Acetylated Form of *Staphylococcal* Poly-N-Acetyl- β -(1-6)-Glucosamine. *Infect Immun* **2007**, *75* (7), 3406–3413. <https://doi.org/10.1128/IAI.00078-07>.
- (101) Cerca, N.; Maira-Litrán, T.; Jefferson, K. K.; Grout, M.; Goldmann, D. A.; Pier, G. B. Protection against *Escherichia Coli* Infection by Antibody to the *Staphylococcus Aureus* Poly-N-Acetylglucosamine Surface Polysaccharide. *Proc Natl Acad Sci U S A* **2007**, *104* (18), 7528–7533. <https://doi.org/10.1073/pnas.0700630104>.

- (102) Jennings, L. K.; Storek, K. M.; Ledvina, H. E.; Coulon, C.; Marmont, L. S.; Sadovskaya, I.; Secor, P. R.; Tseng, B. S.; Scian, M.; Filloux, A.; Wozniak, D. J.; Howell, P. L.; Parsek, M. R. Pel Is a Cationic Exopolysaccharide That Cross-Links Extracellular DNA in the *Pseudomonas Aeruginosa* Biofilm Matrix. *Proceedings of the National Academy of Sciences* **2015**, *112* (36), 11353–11358. <https://doi.org/10.1073/pnas.1503058112>.
- (103) Dal Ferro, M.; Rizzo, S.; Rizzo, E.; Marano, F.; Luisi, I.; Tarasiuk, O.; Sblattero, D. Phage Display Technology for Human Monoclonal Antibodies. In *Human Monoclonal Antibodies: Methods and Protocols*; Steinitz, M., Ed.; Springer: New York, NY, 2019; pp 319–338. https://doi.org/10.1007/978-1-4939-8958-4_15.
- (104) McCombs, J. R.; Owen, S. C. Antibody Drug Conjugates: Design and Selection of Linker, Payload and Conjugation Chemistry. *AAPS J* **2015**, *17* (2), 339–351. <https://doi.org/10.1208/s12248-014-9710-8>.
- (105) Palermo, E. F.; Vemparala, S.; Kuroda, K. Cationic Spacer Arm Design Strategy for Control of Antimicrobial Activity and Conformation of Amphiphilic Methacrylate Random Copolymers. *Biomacromolecules* **2012**, *13* (5), 1632–1641. <https://doi.org/10.1021/bm300342u>.
- (106) Lienkamp, K.; Madkour, A. E.; Musante, A.; Nelson, C. F.; Nüsslein, K.; Tew, G. N. Antimicrobial Polymers Prepared by ROMP with Unprecedented Selectivity: A Molecular Construction Kit Approach. *J. Am. Chem. Soc.* **2008**, *130* (30), 9836–9843. <https://doi.org/10.1021/ja801662y>.
- (107) Zhou, C.; Qi, X.; Li, P.; Chen, W. N.; Mouad, L.; Chang, M. W.; Leong, S. S. J.; Chan-Park, M. B. High Potency and Broad-Spectrum Antimicrobial Peptides Synthesized via Ring-Opening Polymerization of α -Aminoacid-N-Carboxyanhydrides. *Biomacromolecules* **2010**, *11* (1), 60–67. <https://doi.org/10.1021/bm900896h>.
- (108) Salas-Ambrosio, P.; Tronnet, A.; Since, M.; Bourgeade-Delmas, S.; Stigliani, J.-L.; Vax, A.; Lecommandoux, S.; Dupuy, B.; Verhaeghe, P.; Bonduelle, C. Cyclic Poly(α -Peptoid)s by Lithium Bis(Trimethylsilyl)Amide (LiHMDS)-Mediated Ring-Expansion Polymerization: Simple Access to Bioactive Backbones. *J. Am. Chem. Soc.* **2021**, *143* (10), 3697–3702. <https://doi.org/10.1021/jacs.0c13231>.
- (109) Salas-Ambrosio, P.; Tronnet, A.; Badreldin, M.; Reyes, L.; Since, M.; Bourgeade-Delmas, S.; Dupuy, B.; Verhaeghe, P.; Bonduelle, C. Star-like Poly(Peptoid)s with Selective Antibacterial Activity. *Polym. Chem.* **2022**, *13* (5), 600–612. <https://doi.org/10.1039/D1PY01529J>.
- (110) Liu, R.; Suárez, J. M.; Weisblum, B.; Gellman, S. H.; McBride, S. M. Synthetic Polymers Active against *Clostridium Difficile* Vegetative Cell Growth and Spore Outgrowth. *J. Am. Chem. Soc.* **2014**, *136* (41), 14498–14504. <https://doi.org/10.1021/ja506798e>.
- (111) Ergene, C.; Yasuhara, K.; Palermo, E. F. Biomimetic Antimicrobial Polymers: Recent Advances in Molecular Design. *Polym. Chem.* **2018**, *9* (18), 2407–2427. <https://doi.org/10.1039/C8PY00012C>.
- (112) Salas-Ambrosio, P.; Tronnet, A.; Verhaeghe, P.; Bonduelle, C. Synthetic Polypeptide Polymers as Simplified Analogues of Antimicrobial Peptides. *Biomacromolecules* **2021**, *22* (1), 57–75. <https://doi.org/10.1021/acs.biomac.0c00797>.

- (113) Cheng, G.; Xue, H.; Zhang, Z.; Chen, S.; Jiang, S. A Switchable Biocompatible Polymer Surface with Self-Sterilizing and Nonfouling Capabilities. *Angewandte Chemie* **2008**, *120* (46), 8963–8966. <https://doi.org/10.1002/ange.200803570>.
- (114) Fik, C. P.; Krumm, C.; Muennig, C.; Baur, T. I.; Salz, U.; Bock, T.; Tiller, J. C. Impact of Functional Satellite Groups on the Antimicrobial Activity and Hemocompatibility of Telechelic Poly(2-Methyloxazoline)s. *Biomacromolecules* **2012**, *13* (1), 165–172. <https://doi.org/10.1021/bm201403e>.
- (115) Alswieleh, A. M.; Cheng, N.; Canton, I.; Ustbas, B.; Xue, X.; Ladmira, V.; Xia, S.; Ducker, R. E.; El Zubir, O.; Cartron, M. L.; Hunter, C. N.; Leggett, G. J.; Armes, S. P. Zwitterionic Poly(Amino Acid Methacrylate) Brushes. *J. Am. Chem. Soc.* **2014**, *136* (26), 9404–9413. <https://doi.org/10.1021/ja503400r>.
- (116) Bauri, K.; Sayala, K. D.; Roy, R. S.; De, P. Chiral Copoly(Methacrylate)s Carrying Amino Acid Pendants in the Side-Chains. *European Polymer Journal* **2015**, *73*, 237–246. <https://doi.org/10.1016/j.eurpolymj.2015.10.017>.
- (117) Kuroda, K.; Caputo, G. A.; DeGrado, W. F. The Role of Hydrophobicity in the Antimicrobial and Hemolytic Activities of Polymethacrylate Derivatives. *Chemistry – A European Journal* **2009**, *15* (5), 1123–1133. <https://doi.org/10.1002/chem.200801523>.
- (118) Shadmon, H.; Basu, A.; Eckhard, L. H.; Domb, A. J.; Beyth, N. Synthesis, Characterization and Antibacterial Activity of Heterocyclic Quaternary Ammonium Polymers. *New J. Chem.* **2018**, *42* (18), 15427–15435. <https://doi.org/10.1039/C8NJ03392G>.
- (119) Li, G.; Shen, J. A Study of Pyridinium-Type Functional Polymers. IV. Behavioral Features of the Antibacterial Activity of Insoluble Pyridinium-Type Polymers. *Journal of Applied Polymer Science* **2000**, *78* (3), 676–684. [https://doi.org/10.1002/1097-4628\(20001017\)78:3%3C676::AID-APP240%3E3.0.CO;2-E](https://doi.org/10.1002/1097-4628(20001017)78:3%3C676::AID-APP240%3E3.0.CO;2-E).
- (120) Soykan, C.; Coşkun, R.; Delibaş, A. Microbial Screening of Copolymers of N-Vinylimidazole with Phenacyl Methacrylate: Synthesis and Monomer Reactivity Ratios. *Journal of Macromolecular Science, Part A* **2005**, *42* (12), 1603–1619. <https://doi.org/10.1080/10601320500246693>.
- (121) Ng, V. W. L.; Tan, J. P. K.; Leong, J.; Voo, Z. X.; Hedrick, J. L.; Yang, Y. Y. Antimicrobial Polycarbonates: Investigating the Impact of Nitrogen-Containing Heterocycles as Quaternizing Agents. *Macromolecules* **2014**, *47* (4), 1285–1291. <https://doi.org/10.1021/ma402641p>.
- (122) Almeida, A. A. P.; Farah, A.; Silva, D. A. M.; Nunan, E. A.; Glória, M. B. A. Antibacterial Activity of Coffee Extracts and Selected Coffee Chemical Compounds against Enterobacteria. *J. Agric. Food Chem.* **2006**, *54* (23), 8738–8743. <https://doi.org/10.1021/jf0617317>.
- (123) Nonthakaew, A.; Matan, N.; Aewsiri, T. Caffeine in Foods and Its Antimicrobial Activity. *international food research journal* **2015**.
- (124) Gupta, R. S.; Moffat, M. R. K. Synergistic Effect of Purine Derivatives on the Toxicity of Pyrazofurin and 6-azauridine towards Cultured Mammalian Cells. *Journal Cellular Physiology* **1982**, *131* (3), 291–294. <https://doi.org/10.1002/jcp.1041110310>.

- (125) Wang, Y.-N.; Bheemanaboina, R. R. Y.; Cai, G.-X.; Zhou, C.-H. Novel Purine Benzimidazoles as Antimicrobial Agents by Regulating ROS Generation and Targeting Clinically Resistant *Staphylococcus Aureus* DNA Groove. *Bioorganic & Medicinal Chemistry Letters* **2018**, 28 (9), 1621–1628. <https://doi.org/10.1016/j.bmcl.2018.03.046>.
- (126) Fischer, Emil. SYNTHESIS OF DEPSIDES, LICHEN-SUBSTANCES AND TANNINS. *J. Am. Chem. Soc.* **1914**, 36 (6), 1170–1201. <https://doi.org/10.1021/ja02183a009>.
- (127) Rajendran, K.; Poornima, S.; Ponnusamy, P. Antimicrobial and Antiproliferative Activities of Depside Compound Isolated from the Mycobiont Culture of *Parmotrema Austrosinense* (Zahlbr.) Hale. *J Pure Appl Microbiol* **2020**, 14 (4), 2525–2541. <https://doi.org/10.22207/JPAM.14.4.29>.
- (128) Gomes, A. T.; Smania Júnior, A.; Seidel, C.; Smania, E. D. F. A.; Honda, N. K.; Roese, F. M.; Muzzi, R. M. Antibacterial Activity of Orsellinates. *Braz. J. Microbiol.* **2003**, 34 (3). <https://doi.org/10.1590/S1517-83822003000300002>.
- (129) Ureña-Vacas, I.; González-Burgos, E.; Divakar, P. K.; Gómez-Serranillos, M. P. Lichen Depsides and Tridepsides: Progress in Pharmacological Approaches. *JoF* **2023**, 9 (1), 116. <https://doi.org/10.3390/jof9010116>.
- (130) Ibrahim, S. R. M.; Sirwi, A.; Eid, B. G.; Mohamed, S. G. A.; Mohamed, G. A. Fungal Depsides—Naturally Inspiring Molecules: Biosynthesis, Structural Characterization, and Biological Activities. *Metabolites* **2021**, 11 (10), 683. <https://doi.org/10.3390/metabo11100683>.
- (131) Zhou, R.; Yang, Y.; Park, S.-Y.; Nguyen, T. T.; Seo, Y.-W.; Lee, K. H.; Lee, J. H.; Kim, K. K.; Hur, J.-S.; Kim, H. The Lichen Secondary Metabolite Atranorin Suppresses Lung Cancer Cell Motility and Tumorigenesis. *Sci Rep* **2017**, 7 (1), 8136. <https://doi.org/10.1038/s41598-017-08225-1>.
- (132) Kumar Kc, S.; Müller, K. Lichen Metabolites. 1. Inhibitory Action Against Leukotriene B₄ Biosynthesis by a Non-Redox Mechanism. *J. Nat. Prod.* **1999**, 62 (6), 817–820. <https://doi.org/10.1021/np9803777>.
- (133) Müller, K. Pharmaceutically Relevant Metabolites from Lichens. *Applied Microbiology and Biotechnology* **2001**, 56 (1–2), 9–16. <https://doi.org/10.1007/s002530100684>.
- (134) Saetang, P.; Rukachaisirikul, V.; Phongpaichit, S.; Preedanon, S.; Sakayaroj, J.; Hadsadee, S.; Jungsuttiwong, S. Antibacterial and Antifungal Polyketides from the Fungus *Aspergillus Unguis* PSU-MF16. *J. Nat. Prod.* **2021**, 84 (5), 1498–1506. <https://doi.org/10.1021/acs.jnatprod.0c01308>.
- (135) Yılmaz, M.; Türk, A. Ö.; Tay, T.; Kıvanç, M. The Antimicrobial Activity of Extracts of the Lichen *Cladonia Foliacea* and Its (–)-Usnic Acid, Atranorin, and Fumarprotocetraric Acid Constituents. *Zeitschrift für Naturforschung C* **2004**, 59 (3–4), 249–254. <https://doi.org/10.1515/znc-2004-3-423>.
- (136) Phainuphong, P.; Rukachaisirikul, V.; Phongpaichit, S.; Sakayaroj, J.; Kanjanasirirat, P.; Borwornpinyo, S.; Akrimajirachote, N.; Yimnual, C.; Muanprasat, C. Depsides and Depsidones from the Soil-Derived Fungus *Aspergillus Unguis* PSU-RSPG204. *Tetrahedron* **2018**, 74 (39), 5691–5699. <https://doi.org/10.1016/j.tet.2018.07.059>.

- (137) Zhou, H.; Zhan, J.; Watanabe, K.; Xie, X.; Tang, Y. A Polyketide Macrolactone Synthase from the Filamentous Fungus *Gibberella Zeae*. *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105* (17), 6249–6254. <https://doi.org/10.1073/pnas.0800657105>.
- (138) Okorafor, I. C.; Chen, M.; Tang, Y. High-Titer Production of Olivetolic Acid and Analogs in Engineered Fungal Host Using a Nonplant Biosynthetic Pathway. *ACS Synth. Biol.* **2021**, *10* (9), 2159–2166. <https://doi.org/10.1021/acssynbio.1c00309>.
- (139) Liu, Y.; Ding, S.; Shen, J.; Zhu, K. Nonribosomal Antibacterial Peptides That Target Multidrug-Resistant Bacteria. *Natural Product Reports* **2019**, *36* (4), 573–592. <https://doi.org/10.1039/C8NP00031J>.
- (140) Xuan, J.; Feng, W.; Wang, J.; Wang, R.; Zhang, B.; Bo, L.; Chen, Z.-S.; Yang, H.; Sun, L. Antimicrobial Peptides for Combating Drug-Resistant Bacterial Infections. *Drug Resistance Updates* **2023**, *68*, 100954. <https://doi.org/10.1016/j.drug.2023.100954>.
- (141) Roh, J.; Boyer, C.; Kumar, P. V. Uncovering Key Characteristics of Antibacterial Peptides through Machine Learning. *Macromolecular Rapid Communications n/a* (n/a), e00583. <https://doi.org/10.1002/marc.202500583>.
- (142) Kocgozlu, L.; Mutschler, A.; Tallet, L.; Calligaro, C.; Knopf-Marques, H.; Lebaudy, E.; Mathieu, E.; Rabineau, M.; Gribova, V.; Senger, B.; Vrana, N. E.; Laval, P. Cationic Homopolypeptides: A Versatile Tool to Design Multifunctional Antimicrobial Nanocoatings. *Materials Today Bio* **2024**, *28*, 101168. <https://doi.org/10.1016/j.mtbio.2024.101168>.
- (143) Zarrintaj, P.; Ghorbani, S.; Barani, M.; Singh Chauhan, N. P.; Khodadadi Yazdi, M.; Saeb, M. R.; Ramsey, J. D.; Hamblin, M. R.; Mozafari, M.; Mostafavi, E. Polylysine for Skin Regeneration: A Review of Recent Advances and Future Perspectives. *Bioeng Transl Med* **2021**, *7* (1), e10261. <https://doi.org/10.1002/btm2.10261>.
- (144) Hyldgaard, M.; Mygind, T.; Vad, B. S.; Stenvang, M.; Otzen, D. E.; Meyer, R. L. The Antimicrobial Mechanism of Action of Epsilon-Poly-L-Lysine. *Appl Environ Microbiol* **2014**, *80* (24), 7758–7770. <https://doi.org/10.1128/AEM.02204-14>.
- (145) Kondo, K.; Iwasaki, H.; Ueda, N.; Takemoto, K.; Imoto, M. Vinyl Compounds of Nucleic Acid Bases. II. Synthesis and Polymerization of N- β -methacryloyloxyethyl Derivatives of Uracil, Thymine, Theophylline, 2-chloro-6-methylpurine, and Adenine. *Makromol. Chem.* **1968**, *120* (1), 21–26. <https://doi.org/10.1002/macp.1968.021200103>.
- (146) Cao, Z.; Yu, Q.; Xue, H.; Cheng, G.; Jiang, S. Nanoparticles for Drug Delivery Prepared from Amphiphilic PLGA Zwitterionic Block Copolymers with Sharp Contrast in Polarity between Two Blocks. *Angew Chem Int Ed* **2010**, *49* (22), 3771–3776. <https://doi.org/10.1002/anie.200907079>.
- (147) Xu, Q.; He, C.; Xiao, C.; Yu, S.; Chen, X. ϵ -Methacryloyl-L-Lysine Based Polypeptides and Their Thiol–Ene Click Functionalization. *Polym. Chem.* **2015**, *6* (10), 1758–1767. <https://doi.org/10.1039/C4PY01523A>.
- (148) Hewawasam, R. S.; Kalana, U. L. D. I.; Dharmaratne, N. U.; Wright, T. J.; Bannin, T. J.; Kiesewetter, E. T.; Kiesewetter, M. K. Bisurea and Bisthiourea H-Bonding Organocatalysts for Ring-Opening Polymerization: Cues for the Catalyst Design. *Macromolecules* **2019**, *52* (23), 9232–9237. <https://doi.org/10.1021/acs.macromol.9b01875>.

- (149) Evans, J. C.; Klix, R. C.; Bach, R. D. Diels-Alder Approaches to Model Compounds Related to Fredericamycin A. *J. Org. Chem.* **1988**, *53* (23), 5519–5527. <https://doi.org/10.1021/jo00258a022>.
- (150) Yoshino, T.; Sato, I.; Hiram, M. Total Synthesis of Aspercyclides A and B via Intramolecular Oxidative Diaryl Ether Formation. *Org. Lett.* **2012**, *14* (16), 4290–4292. <https://doi.org/10.1021/ol3019727>.
- (151) Kabsch, W. Integration, Scaling, Space-Group Assignment and Post-Refinement. *Acta Crystallogr D Biol Crystallogr* **2010**, *66* (Pt 2), 133–144. <https://doi.org/10.1107/S0907444909047374>.
- (152) Mies, T.; White, A. J. P.; Parsons, P. J.; Barrett, A. G. M. Photolytic Studies on the Generation and Trapping of 6-Oxomethylidenecyclohexa-2,4-Diene-1-One Derivatives with Various Nucleophiles. *Helvetica Chimica Acta* **2021**, *104* (12), e2100189. <https://doi.org/10.1002/hlca.202100189>.
- (153) Liu, N.; Hung, Y.-S.; Gao, S.-S.; Hang, L.; Zou, Y.; Chooi, Y.-H.; Tang, Y. Identification and Heterologous Production of a Benzoyl-Primed Tricarboxylic Acid Polyketide Intermediate from the Zaragozic Acid A Biosynthetic Pathway. *Org Lett* **2017**, *19* (13), 3560–3563. <https://doi.org/10.1021/acs.orglett.7b01534>.
- (154) Deming, T. J. Living Polymerization of α -Amino Acid-N-Carboxyanhydrides. *Journal of Polymer Science Part A: Polymer Chemistry* **2000**, *38* (17), 3011–3018. [https://doi.org/10.1002/1099-0518\(20000901\)38:17%3C3011::AID-POLA10%3E3.0.CO;2-Z](https://doi.org/10.1002/1099-0518(20000901)38:17%3C3011::AID-POLA10%3E3.0.CO;2-Z).
- (155) Kramer, J. R.; Deming, T. J. General Method for Purification of α -Amino Acid-N-Carboxyanhydrides Using Flash Chromatography. *Biomacromolecules* **2010**, *11* (12), 3668–3672. <https://doi.org/10.1021/bm101123k>.
- (156) Deming, T. J.; Curtin, S. A. Chain Initiation Efficiency in Cobalt- and Nickel-Mediated Polypeptide Synthesis. *J. Am. Chem. Soc.* **2000**, *122* (24), 5710–5717. <https://doi.org/10.1021/ja994281q>.
- (157) Benavides, I.; Raftery, E. D.; Bell, A. G.; Evans, D.; Scott, W. A.; Houk, K. N.; Deming, T. J. Poly(Dehydroalanine): Synthesis, Properties, and Functional Diversification of a Fluorescent Polypeptide. *J. Am. Chem. Soc.* **2022**, *144* (9), 4214–4223. <https://doi.org/10.1021/jacs.2c00383>.
- (158) Clauss, Z. S.; Kramer, J. R. Polypeptoids and Peptoid–Peptide Hybrids by Transition Metal Catalysis. *ACS Appl. Mater. Interfaces* **2022**, *14* (20), 22781–22789. <https://doi.org/10.1021/acsami.1c19692>.
- (159) Kowalska-Krochmal, B.; Dudek-Wicher, R. The Minimum Inhibitory Concentration of Antibiotics: Methods, Interpretation, Clinical Relevance. *Pathogens* **2021**, *10* (2), 165. <https://doi.org/10.3390/pathogens10020165>.
- (160) Zhou, Y.-F.; Shi, W.; Yu, Y.; Tao, M.-T.; Xiong, Y. Q.; Sun, J.; Liu, Y.-H. Pharmacokinetic/Pharmacodynamic Correlation of Cefquinome Against Experimental Catheter-Associated Biofilm Infection Due to Staphylococcus Aureus. *Front Microbiol* **2016**, *6*, 1513. <https://doi.org/10.3389/fmicb.2015.01513>.

- (161) Alvi, S. B.; Rajalakshmi, P. S.; Jogdand, A.; Sanjay, A. Y.; B, V.; John, R.; Rengan, A. K. Iontophoresis Mediated Localized Delivery of Liposomal Gold Nanoparticles for Photothermal and Photodynamic Therapy of Acne. *Biomater. Sci.* **2021**, *9* (4), 1421–1430. <https://doi.org/10.1039/D0BM01712D>.
- (162) Nelson, A. M.; Hemp, S. T.; Chau, J.; Long, T. E. Free Radical Polymerization of Caffeine-Containing Methacrylate Monomers. *Journal of Polymer Science Part A: Polymer Chemistry* **2015**, *53* (24), 2829–2837. <https://doi.org/10.1002/pola.27756>.
- (163) Guterman, R.; Antonietti, M.; Yuan, J. Plants to Polyelectrolytes: Theophylline Polymers and Their Microsphere Synthesis. *Macromolecular Rapid Communications* **2017**, *38* (8), 1600748. <https://doi.org/10.1002/marc.201600748>.
- (164) Kumar, K.; Kumar Shyamlal, B. R.; Verma, R.; Kondaiah, P.; Chaudhary, S. Reduction-Triggered Doxorubicin Delivery by Self-Assembled Nanospheres of Lipoylated Caffeine. *ChemMedChem* **2020**, *15* (9), 733–737. <https://doi.org/10.1002/cmdc.202000070>.
- (165) Lin, X.; Jain, P.; Wu, K.; Hong, D.; Hung, H.-C.; O’Kelly, M. B.; Li, B.; Zhang, P.; Yuan, Z.; Jiang, S. Ultralow Fouling and Functionalizable Surface Chemistry Based on Zwitterionic Carboxybetaine Random Copolymers. *Langmuir* **2019**, *35* (5), 1544–1551. <https://doi.org/10.1021/acs.langmuir.8b02540>.
- (166) Hu, W.-C.; Pang, J.; Biswas, S.; Wang, K.; Wang, C.; Xia, X.-H. Ultrasensitive Detection of Bacteria Using a 2D MOF Nanozyme-Amplified Electrochemical Detector. *Anal. Chem.* **2021**, *93* (24), 8544–8552. <https://doi.org/10.1021/acs.analchem.1c01261>.
- (167) Fugolin, A. P.; Lewis, S.; Logan, M. G.; Ferracane, J. L.; Pfeifer, C. S. Methacrylamide–Methacrylate Hybrid Monomers for Dental Applications. *Dental Materials* **2020**, *36* (8), 1028–1037. <https://doi.org/10.1016/j.dental.2020.04.023>.
- (168) Song, X.; Li, R.; Zhang, Q.; He, S.; Wang, Y. Antibacterial Effect and Possible Mechanism of Salicylic Acid Microcapsules against Escherichia Coli and Staphylococcus Aureus. *IJERPH* **2022**, *19* (19), 12761. <https://doi.org/10.3390/ijerph191912761>.
- (169) Bouarab, L.; Degraeve, P.; Bouajila, J.; Cottaz, A.; Jbilou, F.; Joly, C.; Oulahal, N. *Staphylococcus Aureus* Membrane-Damaging Activities of Four Phenolics. *FEMS Microbiology Letters* **2021**, *368* (13), fnab081. <https://doi.org/10.1093/femsle/fnab081>.
- (170) Kim, H. J.; Hillmyer, M. A.; Ellison, C. J. Enhanced Polyester Degradation through Transesterification with Salicylates. *J. Am. Chem. Soc.* **2021**, *143* (38), 15784–15790. <https://doi.org/10.1021/jacs.1c07229>.
- (171) Kim, H. J.; Reddi, Y.; Cramer, C. J.; Hillmyer, M. A.; Ellison, C. J. Readily Degradable Aromatic Polyesters from Salicylic Acid. *ACS Macro Lett.* **2020**, *9* (1), 96–102. <https://doi.org/10.1021/acsmacrolett.9b00890>.
- (172) Ren, F.; Xian, J.; Jia, Z.; Chen, Z.; Fu, H.; Wang, R.; Chu, W.; Pan, X.; Wu, J. Tetrabutylammonium Halides as Selectively Bifunctional Catalysts Enabling the Syntheses of Recyclable High Molecular Weight Salicylic Acid-Based Copolyesters. *Angew Chem Int Ed* **2023**, *62* (44), e202306759. <https://doi.org/10.1002/anie.202306759>.
- (173) Pedna, A.; Rosi, L.; Frediani, M.; Frediani, P. High Glass Transition Temperature Polyester Coatings for the Protection of Stones. *J of Applied Polymer Sci* **2015**, *132* (30), app.42323. <https://doi.org/10.1002/app.42323>.

- (174) Baker, G. L.; Jing, F.; III, M. R. S. Degradable 1,4-Benzodioxepin-3-Hexyl-2,5-Dione Monomer Derived Polymer with a High Glass Transition Temperature. US7923528B2, April 12, 2011. <https://patents.google.com/patent/US7923528B2/en> (accessed 2026-04-07).
- (175) Kim, H. J.; Peng, X.; Shin, Y.; Hillmyer, M. A.; Ellison, C. J. Blend Miscibility of Poly(Ethylene Terephthalate) and Aromatic Polyesters from Salicylic Acid. *J. Phys. Chem. B* **2021**, *125* (1), 450–460. <https://doi.org/10.1021/acs.jpcc.0c09322>.
- (176) Fastnacht, K. V.; Spink, S. S.; Dharmaratne, N. U.; Pothupitiya, J. U.; Datta, P. P.; Kiesewetter, E. T.; Kiesewetter, M. K. Bis- and Tris-Urea H-Bond Donors for Ring-Opening Polymerization: Unprecedented Activity and Control from an Organocatalyst. *ACS Macro Lett.* **2016**, *5* (8), 982–986. <https://doi.org/10.1021/acsmacrolett.6b00527>.
- (177) Salas-Ambrosio, P.; Vexler, S.; P S, R.; Chen, I. A.; Maynard, H. D. Caffeine and Cationic Copolymers with Antimicrobial Properties. *ACS Bio Med Chem Au* **2023**. <https://doi.org/10.1021/acsbiochemau.2c00077>.
- (178) Kanazawa, A.; Ikeda, T.; Endo, T. Antibacterial Activity of Polymeric Sulfonium Salts. *J. Polym. Sci. A Polym. Chem.* **1993**, *31* (11), 2873–2876. <https://doi.org/10.1002/pola.1993.080311126>.
- (179) Kanazawa, A.; Ikeda, T.; Endo, T. Polymeric Phosphonium Salts as a Novel Class of Cationic Biocides. II. Effects of Counter Anion and Molecular Weight on Antibacterial Activity of Polymeric Phosphonium Salts. *J. Polym. Sci. A Polym. Chem.* **1993**, *31* (6), 1441–1447. <https://doi.org/10.1002/pola.1993.080310611>.
- (180) Flemming, H.-C.; Van Hullebusch, E. D.; Neu, T. R.; Nielsen, P. H.; Seviour, T.; Stoodley, P.; Wingender, J.; Wuertz, S. The Biofilm Matrix: Multitasking in a Shared Space. *Nat Rev Microbiol* **2023**, *21* (2), 70–86. <https://doi.org/10.1038/s41579-022-00791-0>.
- (181) Kolpen, M.; Jensen, P. Ø.; Faurholt-Jepsen, D.; Bjarnsholt, T. Prevalence of Biofilms in Acute Infections Challenges a Longstanding Paradigm. *Biofilm* **2022**, *4*, 100080. <https://doi.org/10.1016/j.bioflm.2022.100080>.
- (182) Krumm, C.; Hijazi, M.; Trump, S.; Saal, S.; Richter, L.; Noschmann, G. G. F. K.; Nguyen, T.-D.; Preslikoska, K.; Moll, T.; Tiller, J. C. Highly Active and Selective Telechelic Antimicrobial Poly(2-Oxazoline) Copolymers. *Polymer* **2017**, *118*, 107–115. <https://doi.org/10.1016/j.polymer.2017.04.074>.
- (183) Richter, L.; Hijazi, M.; Arfeen, F.; Krumm, C.; Tiller, J. C. Telechelic, Antimicrobial Hydrophilic Polycations with Two Modes of Action. *Macromolecular Bioscience* **2018**, *18* (4), 1700389. <https://doi.org/10.1002/mabi.201700389>.
- (184) Jung, K.; Corrigan, N.; Wong, E. H. H.; Boyer, C. Bioactive Synthetic Polymers. *Advanced Materials* **2022**, *34* (2), 2105063. <https://doi.org/10.1002/adma.202105063>.
- (185) Manoukian, O. S.; Sardashti, N.; Stedman, T.; Gailunas, K.; Ojha, A.; Penalosa, A.; Mancuso, C.; Hobert, M.; Kumbar, S. G. Biomaterials for Tissue Engineering and Regenerative Medicine. In *Encyclopedia of Biomedical Engineering*; Elsevier, 2019; pp 462–482. <https://doi.org/10.1016/B978-0-12-801238-3.64098-9>.
- (186) Venkatesh, M.; Barathi, V. A.; Goh, E. T. L.; Anggara, R.; Fazil, M. H. U. T.; Ng, A. J. Y.; Harini, S.; Aung, T. T.; Fox, S. J.; Liu, S.; Yang, L.; Barkham, T. M. S.; Loh, X. J.; Verma,

- N. K.; Beuerman, R. W.; Lakshminarayanan, R. Antimicrobial Activity and Cell Selectivity of Synthetic and Biosynthetic Cationic Polymers. *Antimicrobial Agents and Chemotherapy* **2017**, *61* (10), 10.1128/aac.00469-17. <https://doi.org/10.1128/aac.00469-17>.
- (187) Zheng, M.; Pan, M.; Zhang, W.; Lin, H.; Wu, S.; Lu, C.; Tang, S.; Liu, D.; Cai, J. Poly(α -L-Lysine)-Based Nanomaterials for Versatile Biomedical Applications: Current Advances and Perspectives. *Bioact Mater* **2020**, *6* (7), 1878–1909. <https://doi.org/10.1016/j.bioactmat.2020.12.001>.
- (188) Bevilacqua, M. P.; Huang, D. J.; Wall, B. D.; Lane, S. J.; Edwards, C. K.; Hanson, J. A.; Benitez, D.; Solomkin, J. S.; Deming, T. J. Amino Acid Block Copolymers with Broad Antimicrobial Activity and Barrier Properties. *Macromol Biosci* **2017**, *17* (10). <https://doi.org/10.1002/mabi.201600492>.
- (189) Salas-Ambrosio, P.; Vexler, S.; P Sivasankaran, R.; Vlahakis, N.; Lai, R. S.; Johnson, C.; Baas-Maynard, S. I.; Min, D. S.; Lower, H.; Doyle, A. G.; Tang, Y.; Rodriguez, J. A.; Chen, I. A.; Read De Alaniz, J.; Maynard, H. D. Biosourced Functional Hydroxybenzoate-Co -Lactide Polymers with Antimicrobial Activity. *J. Am. Chem. Soc.* **2025**, *147* (22), 19230–19238. <https://doi.org/10.1021/jacs.5c04624>.
- (190) Wan, P.; Wang, Y.; Guo, W.; Song, Z.; Zhang, S.; Wu, H.; Yan, W.; Deng, M.; Xiao, C. Low-Molecular-Weight Polylysines with Excellent Antibacterial Properties and Low Hemolysis. *ACS Biomater. Sci. Eng.* **2022**, *8* (2), 903–911. <https://doi.org/10.1021/acsbiomaterials.1c01527>.
- (191) Kadlecova, Z.; Baldi, L.; Hacker, D.; Wurm, F. M.; Klok, H.-A. Comparative Study on the In Vitro Cytotoxicity of Linear, Dendritic, and Hyperbranched Polylysine Analogues. *Biomacromolecules* **2012**, *13* (10), 3127–3137. <https://doi.org/10.1021/bm300930j>.
- (192) Hall, A.; Wu, L.-P.; Parhamifar, L.; Moghimi, S. M. Differential Modulation of Cellular Bioenergetics by Poly(L-Lysine)s of Different Molecular Weights. *Biomacromolecules* **2015**, *16* (7), 2119–2126. <https://doi.org/10.1021/acs.biomac.5b00533>.
- (193) Peng, H.; Vexler, S.; Xu, S.; Chen, I. A. Real-Time in Vivo Bacterial Imaging by Computed Tomography and Fluorescence Using Phage-Gold Nanorod Bioconjugates as Contrast Agents. *ACS Biomaterials Science & Engineering* **2025**. <https://doi.org/10.1021/acsbiomaterials.4c02190>.
- (194) Sæbø, I. P.; Bjørås, M.; Franzyk, H.; Helgesen, E.; Booth, J. A. Optimization of the Hemolysis Assay for the Assessment of Cytotoxicity. *Int J Mol Sci* **2023**, *24* (3), 2914. <https://doi.org/10.3390/ijms24032914>.
- (195) Seemann, T. Snippy: Rapid Haploid Variant Calling and Core SNP Phylogeny, 2015. <https://github.com/tseemann/snippy> (accessed 2025-09-25).
- (196) Morrison, C. A.; Chan, E. P.; Lee, T.; Deming, T. J. Switchable Coacervate Formation via Amino Acid Functionalization of Poly(Dehydroalanine). *Biomacromolecules* **2024**, *25* (4), 2554–2562. <https://doi.org/10.1021/acs.biomac.4c00048>.
- (197) Yang, Y.; Chen, I. A. Visualization of Engineered M13 Phages Bound to Bacterial Targets by Transmission Electron Microscopy. In *Phage Engineering and Analysis: Methods and Protocols*; Peng, H., Liu, J., Chen, I. A., Eds.; Springer US: New York, NY, 2024; pp 175–183. https://doi.org/10.1007/978-1-0716-3798-2_12.

- (198) Iwasawa, A.; Ayaki, M.; Niwano, Y. Cell Viability Score (CVS) as a Good Indicator of Critical Concentration of Benzalkonium Chloride for Toxicity in Cultured Ocular Surface Cell Lines. *Regulatory Toxicology and Pharmacology* **2013**, *66* (2), 177–183. <https://doi.org/10.1016/j.yrtph.2013.03.014>.
- (199) Pal, T.; Ghosh, B.; Mukherjee, K.; Giri, T. K. Progress and Prospects of Synthetic Antimicrobial Polymers for the Treatment of Infectious Diseases. *Journal of Drug Delivery Science and Technology* **2025**, *111*, 107126. <https://doi.org/10.1016/j.jddst.2025.107126>.
- (200) Williams, S. C.; Chosy, M. B.; Jons, C. K.; Dong, C.; Prossnitz, A. N.; Liu, X.; Hernandez, H. L.; Cegelski, L.; Appel, E. A. Polyacrylamide-Based Antimicrobial Copolymers to Replace or Rescue Antibiotics. *ACS Cent. Sci.* **2025**, *11* (3), 486–496. <https://doi.org/10.1021/acscentsci.4c01973>.
- (201) CLSI. *Performance Standards for Antimicrobial Susceptibility Testing. 30th Edition. CLSI Supplement M100.*; Clinical and Laboratory Standards Institute (CLSI): USA, 2020; Vol. 40.
- (202) MacLowry, J. D.; Jaqua, M. J.; Selepak, S. T. Detailed Methodology and Implementation of a Semiautomated Serial Dilution Microtechnique for Antimicrobial Susceptibility Testing. *Appl Microbiol* **1970**, *20* (1), 46–53. <https://doi.org/10.1128/am.20.1.46-53.1970>.
- (203) Gardete, S.; Wu, S. W.; Gill, S.; Tomasz, A. Role of VraSR in Antibiotic Resistance and Antibiotic-Induced Stress Response in Staphylococcus Aureus. *Antimicrob Agents Chemother* **2006**, *50* (10), 3424–3434. <https://doi.org/10.1128/AAC.00356-06>.
- (204) Alcock, B. P.; Huynh, W.; Chalil, R.; Smith, K. W.; Raphenya, A. R.; Wlodarski, M. A.; Edalatmand, A.; Petkau, A.; Syed, S. A.; Tsang, K. K.; Baker, S. J. C.; Dave, M.; McCarthy, M. C.; Mukiri, K. M.; Nasir, J. A.; Golbon, B.; Imtiaz, H.; Jiang, X.; Kaur, K.; Kwong, M.; Liang, Z. C.; Niu, K. C.; Shan, P.; Yang, J. Y. J.; Gray, K. L.; Hoad, G. R.; Jia, B.; Bhando, T.; Carfrae, L. A.; Farha, M. A.; French, S.; Gordzevich, R.; Rachwalski, K.; Tu, M. M.; Bordeleau, E.; Dooley, D.; Griffiths, E.; Zubyk, H. L.; Brown, E. D.; Maguire, F.; Beiko, R. G.; Hsiao, W. W. L.; Brinkman, F. S. L.; Van Domselaar, G.; McArthur, A. G. CARD 2023: Expanded Curation, Support for Machine Learning, and Resistome Prediction at the Comprehensive Antibiotic Resistance Database. *Nucleic Acids Res* **2023**, *51* (D1), D690–D699. <https://doi.org/10.1093/nar/gkac920>.
- (205) Hanssen, A.-M.; Ericson Sollid, J. U. SCCmec in Staphylococci: Genes on the Move. *FEMS Immunology & Medical Microbiology* **2006**, *46* (1), 8–20. <https://doi.org/10.1111/j.1574-695X.2005.00009.x>.
- (206) Yang, S.-J.; Bayer, A. S.; Mishra, N. N.; Meehl, M.; Ledala, N.; Yeaman, M. R.; Xiong, Y. Q.; Cheung, A. L. The Staphylococcus Aureus Two-Component Regulatory System, GraRS, Senses and Confers Resistance to Selected Cationic Antimicrobial Peptides. *Infect Immun* **2012**, *80* (1), 74–81. <https://doi.org/10.1128/IAI.05669-11>.
- (207) Rutherford, S. T.; Bassler, B. L. Bacterial Quorum Sensing: Its Role in Virulence and Possibilities for Its Control. *Cold Spring Harb Perspect Med* **2012**, *2* (11), a012427. <https://doi.org/10.1101/cshperspect.a012427>.
- (208) Falord, M.; Karimova, G.; Hiron, A.; Msadek, T. GraXSR Proteins Interact with the VraFG ABC Transporter To Form a Five-Component System Required for Cationic

- Antimicrobial Peptide Sensing and Resistance in *Staphylococcus Aureus*. *Antimicrobial Agents and Chemotherapy* **2012**, 56 (2), 1047–1058. <https://doi.org/10.1128/aac.05054-11>.
- (209) Chen, L.; Wang, Z.; Xu, T.; Ge, H.; Zhou, F.; Zhu, X.; Li, X.; Qu, D.; Zheng, C.; Wu, Y.; Zhao, K. The Role of *graRS* in Regulating Virulence and Antimicrobial Resistance in Methicillin-Resistant *Staphylococcus Aureus*. *Front Microbiol* **2021**, 12, 727104. <https://doi.org/10.3389/fmicb.2021.727104>.
- (210) Liu, X.; Wang, Y.; Chang, W.; Dai, Y.; Ma, X. *AgrA* Directly Binds to the Promoter of *vraSR* and Downregulates Its Expression in *Staphylococcus Aureus*. *Antimicrob Agents Chemother* **2024**, 68 (3), e0089323. <https://doi.org/10.1128/aac.00893-23>.
- (211) Cui, L.; Neoh, H.; Shoji, M.; Hiramatsu, K. Contribution of *vraSR* and *graSR* Point Mutations to Vancomycin Resistance in Vancomycin-Intermediate *Staphylococcus Aureus*. *Antimicrob Agents Chemother* **2009**, 53 (3), 1231–1234. <https://doi.org/10.1128/AAC.01173-08>.
- (212) Hagh Ranjbar, H.; Hosseini-Abari, A.; Ghasemi, S. M.; Hafezi Birgani, Z. Antibacterial Activity of Epsilon-Poly-L-Lysine Produced by *Stenotrophomonas Maltophilia* HS4 and *Paenibacillus Polymyxa* HS5, Alone and in Combination with Bacteriophages. *Microbiology (Reading)* **2023**, 169 (7), 001363. <https://doi.org/10.1099/mic.0.001363>.
- (213) Shalitin, C.; Danon, D.; Katchalski, E. Inactivation of *Escherichia Coli* Bacteriophage T2 by Poly-L-Lysine. I. Nature of the Inactivation Process. *Archives of Biochemistry and Biophysics* **1962**, 99 (3), 494–507. [https://doi.org/10.1016/0003-9861\(62\)90298-9](https://doi.org/10.1016/0003-9861(62)90298-9).
- (214) Lamboy, J. A.; Tam, P. Y.; Lee, L. S.; Jackson, P. J.; Avrantinis, S. K.; Lee, H. J.; Corn, R. M.; Weiss, G. A. Chemical and Genetic Wrappers for Improved Phage and RNA Display. *Chembiochem* **2008**, 9 (17), 2846–2852. <https://doi.org/10.1002/cbic.200800366>.
- (215) Cushnie, T. P. T.; Cushnie, B.; Echeverría, J.; Fowsantear, W.; Thammawat, S.; Dodgson, J. L. A.; Law, S.; Clow, S. M. Bioprospecting for Antibacterial Drugs: A Multidisciplinary Perspective on Natural Product Source Material, Bioassay Selection and Avoidable Pitfalls. *Pharm Res* **2020**, 37 (7), 125. <https://doi.org/10.1007/s11095-020-02849-1>.
- (216) Greco, I.; Molchanova, N.; Holmedal, E.; Jenssen, H.; Hummel, B. D.; Watts, J. L.; Håkansson, J.; Hansen, P. R.; Svenson, J. Correlation between Hemolytic Activity, Cytotoxicity and Systemic in Vivo Toxicity of Synthetic Antimicrobial Peptides. *Sci Rep* **2020**, 10, 13206. <https://doi.org/10.1038/s41598-020-69995-9>.
- (217) *COVID-19: U.S. Impact on Antimicrobial Resistance, Special Report 2022*; National Center for Emerging and Zoonotic Infectious Diseases, 2022. <https://doi.org/10.15620/cdc:117915>.
- (218) Emdin, F.; Orubu, E. S. F.; Rogers Van Katwyk, S.; Strong, K.; Shaver, N.; Abdullah, K.; Asamoah, G.; Skidmore, B.; Chan, E.; Poirier, M. J. P. COVID-19 Impact on AMR: A Rapid Scoping Review, Equity Analysis and Evidence Gap Map Study. *BMJ Glob Health* **2025**, 10 (11), e018118. <https://doi.org/10.1136/bmjgh-2024-018118>.
- (219) Zhang, T.; Wu, Y.; Tian, Y.; Wang, Y.; Zhang, P.; Shi, Q.; Fang, Q.; Pan, J.; Jin, Q.; Ji, J. AI-Guided Precise Design of Antimicrobial Polymers through High-Throughput Screening Technology on an Automated Platform. *Bioactive Materials* **2026**, 58, 472–485. <https://doi.org/10.1016/j.bioactmat.2025.12.020>.

- (220) Kundi, V.; Jin, Y.; Chandrasekaran, A.; Muralidhar, A.; Masood, H.; Boyer, C.; Kumar, P. Machine Learning Prediction of Antibacterial Activity of Block Copolymers. *ACS Appl. Nano Mater.* **2024**, 7 (8), 8939–8948. <https://doi.org/10.1021/acsanm.4c00430>.
- (221) Titlow, W. B.; Lee, C.-H.; Ryou, C. Characterization of Toxicological Properties of L-Lysine Polymers in CD-1 Mice. *J Microbiol Biotechnol* **2013**, 23 (7), 1015–1022. <https://doi.org/10.4014/jmb.1302.02055>.
- (222) Fu, Z.; Li, S.; Han, S.; Shi, C.; Zhang, Y. Antibody Drug Conjugate: The “Biological Missile” for Targeted Cancer Therapy. *Sig Transduct Target Ther* **2022**, 7 (1), 93. <https://doi.org/10.1038/s41392-022-00947-7>.
- (223) Di Gregorio, E.; Israel, S.; Staelens, M.; Tankel, G.; Shankar, K.; Tuszyński, J. A. The Distinguishing Electrical Properties of Cancer Cells. *Physics of Life Reviews* **2022**, 43, 139–188. <https://doi.org/10.1016/j.plrev.2022.09.003>.