4-5-2006

Antibiotic-Conjugated Polyacrylate Nanoparticles: New Opportunities for Development of Anti-MRSA Agents

Yang Wang
University of South Florida

Follow this and additional works at: https://digitalcommons.usf.edu/etd

Part of the American Studies Commons

Scholar Commons Citation
Antibiotic-Conjugated Polyacrylate Nanoparticles: New Opportunities for Development of Anti-MRSA Agents

by

Yang Wang

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy
Department of Chemistry
College of Arts and Sciences
University of South Florida

Major Professor: Edward Turos, Ph.D.
Julie P. Harmon, Ph.D.
Bill J. Baker, Ph.D.
Kirpal S. Bisht, Ph.D.

Date of Approval:
April 5th, 2006

Keywords: β-Lactam, Staphylococcus, Drug delivery, Nanoparticle

© Copyright 2006, Yang Wang
ACKNOWLEDGMENTS

The completion of this research would not have been possible without the assistance and guidance of many people within the Departments of Chemistry and Biology at the University of South Florida. First and foremost, I am grateful to my research advisor, Professor Edward Turos, who has taught me the art of organic chemistry and the value of research. Throughout the years, Professor Turos has maintained an environment rich in intellectual thought for which to mature in as a scientist. I am also grateful to him for granting me the freedom to conduct investigations that were of interest despite the occasional failed experiments. The phenomenal experience of working in Dr. Turos' laboratory will serve me well in the future endeavors for which I will always be indebted.

During my studies at the University of South Florida, I was obliged to receive direction from an outstanding committee. Firstly, Professor Kirpal Bisht, Julie Harmon and Bill Baker have been tremendous advisors and have made my research experience over recent years an enjoyable one.

While in Professor Turos' laboratory, I had the privilege to work with a number of outstanding scientists. I am grateful to all my current and former colleagues for their assistance and support. First and foremost, my former labmates who initiated this project: Timothy E. Long, Cristina M. Coates, Bart A. Heldreth, and Jeung-Yeop Shim. In addition, I would like to thank two labmates who joined the group at the same time: Sampath Abeylath and J. Michelle Leslie. In later years, several other remarkable individuals who joined the lab and had the same influence: Dr. Seyoung Jang, Julio Garay, Kerriann Greenhalgh, Marcie Culbreath, Proveen, Kevin Revell, Dr. Ivan Perez, Dr. Suresh Reddy, Dr. Tyler Schertz and Dr. Rajesh Mishra. It was truly a wonderful and pleasurable experience working with them over the years. Finally, I would like to thank the talented undergraduate researchers whom I was honored to assist in their experiments: Casey Gooden, Setu and Erin Brady.

I would also like to acknowledge the technical assistance of Sonja Dickey. Her efforts made all the biological studies possible. Moreover, the microscopy studies were made possible with the aid of Betty Loraamm.

Finally, I want to thank my parents, Fang Wang and Xiuwen Yang, my husband, Tong Luo who also graduated as a PhD in computer science from USF and his parents. They have provided everything needed to succeed in my life, for which I am deeply grateful. Without their love and support, I would never have been able to accomplish any of this work. This is as well extended to all my family members back in China including my sister and brother-in-law and my grandmother. Finally, I would thank my great fellows from China and all international friends, and they make my life in Tampa wonderful and memorable.
TABLE OF CONTENTS

LIST OF TABLES vi
LIST OF FIGURES vii
LIST OF SCHEMES x
LIST OF SPECTRA xii
LIST OF ABBREVIATIONS xvi
ABSTRACT xvii

CHAPTER 1 CLINICAL DEVELOPMENT OF NEW MRSA ANTIBIOTICS 1

1.1 Introduction 1

1.2 Antibiotics Resistance 4
   1.2.1 Intrinsic Resistance and Acquired Resistance 4
   1.2.2 Urgent Problems of Resistance 5

1.3 Clinical Development of Anti-MRSA Antibiotics 5
   1.3.1 \(\beta\)-Lactam Antibiotics 5
   1.3.2 Glycopeptides and Vancomycin 6
   1.3.3 Oxazolidinones 6
   1.3.4 Quinolones and Fluoroquinolones 7

1.4 Conclusions 7

CHAPTER 2 SYNTHESIS AND BIOLOGICAL PROPERTIES OF C3-SUBSTITUTED N-THIOLATED \(\beta\)-LACTAMS 8

2.1 Introduction 8

2.2 Synthesis of C3-Sulfonate N-Thiolated \(\beta\)-Lactams 9
   2.2.1 Synthesis of C-Aryl (imines) 6 10
   2.2.2 Synthesis of N-Aryl Protected \(\beta\)-Lactams by Staudinger Coupling 10
   2.2.3 Hydrolysis of N-Aryl Protected \(\beta\)-Lactams 8a and 8b 12
   2.2.4 Synthesis of C3-Sulfonate N-Aryl Protected \(\beta\)-Lactams 10 12
   2.2.5 Dearylation of C3-Sulfonate N-Aryl Protected \(\beta\)-Lactams 10 with Ceric Ammonium Nitrate 13
   2.2.6 N-Methylation of C3-Sulfonate \(\beta\)-Lactams 13
   2.2.7 The Structure-Activity Profiling of C3-Sulfonated \(\beta\)-Lactam 3 14
   2.2.8 Fluorescent Sulfonated Lactam 18 16

2.3 C3-Amino \(\beta\)-Lactams 18
   2.3.1 Synthesis of C3-Amino N-Thiolated \(\beta\)-Lactams 19a-d and Azido-Lactam 19e 19
   2.3.2 The Structure-Activity Profiling of C3-Amino \(\beta\)-Lactams 19a-d and C3- Azido Lactam 19e 20
2.4 Additional Biological Activity Screening for N-Thiolated β-Lactam Analogues 21
  2.4.1 Other Bacteria Strains: Activity against Bacillus 21
  2.4.2 Activities against Bacillus Strains 22
2.5 Anticancer Studies of C3-Substituted β-Lactams 23
  2.5.1 SAR Studies for C3-Sulfonated Lactams 24
  2.5.2 Synthesis and SAR Studies of Racemic and Optical Active C3-Acrylated Lactams 24
2.6 Animal Testing of Lactam 25
2.7 Conclusions 29

CHAPTER 3 NANOPARTICLE DELIVERY VEHICLES FOR ANTIBIOTICS 30

3.1 Introduction 30
3.2 Classification of Drug Delivery Systems 31
3.3 Major Development of Delivery Systems for Antibiotics 31
  3.3.1 Liposomes 31
  3.3.2 Hydrogels 33
  3.3.3 Nanoparticles 34
3.4 Poly (alkyl cyanoacrylate) (PACA) Nanoparticles 36
  3.4.1 Poly (ethyl cyanoacrylate) Nanoparticles 36
  3.4.2 Ciprofloxacin-Loaded Poly (2-ethylbutyl cyanoacrylate) Nanoparticles 38
  3.4.3 Poly (isobutyl cyanoacrylate) and Poly (isohexyl cyanoacrylate) Nanoparticles 41
3.5 PLGA Nanoparticles 42
  3.5.1 PLGA Nanoparticles Delivering Hydrophobic Drugs 43
  3.5.2 PLA/PLGA Nanoparticles Loading a Hydrophilic Drug: Gentamicin 44
3.6 Surface-Coated Metal Nanoparticles 45
  3.6.1 Drug Coated Gold Nanoparticles 46
  3.6.2 Porous Hollow Silica Nanoparticles 49
3.7 Magnetic Nanoparticles 50
3.8 Conclusions and Future Perspectives in Antibiotics Delivery 50

CHAPTER 4 NOVEL POLYACRYLATE NANOPARTICLES FOR DELIVERY OF β-LACTAM ANTIBIOTICS 52

4.1 Introduction 52
4.2 Emulsion Polymerization 52
  4.2.1 Introduction 52
  4.2.2 Advantages of Emulsion Polymerization and Applications 54
4.3 Microemulsion Polymerization 54
4.4 Main Components for Microemulsion Polymerization 55
  4.4.1 Choice of Monomer 55
  4.4.2 Choice of Co-Monomer 55
  4.4.3 Choice of Surfactant 56
  4.4.4 Choice of Radical Initiator 57
  4.4.5 Choice of Aqueous Media 58
  4.4.6 Choice of Polymerization Temperature 58
4.5 Polyacrylate Emulsion
   4.5.1 Prior Applications of Polyacrylate Emulsion in the Biomedical Area 59
   4.5.2 Recent Development of Polyacrylate Emulsion in Antibacterial Application 59

4.6 Expansion of Polyacrylate Emulsions 64
   4.6.1 Preparation of Poly (butyl acrylate and styrene) Nanoparticles 64
   4.6.2 Synthesis of Poly (butyl acrylate-methyl methacrylate) Nanoparticles \textbf{NP 4} 66
   4.6.3 Poly (butyl acrylate-styrene-lactam 27) \textbf{NP 5b} 67
   4.6.4 Physical Properties of Poly (butyl acrylate-styrene) and Poly (butyl acrylate-
methyl methacrylate) Nanoparticle Emulsions 67

4.7 Choice of Other Acrylated Drug Monomers 77
   4.7.1 Synthesis of C\textsubscript{4}-Acrylate Lactam 37
   4.7.2 Synthesis of Diacrylate Lactam 38
   4.7.3 Synthesis of C\textsubscript{3}-Long Chain Triester Lactam 39

4.8 Preparation of Polyacrylate Nanoparticles with the Three Drug Monomers \textbf{37-39} 81

4.9 Physical Properties of Polyacrylate Nanoparticles \textbf{NP 6-8} 84
   4.9.1 \textsuperscript{1}H NMR 84
   4.9.2 Film Formation 85
   4.9.3 Transmission Electron Microscopy (TEM) 85
   4.9.4 Dynamic Light Scattering (DLS) and Zeta Potential Analysis 86

4.10 Biological Activities of Nanoparticles 86
   4.10.1 Antibacterial Testing 86
   4.10.2 Results and Discussion of Antibacterial Testing 86

4.11 Mode of Action Studies 88

4.12 Conclusions 89

CHAPTER 5 NOVEL POLYACRYLAMIDE NANOPARTICLES FOR DELIVERY OF
\textit{N}-THIOLATED \textit{\beta}-LACTAM ANTIBIOTICS 91

5.1 Introduction 91

5.2 Targeted Functional Monomers 91
   5.2.1 Introduction 91
   5.2.2 Functional Acrylamide Monomer 92

5.3 Synthesis of \textit{N}-Acrylated Amino Acid Monomers 93
   5.3.1 \textit{N}-Acryloyl Glycine (53) 94
   5.3.2 \textit{N}-Acryloyl Alanine (55) 96
   5.3.3 \textit{N}-Acryloyl Valine (57) 98
   5.3.4 \textit{N}-Acryloyl Proline (59) 99
   5.3.5 \textit{N}-Acryloyl Methionine (61) 101
   5.3.6 \textit{N}-Acryloyl Threonine Derivative (64) 103

5.4 Emulsion Polymerization of Acryloyl Amino Acid-Linked Nanoparticles 104
   5.4.1 Procedure and Formulation 104
   5.4.2 Physical Properties of Polyacrylamide Nanoparticles 105

5.5 Biological Activities against \textit{S. aureus} and MRSA 106
   5.5.1 Monomer Activities against \textit{S. aureus} and MRSA 106
   5.5.2 Polymer Activities against \textit{S. aureus} and MRSA 107

5.6 Conclusions and Future Work 112
CHAPTER 6  MATERIALS AND METHODS 113

6.1 Synthetic Procedures 113
6.1.1 Preparation of N-Anisylimines (6) 113
6.1.2 Preparation of Acid Chlorides 113
6.1.3 Preparation of N-4-Anisyl Azetidin-2-ones 8 114
6.1.4 Hydrolysis of N-Aryl Protected β-Lactam 8 115
6.1.5 Substitution of Sulfonyl at C3 Lactam 115
6.1.6 N-Dearylation of N-(4-Methoxyphenyl) Substituted Lactams 116
6.1.7 Procedure for the N-Methylthiolation of Lactams 3 117
6.1.8 Preparation of Sulfur-Transfer Reagents: N-methylthiophthalimide 14 117
6.1.9 Fluorescent lactam 18 118
6.1.10 Amino-Substituted Lactam 119
6.1.11 Synthesis of Azido Lactam 19e 121
6.1.12 Synthesis of Boc-Protected Lactam 25 122
6.1.13 Racemic Lactam 27 122
6.1.14 Enantiopure Acrylate Lactams (+)-27 and (-)-27 123
6.1.15 C3-Acrylate Lactam 37 125
6.1.16 Diacrylate Lactam 38 126
6.1.17 C2-Long Chain Lactam 39 128
6.1.18 Glycine-Attached Lactam 54 129
6.1.19 Alanine-Attached Lactam 56 130
6.1.20 Valine-Attached Lactam 58 130
6.1.21 Proline-Attached Lactam 58 131
6.1.22 Methionine-Attached Lactam 62 132
6.1.23 Threonine-Attached Lactam 65 132
6.1.24 Methionine-Attached Lactam 67 133
6.1.25 Dihydroxy Lactam 68 134

6.2 Emulsion Polymerization 134
6.2.1 General procedure for preparation of emulsion polymerization 134
6.2.2 Formulation of Emulsion Polymerization 135

6.3 Physical Properties Analysis 135
6.3.1 Particle size determination 135
6.3.2 Zeta Potential Analysis 136
6.3.3 Microscopy 136

6.4 Microbiological Test Procedures 136
6.4.1 Antimicrobial Susceptibility Test 136
6.4.2 MIC Determinations 136

6.5 Interaction of Nanoparticles with S. aureus Cells 137
6.5.1 Whole cell analysis 137
6.5.2 Sectioned cell analysis 137

CHAPTER 7  1H and 13 C NMR SPECTRA 138

REFERENCE 175

ABOUT THE AUTHOR
LIST OF TABLES

Table 2.1: Well-diffusion tests against *Bacillus anthracis*: zone of growth inhibition measured in mm. 23

Table 4.1: Antibacterial activity of the antimicrobial monomer (27) and the resulting nanoparticle emulsion NP 5a with the control emulsion NP 1. All zone of inhibition (ZOI) results are reported for 20 µg of both drug monomer and the emulsion. Minimal inhibitory concentration (MIC) results were obtained using either broth dilution or agar dilution assay procedures. 64

Table 4.2: Particle size and zeta potential analysis of nanoparticles NP 2-5. 70

Table 4.3: Dynamic light scattering analysis of poly (butyl acrylate-styrene) NP 3a-3e. 70

Table 4.4: Dynamic light scattering analysis of poly (butyl acrylate-styrene) nanoparticles NP 3a*, NP 3f and NP 3f*. 71

Table 4.5: Average particle size (nm in diameter) for different nanoparticles NP 3f after being stored for 3 days. 71

Table 4.6: Average particle size (nm in diameter) for different nanoparticles NP 5f after being stored for 3 days. 72

Table 4.7: Average particle size (nm in diameter) for different nanoparticles NP 3f and NP 5f after being stored for one month at 5 °C. 72

Table 4.8: Average particle size (nm in diameter) of diluted samples of NP 4 after being stored for 3 days and one month at the indicated temperature. 73

Table 4.9: Comparison of average particle size for nanoparticle NP 3f* in different pH buffer solutions. 75

Table 4.10: Comparison of average particle size for 6-month old nanoparticle emulsions NP 3a in different pH buffer solutions. 75

Table 4.11: Antibacterial activity of acrylated monomers 27 and the resulting nanoparticle emulsions NP 5. All zone of inhibition (ZOI) results are reported for 20 µg of both drug monomers and emulsions. Minimal inhibitory concentration (MIC) results were obtained using either broth dilution or agar dilution assay procedures. 76

Table 4.12: Comparison of particle sizes of nanoparticle emulsion NP 5d after extraction. 84

Table 4.13: Particle size and zeta potential analysis of nanoparticle NP 6-8. 86

Table 4.14: Antibacterial activity of acrylated monomers 37, 38 and 39 and the resulting nanoparticle emulsions NP 6b, NP 7 and NP 8. All zone of inhibition (ZOI) results are reported for samples containing 20 µg of both drug monomers and emulsions. Minimal inhibitory concentration (MIC) results were obtained using either broth dilution or agar dilution assay procedures. 87

Table 5.1: Comparison of average particle size (nm) and surface charge (-mV) of each polyacrylamide nanoparticle. 106
LIST OF FIGURES

Figure 1.1: Picture of inhibition of bacteria on an agar by fungus Penicillium notatum. 1
Figure 1.2: Structures of penicillin G and penicillin F. 1
Figure 1.3: Analogues of penicillins and cephalosporins. 2
Figure 1.4: Structures of families of β-lactam antibiotics. 2
Figure 1.5: Structure of peptidoglycan. 3
Figure 1.6: Conformational representations of penicillin and acyl-D-Ala-D-Ala. 4
Figure 1.7: Structure of N-thiolated β-lactams. 6
Figure 1.8: Structure of vancomycin. 6
Figure 1.9: Structure of linezolid. 6
Figure 1.10: Structures of quinolone DK-507k and olamufloxacin, fluoroquinolones DW-116 and BMS-284756 and nalidixic acid. 7
Figure 2.1: N-thiolated β-lactam 1 and initial lead β-lactam 2. 8
Figure 2.2: C3-sulfonated N-thiolated β-lactam 3 and C3-amino β-lactam 4. 9
Figure 2.3: Kirby-Bauer well diffusion assay on agar plate measuring zone of inhibition. 15
Figure 2.4: Kirby-Bauer data for analogues 3a-3c against S. aureus and MRSA compared with penicillin G (Pen G) and vancomycin. 15
Figure 2.5: FRET pair concept. 18
Figure 2.6: C3-amino-substituted analogues 19a-d and azido-lactam 19e. 19
Figure 2.7: Comparison of the bioactivities of C3-amino β-lactams 19a-e and the reference standard antibiotics. 21
Figure 2.8: Bacillus strains. 21
Figure 2.9: Four C3-sulfonated lactams were selected for anti-cancer studies. 24
Figure 2.10: Structure-activity relationship (SAR) analysis of N-thiolated β-lactams 3b and 18 with DMSO as a control. 24
Figure 2.11: Structures of 27 and its stereoisomers. 25
Figure 2.12: (+)-Lactam 27 induces apoptosis selectively in tumorigenic cells. Leukemic Jurkat T and nontransformed YT cells were treated with lactam 1 or isomers of lactam 27 at 50 µM for 24 h. Cell death is given as a percent of dead cells over total cell population (+SD). 27
Figure 2.13: Both detached and attached VA-13 and WI-38 fibroblast cell populations were collected and stained with the nuclear staining dye Hoechst 33342. Each sample was then analyzed by fluorescence microscopy for nuclear morphology. 27
Figure 2.14: A) β-Lactam 25 inhibits the tumor growth in nude mice implanted by human breast cancer MDA-MB-231 cells. B) TUNEL (Terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling) analysis of tumor tissues in nude mice generated by human breast cancer MDA-MB-231 cells for DNA damages in tumor cells treated by β-lactam 25 at 0.3 mg/kg (low dose) or 3 mg/kg (high dose) or solvent as control for 30 days. Nuclei stained in dark blue indicate TUNEL-positive. 28
Figure 3.1: A) Structure of phospholipids; B) Formation of a phospholipid bilayer structure and C) the liposome. 32
Figure 3.2: A complete three-dimensional liposome structure split in half to show its internal structure. 33
Figure 3.3: A hydrogel formation with drug incorporated. 34
Figure 3.4: Types of nanoparticle delivery systems. 35
Figure 3.5: Structure of ampicillin. 37
Figure 3.6: Structure of surfactant Pluronic F68. 37
Figure 3.7: Structure of amoxicillin. 38
Figure 3.8: Structure of ciprofloxacin. 39
Figure 3.9: Equilibrium between the free base form and the hydrochloride form of the cipro. 40
Figure 3.10: Schematic model for ciprofloxacin-loaded nanoparticle formation, according to the physicochemical studies and as a function of the amount of ciprofloxacin initially added to the medium: 51.5% of ciprofloxacin entrapped inside the matrix as a chemically unmodified form; 18% of unbound ciprofloxacin (hydrochloride form); 30.5% of undetectable ciprofloxacin. 40
Figure 3.11: Structure of gentamicin. 41
Figure 3.12: Release of ciprofloxacin from nanoparticles dispersed in solutions of mannitol or other viscosifying agents. Diamonds stand for nanoparticles dispersed in mannitol solution (reference solution), triangles for dispersion in hydroxyethylcellulose (HEC), circles for dispersion in poloxamer and squares for dispersion in Carbopol 980. 43
Figure 3.13: Structure of benzathine penicillin G. 44
Figure 3.14: Structure of gentamicin. 45
Figure 3.15: Metal nanoparticle can be coated on the surface with bioactive molecules. 46
Figure 3.16: A possible interaction between vancomycin-capped gold nanoparticle and a VRE strain. 47
Figure 3.17: Cipro-gold nanoparticles. 48
Figure 3.18: Structures of gold nanoparticles for cytotoxicity testing. 48
Figure 3.19: Molecular structure of cefradine. 49
Figure 3.20: Formation of porous hollow silica nanoparticle. 49
Figure 3.21: Magnetic nanoparticle delivery system. 50
Figure 4.1: Adopted schematic illustration of an emulsion polymerization. 53
Figure 4.2: Illustration of formation of spherical micelles with an interior composed of the hydrocarbon chains and a surface of the polar head groups facing water. 53
Figure 4.3: Schematic representation of relative stability as a function of droplet size of the three classes of emulsions. 54
Figure 4.4: Structures of the anionic surfactants. 56
Figure 4.5: Structures of the non-ionic surfactants. 57
Figure 4.6: Structures of the cationic surfactants. 57
Figure 4.7: Structures of the zwitterionic surfactants. 57
Figure 4.8: Structures of potassium persulfate initiator. 58
Figure 4.9: A bottle of polyacrylate emulsion. 61
Figure 4.10: NMR for homopoly (ethyl acrylate) NP 1 and poly (ethyl acrylate-lactam 27) NP 5a. 61
Figure 4.11: Adopted illustration of the formation of film from the latex particles. 62
Figure 4.12: Evaporation of emulsion produces a thermoplastic thin film. 62
Figure 4.13: Scanning electron microscopy (SEM) of polyacrylate nanoparticles. 63
Figure 4.14: Formulations and reaction conditions for three polymers. 66
Figure 4.15: Formulation of preparation of NP 4. 66
Figure 4.16: NMR comparison of poly (styrene) NP 2 and poly (butyl acrylate-styrene) NP 3. 68
Figure 4.17: Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) of polyacrylate nanoparticles. 69
Figure 4.18: Particle size and distribution analysis by dynamic light scattering (DLS). 69
Figure 4.19: Particle size vs. concentration for different concentration of freshly-made polymer NP 3f. 71
Figure 4.20: Particle size vs. concentration for different concentrations of freshly-made polymer NP 5f. 72
Figure 4.21: Average particle size vs. concentration for nanoparticle NP 5f stored at 5 °C for one month. 73
Figure 4.22: Average particle sizes of poly (butyl acrylate-methyl methacrylate) nanoparticles NP 4 stored at different temperatures. 74
Figure 4.23: Average particle size of poly (butyl acrylate-methyl methacrylate) NP 4 (10% polymer content) stored at different temperatures for one month. 74
Figure 4.24: Comparison of particle size at different pH values for nanoparticle emulsions NP 3f*. 75
Figure 4.25: Comparison of particle size for nanoparticle emulsions NP 3a at different pH values. 75
Figure 4.26: General features of the desired drug monomer, and the initial β-lactam monomer 27. 77
Figure 4.27: Three monomer targets. 77
Figure 4.28: NMR comparison of polystyrene NP 2 and poly (butyl acrylate-styrene-lactam 37) NP 6a. 85
Figure 4.29: Transmission electron microscopy (TEM) of polyacrylate nanoparticles. 85
Figure 4.30: Comparison of bioactivities of acrylated monomers (27, 37, 38 and 39) and nanoparticles (NP 1, NP 3a, NP 5a, NP 5b, NP 6b, NP 7 and NP 8).

Figure 4.31: Transmission electron microscopy (TEM) image showing the polymer strands.

Figure 4.32: Schematic illustrating modes of delivery of an antibiotic-conjugated nanoparticle to a bacterial cell. In this depiction, the nanoparticle can release drug either while on the surface of the cell or after entry.

Figure 4.33: Transmission electron microscopy (TEM) images of *S. aureus* cells treated with poly (butyl acrylate-styrene) nanoparticles. (A) Sectioned cells with both spherical nanoparticles inside the cell and deformed nanoparticles on the surface. (B) Exterior of intact cells interacting with the control nanoparticles. The textured appearance in the cytoplasm of the non-dehydrated whole cells results from effervescence under the electron beam. (Images taken by Kerriann Greenhalgh)

Figure 5.1: Structure of acrylamide.

Figure 5.2: Proposed acrylamide functional monomer.

Figure 5.3: Structures of amino acid acrylamides used for emulsion polymerization.

Figure 5.4: NMR of lactam 54.

Figure 5.5: $^1$H NMR analysis of N-acryloyl alanine 55.

Figure 5.6: $^1$H NMR analysis of lactam 56.

Figure 5.7: $^1$H NMR analysis of lactam 58.

Figure 5.8: $^1$H NMR analysis of lactam 60.

Figure 5.9: $^1$H NMR analysis of lactam 62.

Figure 5.10: Formulations used for the preparation of polyacrylamide nanoparticles NP 9-12.

Figure 5.11: Comparison of bioactivities of acrylated monomers 54, 56, 58, 60 and 62.

Figure 5.12: Comparison of bioactivities of polyacrylamide nanoparticles versus their monomers.

Figure 5.13: Compounds subjected to anticancer screening in Dr. Q. Ping Dou’s laboratory (Wayne State University).

Figure 5.14: Trypan blue staining of Jurkat cells with the lactams after 24 hours of treatment.

Figure 5.15: Trypan blue staining of the same Jurkat cells after 48 hours of treatment with the lactams.

Figure 5.16: β-Lactam treatment of HL-60 cells after 24 hrs.

Figure 5.17: β-Lactam treatment of HL-60 cells after 48 hours.
LIST OF SCHEMES

Scheme 1.1: Proposed mechanism of the transpeptidation reaction (a) and the inhibition of the transpeptidase enzymes by penicillin (b). 3
Scheme 2.1: General synthetic route to lactam 3. 9
Scheme 2.2: Synthesis of N-aryl (imines) 6. 10
Scheme 2.3: Synthesis of N-aryl protected β-lactam 8 by Staudinger [2+2] condensation. 10
Scheme 2.4: Proposed mechanism of β-lactam formation by Staudinger reaction. 11
Scheme 2.5: Synthesis of acetoxyacetethyl chloride (7). 12
Scheme 2.6: Hydrolysis of N-Aryl Protected β-Lactams 8a and 8b. 12
Scheme 2.7: Formation of C3-Sulfonate N-Aryl Protected β-Lactams 10a-c. 12
Scheme 2.8: Oxidative dearylation of β-lactams 10. 13
Scheme 2.9: Synthesis of N-methylthio β-lactams 3 using N-methylthiophthalamide. 14
Scheme 2.10: Synthesis of N-methylthiophthalamide (14). 14
Scheme 2.11: Synthetic route to make dansyl-substituted β-lactam 11d. 17
Scheme 2.12: Alternative route for the synthesis of dansyl-substituted lactam 18. 17
Scheme 2.13: Synthesis of C3-amino-substituted lactams 19a-d. 19
Scheme 2.14: Synthesis of azido β-lactam 19e. 20
Scheme 2.15: Synthesis of C3-Boc-alanine lactam 25. 22
Scheme 2.16: Enzymatic kinetic resolution of acetoxy lactam 8b. 25
Scheme 2.17: Synthesis of racemic C3-acrylate β-lactam 27. 25
Scheme 2.18: Synthesis of (−)-27. 26
Scheme 2.19: Synthesis of (+)-27. 26
Scheme 3.11: Hypothetical model for liposome drug delivery. 33
Scheme 3.2: Emulsion polymerization of alkyl cyanoacrylate monomer. 36
Scheme 3.3: Formation of cipro-attached polyalkyl cyanoacrylate nanoparticles. 39
Scheme 3.4: Formation of PLGA. 43
Scheme 3.5: Formation of vancomycin-attached gold nanoparticles. 46
Scheme 4.1: Schematic illustration of a polymerization. 55
Scheme 4.2: Formation of polyacrylates. 59
Scheme 4.3: Synthesis of Polymeric N-Halamines. 59
Scheme 4.4: Synthesis of poly (ethyl acrylate) NP 1. 60
Scheme 4.5: General scheme for emulsion polymerization of poly (ethyl acrylate-lactam 27) nanoparticles NP 5a. 60
Scheme 4.6: General scheme for emulsion polymerization of polystyrene NP 2 and poly (butyl acrylate-styrene) NP 3. 65
Scheme 4.7: Procedure for emulsion polymerization of poly (butyl acrylate-methyl methacrylate) NP 4. 66
Scheme 4.8: Poly (butyl acrylate-styrene-lactam 27) NP 5b-f nanoparticles with different % (w/w) of drug content. 67
Scheme 4.9: Attempted synthesis of C4-acrylate N-methylthio β-lactam 37. 78
Scheme 4.10: Alternative route to lactam 37. 78
Scheme 4.11: Illustration of crosslinked polymers. 79
Scheme 4.12: Synthesis of di-acrylated N-methylthio β-lactam 38. 79
Scheme 4.13: Synthesis of C3-acryloyl β-lactam 39. 80
Scheme 4.14: Alternative method to make 51. 81
Scheme 4.15: Poly (butylacrylate-styrene-lactam 37) NP 6 with different % (w/w) of drug content. 81
Scheme 4.16: Poly (butyl acrylate-styrene-lactam 38) NP 7. 82
Scheme 5.1: Preparation of polyacrylamide nanoparticles (NP 9, 10, 11 and 12). 105
Scheme 5.17: Synthesis of lactam 67. 109
Scheme 5.18: Synthesis of di-hydroxy lactam 68. 109
LIST OF SPECTRA

Spectrum 7.1: $^1$H NMR (250 MHz, CDCl$_3$) (6b) 138
Spectrum 7.2: $^{13}$C NMR (63 MHz, CDCl$_3$) (6b) 138
Spectrum 7.3: $^1$H NMR (250 MHz, CDCl$_3$) (6a) 139
Spectrum 7.4: $^1$H NMR (250 MHz, CDCl$_3$) (8a) 139
Spectrum 7.5: $^1$H NMR (250 MHz, CDCl$_3$) (9a) 140
Spectrum 7.6: $^1$H NMR (250 MHz, CDCl$_3$) (10a) 140
Spectrum 7.7: $^1$H NMR (250 MHz, CDCl$_3$) (11a) 141
Spectrum 7.8: $^1$H NMR (250 MHz, CDCl$_3$) (8b) 141
Spectrum 7.9: $^1$H NMR (250 MHz, CDCl$_3$) (3a) 142
Spectrum 7.10: $^{13}$C NMR (63 MHz, CDCl$_3$) (3a) 142
Spectrum 7.11: $^1$H NMR (250 MHz, CDCl$_3$) (10b) 143
Spectrum 7.12: $^{13}$C NMR (63 MHz, CDCl$_3$) (10b) 143
Spectrum 7.13: $^1$H NMR (250 MHz, CDCl$_3$) (3b) 144
Spectrum 7.14: $^{13}$C NMR (63 MHz, CDCl$_3$) (3b) 144
Spectrum 7.15: $^1$H NMR (250 MHz, CDCl$_3$) (10c) 145
Spectrum 7.16: $^{13}$C NMR (63 MHz, CDCl$_3$) (10c) 145
Spectrum 7.17: $^1$H NMR (250 MHz, CDCl$_3$) (3c) 146
Spectrum 7.18: $^{13}$C NMR (63 MHz, CDCl$_3$) (3c) 146
Spectrum 7.19: $^1$H NMR (250 MHz, CDCl$_3$) (15) 147
Spectrum 7.20: $^1$H NMR (100 MHz, CDCl$_3$) (16) 147
Spectrum 7.21: $^1$H NMR (250 MHz, CDCl$_3$) (17) 148
Spectrum 7.22: $^1$H NMR (250 MHz, CDCl$_3$) (17) 148
Spectrum 7.23: $^1$H NMR (250 MHz, CDCl$_3$) (10d) 149
Spectrum 7.24: $^{13}$C NMR (63 MHz, CDCl$_3$) (10d) 149
Spectrum 7.25: $^1$H NMR (250 MHz, CDCl$_3$) (18) 150
Spectrum 7.26: $^1$H NMR (250 MHz, CDCl$_3$) (21a) 150
Spectrum 7.27: $^1$H NMR (250 MHz, CDCl$_3$) (20) 151
Spectrum 7.28: $^{13}$C NMR (63 MHz, CDCl$_3$) (20) 151
Spectrum 7.29: $^1$H NMR (250 MHz, CDCl$_3$) (22a) 152
Spectrum 7.30: $^1$H NMR (250 MHz, CDCl$_3$) (19b) 152
Spectrum 7.31: $^1$H NMR (250 MHz, CDCl$_3$) (21b) 153
Spectrum 7.32: $^{13}$C NMR (63 MHz, CDCl$_3$) (21b) 153
Spectrum 7.33: $^1$H NMR (250 MHz, CDCl$_3$) (22b) 154
Spectrum 7.34: $^{13}$C NMR (63 MHz, CDCl$_3$) (22b) 154
Spectrum 7.35: $^1$H NMR (250 MHz, CDCl$_3$) (21c) 155
Spectrum 7.36: $^{13}$C NMR (63 MHz, CDCl$_3$) (21c) 155
Spectrum 7.37: $^1$H NMR (250 MHz, CDCl$_3$) (22c) 156
Spectrum 7.38: $^1$H NMR (250 MHz, CDCl$_3$) (19c) 156
Spectrum 7.39: $^1$H NMR (250 MHz, CDCl$_3$) (21d) 157
Spectrum 7.40: $^1$H NMR (250 MHz, CDCl$_3$) (19d) 157
Spectrum 7.41: $^1$H NMR (250 MHz, CDCl$_3$) (22d) 158
Spectrum 7.42: $^{13}$C NMR (63 MHz, CDCl$_3$) (22d) 158
Spectrum 7.43: $^1$H NMR (250 MHz, CDCl$_3$) (23) 159
Spectrum 7.44: $^1$H NMR (250 MHz, CDCl$_3$) (19e) 159
Spectrum 7.45: $^{13}$C NMR (100 MHz, CDCl$_3$) (30) 160
Spectrum 7.46: $^1$H NMR (400 MHz, CDCl$_3$) (31) 160
Spectrum 7.47: $^1$H NMR (400 MHz, CDCl$_3$) (27) 161
Spectrum 7.48: $^{13}$C NMR (100 MHz, CDCl$_3$) (27) 161
Spectrum 7.49: $^1$H NMR (250 MHz, CDCl$_3$) (28, 29) 162
Spectrum 7.50: $^1$H NMR (400 MHz, CDCl$_3$) (34) 162
Spectrum 7.51: $^1$H NMR (250 MHz, CDCl$_3$) (29) 163
Spectrum 7.52: $^1$H NMR (400 MHz, CDCl$_3$) (32) 163
Spectrum 7.53: $^1$H NMR (400 MHz, CDCl$_3$) (33) 164
Spectrum 7.54: $^1$H NMR (250 MHz, CDCl$_3$) (25) 164
Spectrum 7.55: $^1$H NMR (250 MHz, CDCl$_3$) (42) 165
Spectrum 7.56: $^{13}$C NMR (100 MHz, CDCl$_3$) (42) 165
Spectrum 7.57: $^1$H NMR (400 MHz, CDCl$_3$) (44) 166
Spectrum 7.58: $^1$H NMR (400 MHz, CDCl$_3$) (45) 166
Spectrum 7.59: $^1$H NMR (400 MHz, CDCl$_3$) (47) 167
Spectrum 7.60: $^{13}$C NMR (100 MHz, CDCl$_3$) (47) 167
Spectrum 7.61: $^{13}$C NMR (100 MHz, CDCl$_3$) (48) 168
Spectrum 7.62: $^1$H NMR (250 MHz, CDCl$_3$) (14) 168
Spectrum 7.63: $^1$H NMR (400 MHz, CDCl$_3$) (49) 169
Spectrum 7.64: $^{13}$C NMR (100 MHz, CDCl$_3$) (49) 169
Spectrum 7.65: $^1$H NMR (400 MHz, CDCl$_3$) (50) 170
Spectrum 7.66: $^{13}$C NMR (100 MHz, CDCl$_3$) (50) 170
Spectrum 7.67: $^{13}$C NMR (100 MHz, CDCl$_3$) (38) 171
Spectrum 7.68: $^{13}$C NMR (100 MHz, CDCl$_3$) (51) 171
Spectrum 7.69: $^{13}$C NMR (100 MHz, CDCl$_3$) (39) 172
Spectrum 7.70: $^1$H NMR (250 MHz, CDCl$_3$) (56) 172
Spectrum 7.71: $^1$H NMR (400 MHz, CDCl$_3$) (57) 173
Spectrum 7.72: $^1$H NMR (400 MHz, CDCl$_3$) (NP 6c) 173
Spectrum 7.73: $^1$H NMR (400 MHz, CDCl$_3$) (NP 7) 174
Spectrum 7.74: $^1$H NMR (400 MHz, CDCl$_3$) (NP 6b) 174
LIST OF ABBREVIATIONS

α           alpha
AFM         atomic microscopy
Ar           aryl
Ac           acetyl
AcO         acetoxy
β           beta
Bn           benzyl
Br           broad
Bu           butyl
Boc         tert-butyloxycarbonyl
bp           boiling point
br           broad (spectral)
Bz           benzoyl

°C           degrees Celsius
¹³C         carbon-13
c           concentration (mg/ml)
CAN         ceric ammonium nitrate
Cbz         carbobenzyloxy
CH₂Cl₂       dichloromethane
Cl₂         chlorine gas
cm⁻¹         wave numbers (reciprocal centimeters)
CSA         camphorsulfonic acid

D           deuterium (¹H)
DBU         1,8-diazabicyclo[5.4.0]undec-7-ene
DCC         dicyclohexyl carbodiimide
DEAD       diethyl azodicarboxylate
DEPC       diethylphosphorou cyanide
DIBAL      diisobutylaluminum hydride
DIPEA      diisopropylethylamine (base catalyst)
DLS         dynamic light scattering
DMAP       4-dimethyaminopyridine
DMSO       dimethylsulfoxide

EDC         1-(3-(di-methylamino)propyl)-3-ethylcarbodiimide
Et           ethyl
Et₃N       triethylamine
EtOAc      ethyl acetate

g           gram(s)
¹H           proton
²H           deuterium
<table>
<thead>
<tr>
<th>Symbol</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^3$H</td>
<td>tritium</td>
</tr>
<tr>
<td>HMDS</td>
<td>hexamethyldisilazide</td>
</tr>
<tr>
<td>HOBT</td>
<td>1-hydroxybenzotriazole</td>
</tr>
<tr>
<td>hr</td>
<td>hour(s)</td>
</tr>
<tr>
<td>Hünig's base</td>
<td>diisopropylethylamine</td>
</tr>
<tr>
<td>Hz</td>
<td>hertz</td>
</tr>
<tr>
<td>IR</td>
<td>infrared</td>
</tr>
<tr>
<td>$J$</td>
<td>coupling-constant(s)</td>
</tr>
<tr>
<td>KOH</td>
<td>potassium hydroxide</td>
</tr>
<tr>
<td>LAH</td>
<td>lithium aluminum hydride</td>
</tr>
<tr>
<td>LDA</td>
<td>lithium diisopropylamide</td>
</tr>
<tr>
<td>Me</td>
<td>methyl</td>
</tr>
<tr>
<td>MeCN</td>
<td>acetonitrile</td>
</tr>
<tr>
<td>MeOH</td>
<td>methanol</td>
</tr>
<tr>
<td>mCPBA</td>
<td>meta-chloroperoxybenzoic acid</td>
</tr>
<tr>
<td>mg</td>
<td>milligram(s)</td>
</tr>
<tr>
<td>µg</td>
<td>microgram(s)</td>
</tr>
<tr>
<td>mM</td>
<td>millimoles per liter</td>
</tr>
<tr>
<td>mmol</td>
<td>millimole(s)</td>
</tr>
<tr>
<td>mol</td>
<td>mole(s)</td>
</tr>
<tr>
<td>MOM</td>
<td>methoxymethyl</td>
</tr>
<tr>
<td>MRSA</td>
<td>methicillin-resistant <em>Staphylococcus aureus</em></td>
</tr>
<tr>
<td>MSSA</td>
<td>methicillin-susceptible <em>Staphylococcus aureus</em></td>
</tr>
<tr>
<td>Ms</td>
<td>methanesulfonyl, mesyl</td>
</tr>
<tr>
<td>N</td>
<td>normal</td>
</tr>
<tr>
<td>NaOH</td>
<td>sodium hydroxide</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>PCC</td>
<td>pyridinium chlorochromate</td>
</tr>
<tr>
<td>Ph</td>
<td>phenyl</td>
</tr>
<tr>
<td>PhH</td>
<td>benzene</td>
</tr>
<tr>
<td>PhMe</td>
<td>toluene</td>
</tr>
<tr>
<td>ppm</td>
<td>parts per million</td>
</tr>
<tr>
<td>NPhth</td>
<td>phthalimido</td>
</tr>
<tr>
<td>PMP</td>
<td>para-methoxyphenyl</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>SEM</td>
<td>scanning electron microscopy</td>
</tr>
<tr>
<td>TBAF</td>
<td>tetra-$n$-butylammonium fluoride</td>
</tr>
<tr>
<td>TBDMS</td>
<td><em>tert</em>-butyldimethylsilyl</td>
</tr>
<tr>
<td>TBDPS</td>
<td><em>tert</em>-butyldiphenylsilyl</td>
</tr>
<tr>
<td>TBS</td>
<td><em>tert</em>-butyldimethylsilyl</td>
</tr>
<tr>
<td>'Bu</td>
<td><em>tert</em>-butoxycarbonyl</td>
</tr>
<tr>
<td>TCA</td>
<td>tichloroacetic acid</td>
</tr>
<tr>
<td>TEM</td>
<td>transmission electron microscopy</td>
</tr>
<tr>
<td>THF</td>
<td>tetrahydrofuran</td>
</tr>
<tr>
<td>TLC</td>
<td>thin layer chromatography</td>
</tr>
</tbody>
</table>
Tf  trifluorosulfonyl (CF$_3$SO$_2$)
TFA  trifluoroacetic acid
Ts  p-toluenesulfonyl (p-CH$_3$C$_6$H$_4$SO$_2$)
ZP ($\zeta$)  zeta potential
Antibiotic-Conjugated Polyacrylate Nanoparticles: New Opportunities for Development of Anti-MRSA Agents

Yang Wang

ABSTRACT

*N*-Thiolated β-lactams represent a novel family of antibacterial agents, whose *in vitro* activity is confined largely to *Staphylococcus* species, including multidrug-resistant forms of *S. aureus*. *N*-Thiolated β-lactams have recently been shown to possess intriguing biological activities which are addressed in Chapter II. Current development of nanoparticles as a new drug delivery vehicle is described in Chapter III. Chapter IV and V described the current research in our laboratories focusing on the synthesis and characterization of emulsified polyacrylate/polyacrylamide nanoparticle antibacterials for drug delivery of water-insoluble antibiotics. These nanoparticles can be prepared in aqueous media directly from acrylate/acrylamide monomers through free radical microemulsion polymerization. These emulsions contain antibiotic-conjugated polyacrylate nanospheres measuring 30-60 nm in diameter and have enhanced antibacterial activity against drug resistant *S. aureus* (MRSA) through what we believe is a novel mechanism.
CHAPTER 1  CLINICAL DEVELOPMENT OF NEW MRSA ANTIBIOTICS

1.1 Introduction

Antibiotics, one of the most significant accomplishments of this past century, have prolonged lives with their ability to control bacterial infections, such as pneumonia, tuberculosis, dysentery and cholera. It was Pasteur who introduced the term of antibiosis meaning that one microorganism kills another, and it was much later that this concept was implemented in the form of an actual antibiotic. In 1929, Alexander Fleming discovered that a broth of *Penicillium notatum* inhibits the growth of *Staphylococcus aureus* on an agar plate and three years later, he named this active substance “Penicillin”. Figure 1.1 (Merck & Co., Inc.) shows how the growth of bacteria on the agar in a culture dish has been inhibited by the three circular colonies of the fungus *Penicillium notatum*. The antibiotic penicillin, diffusing outward from the colonies, is responsible for this effect. Today, penicillin is made from cultures of *Penicillium chrysogenum* that has been specially adapted for high yields. Later on, the first clinical trial with penicillin as an antibacterial therapy was undertaken in 1941.

![Figure 1.1: Picture of inhibition of bacteria on an agar by fungus Penicillium notatum.](image)

In 1945, Dorothy Hodgkin and Barbara Roger-Low determined the molecular structure of penicillin G by crystallography (Figure 1.2). Penicillin F is a structurally related substance studied in Great Britain in the 1940's, which has a different amido side chain arising from the use of 3-hexenoic acid in place of phenylacetic acid in the culture media.

![Figure 1.2: Structures of penicillin G and penicillin F.](image)
Since the 1940s, β-lactam antibiotics have been playing an important role in the treatment of microbial infections as a result of their high antibacterial activity and low toxicity. The β-lactams get their name from the characteristic structure of the four-membered ring. The history of β-lactam antibiotics began with the discovery of the penicillins and continued with the development of cephalosporins. Penicillins include penicillin G, a natural product, and two semi-synthetic products, ampicillin and amoxicillin. Over two dozen of analogues of cephalosporins are in current use and most of them are semi-synthetics derived from the secretion of the mold *Cephalosporium*. Both have been widely used and studied. Structures of analogues of penicillins and cephalosporins are demonstrated in Figure 1.3.

During the 1960s and 70s, other types of β-lactam antibiotics with biological activities against *S. aureus*, as well as other bacteria strains, were discovered either from natural sources or synthetic pathways. Families of clinically-prescribed β-lactam antibiotics are illustrated in Figure 1.4.

---

Figure 1.3: Analogues of penicillins and cephalosporins.

Figure 1.4: Structures of families of β-lactam antibiotics.
β-Lactams work by interfering with bacterial cell wall biosynthesis. The walls of bacteria are made of a complex polymeric material called peptidoglycan, which contains both amino acids and sugars. The sugars are of two kinds, a nitrogen-containing hexose called N-acetylglucosamine (NAG), and N-acetylmuramic acid (NAM) (Figure 1.5). These two generate a linear polymer of NAG alternating with NAM and they are linked by a glycosidic bond. The side chains attached to each NAM contain 4 or 5 amino acids.

β-Lactams bind to and inhibit bacterial enzymes that catalyze cross-linking reactions of peptides on peptidoglycan strands of a growing cell wall. Upon binding, a serine residue within the active site attacks the β-lactam carbonyl, forming an irreversible penicillin-enzyme complex. This acylation reaction renders the enzyme inactive (Scheme 1.1).

Scheme 1.1: Proposed mechanism of the transpeptidation reaction (a) and the inhibition of the transpeptidase enzymes by penicillin (b).
The structural similarity of β-lactams to the natural D-Ala-D-Ala-terminated substrate, and the strong acylating ability of the β-lactam ring, results in the affinity of β-lactams to these transpeptidase. It has been proposed that penicillin imitates a conformation of the enzyme’s substrate, D-alanyl-D-alanine, in the transpeptidase active site. The structural representations of penicillin and acyl-D-Ala-D-Ala are shown in Figure 1.6.

Figure 1.6: Conformational representations of penicillin and acyl-D-Ala-D-Ala.

### 1.2 Antibiotics Resistance

#### 1.2.1 Intrinsic Resistance and Acquired Resistance

The development of antibiotics for clinical use has cured and controlled previously untreatable bacterial diseases, but unfortunately, they may also bring new problems after being used easily and excessively. As with any medication, antibiotics can produce side effects, such as allergic reactions and impairment of organ function, as of the liver or kidneys. Moreover, antibiotic treatment may create resistance in the target organisms that cause infection and against which antibiotic therapy is directed. Thus, over time, antimicrobial agents typically lose their effectiveness as they are used. In the early β-lactam literature, it was reported that many bacteria strains showed increased resistance to the penicillin shortly after it was introduced into the public. In the 1940s, an enzyme named “penicillinase” was isolated from a strain of *Escherichia coli* and found to be responsible for the therapeutic failure of penicillin in *Staphylococcus aureus*, a common cause of hospital infections. Since then, a number of related enzymes (Penicillinases) have been discovered that inactivate β-lactams through bond cleavage.

Another major factor that accounts for bacterial resistance is the inability of β-lactam antibiotics to penetrate the outer bacterial cell membrane. This is a particular problem associated with Gram-negative bacteria as a result of their complex protein, lipid, and lipopolysaccharide membrane. This permeability barrier is responsible for the resistance of *Pseudomonas aeruginosa* to many β-lactam antibiotics. Some bacteria are intrinsically resistant to other antibiotics, for example, Gram-positive bacteria are much less susceptible to polymixins than Gram-negative bacteria.
Many bacteria acquire resistance to one or more of the antibiotics to which they were formerly susceptible. For example, resistance of *Shigella*, causing gastrointestinal illness, to ampicillin grew from 32% to 67% in the decade from 1985-1995 in the U.S. Bacteria develop resistance by acquiring genes encoding proteins that protect them from the effects of the antibiotics. In some cases, the genes arise by mutation; in others, they are acquired from other bacteria that are already resistant to the antibiotic. The genes are often found on plasmids which spread easily from one bacterium to another—even from one species of bacterium to another. As mentioned previously, penicillinase enzymes provide protection against most β-lactam antibiotics by breaking the amide bond on the β-lactam ring.

1.2.2 Urgent Problems of Resistance

Several types of antibiotic resistance are currently undergoing rapid evolution and spread and they may leave current antibiotics with little or no effectiveness against their target organisms. One of the most important microbes to develop resistance is methicillin-resistant *Staphylococcus aureus* (MRSA). It is a multi-drug resistant pathogen whose insensitivity to most antibiotics has evolved over the span of five decades. *Staphylococcus aureus*, a species of bacteria which is often referred to as "staph", can live harmlessly on many skin surfaces, especially around portals such as the nose, mouth, genitals, and rectum. But when the skin is punctured or broken, *S. aureus* can enter the wound and cause an infection. *S. aureus* can cause folliculitis, boils, scalded skin syndrome, impetigo, toxic shock syndrome, cellulitis, and other types of infections. After the discovery of penicillin by Alexander Fleming in 1928 and the scale-up production processes of Flory and Chain, *S. aureus* infections became treatable by penicillin, and eventually by a variety of penicillin analogues. The efficacy of these drugs has steadily declined since their initial conception, due to the ability of *S. aureus* to acquire resistance mechanisms. Methicillin resistant *Staphylococcus aureus* (MRSA), was first discovered in 1961 which was only one year after clinical trials of methicillin. The percentage of *S. aureus* strains with resistance to standard β-lactam antibiotics has doubled over the past fourteen years, from 30% to 60%.

MRSA infections are commonly acquired during treatments in a hospital setting. For example, a patient may visit a hospital for a puncture wound and become infected with MRSA. With low drug efficacies against these infections, it often becomes quite difficult to treat MRSA. Even other types of antibiotics, such as Vancomycin, considered the last line of defense against MRSA, are now exhibiting lower efficacy levels due to acquired resistance.

1.3 Clinical Development of Anti-MRSA Antibiotics

1.3.1 β-Lactam Antibiotics

Recent structure-activity relationship (SAR) studies of cephalosporins and penems have shown enhanced binding to transpeptidases and heightened resistance to β-lactamase enzymes. Traditional cephalosporins are poor inhibitors of penicillin-binding proteins (PBPs) and are considered ineffective for the treatment of MRSA. During the 1990s, new semi-synthetic analogues were produced to enhance bioactivity and stability. At the same time, other anti-MRSA β-lactams, carbapenems, were developed by Merck & Co and SmithKline Beecham. However, the investigations of these newly-designed antibiotics were discontinued or suspended by 2001.

*N*-Thiolated β-lactams (Figure 1.7) are a novel class of synthetic antibiotics for MRSA whose mode of action does not involve the inhibition of cell wall biosynthesis. Further study of this family of antibacterial agents, one of the subjects of this dissertation, is presented in chapter two.
1.3.2 Glycopeptides and Vancomycin

Glycopeptides represent the most successful family of MRSA antibiotics and remain the choice for the treatment of MRSA diseases. Glycopeptides interfere with synthesis of the bacterial cell wall but by a different mechanism than the β-lactams. Vancomycin (Figure 1.8) is a widely-used glycopeptide in the U.S. that acts by binding to the D-alanine on the end of the peptidoglycan bridge to prevent interstrand cross-linking. It has become the antibiotic of last resort as resistance to the other antibiotics has become more and more common.

1.3.3 Oxazolidinones

Oxazolidinones are bacteriostatic antibiotics that inhibit protein synthesis by binding to two separate ribosomal subunits (30S and 50S). Linezolid (Zyvox®) (Figure 1.9) is the first of a new class of antimicrobial agents to be approved for clinical use by the U.S. Food and Drug Administration in 2000. The drug is a total synthetic compound, which lessens the likelihood of naturally occurring resistance mechanisms. It has excellent activity against virtually all important Gram-positive pathogens, including methicillin-resistant \textit{Staphylococci}, penicillin-resistant \textit{Pneumococci}, macrolide-resistant \textit{Streptococci}, and vancomycin-resistant \textit{Enterococci}.

Figure 1.7: Structure of N-thiolated β-lactams.

Figure 1.8: Structure of vancomycin.

Figure 1.9: Structure of linezolid.
1.3.4 Quinolones and Fluoroquinolones

Quinolones and fluoroquinolones form a group of broad-spectrum antibiotics related to nalidixic acid (Figure 1.10). Quinolones act by inhibiting the bacterial DNA gyrase and/or the topoisomerase IV enzyme in S. aureus. Thus, they inhibit DNA replication and act bacteriocidally. They are considered to be a chemotherapeutic agent as opposed to a true antibiotic since they prevent replication of the bacterial cell by interfering with the genetic replication of the bacterium.

![Diagram of chemical structures]

Figure 1.10: Structures of quinolone DK-507k and olamufloxacin, fluoroquinolones DW-116 and BMS-284756 and nalidixic acid.

1.4 Conclusions

The frequency of antibiotic resistance has continued to rise despite concerted efforts to develop new drugs and improve the traditional antibiotics efficiency. Tremendous effort to discover new anti-MRSA antibiotics has been put forth, and β-lactams continue to be the most heavily investigated class due to the enduring ability to fight infection while maintaining low toxicity in humans.

However, future therapies cannot be devised by analogue synthesis of pre-existing antibiotics alone. Compounds with novel modes of action must be developed as S. aureus continues to evolve new mechanisms and new levels of resistance to current antibiotics. In chapter two, the synthesis and structure-activity relationships of the novel anti-MRSA antibiotics called "N-methylthio β-lactams" are described. Recent developments of nanotechnology-based polymer for the delivery of the antibiotics are described in chapter three. A novel synthesized polyacrylate/acrylamide nanoparticle for drug delivery of water-insoluble antibiotics is described in chapters four and five.
CHAPTER 2  SYNTHESIS AND BIOLOGICAL PROPERTIES OF C3-SUBSTITUTED N-ThIOLATED β-LACTAMS

2.1 Introduction

N-Methylthio β-lactams 1 represent a new family of antibiotics that inhibit the growth of Staphylococcus bacteria (Figure 2.1). Previous research in the Turos group has shown that the initial lead compound 2 has antibacterial activity against Staphylococcus aureus and methicillin resistant S. aureus (MRSA). Some initial structure-activity studies were performed to determine what structural features give the N-thiolated β-lactam its antibacterial activity. The N-methylthio substituent is essential for activity; however, the C4 alkynyl substituent can be replaced with E or Z alkenyl, alkyl, aryl, or heteroaryl without the loss of biological activity.

The N-thiolated β-lactams are highly lipophilic molecules which are in contrast to the penicillins, cephalosporins, penems, carbapenems and monobactams, structurally similar β-lactams that contain an acidic or ionic moiety adjacent to the lactam nitrogen. Traditional β-lactam antibiotics require this functional group for binding to the penicillin binding proteins. These new antibiotics do not cause any morphological defects in bacterial cells, which are expected of an inhibitor of cell wall synthesis such as the penicillins. The mode of action, although still ill-defined, clearly differs from the traditional β-lactam antibiotics.

This chapter examines an additional element of the structure-activity relationship (SAR) studies of N-thiolated β-lactams, one in which antibiotics containing sulfonate and amino substituents at the C3 position on the lactam ring (Figure 2.2). Compared to methoxy lactam 1, the sulfonate is greater than methoxy in electron-withdrawing ability, while the amino group is greater than methoxy in basicity and polarity. These changes might affect the biological activities against MRSA. The synthesis and biological evaluation of the lactams against a panel of common bacteria consisting of 9 clinical strains of methicillin-resistant S. aureus (MRSA) is reported, as well as some additional microbes. The information gathered from the experiments presented in this chapter can in effect be applied towards the understanding the lactam's mode of action in bacteria.
2.2 Synthesis of C₃-Sulfonate N-Thiolated β-Lactams

C₃-Sulfonate-substituted β-lactams 3 were prepared by the series of reactions illustrated in Scheme 2.1. The five stage sequence was initiated by the synthesis of N-(4-methoxyphenyl) imine 6 from benzaldehyde (5) and p-anisidine. Staudinger coupling of acetoxyacetyl chloride (7) and imine 6 by a formal [2+2] cycloaddition afforded exclusively the cis-(3S,4R)-substituted β-lactam 8 as a racemic mixture. Base hydrolysis formed alcohol 9 and the following SN₂-type sulfonation provided β-lactams 10. Following oxidative cleavage of the p-anisyl residue by aqueous ceric ammonium nitrate, the N-protio lactam 11 was thiolated using readily available sulfonylating reagents to provide the N-thiolated β-lactams 3 in 6 steps. The yields of the reactions were variable depending on the substituents located at C₃ the ring. The following section details each reaction involved in the synthesis of N-thiolated β-lactams.

Scheme 2.1: General synthetic route to lactam 3.

R = H, Cl
2.2.1 Synthesis of C-Aryl (imines) 6

*N-(4-Methoxyphenyl)imines* 6 were prepared from the condensation of benzaldehyde (5a) or o-chlorobenzaldehyde (5b) and *p*-anisidine (Scheme 2.2). Prior to the reaction, the *p*-anisidine was recrystallized in water heated to ~ 60 °C and dried *in vacuo*. While the two aldehydes were used without further purification, when necessary, the acid contaminant was removed by washing with 10% sodium bicarbonate or distilling at atmospheric pressure. The aldehyde and *p*-anisidine were dissolved in dry methylene chloride and stirred at room temperature for 1-2 hrs. Approximately 1 mg of camphorsulfonic acid (CSA) was added to the mixture and heating to reflux was sometimes applied to promote the condensation of the starting materials. In most instances, conversion to the imine was completed within 1 hour and could be followed by thin layer chromatography (TLC). The resulting imine was then recrystallized in methanol to get rid of the excess of the anisidine and give the final product, *N-(4-methoxyphenyl) imines* 6.

Scheme 2.2: Synthesis of *N*-aryl (imines) 6.

\[
\begin{align*}
\text{R} & \text{O} \quad \text{OCH}_3 \\
\text{R} & \text{NH}_2
\end{align*}
\]

\[
\begin{align*}
\text{5a: R=H} & \\
\text{5b: R=Cl}
\end{align*}
\]

\[
\begin{align*}
\text{R} & \text{OCH}_3
\end{align*}
\]


2.2.2 Synthesis of *N*-Aryl Protected β-Lactams by Staudinger Coupling

The most frequently described procedure for preparing monocyclic β-lactams is Staudinger coupling. This reaction involves a formal [2+2] cycloaddition of acetoxyacetyl chloride (7) or an activated carboxylic acid to an imine (6), and was the methodology employed to synthesize *N*-aryl protected β-lactams 8a and 8b (Scheme 2.3).

The mechanism of β-lactam formation in the synthesis of 8 is depicted in Scheme 2.4. N-Aryl imines 6 exist primarily in the E-configuration\textsuperscript{24} and form cis β-lactams 8 by a kinetic pathway. Orthogonal attack of the imine on the least hindered side of the activated carbonyl center generates a zwitterionic intermediate, which can interconvert via the anionic intermediate, reverts back to the starting materials, or leads to cis and trans β-lactams. Generally, the cis cycloadduct is obtained when the acyl chloride is added dropwise to a solution of the imine and tertiary amine base, and it is the major or exclusive stereoisomer.\textsuperscript{25} On the other hand, it is found that when the tertiary amine base is added to a solution of the imine and the acyl chloride, a variable mixture of the cis and trans cycloadducts are obtained in which the trans configuration is the major or in some cases the exclusive product.\textsuperscript{26,27} The relative stereochemistry of the β-lactam 8 can be determined by $^1$H NMR analysis. For cis β-lactams the coupling constant for the protons on C$_3$ and C$_4$ ring is about 4-5 Hz, and for trans β-lactams the coupling constant is around 2-3 Hz.

Scheme 2.4: Proposed mechanism of β-lactam formation by Staudinger reaction.

The yields of the Staudinger reaction involving acetoxyacetyl chlorides (7) varied greatly depending on the solvent and temperature. Staudinger coupling reactions typically require a tertiary amine base such as triethylamine or ethyldiisopropylamine (e.g., Hüng's base). The former was utilized for most reactions but could be replaced by Hüng's base with little effect on the isolated yields. A minimum of three equivalents of base was needed to enable the reaction to go to completion. Mechanistically, the excess is required to (1) generate the activated carbonyl group, (2) serve as a proton scavenger, and (3) act as a nucleophile (Nu) in formation of the anionic intermediate.

The yields of the Staudinger reaction involving acetoxyacetyl chlorides (7) varied greatly depending on the solvent and temperature. Staudinger coupling reactions typically require a tertiary amine base such as triethylamine or ethyldiisopropylamine (e.g., Hüng's base). The former was utilized for most reactions but could be replaced by Hüng's base with little effect on the isolated yields. A minimum of three equivalents of base was needed to enable the reaction to go to completion. Mechanistically, the excess is required to (1) generate the activated carbonyl group, (2) serve as a proton scavenger, and (3) act as a nucleophile (Nu) in formation of the anionic intermediate.

Synthesis of the ketene precursor (7) is described in Scheme 2.5. Acetoxyacetic acid (12) was prepared from glycolic acid and acetyl chloride in quantitative yield, and reacted with thionyl chloride to form acetoxyacetyl chloride (7) in 75% yield after distillation. The methodology for synthesizing 12 shown in Scheme 2.5 is practical in providing the costly commercially-available product.
Scheme 2.5: Synthesis of acetoxyacetyl chloride (7).

\[
\begin{align*}
\text{HO-} & \quad \text{AcCl} \quad \text{0°C} \quad \text{AcO-} \quad \text{OH} \\
\text{12} & \quad \text{SOCl}_2 \quad \text{0°C-} \quad \text{50°C} \quad \text{AcO-} \quad \text{7} \\
\end{align*}
\]

2.2.3 Hydrolysis of N-Aryl Protected β-Lactams 8a and 8b

Hydrolysis of N-aryl protected β-lactams 8a and 8b was performed in a basic solution, eg. KOH in methanol, at 0 °C, as shown in Scheme 2.6. The yields of the hydrolysis are usually good, ranging from 93% to quantitative.

Scheme 2.6: Hydrolysis of N-Aryl Protected β-Lactams 8a and 8b.

\[
\begin{align*}
\text{AcO} & \quad \text{HO} \quad \text{AcO} \\
\text{8a: R=H} & \quad \text{KOH/MeOH} \\
\text{8b: R=Cl} & \quad \text{Acetone} \quad \text{0°C} \\
\end{align*}
\]

2.2.4 Synthesis of C₃-Sulfonate N-Aryl Protected β-Lactams 10

Three different types of sulfonate groups were introduced onto β-lactams 9a as shown in Scheme 2.7. Sodium hydride in dry methylene chloride was used to deprotonate the hydroxyl proton on the C₃-lactam and the corresponding sulfonyl chloride was added to form an analogue of C₃-sulfonated N-aryl β-lactams 10. Yields of isolated, chromatographed product ranged from 75-93%.

Scheme 2.7: Formation of C₃-Sulfonate N-Aryl Protected β-Lactams 10a-c.

\[
\begin{align*}
\text{OCH₃} & \quad \text{NaH, CH₂Cl₂} \\
\text{9a} & \quad \text{sulfonyl chloride} \\
\end{align*}
\]

\[
\begin{align*}
\text{OCH₃} & \quad \text{R}_1 \text{SO}_2\text{Cl} \\
\text{10a: R}_1 = & \quad \text{Ph} \quad 75\% \\
\text{10b: R}_1 = & \quad \text{Me} \quad 93\% \\
\text{10c: R}_1 = & \quad \text{Ph} \quad 84\% \\
\end{align*}
\]
2.2.5 Dearylation of C₃-Sulfonate N-Aryl Protected β-Lactams 10 with Ceric Ammonium Nitrate

Kronenthal reported a general method to remove the p-anisyl moiety of protected β-lactams using ceric ammonium nitrate (CAN) in aqueous acetonitrile. The same procedure was applied in the synthesis of N-methylthio β-lactams by the conversion of N-anisyl lactams 10 to N-protio lactams 11. The β-lactam 10 was dissolved into acetonitrile and cooled to 0 °C in an ice bath for 15 min. Generally, for 1.0 g of starting material, 30 mL of acetonitrile was used. Three equivalents of ceric ammonium nitrate was then dissolved in 43 mL of water and added dropwise to the solution of the lactam. The reaction mixture turned from clear and colorless to red and clear. During the oxidation, the electron-rich aromatic ring permitted cleavage by a radical generating species such as CAN. The reaction proceeded through intermediate 13 (Scheme 2.8) which decomposed to the dearylated β-lactam and benzoquinone when washed with a 5% sodium bisulfite solution. Attempts to increase the reaction yield included (1) maintaining the reaction temperature at 0 °C; (2) vigorous stirring; (3) diluting the reaction mixture and (4) altering the workup procedure to remove the quinone.

Scheme 2.8: Oxidative dearylation of β-lactams 10.

2.2.6 N-Methylation of C₃-Sulfonate β-Lactams

The method of introducing a methythio substituent onto the lactam nitrogen was reported by Miller using N-methylthiophthalimide (14). The reaction employed a mild base such as triethylamine or Hünig's base under reflux conditions in a low boiling, nonpolar aprotic solvent (Scheme 2.9). The synthesis of 14 was carried out in the following manner (Scheme 2.10). From methyl disulfide, 1.2 equivalents of bromine was used to generate the methylsulfenyl bromide in situ, which was then cannulated into a flask containing 1 equivalent of phthalimide in a mixture of pyridine and acetonitrile.
Scheme 2.9: Synthesis of N-methythio β-lactams 3 using N-methylthiophthalimide.\textsuperscript{10,11}

\[
\begin{align*}
\text{O} & \quad \text{N} \\
\text{R}_1\text{SO}_2\text{O} & \quad \text{H} \\
\text{H} & \quad \text{H} \\
\text{N} & \quad \text{O} \\
\text{SCH}_3 & \quad \text{R}_1\text{SO}_2\text{O} \\
\text{H} & \quad \text{H} \\
\text{11} & \quad \text{3} \\
\end{align*}
\]

\text{\textsuperscript{11}Pr}_2\text{EtN}, \text{CH}_2\text{CH}_2, \text{reflux}

11a: R\textsubscript{1} = \text{Me}  \\
11b: R\textsubscript{1} = \text{Me}  \\
11c: R\textsubscript{1} = \text{Ph}  \\

3a: R\textsubscript{1} = \text{Me} 74\%  \\
3b: R\textsubscript{1} = \text{Me} 64\%  \\
3c: R\textsubscript{1} = \text{Ph} 72\%

Scheme 2.10: Synthesis of N-methylthiophthalimide (14).

\[
\begin{align*}
\text{CH}_3&-\text{S}-\text{S}-\text{CH}_3 \\
\text{Br}_2, \text{CH}_3\text{CN, 0}\text{C} & \quad \text{O} \\
\text{NH} & \quad \text{pyridine} \\
\text{N} & \quad \text{SCH}_3 \\
\text{14} & \\
\end{align*}
\]

Reactions involving sulfur transfer reagent 14 were found to be efficient and reliable, and became the method of choice for the N-methythiolation of all the C3 sulfonated β-lactams prepared in this study.

2.2.7 The Structure-Activity Profiling of C3-Sulfonated β-Lactam 3

2.2.7.1 Microbiological Evaluation of 3a, 3b and 3c Using Kirby-Bauer Testing

The SAR studies were initiated with the microbial screening of C3-sulfonate N-thiolated β-lactams 3a, 3b and 3c. As a control, penicillin G and vancomycin were included in the series for comparison. The β-lactams were individually tested for antibacterial activity against methicillin-susceptible and methicillin-resistant \textit{S. aureus} strains. Compounds were evaluated \textit{in vitro} by well diffusion on agar plates (Kirby-Bauer) in accordance with the guidelines recommended by the National Committee for Clinical Laboratory Standards (NCCLS).\textsuperscript{31} Initially, the well variation of the test was applied to assess the susceptibility. The plates were prepared by cutting 6 mm circular holes into the inoculated medium and applying 20 µg of the test drug in dimethyl sulfoxide (DMSO) to the wells. Following 24 hrs incubation, the zones of growth inhibition were measured in millimeters (from 3 trials) to determine the relative potency of the drug (Figure 2.3).
Nine MRSA strains were used, including one from ATTC sources, and 8 clinical isolates which were obtained from Lakeland Regional Medical Center, and identified as USF 652-659. All were β-lactamase-producing strains. The biological activities of C$_2$-sulfonate lactams are shown in Figure 2.4 with vertical bars representing the growth inhibition zone size; the blue bars are for methicillin-susceptible *S. aureus* (MSSA), while the red bars are for methicillin-resistant *S. aureus* (MRSA).

**Figure 2.4:** Kirby-Bauer data for analogues 3a-3c against *S. aureus* and MRSA compared with penicillin G (Pen G) and vancomycin.
Twenty micrograms of test compound in DMSO solution was used in each case. The values indicate the average diameters in mm (of three trials) for the zone of growth inhibition obtained for each compound after 24 h of incubation at 37 °C, with a margin of error of ±1 mm. For three C3-sulfonated β-lactams, their biological activities are higher than penicillin G and slightly stronger than vancomycin. Compared to the C3-methoxy lactam 1, the sulfonation on the C3 position of the lactam did not enhance the bioactivity. The trend for the activity increases with molecular weight: methyl < phenyl < p-tolyl for the MSSA and MRSA strains.

2.2.7.2 Minimum Inhibitory Concentration (MIC) Determination

Serial dilutions of the selected lactam in define TSB media was the initial method of choice to determine MICs. The results were neither consistent nor comparable to the potency levels seen with the Kirby-Bauer diffusion method. The agar variation, however, provided dependable results that were not obtainable by broth dilutions. The agar media was prepared in 48-well microtiter plates and inoculated by applying a 1 µl suspension of freshly prepared bacterial cultures 10^6 cells/ml to the well. Following a 24 hour incubation period, the plates were examined for growth. The lowest concentration of antibiotic to inhibit the visual growth of bacteria was recorded as the MIC.

The same trend in the biological activities of C3-sulfonated β-lactams 3a-c was observed in broth MIC testing, which ranged from 64-128 µg/mL. For tosyl compound 3a, MIC’s are around 64 µg/mL against both MSSA (ATCC 25923) and one MRSA strain (USF 919), for phenylsulfonyl lactam 3c, between 84-128 µg/mL, and approach 128 µg/mL for mesyl derivative 3b.

2.2.8 Fluorescent Sulfonated Lactam 18

2.2.8.1 Application to the Study of Mode of Action

As discussed in chapter 1, the classical β-lactam antibiotics inhibit bacterial growth by interrupting cell wall biosynthesis. However, studies of the mode of action show that N-thiolated β-lactams do not interrupt the cell wall crosslinking. Evidence to this fact includes no changes in cellular morphologies, via examination by scanning electron microscopy (SEM) of cells treated with N-thiolated β-lactams, and no change in cell wall density as determined by Gram-staining. Dramatic alterations in morphology and cell wall thickness occur for the same cells treated with penicillins. Therefore, it is essential to find out where in the cell these drugs are going, what are they interacting with and how do these interactions produce inhibitive effects.

One method to determine where these drugs may end up in a bacterial cell involved measuring fluorescence uptake. The first idea is to attach a fluorescent group at the C3 position on the β-lactam ring via a sulfonate linkage. Dansyl chloride has been used extensively to determine the N-terminal amino acid residue of proteins and to prepare fluorescent derivatives of drugs, amino acids, oligonucleotides and proteins for detection by numerous chromatographic methods. With this idea in hand, lactam 18 was designed for the study of mode of action which contains a dansyl group as the fluorescent tracer.

2.2.8.2 Synthesis of Dansyl Substituted Lactam 18

Synthesis of lactam 18 followed the same procedure as the synthesis mentioned previously for lactams 3a-c (Scheme 2.11). However, CAN deprotection of the para-methoxyphenyl (PMP) group on the lactam failed due to the decomposition of the lactam ring during the reaction.
Another route examined for the synthesis was to attach the dansyl substituent in the last step. The key intermediate shown in Scheme 2.12 is the C3-hydroxy N-methylthio lactam 17. PMP deprotection of 8b gave N-protio lactam 15, N-thiolation of the deprotected lactam afforded lactam 16, further K2CO3 hydrolysis gave C3 hydroxy-lactam 17 and the final SN2 substitution with dansyl chloride gave the compound 18.

2.2.8.3 Results and Conclusions

Due to the huge steric effect of the dansyl group, it is likely hard for the lactam to diffuse in the agar, thus its bioactivity as determined by Kirby-Bauer assay against MRSA is fairly low, with a zone of growth inhibition of around 10 mm. In addition, the fluorescent activity of the dansyl group (~330-340 nm) is not strong enough to be traced down in the bacteria due to the auto-fluorescence of the bacteria. Therefore, a stronger fluorescent tracer should be selected for the mode of action future studies.
Another method to determine the mode of action of the lactam is radiolabeling, which was done by Dr. Timothy Long. Due to the lowest cost and smallest number of “hot” synthetic steps, tritium was chosen for the synthesis of radiolabeled N-methylthio β-lactam in which all three methyl hydrogens were swapped for labels. Unfortunately, the results from this experiment did not show where the drug went, only where it did not go. These lactams were not detected in any appreciable levels in cellular fractions containing DNA, RNA, or proteins. At the time, low molecular weight targets were not considered and quite possibly the radio-tag was thrown out with the cellular fluid. A repeat experiment done by Dr. Seyoung Jang and Marci also showed that no labeled cellular contents (small proteins) were found even with a large amount of radioactivity.

A similar idea to follow the drug’s pathway into the cell was investigated by Dr. Bart Heldreth. The idea was to attach a fluorophore as part of the sulfur side chain, to trace the path of this fluorophore through the bacterial cell membrane and into the cell. To see precisely the location within the cell where the lactam may interact with its target, a Forster Resonance Energy Transfer (FRET) pair was built into the N-thiolated β-lactam framework. The principle with FRET is that two functional moieties are installed in the framework where the fluorescence wavelength of one, the donor, would overlap with the absorption wavelength of the other, the acceptor. In this way the fluorescence of the donor would be quenched by the acceptor until the two groups detach (Figure 2.5).

![Figure 2.5: FRET pair concept.](image)

Also, this FRET phenomenon is greatly distance-dependent. In practice, however, there were significant problems, for example, it proved to be difficult to introduce two fluorescent side chains onto the lactam due to ring decomposition, thus, preventing formation of the final FRET product. As well, this was only, at best, a model system, as the donor fluorophore is simply not red-shifted enough to be used for cell structures. In the future, it is important to select a suitable candidate for the FRET system with an appropriate fluorescence wavelength: for example, a coumarin/dinitrophenyl FRET paired analogue, if it is possible to overcome the difficulties of the synthesis.

There is an inherent problem with this type of experiment, the lactam reacts with thiols, including CoA, in the cytoplasm, and the SMe may be transferred to different targets- FRET studies would only show the transfer occurring in the cytoplasm.

### 2.3 C₃-Amino β-Lactams

These studies of the C₃-sulfonated lactams 3a-c show that a lipophilic sulfonate group on the lactam ring does not increase the bioactivity against MRSA compared to the C₃-oxygenated lactam 1. Follow-up studies on the structure-activity relationships of C₃ substituted analogues were conducted to examine the effect of a polar amine-containing group on the bioactivity of N-thiolated β-lactams. Five lactams, 19a-e, were selected for these experiments (Figure 2.6).
2.3.1 Synthesis of C₃-Amino N-Thiolated β-Lactams 19a-d and Azido-Lactam 19e

Synthesis of C₃-amino-substituted analogues 22a-d was done in four steps from hydroxy lactam 9b as shown in Scheme 2.13. Moffatt oxidation of 9b with P₂O₅ in DMSO gave ketolactam 19, which underwent reductive amination using an alkyl- or dialkylamine and NaBH(OAc)₃ in a mixture of acetic acid and dichloroethane, to afford amino adducts 20a-d in good yields.¹² The trans stereochemistry was established by ¹H NMR. These PMP-protected compounds were then converted to N-methylthio products 19a-d under the usual N-dearylation/N-methylthiolation conditions. In the case of 22a and 22b, the N-thiolation step occurred cleanly on the lactam nitrogen without affecting the 2º amine at C₃.

Scheme 2.13: Synthesis of C₃-amino-substituted lactams 19a-d.

Conditions: a.) P₂O₅, DMSO; b.) RR’NH, NaBH(OAc)₃, AcOH, ClCH₂CH₂Cl; c.) (NH₄)₂Ce(NO₃)₆, MeCN-H₂O, 0 °C; d.) N-methylthiophthalimide, triethylamine, CH₂Cl₂, reflux.
The synthesis of azido β-lactam 19e is shown in Scheme 2.14. Azido lactams can normally be prepared by Staudinger coupling of an imine with an α-azidoacetyl chloride. However, it is more convenient to access the lactam directly from C3-hydroxy β-lactam 9b. Hydroxy lactam was first converted to its mesylate by reaction of its sodium salt with methanesulfonyl chloride in anhydrous CH₂Cl₂, and then displaced with NaN₃ in DMF at 80 °C. In this case, trans-disubstituted lactam 23 was obtained exclusively, as revealed by the characteristically small proton NMR coupling constant (J = 1.8 Hz) for the β-lactam ring protons. The N-protected lactam 23 was then transformed into the N-methylthio lactam 19e by N-dearylation/N-thiolation as described above.

Scheme 2.14: Synthesis of azido β-lactam 19e.

Conditions: a) NaH, MsCl, 0 °C to r.t.; then NaN₃, DMF, 80 °C; b) (NH₄)₂Ce(NO₃)₆, MeCN-H₂O, 0 °C; c.) N-methylthiophthalimide, triethylamine, CH₂Cl₂, reflux.

2.3.2 The Structure-Activity Profiling of C3-Amino β-Lactams 19a-d and C3-Azido Lactam 19e

The five amino-substituted analogues were tested for biological activities against MSSA and MRSA by Kirby-Bauer disk diffusion (Figure 2.7). β-Lactam 19a-d turned out to be significantly less potent against MRSA than methoxy compound 1 or the C3-azido lactams 19e which had a zone of inhibition of about 24 mm against S. aureus and 21 mm for MRSA. In fact, only the N-benzyl substituted compound 19b displayed any activity at all against MRSA’s, with average zone sizes of ~9 mm in diameter. This weak activity was also observed in the MIC broth testing, with MIC values greater than 256 µg/mL. Thus, amino substituents at C₃ of these N-methylthio lactams appear to significantly diminish anti-MRSA activity.
2.4 Additional Biological Activity Screening for N-Thiolated β-Lactam Analogues

2.4.1 Other Bacteria Strains: Activity against *Bacillus*

*Bacillus* represents a genus of Gram-positive bacteria which is ubiquitous in nature (soil, water, and airborne dust). *B. anthracis* is the bacterium which causes anthrax in cows, sheep, and sometimes humans. The microbe is large, sporeforming rod, 1 - 1.2 µm in width x 3 - 5µm in length having characteristic squared ends (Figure 2.8).

If inhaled, spores of *B. anthracis* rapidly migrate to lymphonodes of the lungs, where they begin to germinate and release toxins that cripple the immune response, causing bacteremia, toxemia, and frequently, death.36 Concerns about the possible use of *B. anthracis* as a biological weapon have led to efforts to prevent or treat anthrax infections with vaccine or antibacterial drug development, and newer methods for detecting the microbe.37,38

Figure 2.8: *Bacillus* strains.
As previously described, \( N \)-thiolated \( \beta \)-lactams, which have a mode of action distinct to that of all other \( \beta \)-lactam antibiotics,\(^{39}\) seem to affect cellular processes through transfer of the \( N \) organothio group to a bacterial thiol. We also note that these lactams exert anti-proliferative properties against only a narrow range of bacteria, most significantly, \( Staphylococcus \) (including MRSA). This selectivity seems to be related to the levels and types of cellular thiols present in each microbe that is sensitive to the lactams, not to whether the microbes are Gram-positive or Gram-negative classes. Given that \( Staphylococcus \) and \( Bacillus \) are both prominent members of the \( Bacillales \) taxonomic order of bacteria, it will be essential to investigate whether these compounds could possess antibacterial properties against \( Bacillus \) spp.

### 2.4.2 Activities against \( Bacillus \) Strains

The lead compound in this study was \( N \)-methylthio-substituted lactam 1, which has fairly good activity against seven \( Bacillus \) species. Three different types of analogues of \( \beta \)-lactams were tested for \( Bacillus \) strains, \( C_3 \)-sulfonated, \( C_3 \)-amino and \( C_3 \)-amino ester. One representative of the \( C_3 \)-amino ester bearing lactams is compound 25, whose synthesis is mentioned in Scheme 2.15. Alanine was first protected by Boc\(_2\)O in the presence of NaOH in aqueous media to give an acid 26, which was then coupled to \( C_3 \)-hydroxy lactam 17 using EDCI/DMAP to afford the product 25 in 83\% yield.

Scheme 2.15: Synthesis of \( C_3 \)-Boc-alanine lactam 25.

A select number of differentially substituted \( \beta \)-lactams were individually tested for antibacterial activity against \( B.\) \( anthracis \) and six other species of \( Bacillus \) by the Kirby-Bauer method of well diffusion on agar plates (Table 2.1). Twenty micrograms of test compound in DMSO solution was used in each case. The values indicate the average diameters in mm (of three trials) for the zone of growth inhibition obtained for each compound after 24 hour of incubation at 37 °C, with a margin of error of ±1 mm.
Table 2.1: Well-diffusion tests against *Bacillus anthracis*: zone of growth inhibition measured in mm

<table>
<thead>
<tr>
<th>Compound</th>
<th>B. anthracis</th>
<th>B. globigii</th>
<th>B. thuringensis</th>
<th>B. megaterium</th>
<th>B. coagulans</th>
<th>B. subtilis</th>
<th>B. cereus</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>25</td>
<td>18</td>
<td>19</td>
<td>16</td>
<td>20</td>
<td>18</td>
<td>21</td>
</tr>
<tr>
<td>3a</td>
<td>19</td>
<td>12</td>
<td>11</td>
<td>11</td>
<td>13</td>
<td>12</td>
<td>13</td>
</tr>
<tr>
<td>3b</td>
<td>10</td>
<td>8</td>
<td>10</td>
<td>0</td>
<td>10</td>
<td>10</td>
<td>14</td>
</tr>
<tr>
<td>3c</td>
<td>18</td>
<td>15</td>
<td>13</td>
<td>15</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>18</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>19a</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>19b</td>
<td>10</td>
<td>9</td>
<td>9</td>
<td>7</td>
<td>0</td>
<td>0</td>
<td>11</td>
</tr>
<tr>
<td>19c</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>19d</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>19e</td>
<td>20</td>
<td>14</td>
<td>14</td>
<td>11</td>
<td>15</td>
<td>14</td>
<td>17</td>
</tr>
<tr>
<td>25</td>
<td>19</td>
<td>18</td>
<td>17</td>
<td>16</td>
<td>17</td>
<td>18</td>
<td>20</td>
</tr>
</tbody>
</table>

The first series of compounds examined in this study consists of C3-sulfonate-bearing derivatives 3a-c. The data show that their bioactivities are dependent on the size and lipophilicity of the sulfonyl side chain. Whereas the methanesulfonyl compound 3b is only weakly active against six of the seven *Bacillus* microbes, and totally inactive against *B. megaterium*, the toluenesulfonyl and phenylsulfonyl variants 3a and 3c are appreciably more active against *B. anthracis* with an average activity around 13 and 15 mm, respectively.

The biological activity trends observed for C3-amino-substituted lactams 19a-d suggest that polar side chains at C3 decrease the anti-anthracis activity. Only N-benzylamino compound 19b possesses any bioactivity. This parallels what was found previously for MRSA. In addition, replacement of the C3 methoxy substituent of lactam 1 for an azido group 19e slightly decreases activity.

Finally, the C3 ester lactam 25 with a protected amino acid attached on the ester side chain maintained good and uniform bioactivity against all seven *Bacillus* species.

### 2.5 Anticancer Studies of C3-Substituted β-Lactams

One of the major goals in drug discovery is to selectively target tumor cells over normal cells. Because of the ease of synthesis and structural manipulation, small molecules with apoptosis-inducing ability have great potential to be novel chemotherapeutic agents.40 Initiation, commitment, and execution are the three fundamental steps of apoptosis.41 In previous studies, one lead N-thiolated β-lactam 1 showed the potential activity in inducing DNA strand breakage, inhibiting DNA replication and inducing apoptosis in a time- and concentration-dependent manner.42 Several additional N-thiolated β-lactams with substitutions at the C3 position have consequently been screened for their structure-activity relationship.
2.5.1 SAR Studies for C₃-Sulfonated Lactams

C₃-Sulfonated lactams were 3a-c and 18 assessed for their anti-proliferative potential in human MCF-7 breast cancer cells (Figure 2.9). These experiments were done in collaboration with Dr. Q. Ping Dou at Wayne State University. MCF-7 cells were treated with 50 µM of selected lactams for 24 h, followed by MTT assay. MCF-7 cells were plated in a 96-well plate and grown to 70-80% confluency followed by addition of 50 mM β-lactam for 24 hours. Cells were then incubated with 1 mg/ml MTT for 3 hours and proliferation rates were determined using a multi-label plate reader (Victor³, Perkin Elmer; ±SD).

![Chemical structures](image)

Figure 2.9: Four C₃-sulfonated lactams were selected for anti-cancer studies.

All four lactams 3a-c, and 18 bear good leaving groups on the C₃ oxygen, and should essentially be equally as potent. However, lactams with large groups, for example, tosyl, phenyl sulfonyl or dansyl have very little effect on proliferation, while the lactam 3b with a small mesyl group is the most active compound. Mesyl lactam 3b inhibited 57% of MCF-7 cell growth, while the other three lactams have very little effect on proliferation (Figure 2.10). A possible explanation for the partial effectiveness is that lactams with large groups, e.g., dansyl in lactam 18, is incapable of crossing the cell membrane.

![Graph](image)

Figure 2.10: Structure-activity relationship (SAR) analysis of N-thiolated β-lactams 3b and 18 with DMSO as a control.

2.5.2 Synthesis and SAR Studies of Racemic and Optical Active C₃-Acrylated Lactams

To this point all of the N-thiolated β-lactams that were screened for anticancer activity have been in the racemic form; therefore, it was necessary to determine whether one enantiomer is more active than the other. Racemic and optical pure forms of C₃-acrylate lactam 27 were selected for the SAR study of the stereochemistry (Figure 2.11).
2.5.2.1 Synthesis of C₃-Acrylate β-Lactam 27 and its Stereoisomers

Our laboratory in collaboration with Dr. Kirpal Bisht’s proved that the racemic β-lactam 8b can be resolved via enzymatic resolution using *Pseudomonas cepacia* lipase (PS-30) to give the (-)-(3S, 4R) hydroxy enantiomer 30 and the (+)-(3R, 4S) acetoxy enantiomer 31 in 99% ee (Scheme 2.16).43

Scheme 2.16: Enzymatic kinetic resolution of acetoxy lactam 8b.

The enantiomeric purity was determined by ¹H NMR in the presence of a chiral shift reagent, (+)-Eu-(hfc)₃. The two compounds 28 and 29 were then separated by column chromatography (EtOAc: Hex= 1:2), and lactam 28 was then acrylated with acryloyl chloride/NaH in dry methylene chloride for the synthesis of (-)-29.

The synthesis of racemic C₃-acrylate β-lactam 27 started with C₃-hydroxy lactam 9b shown in Scheme 2.17. Lactam 9b was acrylated with acryloyl chloride/NaH in dry methylene chloride to give compound 30, dearylation of PMP group on the lactam ring afforded 31 and N-thiolation gave the final compound 27.

Scheme 2.17: Synthesis of racemic C₃-acrylate β-lactam 27.

Conditions: a.) Acryloyl chloride, NaH, CH₂Cl₂; b.) ceric ammonium nitrate, CH₃CN, H₂O, 0 °C; c.) N-methylthiophthalimide, CH₂Cl₂, Hunig’s base, reflux.
Synthesis of the optical pure lactam (-)-27 is shown in Scheme 2.18 and (+)-27 is illustrated in Scheme 2.19. The acrylate group was introduced directly into hydroxy lactam 28 to give compound 32, while lactam 29 had to be hydrolyzed to hydroxy lactam 34 before the acylation occurred. These PMP-protected lactams were then converted to N-methylthio products under the usual N-dearylation/N-methylthiolation conditions.

Scheme 2.18: Synthesis of (-)-27.

\[
\begin{align*}
\text{28} & \xrightarrow{\text{acyloyl chloride, NaH, CH}_2\text{Cl}_2, \text{rt}} \text{32} \\
\text{32} & \xrightarrow{\text{CAN/CH}_3\text{CN}} \text{33}
\end{align*}
\]

\[
\begin{align*}
\text{14, NEt}_3, & \quad 78\% \\
\end{align*}
\]

Scheme 2.19: Synthesis of (+)-27.

\[
\begin{align*}
\text{29} & \xrightarrow{\text{KOH/MeOH}} \text{34} \\
\text{34} & \xrightarrow{\text{acyloyl chloride, NaH, CH}_2\text{Cl}_2, \text{rt}} \text{35} \\
\text{35} & \xrightarrow{\text{CAN/CH}_3\text{CN}} \text{36} \\
\text{36} & \xrightarrow{14, \text{NEt}_3,} \text{(+)-27}
\end{align*}
\]

\[
\begin{align*}
\text{14, NEt}_3, & \quad 82\% \\
\end{align*}
\]
2.5.2.2 SAR Anti-Cancer Studies of 27

Jurkat and YT cells were treated with 59 µM concentration of lactam 1, lactam 27, (+)-lactam 27 and (-)-lactam 27 for 24 h followed by trypan blue dye exclusion assay (Figure 2.12). It is obvious that stereochemistry plays an important role in the activity of N-thiolated β-lactams in their anti-proliferative, S/G2-M cell cycle arrest, and apoptosis-inducing properties against cultured human transformed cancer cells, but not to the human normal cell line. Lactam 27, which possesses an acrylate moiety on the lactam ring, is twice as potent as lactam 1.

![Figure 2.12: (+)-Lactam 27 induces apoptosis selectively in tumorigenic cells. Leukemic Jurkat T and nontransformed YT cells were treated with lactam 1 or isomers of lactam 27 at 50 µM for 24 h. Cell death is given as a percent of dead cells over total cell population (±SD).](image)

(+)-Lactam 27 induced a very high amount of cell death compared to its isomer (-)-lactam 27, 98% and 58% respectively. Interestingly, the racemic lactam 27 was almost equally as strong as the R-isomer. Normal, non-transformed YT cells did not undergo cell death after β-lactam treatment.

Another experiment using a SV-40 transformed (VA-13) and normal (WI-38) human fibroblasts demonstrates that (-)-lactam 27 is not an active isomer. A DNA stain of VA-13 and WI-38 fibroblast cell lines treated with 50 µM of each lactam for 24 h shows that there is a high degree of detachment and DNA condensation in lactam 1, lactam 27 and (+)-lactam 27 treated cells, which are characteristics indicative of apoptosis (Figure 2.13).

![Figure 2.13: Both detached and attached VA-13 and WI-38 fibroblast cell populations were collected and stained with the nuclear staining dye Hoechst 33342. Each sample was then analyzed by fluorescence microscopy for nuclear morphology.](image)
(-)-Lactam 27 showed decreased activity when compared with the racemic lactam 27 and (+)-lactam 27. There was a very minor amount of cellular detachment observed in the normal WI-38 fibroblasts treated with (+)-lactam 27, but all the other lactams showed a high specificity of cell killing to the transformed VA-13 cells.

### 2.6 Animal Testing of Lactam 25

The initial screening for lactam 25 (shown in Figure 2.14a) showed that it was effective in tumorigenic cells. Thus, a further study of anti-cancer activity was performed in an animal trial. Female athymic nude mice (NCRNU-M) were xenografted by injection of 6 X 106 MDA-MB-231 cells. 15 days after the injection, the mice were divided into three groups: solvent control, low dose (0.3 mg/kg) and high dose (3 mg/kg) treatment with β-lactam 25 by subcutaneous injection daily. Tumor size was measured every 5 days and tumor volume (V) was determined by the equation: \( V = (L \times W^2) \times 0.5 \), where \( L \) is the length and \( W \) is the width of a tumor. Tumor volume was calculated and expressed as cubic millimeters (Figure 2.14b). TUNEL (Terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling) analysis of tumor tissues in nude mice generated by human breast cancer MDA-MB-231 cells for DNA damages in tumor cells treated by β-lactam HY-14 at 0.3 mg/kg (low dose) or 3 mg/kg (high dose) or solvent as control for 30 days. Nuclei stained in dark blue indicate TUNEL-positive.

![Figure 2.14: A) β-Lactam 25 inhibits the tumor growth in nude mice implanted by human breast cancer MDA-MB-231 cells. B) TUNEL (Terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling) analysis of tumor tissues in nude mice generated by human breast cancer MDA-MB-231 cells for DNA damages in tumor cells treated by β-lactam 25 at 0.3 mg/kg (low dose) or 3 mg/kg (high dose) or solvent as control for 30 days. Nuclei stained in dark blue indicate TUNEL-positive.](image)
In conclusion, N-Methylthio β-lactam 25 inhibits 50% of the tumor growth in nude mice treated with 3 mg/kg of lactam 25, this demonstrated unique anticancer property against human leukemic and solid tumor cell lines.

### 2.7 Conclusions

Compared to the lactam 1, three synthesized C3-sulfonated β-lactams 3a-c show slightly decreased activities against for MSSA and MRSA strains. Activity increases with molecular weight: methyl < phenyl < p-tolyl. Another sulfonated fluorescent lactam 18 has low bioactivities against MRSA with zone of inhibition less than 10 mm. β-Lactams 19a-d are significantly less potent against MRSA than methoxy lactam 1. C3-azido lactam 19e has a zone of inhibition of 24 mm against S. aureus and 21 mm against for MRSA. Thus, putting on a more polar group at the C3 position of the lactam dramatically decreases the bioactivities against MRSA.

Same results were also obtained for the bioactivity screening against Bacillus strains. Lactams 3a-c and 19e show some activities against seven Bacillus strains, while the lactams 19a-d have no bioactivity.

Among four sulfonated lactam 3a-c and 18, compound 3b shows the highest activity against cancer cell MCF-7. C3-Acrylate lactam 27 induces equal activity as one of its isomers, (+)-27, while the other isomer (-)-27 shows decreased activity. A further investigation in an animal model shows that N-methylthio β-lactam 25 inhibits 50% of the tumor growth in nude mice treated with 3 mg/kg of lactam 25; this demonstrated unique anticancer properties against human leukemic and solid tumor cell lines.
CHAPTER 3  NANOPARTICLE DELIVERY VEHICLES FOR ANTIBIOTICS

3.1 Introduction

Antibiotics, one of the great innovations of the modern world, were first developed clinically in the late 1940s for the treatment of bacterial diseases, and at that time many believed that scourges such as tuberculosis, dysentery, cholera, pneumonia, and enteric diseases would no longer threaten humankind. However, misuse and overuse of antibiotics over the past 60 years have resulted in increased bacterial resistance, which has been recognized for many decades. In order to control the development of antibiotic resistance, it is essential to understand the earliest stages of resistance formation. Resistance is rapidly spreading, particularly in hospitals where antibiotics are heavily used. The problem is that the more antibiotics are used, the more resistance develops.

Recently there has been a rapid growth in the drug discovery area facilitated by some novel technologies such as combinatorial chemistry. Numerous drug candidates have been designed and many of these analogues show promising biological activities in vitro and in vivo. However, most of them fail to be commercialized because of the problems of unwanted cytotoxicity, no targeting and no controlled release, very poor water solubility and low bioavailability. Thus, new antibacterial discovery projects have been cut since companies have chosen to move into some more lucrative areas, such as chronic illnesses and mood disorders for the reasons (1) microbial resistance increases the demand for new antibiotics, but at the same time new antibiotics create more resistance and it shortens their useful lifetime; (2) the best antibiotics are often reserved by the doctors so that a new antibiotic to which there is less resistance will be little used. Therefore, a profit-making company is not likely to be willing to develop new antibiotics for the public. Furthermore, since antibiotics generally cure the disease, companies find it more lucrative to fund research into treatments for chronic conditions, such as high cholesterol or rheumatoid arthritis, for which patients take the drug over years or a lifetime, rather than for just a week or two.

Although the traditional antibiotics possess some drawbacks as mentioned earlier, many of them are still in use and could perhaps be made more effective against drug-resistant forms of the pathogen they target. Traditionally, these antibiotics are administered orally by capsule or tablet, due to poor stability of many antibiotics in the acidic pH of the stomach or poor permeability through the mucus layer, sub-therapeutic concentrations of the antibiotics often end up reaching the infection site. Thus, better methods for delivering antibacterials for either local or sustained release may allow antibiotics to have enhanced effectiveness with fewer adverse effects.

Polymer-based drug delivery has proven to be an exciting method for improving performance of various antibacterial agents, offering improved solubility and stability, lowering their cytotoxicity, affording controlled release and in some cases, targeting to the infection sites. One of the important polymer-based delivery systems developed over the past 30 years is the nanoparticle, which offers a sustained means of delivering small molecular weight drugs. This chapter will discuss several major drug delivery systems for antibiotics, including nanoparticles.
3.2 Classification of Drug Delivery Systems

Drug delivery systems can be classified into three generations based on their applications. The first-generation systems were developed in the early 1970s. Microcapsules and microspheres belong to this class. Although these are capable of delivering an active substance specifically to the target, they are not drug ‘carriers’ since they have to be implanted as closely as possible to the site of action.

The second-generation of drug delivery systems, also developed in early 1970s and they are true carriers comprised of colloidal particulates less than 1 mm in diameter. Liposomes, nanocapsules, and nanospheres, belong to this category. They are capable not only of releasing a drug at the target but also of carrying it by a general administration. However, after intravenous administration, most colloidal carriers are rapidly removed from the circulation by phagocytic cells in the liver and spleen. Therefore, systemic clearance of the particulates limits their potential to deliver the drug molecule to the specific sites. In the past 10 years, efforts have been made to modify the surface properties of these drug delivery systems to reduce the deposition of plasma proteins onto the particle and diminish the recognition by phagocytes. These delivery systems are now termed as ‘Stealth’ carriers and may remain in the blood stream for a longer period of time.

The third-generation of drug delivery systems are also true carriers and, furthermore, are capable of specific recognition by the target. For example, monoclonal antibodies, liposomes and nanospheres conjugated to monoclonal antibodies or other ligands belong to this category. Targeted drug delivery carriers can be molecular or particulate and the molecular carriers include soluble polymers to which drug molecules are covalently attached, sometimes with targeting moieties bound to the same molecule. Numerous reviews have focused on polymer-based delivery systems for bioactive molecules, such as anticancer agents.

3.3 Major Development of Delivery Systems for Antibiotics

Many drug delivery systems are designed to improve an antibiotic’s pharmacokinetics and bioavailability by providing sustained release mechanisms. The most notable examples include liposomes and other lipid-based polymers, hydrogels and nanoparticles.

3.3.1 Liposomes

Liposomes consist of one or more phospholipid bilayers enclosing an aqueous phase. Figure 3.1A shows the major groups of phospholipids which have a hydrophilic head and hydrophobic tail. When phospholipids are immersed in water they arrange themselves so that their hydrophilic regions point toward the water and their hydrophobic regions point away from the water. This unique simultaneously hydrophilic/hydrophobic structure of phospholipids is the key to their ability to organize as a bilayer formation (Figure 3.1B). Phospholipid bilayers are the core structure of a liposome (Figure 3.1C).
Figure 3.1: A) Structure of phospholipids; B) Formation of a phospholipid bilayer structure and C) the liposome.

Liposomes were first proposed as carriers of biologically active substances in 1971, and have since been comprehensively studied for applications in drug delivery. They can be classified as large multilamellar liposomes (MLVs), small unilamellar vesicles (SUVs), or large unilamellar vesicles (LUVs) depending on their size and the number of lipid bilayers. Water-soluble drugs can be included within the aqueous compartments, while lipophilic or amphiphilic compounds can be associated with the lipid bilayers (Figure 3.2). In some cases, the resulting structures are better described as lipid complexes rather than liposomes, since they do not contain an internal aqueous phase.
Figure 3.2: A complete three-dimensional liposome structure split in half to show its internal structure.

Liposomes can incorporate water-soluble materials as well as oil-soluble materials in different regions of the assembly. The mechanism of how a liposome may deliver a drug to a bacterial cell is thought to occur through a process called endocytosis. Scheme 3.1 shows a liposome fusing with a cell, which goes through a three step sequence. First the outer layer of the liposome (red) fuses with the outer layer of the plasma membrane (brown). Then, the two fused membranes coalesce. Finally, the two inner layers fuse so that the drug (green) has access to the cytoplasm.

Scheme 3.1: Hypothetical model for liposome drug delivery.

Progress has been made for the application of liposome delivery systems for antibiotics. There are many recent reviews on liposomes used as drug carriers for antibiotics. However, the stability of liposomes is often contested and other types of carriers have been studied to improve control in the delivery of drugs.

3.3.2 Hydrogels

Hydrogels are three-dimensional structures of polymer chains that are water-soluble, sometimes found as a colloidal gel in which water is the dispersing medium. Hydrogels have the property of swelling in aqueous medium but do not dissolve in water. The highly swollen environment maintains a high water content which helps to promote cell proliferation and cell function. Due to their soft tissue biocompatibility and the ease that many bioactive molecules have dispersing in the crosslinked matrix, hydrogels are frequently used in controlled release applications for drug delivery (Figure 3.3).
Several applications of hydrogel-based delivery systems for antibiotics have been proved to be effective in vitro. For example, two antibiotics, tylosin tartrate and oxytetracycline hydrochloride, entrapped in poly (vinyl alcohol) hydrogel have shown good in vitro properties suitable for serving as a controlled release system.

3.3.3 Nanoparticles

A nanoparticle is a sub-microscopic substance whose diameter is between 1 and 100 nm. In 1976, Kreuter and Speiser first successfully prepared polyacrylamide nanoparticles in the presence of antigens, leading to the development of a vaccine adjuvant. Three years later, Couvreur developed a series of poly (alkylcyanoacrylate) nanoparticles which are bioresorbable, and are still being used as surgical glues. Several methods have been used in the past for the manufacture of drug nanoparticles. Some of the conventional techniques include spray drying and ultra-fine milling, in which particles with a broad distribution can be produced. Drug nanoparticles can also be prepared by a precipitation process in which polymer being formed is insoluble in its own monomer or in a particular monomer-solvent combination and thus precipitates out as it is formed. This normally leads to the formation of a hydrosol which is a colloid with water as the dispersing medium. In this case, the nanoparticle growth is difficult to control.

Compared to the development of other delivery systems for antibiotics, there is less attention to the development of nanoparticles for delivering antimicrobial agents, and thus few reviews have focused on this area. Due to their polymeric properties, nanoparticles may be more stable than liposomes in biological fluids and storage. Nanoparticles are colloidal polymers that can be used as drug vehicles which may give antibacterials a second lease on life. First, some nanoparticle delivery systems exhibit a property of controlled release of antibiotics. Furthermore, they can increase an antibiotic’s therapeutic efficacy, in vivo stabilities, bioavailability, targetability, biodistribution and reduce toxicity. Another major advantage of nanoparticles is that they are better at reaching the desired target organs when compared to larger particles, since particles bigger than 100 nm do not reach the bone marrow and those larger than 300 nm are unable to penetrate heart and lung tissues. Nanoparticle delivery systems so far include polymer nanoparticles having the drug dispersed within the polymer matrix or adsorbed on the surface, and those coated with a biodegradable polymer and nanoparticle suspensions for poorly soluble drugs.
Nanoparticles can normally be delivered as an aqueous dispersion by oral administration if absorption of the nanoparticle-sized drug carriers can be achieved in the gastrointestinal (GI) tract in useful quantities. Nanoparticles protect the labile drug molecules from degradation in the gastrointestinal (GI) tract and help in targeting therapeutic agents to the infected sites. They also act as a long-circulating drug reservoir from which drugs can be slowly released over a prolonged period. Apart from oral administration, nanoparticles can also be delivered parenterally through intravenous, subcutaneous, and intraperitoneal means. Nanoparticles have a higher dissolution rate, higher saturation solubility, and a greater adhesion when compared to microparticles. Overall, the oral form of immunization and drug delivery has obvious advantages over the parenteral form.

Several types of nanoparticles have been investigated for antibacterial applications including polyalkylycyanoacrylate, polylactide-co-glycolide (PLGA), and surface-coated metal and magnetic nanoparticles (Figure 3.4). The discussion that follows describes the efforts to develop each nanoparticle system for antibiotics delivery.

![Polyalkyl cyanoacrylate](image1)

![Polylactide (PLA)](image2)

![Polylactide-co-glycolide (PLGA)](image3)

![Surface-Charged Metal Nanoparticles](image4)

![Magnetic Nanoparticles](image5)

Figure 3.4: Types of nanoparticle delivery systems.
3.4 Poly (alkyl cyanoacrylate) (PACA) Nanoparticles

Poly (alkyl cyanoacrylate) nanoparticles were first developed by Couvreur et al.\textsuperscript{85} Due to their size, structure, biodegradability and ability to entrap a variety of biologically active substances; poly (alkyl cyanoacrylates) (PACA) have been used for drug delivery.\textsuperscript{86} These nanoparticles have proved to be effective as carriers of oral antibiotics to overcome problems of instability of drugs that are unstable in the gastrointestinal tract or are inadequately adsorbed.\textsuperscript{87} Recent studies show that poly (alkyl cyanoacrylate) nanoparticles are biodegradable carriers, and could be an effective colloidal system able to avoid mononuclear phagocyte recognition after intravenous injection. For example, poly (ethyl cyanoacrylate) nanoparticles coated with polyethyleneglycol (PEG) showed a reduction of phagocytic uptake of 50\% after 90 minutes.

Poly (alkyl cyanoacrylate) nanoparticles are usually prepared by emulsion polymerization at a pH below 3 as shown in Scheme 3.2.\textsuperscript{88} The polymerization is initiated by a base, for example OH\textsuperscript{-} from the dissociation of water. Therefore, the pH for the reaction has to be acidic in order to prevent excessively rapid polymerization and to enable the formation of nanoparticles.

Surfactants have to be included in the polymerization to prevent the aggregation of nanoparticles in the early stage and facilitate the formation of uniformly-sized nanoparticles.\textsuperscript{89} There are several types of poly (alkyl cyanoacrylate) nanoparticles, including poly (ethyl cyanoacrylate), poly (2-ethylbutyl cyanoacrylate), poly(isohexyl cyanoacrylate) and poly (isobutyl cyanoacrylate).

Scheme 3.2: Emulsion polymerization of alkyl cyanoacrylate monomer.

3.4.1 Poly (ethyl cyanoacrylate) Nanoparticles

A recent study in Fontana’s group has shown that β-lactam antibiotics can be entrapped in polyethylecyanocrylate (PECA) nanoparticles and utilized in aqueous emulsions.\textsuperscript{90} Various concentrations of ampicillin, which is shown in Figure 3.5, was used for the emulsion polymerization using of two different surfactants, Pluronic F108 and Pluronic F68 which are nonionic poly polyoxyethylene-polyoxypropylene block co-polymers with similar hydrophobic-lipophilic balance (HLB) value but differing in molecular weight, 8,300 and 14,000, respectively. The general formula of Pluronic surfactant is HO(C$_2$H$_4$O)$_n$(-C$_3$H$_7$O)$_m$(C$_2$H$_4$O)$_n$H and the structure is shown in Figure 3.6. They are available in different grades which vary from liquids to solids. It is used as an emulsifying agent, solubilising agent, surfactant, and wetting agent for antibiotics.
Each sample was studied in terms of particle dimensions, stability, loading capacity, drug release profile and antimicrobial activities. Ampicillin-encapsulated PECA nanoparticles have an average size around 350 nm, and different drug concentrations in the polymerization do not affect the particle dimensions. Studies of degradation of the formed nanoparticles showed that ampicillin-loaded PECA nanoparticles were found to be stable at pH 7.4 and the degradation was almost negligible within 5 hr; however, the degradation was much quicker at pH 1.1 than degradation at neutral pH; around 70% of ampicillin remained intact in the polymer after 3 hr and only 50% of the drug survived degradation after 5 hr.

In terms of loading efficiency, it was found that the higher the amount of drug added to the polymerization, the higher the loading capacity no matter what surfactants were used for polymerization. With 10% concentration of ampicillin initially loaded, the loading capacity was 13.2%; while only 2% of drug loading efficiency was obtained with drug concentration being 1%.

Ampicillin release from PECA nanoparticles was studied under various conditions by HPLC analysis. First, drug release studies performed at pH 7.4 demonstrate that surfactant strongly influenced the release rate of the drug from the polymer. Drug release ability was increased with decreasing the hydrophilic properties of the surfactant and with increasing the molecular weight of the surfactants. Study of drug release was also performed in presence of esterase at pH 7.4. Since the hydrolysis of ester side chain is the main route of degradation of PACA nanoparticles\textsuperscript{91}, the degradation process can be catalyzed by esterase enzyme.

In particular, the rate of drug release was considerably enhanced and all prepared PECA nanoparticles delivered 100% ampicillin. Another drug release profile was done in buffer solution at pH 1.1 and results show that 70% of drug released within 20 mins, and the release rate is decreased and 50% of ampicillin was released after 3 hr and then the amount of drug entrapped in the nanoparticle remains constant.

The antimicrobial activity of PECA nanoparticles against \textit{E.coli}, \textit{S. aureus}, \textit{E. faecalis} and \textit{S. epidermidis} was also examined. The MIC values of the nanoparticles showed that antibiotic activity of drug-loaded nanoparticles were equal to or slightly higher than that of free ampicillin. For example, ampicillin has MICs around 4 µg/mL against \textit{E. coli} while the nanoparticle slightly enhanced the activity with MICs around 2 µg/mL. The same result was also obtained from bioactivity assay for \textit{S. epidermidis}.
Three years after the development of ampicillin-loaded poly (ethyl cyanoacrylate) nanoparticles, Fontana et al. also reported amoxicillin (Figure 3.7) encapsulated PECA nanoparticles coated by polyethyleneglycol (PEG) via emulsion polymerization using surfactant Pluronic F68.92

![Figure 3.7: Structure of amoxicillin.](image)

The particle sizes and the surface charge were analyzed for different batches of PECA nanoparticles. The experimental data showed that average particle size dropped as the molecular weight of PEG increased. For example, nanoparticles prepared in the absence of PEG coating has an average size of 320 nm, while nanoparticles coated with PEG 600 has an average size of 280 nm, and when the molecular weight of PEG increased to 4000, the average size dropped to 220 nm. Same trend was found for the surface charge. Zeta potential value dropped from -18.9 ± 1.5 to -5.1 ± 1.1 as PEG molecular weight increased from 600 to 4000.

Amoxicillin release from PECA nanoparticles was studied by HPLC analysis and showed that at pH 7.4 the rate of drug release rises as the molecular weight of the PEG increases; however, in human plasma, drug release is decreased as molecular weight of PEG increases. This can be explained that in human plasma, esterases catalyze the degradation of the nanoparticles by hydrolysis of the ester linkage of the side chain. The study of the amount of the drug entrapped in PECA nanoparticles at pH 1.1 showed that the drug release is complete within 20 min and the amount of residual entrapped amoxicillin remains constant. This observation implies that PECA nanoparticles can be used for site-specific antibiotic delivery in the stomach.

For the design of colloidal drug delivery systems, it is essential to avoid the phagocytic cell populations within the mononuclear phagocyte system (MPS). Studies on the phagocytic uptake of PECA nanoparticles by murine macrophages showed that PEG-coated nanoparticles were less subjected to phagocytosis than PEG-uncoated nanoparticles. The phagocytic uptake of PECA nanoparticles coated with PEG 2000 and PEG 4000 was reduced 50% and 70%, respectively after 90 min compared to the uncoated PECA nanoparticles. This illustrates that PEG-coated PECA nanoparticles could be a colloidal system which is able to avoid MPS recognition.

### 3.4.2 Ciprofloxacin-Loaded Poly (2-ethylbutyl cyanoacrylate) Nanoparticles

Couvreur et al. also studied the effectiveness of cipro-encapsulated poly (2-ethylbutyl cyanoacrylate) (PEBCA) nanoparticles, (Figure 3.8) which were prepared by anionic emulsion polymerization) as shown in Scheme 3.3. Nanoparticles were freeze-dried for 24 hour under a vacuum in order to stabilize the preparation during storage.93
The particle size of the PEBCA, which was prepared in the presence of ciprofloxacin, was dramatically increased from 130 nm to 275 nm compared with that obtained in the absence of the drug. Comparative size measurements before and after freeze-drying showed no significant modifications in either the drug-loaded and unloaded nanoparticle size.

Cipro-loading efficiency was analyzed by HPLC method. After complete polymerization initiated with 0.5 mg/mL concentration of drug and enzymatic degradation, 51.5% of ciprofloxacin was chemically intact in the nanoparticle suspension. 18% was the free drug which was unbound to the nanoparticle and the rest 30.5% was undetectable by HPLC. The 30.5% of undetectable cipro might covalently bind to the polymer, since cipro could act as a polymerization initiator, and this would not be released in the presence of esterase and not recovered by HPLC analysis. However, it is still debatable as to whether this 30.5% of undetectable cipro correlates to the drug covalently attached to PEBCA nanoparticle, or whether the drug is chemically degraded during the polymerization.
Drug release from the colloidal carrier in medium containing esterase was found to be very slow in the esterase-enriched medium, while in the absence of esterase, no dramatic drug release was observed after 48 hr. This suggests that the release resulted from bioerosion of the polymer matrix. Only 22.3% of cipro drug was released after 6 hours and the release reached a maximum of 62.7% after 48 hours.

Figure 3.9 shows the equilibrium between the free form of cipro and its zwitterionic form. Since polymerization was processed by an anionic mechanism and the nitrogen of the free amine on cipro is able to provide an electron pair, the following nucleophilic attack occurs to form a bond between the methylene group of the monomer and the cipro.

Figure 3.9: Equilibrium between the free base form and the hydrochloride form of the cipro.

$^{19}$F-NMR analysis confirmed that ciprofloxacin entrapped into nanoparticles was only in the zwitterionic form. The measurements of molecular weight suggest the participation of the antibiotic as an anionic polymerization initiator, leading to the formation of a chemical bond between the drug and polymer. Figure 3.10 shows a proposed model describing the association of ciprofloxacin with poly (alkyl cyanoacrylate) nanoparticles obtained by emulsion polymerization.

Figure 3.10: Schematic model for ciprofloxacin-loaded nanoparticle formation, according to the physicochemical studies and as a function of the amount of ciprofloxacin initially added to the medium: 51.5% of ciprofloxacin entrapped inside the matrix as a chemically unmodified form; 18% of unbound ciprofloxacin (hydrochloride form); 30.5% of undetectable ciprofloxacin.
Studies of microbiological activities of cipro-incorporated PEBCA nanoparticles demonstrated that the binding of ciprofloxacin drug to the nanoparticles did not change either its MIC or MBC for *S. typhimurium* strain C53. Indeed, the MIC value was 0.062 µg/mL and the MBC value was 0.5 µg/mL for both the nanoparticles and the free form of the drug.

### 3.4.3 Poly (isobutyl cyanoacrylate) and Poly (isohexyl cyanoacrylate) Nanoparticles

#### 3.4.3.1 β-Lactam Loaded Nanoparticles

Couvreur et al prepared polymerizations of poly (isobutyl cyanoacrylate) (PIBCA) and poly (isohexyl cyanoacrylate) (PIHCA) nanoparticles with ampicillin and gentamicin (Figure 3.11) entrapped. Both drug-encapsulated nanoparticles were then freeze-dried for 48 hr and resuspension of the solid nanoparticle was carried out by addition of D.I. water.

![Figure 3.11: Structure of gentamicin.](image)

For drug-unloaded poly (isobutyl cyanoacrylate) (PIBCA) and poly (isohexyl cyanoacrylate) (PIHCA) nanoparticles, the average diameters of particle size were 132 nm and 149 nm, respectively. The particle size for both systems was increased dramatically as the initial amount of drug loading was increased. For example, an average size of 145 nm nanoparticle was obtained with 250 µg/mL of initial amount of ampicillin was loaded, with the concentration of drug loading increased to 2000 µg/mL, the particle size was increased to 200 nm. As mentioned earlier, drug-entrapped PIBCA and PIHCA nanoparticles were easily resuspended in water after the freeze-drying process, and there is no significant change of particle size before or after freeze-drying.

Analysis of the drug loading efficiency was then carried out on the supernatant liquid of drug-loaded nanoparticles after ultracentrifugation for 1 hr. A reverse-phase HPLC profile showed that 75% of the quantity of ampicillin (up to 1 mg/mL) could be encapsulated within the nanoparticles, for poly (isobutyl cyanoacrylate) nanoparticles. For poly (isohexyl cyanoacrylate) nanoparticles, 82% of the quantity of drug was bound in the nanoparticles, using an initial concentration of 1 mg/mL. Due to the possible degradation of ampicillin in the aqueous acidic media of the polymerization, a freeze-dried formulation was also developed to be able to reproduce with respect to both size and drug content.

The polymer degradation rate was dependent on the length of the alkyl chain, while bio-erosion was prolonged with increasing alkyl chain length. Furthermore, the rate of drug release also depended on the polymer degradation which may occur through an enzymatic pathway. In the presence of esterases, the release of ampicillin was about 60% after 10 hr, and only 20% of drug was released from the nanoparticle in the absence of esterase. Thus, esterase significantly increased the release of drug from polyisobutylcyanoacrylate nanoparticles. On the contrary, the enzyme did not affect the drug release profile for poly (isohexyl cyanoacrylate) nanoparticles. 35% of the drug content was released after 24 hr in the presence of esterase, and about 34% without esterase. This may be due to the steric overcrowding of the longer alkyl chain in PIHCA that reduces the accessibility of the ester function to the esterases.
Entrapment of ampicillin into poly (isohexyl cyanoacrylate) nanoparticles was found to improve dramatically the therapeutic efficiency of drug in intracellular infections of the mouse. For Salmonella typhimurium infected mice, a total dose of 0.8 mg of ampicillin bound to nanoparticles had the same effect as three doses of 32 mg each of the free drug. The therapeutic index of ampicillin, based on mouse mortality, was increased by 120-fold when encapsulated in poly (isohexyl cyanoacrylate) nanoparticles. Toward Listeria monocytogenes infection, the therapeutic index of ampicillin based on liver bacterial counts rose at least 20-fold after its linkage to nanoparticles. Ultrastructural autoradiography showed that the ampicillin-loaded PIHCA nanoparticle could affect the bacterial wall and act by diffusing through the cell. Thus, the enhanced antibacterial effect may have resulted from both direct targeting of bacteria by antibiotic-loaded nanoparticles and by diffusion of the antibiotic into the cell. One explanation for this tremendous enhancement of efficacy of ampicillin-loaded nanoparticles observed in both Salmonella typhimurium and Listeria monocytogenes infected mice is that the cellular uptake of ampicillin was much better when it was encapsulated in nanoparticles when it was free. Previous observations have suggested that nanoparticles are internalized via endocytosis. The free drug does not diffuse through the lysosomal membrane due to their ionic character at neutral extracellular pH. Another explanation for the high efficiency of drug-encapsulated nanoparticles may be the fact that nanoparticles were found to be rapidly captured by the liver and the spleen, resulting in an increase of ampicillin concentration in these organs.

3.4.3.2 Cipro-Loaded PIBCA and PIHCA Nanoparticles

Since β-lactam antibiotics are only active upon dividing bacteria, other antibacterial agents that act independently are required for eradicating both dividing and non-dividing bacteria. Ciprofloxacin, a monofluorinated quinolone, is an excellent combination drug with β-lactams because of its mode of action and good tissue diffusion. Couvreur et al and Fawaz et al studied the effectiveness of cipro-encapsulated poly (isohexyl cyanoacrylate) (PIHCA)/poly (isobutyl cyanoacrylate) (PIBCA) nanoparticles, which were prepared by emulsion polymerization.

The nanoparticle size was measured to be 305 nm for cipro-loaded PIHCA nanoparticle and 195 nm for PIBCA nanoparticles. The size of ciprofloxacin-entrapped nanoparticle was larger than unloaded nanoparticle which has an average of 180 nm. The drug entrapment efficiency for cipro-loaded PIHCA nanoparticles was 82% when 500 µg/mL concentration of initial drug was loaded at pH 2.5.

Studies of efficacy against Mycobacterium avium complex (MAC) in human macrophages proved that the association of ciprofloxacin with PIBCA nanoparticles resulted in an increase compared to the solution of free drug. Antimicrobial activity of cipro-loaded PIBCA nanoparticles against MAC in human macrophages was higher compared with a solution of free drug. When a concentration of 8 µg/mL of cipro was loaded to the nanoparticles, cfu was reduced by 1.1 and 1.2 log units at days 4 and 7, respectively. While for the solution of free drug, cfu only reduced by 0.65 and 0.85.

3.5 PLGA Nanoparticles

Polylactide/polylactide-co-glycolide (PLA/PLGA) nanoparticles can be prepared as biodegradable nanospheres which are useful for prolonged drug release and targeting drugs to specific infection sites (Scheme 3.4). PLGA nanoparticles are normally prepared by means of a water-in-oil-in-water (W/O/W) emulsification-solvent evaporation method. PLGA nanoparticles could be used in many applications including biodegradable delivery of ophthalmic drugs that they can enhance the bioavailability of the antibiotic in eye drops. Due to the biocompatibility and biodegradability of PLA/PLGA nanoparticles, different types of drugs have been associated into these polymeric materials for delivery, including ciprofloxacin, penicillin G and gentamicin.
Scheme 3.4: Formation of PLGA.

Polylactide-co-glycolide (PLGA)

3.5.1 PLGA Nanoparticles Delivering Hydrophobic Drugs

3.5.1.1 Ciprofloxacin-Loaded PLGA Nanoparticles

Ludwig first reported that ciprofloxacin could be incorporated into PLGA nanoparticles by W/O/W emulsification-solvent evaporation followed by high-pressure homogenization. Different preparation parameters slightly affect the physicochemical properties of the nanoparticles. The average particle size of the nanoparticles was 234.68 ± 2.5 nm which was reduced to 188.66 nm when the particles were homogenized for two more cycles. The zeta potential of the nanoparticles was -6.01 mV. The entrapment efficiency of PLGA nanoparticle with 2.5% concentration of ciprofloxacin initially loaded to the nanoparticles is 61%, which corresponds to an absolute drug loading of 25 μg of ciprofloxacin HCl/mg of nanoparticles.

The cipro release profile was determined based on different nanoparticle preparation conditions as well. Four different preparation formulations were used to form PLGA nanoparticles including different ratios of oil to water (O:W) with different homogenization cycles. Experimental data demonstrated that nanoparticles prepared with an O:W ratio 2.5 with one homogenization cycle had the highest rate of drug release. On the contrary, the lowest rate of cipro release was observed from nanoparticles prepared with an O:W ratio of 1.5 with three homogenization cycles. Therefore, the drug release from the PLGA nanoparticles can be controlled by varying the number of homogenization cycles and the O:W ratio.

The cipro release patterns from the nanoparticles were also measured by dispersing in four different solutions before gamma-sterilization (Figure 3.12). The drug release in hydroxyethylcellulose (HEC) solution, which is a cellulose ether, was comparable and slightly slower than in mannitol, a non-viscous reference solution. Carbopol® (CP) 980 caused a small decrease in the rate of drug release, and the release kinetics was slowest when the nanoparticles were dispersed in Poloxamer.

Figure 3.12: Release of ciprofloxacin from nanoparticles dispersed in solutions of mannitol or other viscousifying agents. Diamonds stand for nanoparticles dispersed in mannitol solution (reference solution), triangles for dispersion in hydroxyethylcellulose (HEC), circles for dispersion in poloxamer and squares for dispersion in Carbopol 980.
The antibacterial activities of the cipro-loaded nanoparticles were assessed in comparison with a ciprofloxacin aqueous solution and nanoparticles without drug attached by measuring the minimum inhibitory concentrations (MICs) and minimal bactericidal concentrations (MBCs) against *P. aeruginosa* and *S. aureus* for 4 days. The experimental data showed that cipro-loaded nanoparticles have similar activities against both bacteria as the free drug itself, although 100% of the drug was not been released for 100% after 24 hr. Therefore, the ciprofloxacin concentration in the cipro-attached nanoparticles was enough to kill both bacteria to the same extent as in the aqueous solution.

### 3.5.1.2 Benzathine Penicillin G Loaded PLGA Nanoemulsions and Nanocapsules

Emulsions of benzathine penicillin G-encapsulated nanoparticles (Figure 3.13) can be produced by the spontaneous emulsification process, which is a phenomenon that occurs when two immiscible liquids are placed in contact with each other and emulsify without the aid of any external thermal or mechanical energy source. Nanocapsules of PLGA were prepared according to the interfacial deposition of a pre-formed polymer shown in. The formed nanoemulsion had a mean diameter of 207 ± 8 nm, and the nanocapsules were spherical in shape and had an average diameter of 180 ± 52 nm. The penicillin encapsulation efficiency in the nanocapsules was very high, 85%. Nanoemulsion maintained stability for more than 5 months at 4°C with practical no change in the particle size, ranging from 207 nm to 216 nm. The nanocapsules were only stable for 120 days at either 25°C or 4°C. After this time, the nanocapsule suspension started to degrade and it was proved by transmission electron microscopy (TEM) showing that a second particle population of 2 µm appeared.

![Figure 3.13: Structure of benzathine penicillin G.](image)

The release studies of Penicillin G from nanoemulsions and nanocapsules in vitro showed a similar trend. However, the release rate from nanocapsules was a little faster than from nanoemulsion due to the lower stability of nanocapsules. The whole amount of drug was released within 120 min and 180 min, for nanoemulsion and nanocapsule, respectively. This might be due to the difference of the oily phase property that alter the oil-water partition coefficient. In order to slow the degradation of PenG and the nanocapsules, the freeze-drying method could be used as an alternative way for storage of the nanocapsules.

### 3.5.2 PLA/PLGA Nanoparticles Loading a Hydrophilic Drug: Gentamicin

Gamazo et al. has developed polylactide (PLA) and polylactide-co-glycolide (PLGA) nanoparticles as a delivery system for the cationic and highly hydrophilic antibiotic gentamicin sulfate (GS). Gentamicin sulfate consists of gentamicin C1, C2, C1a, C2a, shown in Figure 3.14.
Gentamicin sulfate (GS) was encapsulated in PLGA microspheres (MS) by spray drying and water/oil/water (W1/O)/W2 solvent evaporation. Two PLGA 50:50 formulations with 2 and 10% GS loading were prepared under these two methods. Both preparations yielded a comparable distribution of particle sizes averaging approximately 300 nm. Conversely, encapsulation efficiency was significantly higher with spray drying, 45% with 8.9 µg/mg concentration of GS loaded initially, than with solvent evaporation which is up to 13% when 2.7 µg/mg concentration of GS loaded.

For the nanoparticles prepared by spray drying using different five types of PLA/PLGA microspheres of similar molecular weight but different hydrophobicity, the encapsulation efficiency generally increased with polymer hydrophilicity, except for the hydrophilic copolymer PLGA 50:50H (letter H names end-group uncapped polymers) carrying carboxylic end-groups.

GS encapsulated efficiency (EE) was also depended on normal drug loading, for example, PLGA 50:50 with 2% loading at a pH 6.0 buffer solution had the highest efficiency, 45%; with 6% of loading, EE dropped to 15%, and further loading up to 10% concentration did not affect the EE.

Finally, the encapsulated-gentamicin PLGA nanospheres were studied by an antibacterial assay using an aqueous incubation medium. The assay measured the growth inhibition of *S. aureus* on Muller Hinton agar. The bioactivity was found to remain the same level after microencapsulation, nanosphere storage and incubation in aqueous media. The results suggest that PLA/PLGA nanospheres prepared by spray drying may be an appropriate delivery system for this amino glycoside antibiotic, since the appropriate particle size should be suitable for monocyte-macrophage uptake and may therefore be useful in the treatment of intracellular *Brucella* infections.

### 3.6 Surface-Coated Metal Nanoparticles

There is currently considerable interest in the size-dependent chemical, physical, and structural properties of metal nanoparticles, triggered in part by specific applications in catalysts, sensors, and microelectronics. To achieve these unique metal nanoparticles, one must be able to tailor only the surface structures of a nanoparticle (Figure 3.15). Gold and silicon have been selected to be coated with different antibiotics as a means for enhanced drug delivery and the discussion that follows describes the details of each development.
3.6.1 Drug Coated Gold Nanoparticles

The attractiveness of using gold as a nanoparticle substance includes the ability to prepare monodispersed colloidal nanoparticles having a certain diameter range, along with the availability of surface-enhanced Raman scattering.113 Gold nanoparticles have served as a versatile platform for exploring many basic applications including DNA, proteins, or small bioactive molecules delivery.114,115 Antibiotics have been surface-coated onto gold nanoparticles as a model system against antimicrobial drug resistance. Comparing with antibiotics that are attached to flexible polymers, gold nanoparticles have a well-developed surface chemistry, a controllable geometry, rigidity, and chemical stability.116,117 Moreover, unlike other polymeric-based nanoparticles, gold nanoparticles not only are smaller in size (4 to 5 nm in diameter, ~10^3 times smaller) than a typical bacterium, but also maintain a constant shape and size in solution.

3.6.1.1 Vancomycin on gold nanoparticles

Xu’s group successfully synthesized vancomycin-attached gold nanoparticles (Scheme 3.5) and illustrated in vitro antibacterial activities against vancomycin-resistant enterococci (VRE).118 These nanoparticles were prepared by treatment of 4-5 nm gold nanoparticles with an aqueous solution of bis(vancomycin) cystamide.

Scheme 3.5: Formation of vancomycin-attached gold nanoparticles.
X-ray photoelectron spectroscopy (XPS) of Van-gold nanoparticle proved the formation of a gold-S bond. This was also confirmed by the UV-vis spectrum of an aqueous solution of Van-coated gold nanoparticles indicating that vancomycin was attached to the gold nanoparticle.

Figure 3.16 shows a possible multivalent interaction between a vancomycin-capped gold nanoparticle and a vancomycin-resistance enterococci (VRE) strain. Vancomycin prevents the biosynthesis of the peptidoglycan layer of the bacteria cell wall by binding to \( \text{L-Lys-}D\text{-Ala-}D\text{-Ala} \) termini of the peptidoglycan.\(^{119}\) VRE is resistant to vancomycin because of its mutated its terminal pentapeptide structure from \( D\text{-Ala-}D\text{-Ala} \) to \( D\text{-Ala-}D\text{-Lac} \), which substantially lowers its affinity to vancomycin about \( 10^3 \) times decrease.\(^{120}\) Thus, use of a more rigid vancomycin-linked metal nanoparticle allows for higher binding with VRE strains and exhibits enhanced to potent activity against VRE.

![Diagram of vancomycin-capped gold nanoparticle and interaction with VRE](image)

Figure 3.16: A possible interaction between vancomycin-capped gold nanoparticle and a VRE strain.

An in vitro activity study showed that the free drug, vancomycin, has little activity against all three types of VRE strains; however, Van-attached gold nanoparticles exhibit excellent activity against all VRE strains, and presumably act as a rigid polyvalent inhibitor of vancomycin-resistant enterococci (VRE). Minimum inhibition concentrations (MICs) of Van-gold nanoparticles are around 2-4 \( \mu \text{g/mL} \) while the MICs for the free form of vancomycin is around 64-128 \( \mu \text{g/mL} \). Van-gold nanoparticles also have good activity against an \( E. coli \) strain with MICs of 8 \( \mu \text{g/mL} \). This implies that this nanoparticle system may provide a useful model for the determination of multi/polyvalent of ligand-receptor pairs.

### 3.6.1.2 Ciprofloxacin Attached Gold Nanoparticles

The antibacterial drug ciprofloxacin has also been coated onto gold nanoparticles.\(^{121}\) Two different gold nanoparticles with mean diameters, 4 and 20 nm, were coated with about 65 and 585 drug molecules, respectively. The cipro-gold particles are stable in the dry state as well as at room temperature.

Voltammetric and spectroscopic studies showed that the nitrogen on the piperazine moiety of ciprofloxacin was responsible for binding to the gold nanoparticles (Figure 3.17), and the voltammetric characteristics of cipro adsorbed on 4 nm sized gold nanoparticle were similar to that of the nanoparticles with 20 nm size.
Moreover, the rate of release of the cipro from gold nanoparticles is faster in basic medium than at pH 7 and the kinetics depend on the particle size. For instance, gold nanoparticle of small size (4 nm) release molecules faster than the larger particles (20 nm). Since the bound cipro is fluorescent, this property could be used in biological investigations.

### 3.6.1.3 Toxicity Studies of Gold Nanoparticles

Recent development of gold nanoparticles demonstrates the versatility of these systems in biological applications. Their interactions with cells have been studied in detail by Rotello et al. To investigate the toxicity of gold nanoparticles, one cationic nanoparticle and one anionic nanoparticle were fabricated for testing with each monolayer consisting of 70 charged thiols and 30 unsubstituted thiols attached (Figure 3.18).

Both gold nanoparticles were tested for toxicity in red blood cell and bacteria cell (E. coli). The LC 50 values showed a 2-3 fold increase was required for the bacterial cells (3.1 µg/mL and > 28 µg/mL, respectively). This moderate increase can be explained as being due to the nature of the bacterial assay, in which a low-density cross-linked agar will decrease the mobility of both cells and nanoparticles. Another explanation could be the increase protection by the outer membrane and the cell wall of E. coli, requiring a
higher nanoparticle concentration to completely kill the bacteria. In summary, gold nanoparticles functionalized with cationic side chains are moderately toxic while anionic nanoparticles are nontoxic, and the toxicity is related to their interaction with the cell membrane.

3.6.2 Porous Hollow Silica Nanoparticles

Owing to high chemical and thermal stabilities, as well as large surface areas and good compatibilities with other materials, porous silica has found wide applications in the fields of selective separation, catalysis and dielectric materials. A growing interest has also been directed toward drug delivery. Chen et al. first presented a novel pathway for the synthesis of porous hollow silica nanoparticles (PHSNP), which adopted nanosized calcium carbonate as a structure-directing template. Antibiotic Cefradine (Figure 3.19) was then employed as a drug molecule for the PHSNP system Figure 3.20.

![Figure 3.19: Molecular structure of cefradine.](image1)

![Figure 3.20: Formation of porous hollow silica nanoparticle.](image2)

Preparation of drug adsorbed porous hollow silica nanoparticles (PHSNP) involves mixing the synthesized PHSNP powder and an aqueous solution of cefradine in a suspension followed by evaporation and drying. Transmission electron microscopy (TEM) images of synthesized cefradine-adsorbed PHSNP showed that spherical nanoparticles with a diameter of 60–70 nm and wall thickness of approximately 10 nm were formed. Brunauer, Emmett, and Teller (BET) surface areas indicated that the pore volume of the carrier was reduced greatly, from 867 to 277 m$^3$/g after entrapping cefradine, which revealed that most pores in the wall of PHSNP had been filled with cefradine.

In vitro release studies of cefradine indicated that PHSNP followed a three-stage pattern. The first stage was a rapid release of cefradine with around 74% in 20 minutes. The following stage was a slow increase of cefradine release from 74% to 82% within 10 hours, at which point a plateau was reached. The release profile indicated that cefradine was released from the pore channels in the wall and inside the hollow part of PHSNP, respectively. Therefore, the PHSNP carrier exhibited a controlled release effect that can perhaps be exploited for drug delivery.
3.7 Magnetic Nanoparticles

Magnetic nanoparticles offer exciting new opportunities toward developing effective drug delivery systems, as it is feasible to produce, characterize, and specifically tailor their functional properties for drug delivery applications. Several magnetic nanoparticle have been synthesized, such magnetite ($\text{Fe}_3\text{O}_4$), and recent reviews on this area describe the applications in biomedicine field using magnetic nanoparticles. First, magnetic nanoparticles have sizes from 2 to 20 nm that place them at dimensions comparable to those of a virus (20–500 nm), a protein (5–50 nm) or a gene (2 nm wide and 10–100 nm long). Second, the nanoparticles are magnetic, which means that they obey Coulomb's law, and can be manipulated by an external magnetic field gradient (Figure 3.21). Additionally, nanoparticles have a large surface area that can be modified to attach biological agents, i.e. a bioactive molecule, or a ligand for targeting. The most frequently used synthetic routes for the preparation of magnetic nanoparticles include synthesis in solution and by aerosol/vapor methods.

Xu et al. reported that FePt magnetic nanoparticles could capture and detect vancomycin-resistant enterococci (VRE) and other Gram-positive bacteria at a concentration of $\sim 10^1$ cfu/mL within an hour. In their studies, a broad spectrum antibiotic, vancomycin was attached to the surface of the FePt nanoparticles (3-4 nm in diameter) by binding to the terminal peptide, D-Ala-D-Ala which is on the cell wall of a Gram-positive bacterium via hydrogen bonding. The conjugate of Van-FePt magnetic nanoparticle showed a high selectivity for Gram-positive bacteria such as *Staphylococcus aureus* (8 cfu/mL), *S. epidermidis* (10 cfu/mL) and a coagulase negative staphylococci (4 cfu/mL). Also, optical and scanning electron microscopy (SEM) profiles proved that the magnetic nanoparticles captured VRE, a life-threatening pathogen, at a concentration of $10^1$-$10^2$ cfu/mL via polyvalent interactions.

3.8 Conclusions and Future Perspectives in Antibiotics Delivery

It is obvious from this brief review of the current literature that modern drug delivery developments are being applied to antimicrobial therapy, including liposomes and nanoparticles. They show an improved therapeutic index compared with the free antibiotics. Their higher efficiency could be explained by the natural targeting of colloidal carriers certain cells that mainly dominates bacteria and are
normally not reachable by the free antibiotics. Another major advantage of drug delivery systems is reduced toxicity and other side effects compared with the traditional antimicrobial agents. However, each system has its own advantages and the limitations as well and this makes the revolution in treatment of infectious diseases seem somehow distant. Due to the limited range of cellular target and the fast-growing planktonical resistance in vivo, most drug delivery systems have not successfully survived in vivo studies although they have shown great enhancement of in vitro activity. Attempts to improve the biocompatibility of drug delivery systems in vivo have to be made and other major issues, such as toxicity, are also need to be resolved.
CHAPTER 4  NOVEL POLYACRYLATE NANOPARTICLES FOR DELIVERY OF β-LACTAM ANTIBIOTICS

4.1 Introduction

There are significant efforts underway to develop polymer-based nanotechnology for drug delivery. New drug delivery vehicles, such as nanoparticles, offer a promising way to improve the bioavailability, efficacy of the pharmaceutical compounds and in some cases, targeting to the specific sites. Most applications for nanoparticle-based delivery are for anticancer molecules, but less attention is being put into the antibiotics area. Several systems have been successfully applied to delivery of antimicrobial agent, including antibiotic-encapsulated poly (alkyl cyanoacrylate) nanoparticles, biodegradable PLGA’s, surface-coated metal nanoparticles and magnetic nanoparticles, each of which possess their own advantages and limitations. Recently, the Turos group investigated a conceptually different strategy for delivery of anti-MRSA antibiotics. This newly-designed system is based on synthetic polyacrylate nanoparticles that are made by emulsion polymerization, and the unique feature of the methodology is that the nanoparticle is built from its constituents in one step without further chemical modification. In this way, the antibiotic is covalently attached to the polymer backbone instead of being encapsulated. Such nanoparticle antibiotics provide an innovative approach for treating infections caused by methicillin-resistant Staphylococcus aureus. This chapter will present the preparation, physical and biological properties of the antibiotic-attached polyacrylate nanoparticles.

4.2 Emulsion Polymerization

4.2.1 Introduction

Emulsion polymerization is a technologically and commercially important reaction initially developed for the production of synthetic rubber latex during the Second World War. Nowadays, emulsion polymerization is the basis of a massive global industry that continues to explore its versatility for the control of the properties of polymer latexes. Some of the major applications of emulsion polymers available in the market include polyvinyl acetate homopolymers and copolymers, styrene butadiene latexes, and acrylic emulsions. These emulsions are mostly applied in adhesives, paints, paper coating and textile coatings.

An emulsion is a stable colloidal suspension consisting of an immiscible liquid dispersed and held in another liquid by a substance called an “emulsifier”. Emulsion polymerization takes place with a mixture of two immiscible liquids. The bulk phase is typically aqueous, and the oil phase comprises the monomer. A surfactant is added to generate the micelles. The product of emulsion polymerization is called a “latex”. The most common type of emulsion polymerization is an oil-in-water emulsion, in which droplets of monomer (the oil) are emulsified with surfactants in a continuous phase of water. Water soluble polymers, such as certain polyvinyl alcohols or hydroxyethyl cellulos, can also be used to act as emulsifiers/stabilizers.
Most emulsion polymerizations are performed under free radical conditions. The leading theory for the mechanism of free-radical emulsion polymerization is summarized in Figure 4.1. First, surfactants emulsify the monomer in a water continuous phase and help create micelles in the water. Micelles only form when the concentration of surfactant is greater than the critical micellar concentration (CMC), and the temperature of the system is greater than the critical micellar temperature, or Krafft temperature.

![Figure 4.1: Adapted schematic illustration of an emulsion polymerization.](image)

In aqueous solution, water molecules surrounding hydrophobic parts of surfactant molecules form hydrogen bonds to make a "cage" in which the hydrophobic part of surfactant molecule (Figure 4.2) is enclosed. When micelles are formed, the hydrophobic parts of the surfactant are no longer in contact with the surrounding water. Micelles can exist in different shapes, including spherical, cylindrical, discoidal, or even vesicular. They are largely controlled by the molecular geometry of the surfactant and the solution conditions (temperature, salt type and concentration, etc.) as well.

![Figure 4.2: Illustration of formation of spherical micelles with an interior composed of the hydrocarbon chains and a surface of the polar head groups facing water.](image)

Next, small amounts of monomer diffuse through the water to the micelle. Initiator, which is usually water-soluble and introduced into the water phase, reacts with the monomer in the micelles. Initiator typically reacts with the micelle and not the monomer droplets since the micelles in total contain a much larger surface area than the fewer, larger monomer droplets. Once polymerization starts, the micelle is referred to as a particle. Polymer particles can grow to extremely high molecular weights,
especially if the initiator concentration is low. That makes the radical concentration and the rate of
termination low as well. Sometimes a chain transfer agent is added to the mix to keep the molecular weight
from getting too high. The propagation process involves the monomer migrating from the large monomer
droplets to the micelles for sustaining polymerization. More monomer from the droplets diffuses to the
growing micelle/particle, where more initiators will eventually react. Monomer droplets and initiator are
continuously and slowly added to maintain their levels in the system as the particles grow. When the
monomer droplets have been completely consumed, the initiator is typically added in for a little while
longer to consume any residual monomer. The final product is a 'dispersion' of polymer particles in water,
also known as a polymer colloid, or a latex.

4.2.2 Advantages of Emulsion Polymerization and Applications

The continuous water phase is an excellent conductor of heat of the reaction and allows the heat of
the reaction to be removed from the system. Since polymer molecules are contained within the particles,
viscosity remains close to that of water and is not dependent on molecular weight. The final product can be
used as is and does not generally need to be altered. Most emulsion polymerizations using a free-radical
polymerization method can be carried out as a batch reaction, in which all reactants are completely added
to the reaction vessel at the start of the polymerization. In many cases, emulsion polymerization is
performed as a starve-fed reaction in which a portion of all monomers is added to the reactor over time
and the copolymer composition will approximate the relative proportions of the monomers in the feed. This
process normally insures a good distribution of monomers into the polymer backbone chain.

4.3 Microemulsion Polymerization

Emulsions are typically classified into three types based on the characteristics: macroemulsion,
miniemulsion or microemulsion. A major distinction between these three categories is the size of the
droplets. A second major difference is the stability of the emulsion. Figure 4.3 shows a general trend in the
relationship between size and stability of these three types.

![Figure 4.3: Schematic representation of relative stability as a function of droplet size of the three classes of
emulsions.](image)

Microemulsion polymerization is the latest in the family of polymerization techniques, discovered
in 1943 by Hoar and Schulman. Microemulsion is an optically transparent, isotropic, and
thermodynamically stable microdispersion consisting of water, oil (monomer), and amphiphile (emulsifier,
coemulsifier, etc.). These transparent oil-in-water micro-dispersions have small spherical droplets varying
from 10 to 100 nm, and microemulsion polymerization is the only technique that can produce uniform
particle size in this range.
There are four components in a typical microemulsion polymerization: the continuous phase, a surfactant, an initiator and the monomer. The bulk phase is typically water. Surfactants that are used to create the micelles which retain the oil soluble monomer, are essential for preparing these emulsions. The surfactants charge can be altered with cationic, anionic and nonionic ionic, and these can also be mixed to alter intrinsic characteristics of the specific surfactants in solution.\textsuperscript{142} Surfactant concentration also has a strong effect on the polymer produced.\textsuperscript{143} The need for high surfactant concentrations, which can become costly, is a disadvantage of microemulsion polymerization. There have been recent attempts to reduce the amount of surfactant to a few percent.\textsuperscript{144} In microemulsion polymerization, radical initiators are the most common. The position of radical initiators within the system can be strategically placed. For example, initiators can be soluble in the continuous phase, inside the micelle, and also at the micelle interface itself.\textsuperscript{145} Different monomers of varying solubility and functionality also can be used.\textsuperscript{146} Other chemicals can be added to the microemulsion to alter the characteristics of the microemulsion or the properties of the polymerized product, such as salts or cosurfactants for the use of forming condensed surface of the microdroplet.\textsuperscript{147}

### 4.4 Main Components for Microemulsion Polymerization

#### 4.4.1 Choice of Monomer

Polymerization involves the linking together of small molecules of monomer to form a long chain polymer molecule known as a macromolecule. Scheme 4.1 depicts a polymerization where M represents both a monomer and a monomer unit in the polymer chain.

**Scheme 4.1: Schematic illustration of a polymerization.**

\[
x \text{M} \xrightarrow{\text{polymerization}} \underbrace{\text{M}_x}_{\text{Polymer}}
\]

Monomers in latex have to meet several requirements: (1) satisfy the polymer end-use needs for certain properties; (2) assure latex compatibility with pigments and fillers and (3) the latex have to be stable during pumping, blending, etc. Moreover, the reactivity of a monomer is strongly dependent on the ability of its substituent group(s) to stabilize the corresponding polymeric radical. The most reactive are monomers having substituent group(s) which can stabilize a radical by delocalization of the unpaired electron (i.e. resonance).

A variety of functional monomers can be copolymerized with the principle monomers as well. These functional monomers are usually used in a small amount, typically 1-3% in a formulation.\textsuperscript{148} In this research, microemulsion polymerization was carried out with a highly water-insoluble antibiotic as the functional monomer in aqueous solution.

#### 4.4.2 Choice of Co-Monomer

A monomer which is used to mix with another monomer for a polymerization reaction is being defined as a co-monomer. Most commercial polymers prepared by free-radical polymerization are in fact copolymers formed by the simultaneous polymerization of two or more monomers, and the inclusion of a second monomer greatly complicates the reaction kinetics. Usually the desired bulk properties of polymers can not be achieved by using only one monomer, and therefore comonomer(s) are essential for polymerization.
Choice of co-monomers is entirely dependent on the chemical and physical properties of the drug monomer. It is critical that the commercial or synthetically modified monomers are able to play two roles in the polymerization process: (1) as the co-monomers for the radical polymerization and (2) as the solvent for dissolving the synthetically modified drug monomers in order to make homogenous liquid phase.

### 4.4.3 Choice of Surfactant

Surfactant is an abbreviation for surface-active agent, which is characterized by its tendency to accumulate at interfaces. All surfactant molecules consist of at least two parts, one which is soluble in a specific fluid and one which is insoluble. The hydrophilic part is referred to as the head group and the hydrophobic part as the tail.149 Hydrophilic head groups of the surfactants are normally ionic, and the most common ionic surfactants are sodium oleate, hexadecyltrimethylammonium bromide, cetyltrimethylammonium bromide (CTAB), and sodium dodecylsulfate (SDS).150 There are also nonionic surfactants such as poly (ethylene oxide) glycol ethers.

Surfactants are a key formulation variable in emulsion polymerization. They act to stabilize the monomer droplets in an emulsion form, serve to solubilize monomer within surfactant micelles and stabilize the formed latex particles as well as the particles which continue to grow during polymerization.

Surfactants also determine the size and the size distribution of the particles formed during emulsion polymerization. Generally, polymerization carried out at or below the critical micelle concentration (CMC) will lead to the formation of more uniformly-sized particles. Surfactants are usually used in the range of 1-6% by weight to the monomer.

The primary classification of surfactants is based on the charge of the polar head groups. It is common practice to divide surfactants into four classes: anionics, cationics, non-ionics and zwitterionics. The latter class contains both an anionic and a cationic charge under normal conditions. Carboxylate, sulfate, sulfonate and phosphate are the polar groups found in anionic surfactants (Figure 4.4).

![Alkyl sulfate](image1)

![Dialkyl sulfosuccinate](image2)

Figure 4.4: Structures of the anionic surfactants.

Non-ionics surfactants have either polyether or polyhydroxyl as the polar group as shown in Figure 4.5.
The vast majority of cationic surfactants are based on the nitrogen atom carrying the cationic charge (Figure 4.6). Both amine and quaternary ammonium-based products are commonly used.

Zwitterionic surfactants contain two charged groups of different sign. Whereas the positive charge is mostly ammonium, the negative charge may vary, although carboxylate is by far the most common. (Figure 4.7)

### 4.4.4 Choice of Radical Initiator

The initiator is normally water-soluble and the most commonly used water-soluble initiator is an inorganic salt of persulfuric acid, such as potassium persulfate (Figure 4.8). It dissociates into two sulfate radical anions which can initiate the polymerization.
Figure 4.8: Structures of potassium persulfate initiator.

Redox radicals, typically generated by an oxidizing agent and a reducing agent, are usually used for polymerization at low temperatures. The formation of radicals in aqueous solution requires that molecular oxygen must be purged from the reaction with an inert gas, such as nitrogen. Oxygen has been shown to be effective in the consumption of radicals. Therefore, the removal of oxygen from the solution immediately prior to initiation prolongs the lifetime of the radicals.

4.4.5 Choice of Aqueous Media

The major component of an emulsion polymerization is the aqueous media. As a continuous phase, although inert, water acts to maintain a low viscosity and provides for good heat transfer. Water also acts as the medium of transfer from the monomer droplets to particles, the locus of initiator decomposition and the medium of dynamic exchange of surfactant between the phase.

It is necessary to use de-ionized water or nano-pure water for emulsion polymerization since the ions or metals can be normally the radical scavenger. In some cases, buffer solution is used depending on the surfactant and the particle stability.

4.4.6 Choice of Polymerization Temperature

Effect of temperature upon the rate of emulsion polymerization must be considered. The reaction temperature that was applied in our polymerization was kept at 70 °C, and it was found that higher or lower temperature did not give the desired nanoparticle morphology and uniform size.

4.5 Polyacrylate Emulsion

Acrylates, or esters of acrylic acid, are sufficiently water soluble to be used in emulsion polymerization. Because these monomers are low-viscosity liquids with reasonably high boiling points, they are conveniently handled on an industrial scale. These monomers yield rubbery polymers when copolymerized due to their flexibility, adhesion, tackiness and weatherability. In most cases, the monomers are mixed with water and surfactant to form a pre-emulsion which is then fed into the reactor where the polymerization is run, typically at atmospheric pressure using temperatures between 60 and 90 °C, and at a pH between approximately 3 to 9. At greater extremes of pH, acrylic monomers are subject to hydrolysis. The polymers, however, are usually quite stable to acidic and basic conditions.

Emulsion polymerization of acrylates shown in Scheme 4.2 generally involves the use of low molecular weight, liquid acrylate monomers, and consequently more sophisticated acrylate monomers could undergo emulsion polymerization with mild reaction conditions. Due to their stability and various functionalities, polyacrylates have been developed as surgical adhesives and for controlled drug release.
4.5.1 Prior Applications of Polyacrylate Emulsion in the Biomedical Area

Prior work by Worley et al. showed that oxazolidinone acrylates undergo radical-promoted emulsion polymerization, and they are efficient biocides in their monomeric form in aqueous solution, and when polymerized, they can be coated on glass, plastic, and fibrous materials for use as surface-active disinfection agents. These substituted oxazolidinones are reacted with several acryloyl chloride derivatives to give the desired monomers that can then homopolymerize to give polymeric oxazolidinones which are then activated by halogenating (Scheme 4.3). This suggests that additional types of lipophilic groupings that might include an active antibacterial drug could likewise be introduced in this way.

Scheme 4.3: Synthesis of Polymeric N-Halamines.

4.5.2 Recent Development of Polyacrylate Emulsion in Antibacterial Application

More recently, in his dissertation, Dr. Jeung-Yeop Shim described the first application of a methodology to synthesize poly (ethyl acrylate) nanoparticle containing an $N$-thiolated $\beta$-lactam antibiotic. The reaction he described is under free-radical emulsion polymerization condition shown in Scheme 4.4 and Scheme 4.5. Enough amount of drug monomer (5%) had to be incorporated into the polymer for characterization by NMR. The maxima amount of lactam that could be dissolved in ethyl acrylate comonomer was around 30% (w/w) for successful preparation of poly (ethyl acrylate-lactam).
Scheme 4.4: Synthesis of poly (ethyl acrylate) NP 1.

\[
\text{Control nanoparticle NP 1}
\]

Formulation and Rxn Conditions

<table>
<thead>
<tr>
<th>components</th>
<th>amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethylacrylate</td>
<td>1000 mg</td>
</tr>
<tr>
<td>Surfactant</td>
<td>30 mg</td>
</tr>
<tr>
<td>Radical initiator</td>
<td>10 mg</td>
</tr>
<tr>
<td>Water</td>
<td>4 ml</td>
</tr>
<tr>
<td>Rxn Temp</td>
<td>70 °C</td>
</tr>
<tr>
<td>Rxn Time</td>
<td>6 hr</td>
</tr>
</tbody>
</table>

Scheme 4.5: General scheme for emulsion polymerization of poly (ethyl acrylate-lactam 27) nanoparticles NP 5a.

Formulation and Rxn Conditions

<table>
<thead>
<tr>
<th>components</th>
<th>amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-lactam 27</td>
<td>298 mg</td>
</tr>
<tr>
<td>Ethyl acrylate</td>
<td>1000 mg</td>
</tr>
<tr>
<td>Surfactant</td>
<td>39 mg</td>
</tr>
<tr>
<td>Radical initiator</td>
<td>13 mg</td>
</tr>
<tr>
<td>Water</td>
<td>4 ml</td>
</tr>
<tr>
<td>Rxn Temp</td>
<td>70 °C</td>
</tr>
</tbody>
</table>

The water-insoluble, highly crystalline N-methyliothio β-lactam acrylate 27 along with a comonomer, ethyl acrylate undergoes an emulsion polymerization in water which leads to the formation of a latex suspension. In this process, the lactam antibacterial drug is covalently attached to the polyacrylate chain. A vial containing this latex shows the paint-like consistency of this emulsified mixture (Figure 4.9).
Analysis of the emulsion polymers prepared above was done by $^1$H NMR and scanning electron microscopy (SEM). The biological activity of polyacrylate nanoparticles containing drug 27 was also described in his dissertation.

### 4.5.2.1 $^1$H NMR

$^1$H NMR spectra for the dry films obtained by coalescing the nanoparticle emulsions of homopoly (ethyl acrylate) **NP 1** and the copolymer **NP 5a** are shown in Figure 4.10. Signals at 2.6, 5.6 and 6.1 ppm are assigned to SCH$_3$, C$_4$-H and C$_3$-H of the polymerized β-lactam, respectively. The olefin protons of the original acrylate in the range of 5.8-6.2 ppm do not show in the spectrum. That indicates that all of the monomeric lactam acrylate and ethyl acrylate was converted to polymeric particles.

![NMR spectra](image)

Figure 4.10: NMR for homopoly (ethyl acrylate) **NP 1** and poly (ethyl acrylate-lactam 27) **NP 5a**.

### 4.5.2.2 Film formation

Film formation refers to the entire sequence from aqueous latex dispersion to the fully developed coating. It involves three major steps. First, evaporation of water from the emulsion brings the particles close enough. Second, as more water evaporates, the particles undergo deformation to form a void-free solid structure. Finally, fusion occurs among these particles to give a mechanically strong film. A drawing depicting this process was shown in Figure 4.11.
In the case of the polyacrylate nanoparticle emulsions, J.Y. Shim showed that a dried film can be easily prepared by slow evaporating, either on glass or any solid surface presented in Figure 4.12.

Figure 4.12: Evaporation of emulsion produces a thermoplastic thin film.

### 4.5.2.3 Scanning Electron Microscopy (SEM)

The morphology and the particle size of the nanoparticles were analyzed by scanning electron microscopy (SEM) in the College of Engineering at University of South Florida. For scanning electron microscopy analysis, a sample of the nanoparticle emulsion was prepared by high dilution (3,000-fold) of the emulsion in D.I. water with evaporation under a N\(_2\)-stream prior to coating by gold sputter under high vacuum. The gold-coated nanoparticles were then observed by SEM (Hitachi S 800).
Figure 4.13: Scanning electron microscopy (SEM) of polyacrylate nanoparticles.

Scanning electron microscopy indicates that the polyacrylate emulsions are comprised of spherical nanoparticles, with particle diameters being uniformly within the 40 to 100 nm range. The average sizes and size distribution of the particles are somewhat dependent on the experimental conditions of polymerization, and on the ratios of the acrylate monomers.

4.5.2.4 Studies of the Microbiological Activities of Nanoparticle Emulsions

The studies of the microbiological activities were performed with the microbial screening of nanoparticle emulsions and the monomer drug 27. As a control, homopoly (ethyl acrylate) NP 1 was included in the series for comparison. The β-lactam monomer and its polymer were individually tested for antibacterial activity against methicillin-susceptible and methicillin-resistant S. aureus strains. All media and supplies were purchased from Fischer Scientific. All bacteria analyzed were purchased from Hardy Diagnostics (from ATCC sources), or were clinical isolates obtained from Lakeland Regional Medical Center and identified as USF 652-659.

1. Kirby-Bauer Assay

Compounds were evaluated in vitro by well diffusion on agar plates (Kirby-Bauer) in accordance with the guidelines recommended by the National Committee for Clinical Laboratory Standards (NCCLS). Five milliliters of sterile phosphate buffered saline (PBS) was inoculated with the desired bacteria and the concentration was adjusted to 0.5 McFarland Standard. The bacteria-containing solution was then streaked evenly onto a Muller-Hinton agar plate and wells were drilled into the agar using sterile 1-3 µL pipette tips. Usually 1 mg of drug monomer was dissolved in 1 mL of DMSO, and for nanoparticle emulsion containing drugs, the original emulsion has to be diluted to 1 mg/mL which corresponds to the drug concentration. The wells were filled with 20 µL of antimicrobial solution or nanoparticle emulsion and the plates were then incubated for 24 hours at 37 °C. Zones of inhibition were determined for each well using a millimeter scale.

2. Broth Dilution Minimal Inhibitory Concentration (MIC) Assay

Lactam monomers and nanoparticles were also evaluated in vitro by broth dilution minimal inhibitory concentration assay using Standard NCCLS protocol M7-A5. Comparison of the resulting zones of inhibition and broth MIC values of all monomers and nanoparticles NP 1, NP 5a against S. aureus (ATCC 25923) and a strain of MRSA (ATCC 43300) are illustrated in Table 4.1. Homopoly (ethyl acrylate) NP 1 is the control experiment and NP 5a contains 30% w/w of lactam drug 27 in the nanoparticle.
Table 4.1: Antibacterial activity of the antimicrobial monomer (27) and the resulting nanoparticle emulsion NP 5a with the control emulsion NP 1. All zone of inhibition (ZOI) results are reported for 20 µg of both drug monomer and the emulsion. Minimal inhibitory concentration (MIC) results were obtained using either broth dilution or agar dilution assay procedures.

<table>
<thead>
<tr>
<th>Compound</th>
<th>MRSA (919)</th>
<th>MIC (µg/mL)</th>
<th>S. aureus (849)</th>
<th>ZOI</th>
<th>MIC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ZOI (mm)</td>
<td></td>
<td></td>
<td>ZOI</td>
<td>MIC</td>
</tr>
<tr>
<td>NP 1</td>
<td>0</td>
<td>&gt;256</td>
<td>0</td>
<td>&gt;256</td>
<td></td>
</tr>
<tr>
<td>27</td>
<td>14</td>
<td>128</td>
<td>14</td>
<td>128</td>
<td></td>
</tr>
<tr>
<td>NP 5a</td>
<td>0</td>
<td>64</td>
<td>0</td>
<td>64</td>
<td></td>
</tr>
</tbody>
</table>

The data in Table 4.1 indicates that the antibiotic-conjugated polyacrylate nanoparticle NP 5a has slightly enhanced anti-MRSA activities compared to the free acrylated forms of lactam 27 against S. aureus and MRSA. Moreover, homopoly (ethyl acrylate) NP 1 has no activity at all and its MICs are greater than 256 µg/mL. There is also another crucial observation that the antibacterial testing of poly (ethyl acrylate-lactam 27) nanoparticles was not successful because the samples were not stable. This is also proved by the fact that the emulsion would precipitate heavily when the broth MIC assays in broth were performed. The precipitate would form in the broth, not on top of the emulsion, and thus cloud the broth more than the bacterial growth. Since the amount of bacterial growth is determined by optical density (OD), we could not accurately determine if a high OD value was from bacterial growth or if it was from the precipitated emulsion. Thus, polymer component will be changed from ethyl acrylate to butyl acrylate-styrene for further investigation.

4.6 Expansion of Polyacrylate Emulsions

4.6.1 Preparation of Poly (butyl acrylate and styrene) Nanoparticles

As mentioned previously, poly (ethyl acrylate) nanoparticle with lactam 27 covalently attached does not exhibit a significant enhancement in the bioactivity against S. aureus and MRSA. Moreover, the solubility of β-lactam 27 in ethyl acrylate is fairly low, and considerable ethyl acrylate co-monomer is required to completely dissolve the drug monomer, for example, 10:1 is the minimum molar ratio of ethyl acrylate to lactam. Thus, a better polyacrylate emulsion formulation with different monomer components was needed for the successful preparation of the emulsions and the determination of bioactivity.

Styrene is a relatively high boiling liquid and can be copolymerized with many monomers such as acrylates. Styrene is comparable tough and hard as a comonomer and it may provide a different physical properties of the film dried from the emulsion. One typical example of poly (styrene-acrylate) copolymer is poly (styrene-butyl acrylate) latex, which has been widely used in coatings, adhesives, and so on due to its good performance and relatively cheap price. Scheme 4.6 illustrates the preparation of poly (butylacrylate-styrene) nanoparticles NP 3.

The general emulsion polymerization procedure employed for this system involves a mixture of liquid acrylates, for example, butyl acrylate and styrene, heated at 70°C, and the mixture is then pre-emulsified in purified water containing 3% w/w of sodium dodecylsulfate, a surfactant typically used in styrene-acrylic latexes, with rapid stirring. The resulting homogenous solution of micelles is then treated with potassium persulfate (1% w/w), a radical initiator, to induce free radical polymerization.
Scheme 4.6: General scheme for emulsion polymerization of polystyrene NP 2 and poly (butyl acrylate-styrene) NP 3.

To determine if the particle size of the polyacrylate emulsion can be uniform and reproducible, a series of five poly (butyl acrylate-styrene) emulsions were prepared under the same formulation and reaction condition named as NP 3a-3e.

To investigate the effect of the reaction time and the formulation on particle size, another three poly (butyl acrylate-styrene) copolymers NP 3a* was prepared using a longer reaction time (12 hr), while NP 3f and NP 3f* were prepared with less surfactant (1%) and initiator (0.5%) and NP 3f* was reacted for a longer time (12 hr). Detailed formulation and reaction conditions are shown in Figure 4.14.
Butyl acrylate
Styrene
Surfactant
Radical initiator
Water
Rxn Temp
Rxn Time

components amount
Butyl acrylate 700 mg
Styrene 300 mg
Surfactant 30 mg
Radical initiator 10 mg
Water 4 ml
Rxn Temp 70 °C
Rxn Time 12hr

Formulation for NP 3a*

Butyl acrylate 700 mg
Styrene 300 mg
Surfactant 10 mg
Radical initiator 5 mg
Water 4 ml
Rxn Temp 70 °C
Rxn Time 6hr

Formulation for NP 3f

Butyl acrylate 700 mg
Styrene 300 mg
Surfactant 10 mg
Radical initiator 5 mg
Water 4 ml
Rxn Temp 70 °C
Rxn Time 12hr

Formulation for NP 3f*

Figure 4.14: Formulations and reaction conditions for three polymers.

4.6.2 Synthesis of Poly (butyl acrylate-methyl methacrylate) Nanoparticles NP 4

Another combination of comonomers is butyl acrylate and methyl methacrylate. Methyl methacrylate (MMA) is a clear and colorless liquid and its outstanding properties include weatherability and scratch resistance. Scheme 4.7 illustrates the preparation of poly (butyl acrylate-styrene) nanoparticles NP 3 and poly (butyl acrylate-methyl methacrylate) nanoparticle NP 4.

Scheme 4.7: Procedure for emulsion polymerization of poly (butyl acrylate-methyl methacrylate) NP 4.

+ \[ \text{methyl methacrylate (MMA)} \]

methyl methacrylate (MMA)

Figure 4.15: Formulation of preparation of NP 4.
4.6.3 Poly (butyl acrylate-styrene-lactam 27) NP 5b

Different amounts of lactam 27 (1, 2, 3 and 4 %) (w/w) were used for preparation of poly (butylacrylate-styrene-lactam) nanoparticles NP 5b-f (Scheme 4.8).

Scheme 4.8: Poly (butyl acrylate-styrene-lactam 27) NP 5b-f nanoparticles with different % (w/w) of drug content.

<table>
<thead>
<tr>
<th>Formulation and Rxn Conditions</th>
<th>amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-lactam</td>
<td>X mg</td>
</tr>
<tr>
<td>Butyl acrylate</td>
<td>700 mg</td>
</tr>
<tr>
<td>Styrene</td>
<td>300 mg</td>
</tr>
<tr>
<td>Surfactant</td>
<td>30 mg</td>
</tr>
<tr>
<td>Radical initiator</td>
<td>10 mg</td>
</tr>
<tr>
<td>Water</td>
<td>4 ml</td>
</tr>
<tr>
<td>Rxn Temp</td>
<td>70 °C</td>
</tr>
</tbody>
</table>

NP 5b: X = 10, 1%
NP 5c: X = 20, 2%
NP 5d: X = 30, 3%
NP 5e: X = 40, 4%

4.6.4 Physical Properties of Poly (butyl acrylate-styrene) and Poly (butyl acrylate-methyl methacrylate) Nanoparticle Emulsions

Analysis of the emulsion polymers prepared above was done by $^1$H NMR, scanning electron microscopy (SEM), transmission electron microscopy (TEM), dynamic light scattering (DLS), and zeta potential analysis. Stability studies were also performed at different temperatures and in different pH buffer solutions.

4.6.4.1 $^1$H NMR Analysis
$^1$H NMR spectra comparison for emulsions of homopoly (styrene) NP 2 and poly (butyl acrylate-styrene) NP 3 is shown in Figure 4.16. Signals between 6.5 and 7.3 represent the styrene phenyl ring, and signals between 3.65 and 4.1 represent the butyl group of butyl acrylate. No acrylate moiety from monomers appears in both NMR spectra, indicating the complete conversion of monomers to polymer.

![NMR spectra comparison](image)

Figure 4.16: NMR comparison of poly (styrene) NP 2 and poly (butyl acrylate-styrene) NP 3.

4.6.4.2 Film Formation

A similar film was formed by evaporation of water from the emulsions NP 3, NP 5, while a white powder was formed from the poly (styrene) NP 2. The film prepared by slow evaporation of poly (butyl acrylate-methyl methacrylate) nanoparticle emulsions NP 4 seems to be stronger and more stretchy than the others.

4.6.4.3 Scanning and Transmission Electron Microscopy (SEM and TEM)

Scanning electron microscopy (SEM) analysis of the particle size of the emulsions was done with the same procedure as described in section 4.5.2.3. Since the nanoparticles were normally coated with a gold layer for SEM analysis, the particle size is slight larger than itself. A more accurate methodology is performed by transmission electron microscopy (TEM). Transmission electron microscopy (TEM) analysis was performed on a FEI Morgagni 268D Electron Microscope in the biology department at University of South Florida. The initial emulsion solution is diluted down to a $10^{-10}$ concentration using nanopure water, then the solution is drop cast onto a Formvar-coated copper grid. The water content is evaporated by applying a cool stream of air to the drop, and the grid is subsequently view on the microscope.

Scanning and transmission electron microscopy indicate that poly (butyl acrylate-styrene) nanoparticles NP 3, nanoparticles NP 5b-f containing drugs and poly (butyl acrylate-methyl methacrylate) nanoparticles NP 4 formed in this manner are all comprised of spherical nanoparticles, with particle diameters being uniformly within the 40 to 100 nm range (Figure 4.17). The average sizes and size distribution of the particles are somewhat dependent on the experimental conditions of polymerization, and on the ratios of the acrylate monomers. Further studies of particle size of the nanoparticles prepared under different formulations were done by DLS.
4.6.4.4 Dynamic Light Scattering (DLS)

To investigate the size distribution of the nanoparticle emulsions, a dynamic light scattering (DLS) analysis was performed on a UPA 150 Honeywell MicroTrac at the University of Florida Particle Engineering Research Center. One drop of concentrated emulsion solution (20% solid content) was placed in a 10 mL well filled with nanopure water. Analysis was performed for 3 runs of 180 seconds per run per sample. The theory behind the dynamic light scattering analysis is that when a beam of light passes through a colloidal dispersion, the particles or droplets scatter some of the light in all directions. When the particles are very small compared with the wavelength of the light, the intensity of the scattered light is uniform in all directions (Rayleigh scattering); for larger particles which are above approximately 250 nm in diameter, the intensity is angle dependent (Mie scattering).

Figure 4.18 demonstrates dynamic light scattering of a nanoparticle containing an average particle size of 40 nm in diameter with a narrow distribution.

4.6.4.5 Zeta Potential Analysis

Zeta potential is a function of the surface charge of the particle which is dependent on the interface and the nature and composition of the surrounding medium in which the particle is suspended. The principal of determining zeta potential by micro electrophoresis is very simple. A controlled electric field is applied via electrodes immersed in the sample suspension and this causes the charged particles to move towards the electrode of opposite polarity. Viscous forces acting upon the moving particle tend to oppose this motion and equilibrium is rapidly established between the effects of the electrostatic attraction and the viscous drag. The particles therefore reach a constant "terminal" velocity.
The zeta potential value of each emulsion sample was obtained on a Brookhaven ZetaPALS. The emulsion solution was diluted to 1.5% (~ pH = 7) of solid content using nanopure water for analysis. For each analysis, 2 × 10 runs per sample was performed. The dispersant viscosity of the emulsion solution was determined to be 0.8872cP, and the dielectric constant of the medium was 78.55. Table 4.2 summarizes the particle size and zeta potential value for each prepared polyacrylate nanoparticles NP 2-5.

<table>
<thead>
<tr>
<th>Nanoparticles</th>
<th>Nanoparticle Description</th>
<th>Average Particle Size (nm)</th>
<th>Zeta Potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NP 2</td>
<td>Polystyrene</td>
<td>38.2</td>
<td>ND</td>
</tr>
<tr>
<td>NP 3</td>
<td>Poly (BA-sty) §</td>
<td>See table 4.3</td>
<td>See table 4.3</td>
</tr>
<tr>
<td>NP 4</td>
<td>Poly (BA-MMA)</td>
<td>52.2</td>
<td>ND</td>
</tr>
<tr>
<td>NP 5b</td>
<td>Poly (BA-sty) with drug 27 (1%)</td>
<td>44.6</td>
<td>-34.9 ± 3.33</td>
</tr>
<tr>
<td>NP 5c</td>
<td>Poly (BA-sty) with drug 27 (2%)</td>
<td>29.3</td>
<td>-112.51 ± 4.70</td>
</tr>
<tr>
<td>NP 5d</td>
<td>Poly (BA-sty) with drug 27 (3%)</td>
<td>33.4</td>
<td>ND</td>
</tr>
<tr>
<td>NP 5e</td>
<td>Poly (BA-sty) with drug 27 (4%)</td>
<td>29.0</td>
<td>-63.53 ± 3.89</td>
</tr>
<tr>
<td>NP 5f</td>
<td>Poly (BA-sty) with drug 27 (1%) δ</td>
<td>59.3</td>
<td>ND</td>
</tr>
</tbody>
</table>

Poly (BA-sty) §: poly (butylacrylate-styrene)
Poly (BA-MMA): poly (butyl acrylate-methyl methacrylate)
Poly (BA-sty) with drug 27 (1%) δ: polymer prepared with 1% of surfactant and 0.5% of initiator.

The particle sizes of poly (butyl acrylate-styrene) emulsions prepared either with the drug incorporated (NP 5) or without (NP 3) have an average diameter of 35 nm, under the normal conditions in Scheme 4.6 as for NP 3. While for poly (butyl acrylate-methyl methacrylate) nanoparticles NP 4, the average particle size slightly increased to 52 nm. The zeta potential analysis shows that the polyacrylate nanoparticles have a negative charge on the surface, although no clear trend can be drawn from the data shown above.

### 4.6.4.6 Reproducibility Studies of Poly (butyl acrylate-styrene) Nanoparticle Synthesis

Five batches of poly (butyl acrylate-styrene) nanoparticles NP 3a-3e were prepared for the investigation of reproducibility of the polymerization formulation and conditions. The average particle size of these five polyacrylate nanoparticles (NP 3a-3e) are shown in Table 4.3.

<table>
<thead>
<tr>
<th>Nanoparticles</th>
<th>DLS (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NP 3a</td>
<td>35.2</td>
</tr>
<tr>
<td>NP 3b</td>
<td>39.7</td>
</tr>
<tr>
<td>NP 3c</td>
<td>39.6</td>
</tr>
<tr>
<td>NP 3d</td>
<td>35.6</td>
</tr>
<tr>
<td>NP 3e</td>
<td>38.3</td>
</tr>
</tbody>
</table>

### 4.6.4.7 Study of Polymerization Formulations and Reaction Conditions

To investigate the effect of the reaction conditions and the formulation on particle size, further studies were performed with nanoparticle NP 3a* prepared using longer reaction time (12 hr), while nanoparticles NP 3f and NP 3f* were prepared with less surfactant (1%) and initiator (0.5%). A longer reaction time (12 hr) was carried out for preparation of NP 3f* (Table 4.4).
Table 4.4: Dynamic light scattering analysis of poly (butyl acrylate-styrene) nanoparticles NP 3a*, NP 3f and NP 3f*.

<table>
<thead>
<tr>
<th></th>
<th>NP 3a</th>
<th>NP 3a*</th>
<th>NP 3f</th>
<th>NP 3f*</th>
</tr>
</thead>
<tbody>
<tr>
<td>DLS (nm)</td>
<td>35.2</td>
<td>37.3</td>
<td>57.2</td>
<td>52.5</td>
</tr>
</tbody>
</table>

In summary, the preparation of polyacrylate nanoparticle is highly reproducible since an average particle size of 35.2 nm was obtained from 5 batches of poly (butyl acrylate-styrene) nanoparticles under the same conditions and formulation. Lengthening the reaction time from 6 hr to 12 hr does not seem to change the particle size, but it rather forms more precipitates from the emulsion and decreases the aqueous volume of the emulsion due to evaporation. Decreasing the amount of both surfactant and initiator does increase the particle size from 35 nm to 57 nm, as shown in Table 4.4 for NP 3f and NP 3f*.

4.6.4.8 Stability Testing

1. Stability Testing for Poly (butyl acrylate-styrene) Nanoparticles

Three samples were prepared for the studies on the stability of nanoparticle emulsions stored at different temperatures. The first sample is the poly (butyl acrylate-styrene) NP 3f, the second sample is the poly (butyl acrylate-methyl methacrylate) NP 4 and the last sample is poly (butyl acrylate-styrene) with drug 27 (1%) NP 5f prepared with 1% of surfactant and 0.5% of initiator. Each sample was then diluted to four different concentrations (10%, 5%, 2.5% and 1.25%) using D.I. water. Each dilution was stored at different temperatures: 40 °C, r.t. and 5 °C. Table 4.5, Table 4.6 and Figure 4.19, Figure 4.20 show the comparison of average particle size for each dilution of NP 3f and NP 5f after being stored for 3 days.

Table 4.5: Average particle size (nm in diameter) for different nanoparticles NP 3f after being stored for 3 days.

<table>
<thead>
<tr>
<th>NP 3f</th>
<th>1.25%</th>
<th>2.5%</th>
<th>5%</th>
<th>10%</th>
</tr>
</thead>
<tbody>
<tr>
<td>5°C</td>
<td>62.1</td>
<td>63.4</td>
<td>62.2</td>
<td>63.3</td>
</tr>
<tr>
<td>r.t.</td>
<td>61.7</td>
<td>62.0</td>
<td>63.5</td>
<td>63.3</td>
</tr>
<tr>
<td>40°C</td>
<td>60.9</td>
<td>65.7</td>
<td>64.2</td>
<td>63.7</td>
</tr>
</tbody>
</table>

Figure 4.19: Particle size vs. concentration for different concentration of freshly-made polymer NP 3f.
Table 4.6: Average particle size (nm in diameter) for different nanoparticles \textbf{NP 5f} after being stored for 3 days.

<table>
<thead>
<tr>
<th>NP 5f</th>
<th>1.25%</th>
<th>2.5%</th>
<th>5%</th>
<th>10%</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 °C</td>
<td>62.1</td>
<td>63.4</td>
<td>62.2</td>
<td>63.3</td>
</tr>
<tr>
<td>r.t.</td>
<td>61.7</td>
<td>62.0</td>
<td>63.5</td>
<td>63.3</td>
</tr>
<tr>
<td>40 °C</td>
<td>60.9</td>
<td>65.7</td>
<td>64.2</td>
<td>63.7</td>
</tr>
</tbody>
</table>

Figure 4.20: Particle size vs. concentration for different concentrations of freshly-made polymer \textbf{NP 5f}.

Each dilution of these two samples was then stored at different temperatures (40 °C, r.t. and 5 °C) up to four weeks. Table 4.7 and Figure 4.21 shows a comparison of the average particle size for each dilution of \textbf{NP 3f} and \textbf{NP 5f} after being stored at 5 °C for one month.

Table 4.7: Average particle size (nm in diameter) for different nanoparticles \textbf{NP 3f} and \textbf{NP 5f} after being stored for one month at 5 °C.

<table>
<thead>
<tr>
<th>NP 3f</th>
<th>1.25%</th>
<th>2.5%</th>
<th>5%</th>
<th>10%</th>
</tr>
</thead>
<tbody>
<tr>
<td>DLS (nm)</td>
<td>59.1</td>
<td>57.7</td>
<td>59.8</td>
<td>60.2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>NP 5f</th>
<th>1.25%</th>
<th>2.5%</th>
<th>5%</th>
<th>10%</th>
</tr>
</thead>
<tbody>
<tr>
<td>DLS (nm)</td>
<td>59.2</td>
<td>55.7</td>
<td>59.0</td>
<td>60.0</td>
</tr>
</tbody>
</table>
Since these was no significant change in the average particle size between the freshly-made nanoparticles and the one stored at 5 °C for one month. In summary, poly (butyl acrylate-styrene) nanoparticles are stable at different temperatures over a period of time. In addition, poly (butyl acrylate-styrene) has a similar average particle size with the nanoparticles \textbf{NP 5f} which contains covalently attached lactam drug 27.

2. Stability Testing for Poly (butyl acrylate-methyl methacrylate) Nanoparticles \textbf{NP 4}

Poly (butyl acrylate-methyl methacrylate) nanoparticle sample \textbf{NP 4} was also prepared. This sample was diluted to four different concentrations (10%, 5%, 2.5% and 1.25%) using D.I. water. All dilutions of \textbf{NP 4} were stored at 40 °C, r.t. and 5 °C for one month and their particle sizes were then measured. Table 4.8 and Figure 4.22 and 4.23 showed the comparison of average particle size for each dilution of \textbf{NP 4} after being stored for 3 days and one month.

Table 4.8: Average particle size (nm in diameter) of diluted samples of \textbf{NP 4} after being stored for 3 days and one month at the indicated temperature.

<table>
<thead>
<tr>
<th></th>
<th>1.25%</th>
<th>2.5%</th>
<th>5%</th>
<th>10%</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textbf{NP 4} (5 °C)</td>
<td>51.6</td>
<td>53.5</td>
<td>47.0</td>
<td>37.6</td>
</tr>
<tr>
<td>r.t</td>
<td>52.9</td>
<td>48.7</td>
<td>67.8</td>
<td>42.5</td>
</tr>
<tr>
<td>40 °C</td>
<td>70.4</td>
<td>44.5</td>
<td>50.6</td>
<td>102</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>1.25%</th>
<th>2.5%</th>
<th>5%</th>
<th>10%</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textbf{NP 4} (5 °C)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>894.9</td>
</tr>
<tr>
<td>r.t</td>
<td>ND</td>
<td>48.0</td>
<td>ND</td>
<td>45.3(46.0)</td>
</tr>
<tr>
<td>40 °C</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>418.5</td>
</tr>
</tbody>
</table>
The average sizes of 10% solid-content poly (butyl acrylate-methyl methacrylate) nanoparticle polymers stored at either 40 °C, or at 5 °C, were increased dramatically from 40 nm to 400 nm; some of them even reached 1 µm. The nanoparticles of different concentrations stored at room temperature seem to be stable. In conclusion, poly (butyl acrylate-methyl methacrylate) NP 4 are not stable at 40 °C or 5 °C after a month, compared to poly (butyl acrylate-styrene) emulsions, since dramatic changes in particle size were observed for poly (butyl acrylate-methyl methacrylate) NP 4. Poly (butyl acrylate-styrene) nanoparticles seem to be much more stable at different storage temperatures than poly (butyl acrylate-methyl methacrylate) emulsions.

3. Stability Testing at Different pH Values

Another stability study was performed to evaluate nanoparticle stability at different pH values. Two nanoparticles, NP 3F (poly (butylacrylate-styrene) with 1% of surfactant and 0.5% of initiator, 12 hr reaction time) and NP 3a (poly (butyl acrylate-styrene with 3% of surfactant and 1% of initiator), which was in storage at room temperature for 6 months, were used. pH buffer solutions ranging from pH 1-12 were freshly made and used for stability testing.
To prepare these samples, 0.1 mL of these two concentrated emulsions was added to 0.1mL of each buffer solution. The resulting solution retained the appearance of the original emulsion, and further analysis of particle size by DLS showed that the nanoparticles were stable at both acidic (pH = 1) and basic solution (pH = 12) (Table 4.9, 4.10 and Figure 4.24, 4.25). The average particle sizes of the original nanoparticle emulsions of both samples are also listed as the reference.

Table 4.9: Comparison of average particle size for nanoparticle NP 3f in different pH buffer solutions.

<table>
<thead>
<tr>
<th></th>
<th>Original (Ref.)</th>
<th>pH 1</th>
<th>pH 2</th>
<th>pH 11</th>
<th>pH 12</th>
</tr>
</thead>
<tbody>
<tr>
<td>DLS (nm)</td>
<td>55.2</td>
<td>50.0</td>
<td>51.3</td>
<td>55.1</td>
<td>58.7</td>
</tr>
</tbody>
</table>

Figure 4.24: Comparison of particle size at different pH values for nanoparticle emulsions NP 3f.

Table 4.10: Comparison of average particle size for 6-month old nanoparticle emulsions NP 3a in different pH buffer solutions.

<table>
<thead>
<tr>
<th></th>
<th>Original (Ref.)</th>
<th>pH 1</th>
<th>pH 2</th>
<th>pH 11</th>
<th>pH 12</th>
</tr>
</thead>
<tbody>
<tr>
<td>DLS (nm)</td>
<td>43.0 nm</td>
<td>42.5</td>
<td>43.7</td>
<td>42.9</td>
<td>39.2</td>
</tr>
</tbody>
</table>

Figure 4.25: Comparison of particle size for nanoparticle emulsions NP 3a at different pH values.
From the data above, both samples of poly (butyl acrylate-styrene) nanoparticles NP 3a and NP 3f* are stable at different pH values, ranging from 1-12. There is no dramatic change for the average particle sizes of these two samples and the emulsions after treatment in different pH buffer solutions still retain the appearance. In summary, poly (butyl acrylate-styrene) nanoparticles are spherical in size with an average of 40 nm. They are stable to storage for one month at different temperatures from 5 °C to 40 °C, and to different pH buffer solutions, ranging from pH 1-12.

4.6.4.9 Studies of the Microbiological Activity of Nanoparticles NP 5

Kirby-Bauer testing and broth MIC assays were used to investigate the biological activities of poly (butyl acrylate-styrene-lactam) nanoparticles NP 5. Agar dilution minimal inhibitory concentration (MIC) assays were also performed for the bioactivity against S. aureus. The concentrations of the emulsions analyzed were based on the drug content incorporated in the emulsions, and these concentrations were placed in a well of a 24 well plate. Phenol red mannitol agar was added to each well in liquid form to produce a total well volume of 1.5 mL. The contents of each well were thoroughly stirred to evenly distribute the antimicrobial within the agar. Once solidified, 10 µL of saline containing 10^7 CFU/ml of the desired bacteria is pipetted on top of each agar and the plates are then incubated for 24 hours at 37 °C. Bacterial growth is assessed by both visual observation and by colorimetric assay.

Comparison of the zones of inhibition and MIC values of monomer 27 and nanoparticles NP 5a-c and 5e against S. aureus (ATCC 25923) and a strain of MRSA (ATCC 43300) are shown in Table 4.11. NP 5a contains 30% w/w of lactam drug 27 in the nanoparticle and is the control. NP 5b, 5c and 5e have 1%, 2% and 4% (w/w) drug in the nanoparticle. MIC values refer to the concentration of bound drug (µg/mL) needed to completely inhibit bacterial growth for 24 hours.

Table 4.11: Antibacterial activity of acrylated monomers 27 and the resulting nanoparticle emulsions NP 5. All zone of inhibition (ZOI) results are reported for 20 µg of both drug monomers and emulsions. Minimal inhibitory concentration (MIC) results were obtained using either broth dilution or agar dilution assay procedures.

<table>
<thead>
<tr>
<th>Compound</th>
<th>MRSA (919)</th>
<th>S. aureus (849)</th>
<th>MIC (µg/mL)</th>
<th>ZOI (mm)</th>
<th>MIC</th>
<th>ZOI</th>
<th>MIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monomer 27</td>
<td>14</td>
<td>128</td>
<td>14</td>
<td>128</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NP 5a</td>
<td>0</td>
<td>64</td>
<td>0</td>
<td>64</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NP 5b</td>
<td>7</td>
<td>32</td>
<td>7</td>
<td>32</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NP 5c</td>
<td>8</td>
<td>32</td>
<td>7</td>
<td>32</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NP 5e</td>
<td>7</td>
<td>32</td>
<td>7</td>
<td>32</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

As shown in Table 4.11, the antibiotic-conjugated poly (butyl acrylate-styrene) nanoparticles NP 5b,c and e showed enhanced anti-MRSA activities compared to the free acrylated forms of the water-insoluble lactam drug 27 and poly (ethyl acrylate-lactam 27) nanoparticle emulsion NP 5a. Zone of growth inhibition of nanoparticle emulsions NP 5b, 5c and 5e are fairly small due to the poor diffusion of the emulsions. However, the MIC assay which is a more accurate measurement shows their values are all around 32 µg/mL, greater than those of the monomer 27 (128 µg/mL) and the nanoparticle NP 5a (64 µg/mL). This indicates that the copolymer of butyl acrylate and styrene seems to have increased the bioactivity relative to the acrylated monomer 27, and the poly (ethyl acrylate-lactam) nanoparticles NP 5a. Finally, these data suggest that butyl acrylate and styrene are the optimal candidate comonomers for further investigations of polyacrylate nanoparticles.
4.7 Choice of Other Acrylated Drug Monomers

*N*-Thiolated β-lactam 27 is the starting drug monomer for these investigations, and contains an acrylate moiety directly at the C3 position of the lactam ring. Water-insoluble β-lactam 27 represents a new family of antibiotics for the treatment of MRSA and it is a good drug monomer for covalent attachment to the polyacrylate backbone via emulsion polymerization. To develop more drug candidates, three essential factors have to be potentially considered. The first one is the drug itself which has to consist of an acrylic moiety for radical emulsion polymerization; the second factor is that the selected drug monomers have to be biologically active after being cleaved off the polymer. The third factor is the linker that connects the drug to the polymer, which should be able to break down chemically or enzymatically to release the drug. The general features of desired drug monomer are shown in Figure 4.26.

![Figure 4.26: General features of the desired drug monomer, and the initial β-lactam monomer 27.](image)

In this study, three other β-lactam antibiotics 37-39 served as the monomer targets for the emulsion polymerization (Figure 4.27). Changing the acrylate group from C3 to the C4-phenolic position is another interesting model (lactam 37) for study. Also, having two acrylate moieties on one molecule (lactam 38) or lengthening the C3 side chain (lactam 39) provide two additional targets that will enable us to the effect of different drug entities and linker units on bioactivity.

![Figure 4.27: Three monomer targets.](image)

4.7.1 Synthesis of C4-Acrylate Lactam 37

Changing the location of the acrylate moiety from C3 in lactam 27 to C4 in lactam 37 provides an alternative drug monomer for polymerization and biological evaluations.
Scheme 4.9 shows the preparation of C₄-acrylate lactam 37. C₄-Hydroxy benzaldehyde was coupled with p-anisidine to give imine 40. Further acrylation of the hydroxyl group on imine 40 gave protected imine 41. Staudinger coupling of methoxyacetyl chloride with the protected imine 41, however, failed to afford the desired β-lactam. Another route shown in Scheme 4.10 illustrates that Staudinger coupling of methoxyacetyl chloride with acetate protected imine 42 successfully gave the C₃-methoxy N-aryl protected β-lactam 43 as a single diastereomer (cis). Hydrolysis of the acetoxyl group on the phenyl ring under basic conditions gave the C₄-phenolic β-lactam 44. Acrylation of the free hydroxyl moiety on the β-lactam with acryloyl chloride gave the C₄ acryloyl N-aryl protected β-lactam 45. Removal of the PMP group with ceric ammonium nitrate (CAN) gave N-dearylated lactam 46. Methylthiolation of 46 with N-methylthiophthalimide (14) afforded C₄-acryloyl N-methylthio β-lactam 37 as a white solid.

Scheme 4.9: Attempted synthesis of C₄-acrylate N-methylthio β-lactam 37.

Scheme 4.10: Alternative route to lactam 37.
Conditions: (a) acetylchloride, triethylamine, CH₂Cl₂, 0 °C-rt, 92%; (b) methoxyacetyl chloride, triethylamine, CH₂Cl₂, rt, 62%; (c) KOH, MeOH, acetone, 0 °C-rt, 87%; (d) triethylamine, acryloyl chloride, CH₂Cl₂, rt, 82%; (e) ceric ammonium nitrate, MeCN, 0 °C, 83%; (f) Hunig’s base, N-methylthiophthalimide (14), CH₂Cl₂, reflux, 76%.

4.7.2 Synthesis of Diacrylate Lactam 38

At the initiation of these studies, it was thought the perhaps a more highly crosslinked polymer could be formed in the emulsion polymerization if the monomer containing difunctional or multifunctional moieties is polymerized together with comonomer(s)\(^{161}\) (Scheme 4.11). Crosslinked particles are of value for chromatographic column packings and for thermally stable matting agents.\(^{162}\) Thus, attaching two acrylates onto the lactam ring would ideally provide a highly crosslinked polymer via emulsion polymerization. This type of crosslinking is usually called homogeneous crosslinking. This was the objective of using diacrylate lactam 38 in the polymerization of the nanoparticles.

Scheme 4.11: Illustration of crosslinked polymers.

Synthesis of diacrylated lactam 38 is described in Scheme 4.12 starting from imine 42. Staudinger coupling of acetoxyacetyl chloride with imine 42 gave C₃-acetoxy N-aryl protected β-lactam 47 in 65% yield. Hydrolysis of the two acetoxy groups under basic conditions gave the dihydroxy β-lactam 48 in 93% yield. Acrylation of free hydroxyl β-lactam with two equivalents of acryloyl chloride under the basic condition gave C₃- and C₄-acryloyl N-aryl protected β-lactam 49 in 92% yield. Deprotection of the PMP group on the β-lactam with ceric ammonium nitrate (CAN) gave N-dearylated lactam 50, followed by methylthiolation with N-methylthiophthalimide (14) to afford C₃ and C₄ di-acryloyl N-methylthio β-lactam 38 as a white solid in 72% yield.

Conditions: (a) Triethylamine, CH₂Cl₂, rt, 65%; (d) KOH, MeOH, acetone, 0 °C-rt, 93%; (e) triethylamine, acryloyl chloride, CH₂Cl₂, rt, 92%; (f) ceric ammonium nitrate, MeCN/H₂O, 0°C, 84%; (g) Hunig’s base, N-methylthiophthalimide (14), CH₂Cl₂, reflux, 65%.

4.7.3 Synthesis of C₃-Long Chain Triester Lactam 39

It was next considered important to investigate the properties of the nanoparticles with a triester linkage between the drug and polymer backbone, since a longer chain may influence bioactivity and the rate of drug release. Thus, lactam 39 was chosen as an additional target for polymerization.

Synthesis of lactam 39 is illustrated in Scheme 4.13. C₃-Hydroxy β-lactam 9b was reacted with succinic anhydride to afford an acid which was found to be unstable. Without any workup, the resulting acid was treated with 2-hydroxyethyl acrylate to give a tri-ester β-lactam 51. Another route to make this intermediate was studied using mono-2-acryloyloxyethyl succinate for coupling with the alcohol 9b. (Scheme 4.14). Removal of the para-methoxyphenyl (PMP) group with ceric ammonium nitrate (CAN) gave the N-dearylated lactam 51. Methylthiolation of 51 with N-methylthiophthalimide (14) afforded C₃-acryloyl β-lactam 39 as a colorless oil.

Scheme 4.14: Alternative method to make 51.

Conditions: a) mono-2-acryloyloxyethyl succinate, EDCI, DMAP, CH₂Cl₂, r.t, 78%.

4.8 Preparation of Polyacrylate Nanoparticles with the Three Drug Monomers 37-39

The general emulsion polymerization procedure employed involves dissolving a water-insoluble acrylated drug in a butyl acrylate and styrene, at 70°C, and the mixture is then pre-emulsified in purified water containing 3% w/w of sodium dodecylsulfate, a surfactant, with rapid stirring. The resulting homogenous solution of micelles is then treated with potassium persulfate (1% w/w), a radical initiator, to induce free radical polymerization. Different amounts of lactam 37 (1 and 2 %) (w/w) were dissolved in different ratios of butyl acrylate/styrene comonomers or ethyl acrylate comonomer for preparation of four types of lactam-attached polyacrylate nanoparticles NP 6 (Scheme 4.15).

Scheme 4.15: Poly (butylacrylate-styrene-lactam 37) NP 6 with different % (w/w) of drug content.
Lactams 38 and 39 (1 or 2% w/w) were dissolved in butyl acrylate/styrene comonomers for preparation of poly (butyl acrylate-styrene-lactam) nanoparticles NP 6 and 7. (Scheme 4.16 and Scheme 4.17)

Scheme 4.16: Poly (butyl acrylate-styrene-lactam 38) NP 7.
Formulation for NP 7
(1% drug content)

<table>
<thead>
<tr>
<th>components</th>
<th>amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-lactam 38</td>
<td>10 mg</td>
</tr>
<tr>
<td>Butyl acrylate</td>
<td>700 mg</td>
</tr>
<tr>
<td>Styrene</td>
<td>300 mg</td>
</tr>
<tr>
<td>Surfactant</td>
<td>30 mg</td>
</tr>
<tr>
<td>Radical initiator</td>
<td>10 mg</td>
</tr>
<tr>
<td>Water</td>
<td>4 ml</td>
</tr>
<tr>
<td>Rxn Temp</td>
<td>70 °C</td>
</tr>
</tbody>
</table>


Formulation for NP 8
(1% drug content)

<table>
<thead>
<tr>
<th>components</th>
<th>amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-lactam 39</td>
<td>10 mg</td>
</tr>
<tr>
<td>Butyl acrylate</td>
<td>700 mg</td>
</tr>
<tr>
<td>Styrene</td>
<td>300 mg</td>
</tr>
<tr>
<td>Surfactant</td>
<td>30 mg</td>
</tr>
<tr>
<td>Radical initiator</td>
<td>10 mg</td>
</tr>
<tr>
<td>Water</td>
<td>4 ml</td>
</tr>
<tr>
<td>Rxn Temp</td>
<td>70 °C</td>
</tr>
</tbody>
</table>
To determine whether there is any unreacted lactam monomer or organic impurities in the poly(butyl acrylate-styrene-lactam) nanoparticles, continuous extraction of the product emulsions using cyclohexane was performed for three continuous days after the preparation of poly (butylacrylate-styrene-lactam) NP 5d to determine the efficiency of the drug monomer incorporated in the polymerization. About 1.5 mL of purified emulsion was saved after being extracted every day. Both purified and unpurified samples were tested for further physical properties, such as average particle size. Particle sizes of the each emulsion after being extracted with cyclohexane were measured by dynamic light scattering (DLS) (Table 4.12).

Table 4.12: Comparison of particle sizes of nanoparticle emulsion NP 5d after extraction.

<table>
<thead>
<tr>
<th></th>
<th>Mv (nm)</th>
<th>Mn (nm)</th>
<th>Ma (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NP 5d original</td>
<td>39.0</td>
<td>34.7</td>
<td>37.4</td>
</tr>
<tr>
<td>NP 5d original-1st trial</td>
<td>40.1</td>
<td>35.5</td>
<td>38.5</td>
</tr>
<tr>
<td>NP 5d after 1 day extraction</td>
<td>104.0</td>
<td>31.3</td>
<td>36.9</td>
</tr>
<tr>
<td>NP 5d after 2 day extraction-1st trial</td>
<td>154.9</td>
<td>34.1</td>
<td>39.5</td>
</tr>
<tr>
<td>NP 5d after 2 day extraction-2nd trial</td>
<td>163.7</td>
<td>35.5</td>
<td>40.9</td>
</tr>
<tr>
<td>NP 5d after 3 day extraction</td>
<td>155.4</td>
<td>35.0</td>
<td>40.6</td>
</tr>
</tbody>
</table>

The data in Table 4.12 indicates that there is no dramatic change in particle size after extraction of the original emulsion with cyclohexane. The residue from the cyclohexane layer contains mainly the impurities from the cyclohexane solvent and there is also no clear indication of unreacted lactam monomer that was extracted from the emulsion. What was learned from this study was that purification of the nanoparticle emulsions by continuous extraction requires distilled cyclohexane or other solvents, which should be done for all future nanoparticle preparations to remove residual monomer or organic impurities.

4.9 Physical Properties of Polyacrylate Nanoparticles NP 6-8

Analysis of the emulsion nanoparticles NP 6-8 was done by $^1$H NMR, scanning electron microscopy (SEM), transmission electron microscopy (TEM), dynamic light scattering (DLS), and zeta potential analysis. These methods reveal different information about particle size, morphology, and composition.

4.9.1 $^1$H NMR

$^1$H NMR spectra for the dry films obtained by coalescing the nanoparticle emulsions of poly styrene NP 2 and the copolymer NP 6a are shown in Figure 4.28. Signals at 3.2 ppm (b) and 4.8 (a) ppm are assigned to the CH$_3$O group at the C$_3$ position of the polymerized β-lactam ring and the vicinal ring protons, respectively. The olefinic protons of the original acrylate in the range of 5.8-6.2 ppm do not show in the spectrum. That indicates that all of the monomeric lactam acrylate and ethyl acrylate was converted to polymeric particles.
4.9.2 Film Formation

Similar films were obtained from emulsions NP 6-8 as illustrated in Figure 4.12.

4.9.3 Transmission Electron Microscopy (TEM)

Particle sizes of nanoparticle emulsions NP 6-8 were measured by transmission electron microscopy (TEM). TEM images shown in Figure 4.29 indicate that polyacrylate emulsions formed in this manner are comprised of spherical nanoparticles, with particle diameters being uniformly within the 30 to 60 nm range. The average sizes and size distribution of the particles are somewhat dependent on the experimental conditions of polymerization, and on the ratios of the acrylate monomers. Further studies of particle size of the nanoparticles prepared under different formulations were done by DLS.

Figure 4.29: Transmission electron microscopy (TEM) of polyacrylate nanoparticles.
4.9.4 Dynamic Light Scattering (DLS) and Zeta Potential Analysis

Dynamic light scattering and zeta potential value of each emulsion were obtained on a Honeywell UPA 150 and Brookhaven ZetaPALS. Table 4.13 summarizes the particle size and zeta potential value for polyacrylate nanoparticles NP 6-8.

Table 4.13: Particle size and zeta potential analysis of nanoparticle NP 6-8.

<table>
<thead>
<tr>
<th>Nanoparticles</th>
<th>Nanoparticle Description</th>
<th>Average Particle Size (nm)</th>
<th>Zeta Potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NP 6a</td>
<td>Poly (BA-sty) with drug 37 (2%)</td>
<td>34.4</td>
<td>-73.78 ± 4.28</td>
</tr>
<tr>
<td>NP 6b</td>
<td>Poly (BA-sty)a with drug 37 (1%)</td>
<td>29.2</td>
<td>-74.47 ± 2.55</td>
</tr>
<tr>
<td>NP 6c</td>
<td>Poly (BA-sty)b with drug 37 (1%)</td>
<td>41.8</td>
<td>-57.06 ± 5.23</td>
</tr>
<tr>
<td>NP 6d</td>
<td>Poly (EA) with drug 37 (1%)</td>
<td>54.9</td>
<td>-55.02 ± 7.06</td>
</tr>
<tr>
<td>NP 7</td>
<td>Poly (BA-sty) with drug 38 (1%)</td>
<td>33.2</td>
<td>-61.42 ± 3.63</td>
</tr>
<tr>
<td>NP 8</td>
<td>Poly (BA-sty) with drug 39 (1%)</td>
<td>43.8</td>
<td>-68.76 ± 3.66</td>
</tr>
</tbody>
</table>

Poly (BA-sty)a: Poly (butylacrylate-styrene = 8:2)
Poly (BA-sty)b: Poly (butylacrylate-styrene = 7:3)

Average particle size of poly (butyl acrylate-styrene) nanoparticles NP 6a-c and NP 7, 8 with a lactam drug covalently attached have similar sizes of 30-40 nm, while nanoparticles NP 6d prepared with poly (ethyl acrylate-lactam) have a slightly larger size, 55 nm. Zeta potential values of each nanoparticle indicate that there is a negative charge on the surface.

4.10 Biological Activities of Nanoparticles

4.10.1 Antibacterial Testing

Microbiological activities of poly (butyl acrylate-styrene-lactam) nanoparticles NP 6-8 against S. aureus and MRSA were investigated by Kirby-Bauer disk diffusion, broth MIC assay and agar dilution minimal inhibitory concentration (MIC) determination. All media and supplies were purchased from Fisher Scientific. All bacteria analyzed were purchased from Hardy Diagnostics, (from ATCC sources), or were clinical isolates obtained from Lakeland Regional Medical Center.

4.10.2 Results and Discussion of Antibacterial Testing

The zone of inhibition values and MIC values of the monomers 37, 38 and 39 and the nanoparticles NP 6-8 against S. aureus (ATCC 25923) and a strain of MRSA (ATCC 43300) are given in Table 4.14. Poly (butyl acrylate-styrene) NP 3a was included as a control. NP 6b, NP 7 and NP 8 have 1% w/w drug content in the nanoparticle. MIC results refer to the concentration of bound drug (µg/mL) needed to completely inhibit bacterial growth for 24 hours.
Table 4.14: Antibacterial activity of acrylated monomers 37, 38 and 39 and the resulting nanoparticle emulsions NP 6b, NP 7 and NP 8. All zone of inhibition (ZOI) results are reported for samples containing 20 µg of both drug monomers and emulsions. Minimal inhibitory concentration (MIC) results were obtained using either broth dilution or agar dilution assay procedures.

<table>
<thead>
<tr>
<th>Compound</th>
<th>MRSA (919)</th>
<th>S. aureus (849)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ZOI (mm)</td>
<td>MIC (µg/mL)</td>
</tr>
<tr>
<td>Monomer 37 NP 6b</td>
<td>12</td>
<td>128</td>
</tr>
<tr>
<td>Monomer 38 NP 7</td>
<td>13</td>
<td>128</td>
</tr>
<tr>
<td>Monomer 39 NP 8</td>
<td>13</td>
<td>64</td>
</tr>
<tr>
<td>NP 3a (control)</td>
<td>0</td>
<td>&gt;256</td>
</tr>
</tbody>
</table>

The data in Table 4.14 indicates that the antibiotic-conjugated polyacrylate nanoparticles NP 6b, NP 7 and NP 8 have enhanced anti-MRSA activities compared to the free acrylated forms of the water-insoluble drugs 37, 38 and 39. Moreover, poly (butylacrylate-styrene) NP 3a control has no activity at all and MICs are greater than 256 µg/mL. Figure 4.30 gives a comparison of the MICs of the drug monomers versus their resulting polyacrylate emulsions.

It is apparent that all N-methythio β-lactam-containing nanoparticles are active against MRSA strains as well as the non-resistant strain, S. aureus 849. Increasing the length of the linkage holding the antibiotic onto the polymer backbone also seems to affect bioactivity of the nanoparticle. NP 8, which has a longer, more flexible linker, has the strongest anti-MRSA properties among all nanoparticles. However, changing the location of this linker, as seen in NP 6b, does not appear to alter the activity of the nanoparticles. Also, NP 7 prepared from lactam 38, whose two acrylates allow for inter- and intra-strand crosslinking, showed the same activity against MRSA as NP 5b. This data suggests that the polyacrylate nanoparticles NP 3-8 are all highly crosslinked during the emulsion polymerization process.
4.11 Mode of Action Studies

The mechanism for antibacterial activity of these nanoparticles is currently under investigation, but is likely related to the overall physical properties of the polymer. The nanoparticles can be viewed as interlinked strands of polyacrylates woven into a nanoball, with most of the drug content residing on the interior. Spherical morphology is confirmed from the SEM and TEM images, and presumably held together by the surfactant, which also prevents aggregation, as well as by covalent crosslinks within the polymer matrix (Figure 4.31). This was also observed in atomic force microscopy (AFM) images taken by Kerriann Greenhalgh with the assistance from the College of Engineering at University of South Florida, showing that the nanoparticles appear as solid spheres with high rigidity.

Figure 4.31: Transmission electron microscopy (TEM) image showing the polymer strands.

Attempts to further increase the degree of crosslinking in the nanoparticle matrix through the use of diacrylated drug 38 does not significantly affect particle size, morphology, or antibacterial activity; NP 7 has similar physical characteristics as NP 4 - NP 8. (Scheme 4.18)

Scheme 4.18: Poly (butylacrylate-styrene-lactam 38) NP 7.

Our experiments confirm that the nanoparticle itself, without the antibiotic attached, has no antibacterial properties of its own. Thus, the antibacterial activity observed is not simply an effect of membrane dissolution by the surfactant or the polymer. Two possible mechanisms of delivery are depicted in Figure 4.32 for the drug-conjugated nanoparticle system.
Figure 4.32: Schematic illustrating modes of delivery of an antibiotic-conjugated nanoparticle to a bacterial cell. In this depiction, the nanoparticle can release drug either while on the surface of the cell or after entry.

Upon interaction with the bacterial cell, the nanoparticle may undergo morphological deformation on the surface of the membrane, exposing the attached drug to bacterial enzymes that hydrolyze the active drug from the polymer backbone. From here, the drug can then enter the cell through its usual route. Alternatively, the nanoparticles may endocytose into the cell before releasing the antibiotic. Electron microscopy studies provide evidence that both pathways are likely occurring for the polyacrylate nanoparticles. TEM images of *S. aureus* cells grown in the presence of the poly (butyl acrylate-styrene) nanoparticles NP 3 (without drug) reveal the presence of the intact nanoparticles on the membrane surface and within the cell wall, or collapsed against the cell’s surface (Figure 4.33). However, for *S. aureus* cells treated with drug-conjugated poly (butyl acrylate-styrene) nanoparticles NP 5b, no nanoparticles are observed inside the cytoplasm of surviving cells, which we speculate could be due to rapid destruction of the bacterium that ingests the drug nanoparticle during cell division. In vitro screens show that these polyacrylate nanoparticles are non-cytotoxic toward human dermal fibroblasts, augmenting the favorable properties and biocompatibilities of polyacrylate biomaterials.

Figure 4.33: Transmission electron microscopy (TEM) images of *S. aureus* cells treated with poly (butyl acrylate-styrene) nanoparticles. (A) Sectioned cells with both spherical nanoparticles inside the cell and deformed nanoparticles on the surface. (B) Exterior of intact cells interacting with the control nanoparticles. The textured appearance in the cytoplasm of the non-dehydrated whole cells results from effervescence under the electron beam. (Images taken by Kerriann Greenhalgh)

4.12 Conclusions
In this chapter, experiments were conducted to prepare, characterize, and assess the bioactivity of spherical nanoparticles containing antibacterial agents covalently attached to the polyacrylate backbone. This work extends the preliminary experiments of Jeung-Yeop Shim who first developed the technique of drug-conjugated nanoparticle polymerization in the Turos laboratory. The primary advantages of this process is that poly (acrylate-\textit{co}-lactam acrylate) nanoparticle spheres prepared by emulsion polymerization 1) are highly uniform in shape and size, 2) have diameters in the range of 30-70 nm in diameter, 3) possess a highly hydrophilic outer surface, and 4) are stable in the temperature range of 5-40 °C and pH range of 1-12. The most significant biological importance of these antibiotics-conjugated nanoparticles is that they have enhanced bioactivity against \textit{S. aureus} and MRSA.

The overriding premise of this methodology is that it allows for the potential enhancement of bioavailability and bioactivity of antibiotic drugs which may otherwise possess poor water solubility or are rapidly deactivated in vivo. Secondly, since the target drug is presumably covalently bound within the interior of the nanoparticles rather than to the exterior surface, the particle may act to secure the drug from chemical or enzymatic destruction until safely transported into the cytoplasm of the target cell where the drug is released for action. Compared to the prior drug delivery systems which use the bio-active materials without modifying its structure, covalently attaching the drug onto the nanoparticles ensures the accumulation of a high local concentration within the bacterial cell prior to release. In addition to improving the membrane permeability of water-soluble drugs into sites within bacterial cells, possibly altering their usual mode of action, this may also be a particularly effective method for enhancing the bioavailability of water-insoluble antibiotics in aqueous media and reducing the side reactions that are the main drawbacks of the other drug delivery systems.

In the next chapter, further studies focus on use of amino ester linkages as a means to attach antibacterial agents to the polymer matrix in these nanoparticles, and the effect this might have on microbiological activity.
CHAPTER 5  NOVEL POLYACRYLAMIDE
NANOPARTICLES FOR DELIVERY OF N-THIOLATED
β-LACTAM ANTIBIOTICS

5.1 Introduction

Polymers based on polyacrylamide (PAM) and its derivatives are widely used for their thickening properties in paints and as pushing fluids in tertiary oil recovery as drag reduction agents and drilling fluids.\(^{165}\) The first polyacrylamide nanoparticle was successfully developed by Kreuter and Speiser in 1976.\(^{166}\) Recent developments of polyacrylamides show that they are capable of binding different proteins, resulting in a wide range of biological activities.

The previous chapter described a new technology based on synthetic polyacrylate nanoparticles for delivery of β-lactam antibiotics. Due to the structural similarity between acrylates and acrylamides, it was decided to extend this research to another family of polymers based on synthetic functional acrylamides, whose properties might make them suitable for a variety of applications related to polymer therapeutics.

5.2 Targeted Functional Monomers

Monomers are building blocks for making polymers, and the desired bulk properties of the polymers usually cannot be achieved by using only one monomer. Thus, comonomers or functional monomers are needed to each specific application. Herein, a series of functionalized acrylamide monomers containing an N-thiolated β-lactam antibiotic are to be prepared for utilization in emulsion polymerization, in the manner described in Chapter 4.

5.2.1 Introduction

Acrylamide is an organic compound of white, odorless, flake-like crystals. The greatest use of acrylamide is as a coagulant aid in drinking water treatment. Other uses include (1) in improving production from oil wells; (2) in making organic chemicals and dyes; (3) in the sizing of paper and textiles; (4) in ore processing; (5) in the construction of dam foundations and tunnels. A typical acrylamide is shown in Figure 5.1, in which R and R’ can be H, alkyl, aryl, or a variety of other substituents.

![Figure 5.1: Structure of acrylamide.](image-url)
Polyacrylamides are usually produced by free-radical chain polymerization of acrylamide monomers. In this process, an initiator is decomposed via thermal energy or UV light to form one or more free radicals that react with a vinyl group CH₂=CH-R. The resulting adduct where the unpaired electron, or radical, is on the CH-R carbon reacts with another monomer in a chain reaction which leads quickly to a high molecular weight material. The polymerization of acrylamide normally takes place in aqueous media. As the reaction proceeds, the solution becomes more viscous, and this corresponds to the use of acrylamide as a water thickener. In this research, the method of microemulsion polymerization described in chapter 4 was applied for generating drug-attached poly (acrylamide) nanoparticles for further investigation of their antibacterial properties (Scheme 5.1).

Scheme 5.1: Polymerization of acrylamide for drug delivery design.

![Scheme 5.1: Polymerization of acrylamide for drug delivery design.](image)

The synthetic polymeric acrylamides are usually submicron in size and have many advantages in drug delivery. In general, these polyacrylamides can be used to provide cellular or tissue delivery of drugs, to improve oral bioavailability, and enhance the water solubility of drugs for intravascular delivery and improve the stability of the drug against enzymatic degradation. Due to their polymeric characteristics, one can control the release of the drug from the polymer to achieve a desired therapeutic level in target sites for optimal therapeutic effects. Moreover, the linkage connecting the drug with the polymer might bind to a biospecific ligand by conjugation which could direct the polymer to the target tissue or organ.

5.2.2 Functional Acrylamide Monomer

5.2.2.1 Design of Drug Monomer

Functional acrylamide monomers have been used previously for preparing polymers and copolymers as basic raw materials for water treating, paper, mining, and oil field chemicals, coatings and adhesives. Few applications in the biomedical area using acrylamide have however been explored.

The target functional acrylamide monomers shown in Figure 5.2 consist of an acrylamide moiety for radical emulsion polymerization, and a biologically active drug (N-thiolated β-lactam) attached through a linker that can potentially break down chemically or enzymatically to release the drug from the backbone of the nanoparticle.

![Figure 5.2: Proposed acrylamide functional monomer.](image)
5.2.2.2 Choice of Linkage

A variety of groups can in principle be used as the linkage for connecting the drug entity to the acrylamide functional group. An amino acid, short peptide or peptide are among these choices. Twenty \( \alpha \)-amino acids are building blocks of proteins that make up the bulk of cell structure and act as enzymes for catalyzing cellular reactions. Some of these amino acids found in proteins also serve functions distinct from the formation of peptides and proteins, e.g., tyrosine in the formation of thyroid hormones or glutamate acting as a neurotransmitter. There are other amino acids found in the body in either free or combined states (i.e. not associated with peptides or proteins). These non-protein associated amino acids perform specialized functions.

The \( \alpha \)-amino acids in peptides and proteins (excluding proline) consist of a carboxylic acid (-COOH) and an amino (-NH\(_2\)) functional group attached to the same tetrahedral carbon atom. This carbon is the \( \alpha \)-carbon. Distinct R groups, that distinguish one amino acid from another, also are attached to the alpha-carbon (except in the case of glycine where the R group is hydrogen). Based on the variety of R substituents that can be present on the \( \alpha \)-carbon, those amino acids having a short chain, a branched chain, a longer chain or bearing a ring were selected for structure and activity relationship (SAR) studies. The representative amino acid used to prepare each type of acrylamide monomer is glycine, alanine, valine, proline, methionine and threonine (Figure 5.3).

![Structures of amino acid acrylamides](image)

Figure 5.3: Structures of amino acid acrylamides used for emulsion polymerization.

5.3 Synthesis of \( N \)-Acrylated Amino Acid Monomers

\( N \)-Acrylated amino acids or their methyl esters were prepared from the reaction between acryloyl chloride and the desired amino acid, either in aqueous solution or in a dry organic solvent. Attempts to synthesize these six \( N \)-acrylated amino acid monomers by direct amidation of acryloyl chloride with the amino acid in aqueous solutions afforded satisfactory results only when glycine, alanine, valine, proline and methionine were used. For preparation of the threonine derivative, it was necessary to employ the amino acid methyl ester instead. The methyl ester was easily obtained by treatment of threonine with SOCl\(_2\) in methanol. Detailed synthetic procedures for each acrylamide monomer will be described in the following section.
5.3.1 N-Acryloyl Glycine (53)

Glycine is a neutral, genetically coded amino acid. It is the only protein-forming amino acid without a center of chirality. Glycine helps trigger the release of oxygen to the energy requiring cell-making process and it is also important in the manufacturing of hormones responsible for a strong immune system.

The procedure for the synthesis of acryloyl-glycine is shown in Scheme 5.2. To a mixture of glycine and NaOH (2 equivalents) in chilled water was added dropwise 1.2 equivalents of acryloyl chloride under cooling at 0 °C. The reaction was stirred at room temperature for 1 hour, and the mixture was neutralized with concentrated HCl to pH ~ 3. The precipitated product was filtered with suction and the melting point of the solid was determined to be 111-112 °C.

Scheme 5.2: Synthesis of N-acryloyl glycine 53.

Numerous methods have been applied for generating ester bonds between a carboxylic acid and an alcohol using different coupling reagents, such as 1,3-dicyclohexylcarbodiimide (DCC)/dimethylaminopyridine(DMAP), 1-(3-(dimethylamino)propyl)-3-ethylcarbodiimide (EDC)/DMAP, 1-hydroxybenzotriazole (HOBt)/DMAP or diethylphosphoryl cyanide (DEPC) with base. The general mechanism involving this coupling reaction starts with the activation of the acid by DCC via 1,2-addition, and the resulting O-acrylisourea intermediate is similar in structure to an acid anhydride and acts as an acylating agents. Further attack by the alcohol on the activated carbonyl group leads to the ester (Scheme 5.3). In this case, EDC/DMAP was used due to the ease of the workup.

With the N-acryloyl glycine 53 in hand, the coupling reaction with racemic C3-hydroxy methylthio β-lactam 17 was performed under EDC/DMAP conditions to afford lactam 54 in 82% yield (Scheme 5.4).
Scheme 5.3: Proposed mechanism of coupling reaction of an acid and an alcohol.

Scheme 5.4: Coupling reaction of 53 with lactam 17.
The structure of amino acid 54 was corroborated by $^1$H NMR (Figure 5.4).

Figure 5.4: NMR of lactam 54.

5.3.2 $\text{N}$-Acryloyl Alanine (55)

Alanine is used by the body to build protein and was first isolated in 1879.\textsuperscript{175} The alpha-carbon in alanine is substituted with a methyl group, making it one of the simplest amino acids with respect to molecular structure and is one of the most widely used in protein construction. It is also an important source of energy for muscle tissue, the brain and central nervous system. It strengthens the immune system by producing antibodies and helps in the metabolism of sugars and organic acids.

L-Alanine was dissolved in two equivalents of NaOH solution at 0 $^\circ$C. To the solution, 1.2 equivalents of acryloyl chloride was added dropwise. The reaction was stirred at room temperature for 1 hr, and the mixture was neutralized with concentrated HCl to pH $\sim$ 3. The precipitated product was filtered with suction and the melting point was determined to be 126-128 $^\circ$C (literature 130 $^\circ$C)\textsuperscript{176} (Scheme 5.5).

Scheme 5.5: Synthesis of $\text{N}$-acryloyl L-alanine 55.

The structure of amino acid 55 was corroborated by $^1$H NMR (Figure 5.5). $^1$H NMR analysis demonstrated that the formed amino acid monomer possessed the pattern for the vinyl moiety. Proton Hc is downfield shifted (6.21 ppm, d, $J = 4.4$ Hz) because of its proximity to the carbonyl group. Protons Ha (5.69 ppm, d, $J = 4.0$ Hz) and Hb (6.05 ppm, d, $J = 4.4$ Hz) are nonequivalent due to the planar geometry of the amide bond. Hence, their coupling to Hc produced split doublet signals.
Figure 5.5: $^1$H NMR analysis of N-acryloyl alanine 55.

The formed N-acryloyl alanine 55 was then reacted with the racemic alcohol 17 using EDC and DMAP to afford the lactam 56 in 91% yield (Scheme 5.6).

Scheme 5.6: Coupling of 55 with lactam 17.

$^1$H NMR analysis of lactam 56 confirmed the structure that the split pattern for the proton on the $\alpha$-carbon of the alanine moiety was a quartet (Figure 5.6).
5.3.3 \textit{N}-Acryloyl Valine (57)

Valine is one of the natural amino acids and is coded for in DNA. Valine is an aliphatic amino acid that is closely related to leucine and isoleucine both in structure and function. These amino acids are extremely hydrophobic and are almost always found in the interior of proteins. Nutritionally, valine is an essential amino acid and must be obtained in the diet. Important sources of valine include soy flour, cottage cheese, fish, meats, and vegetables. Valine is incorporated into proteins and enzymes at the molar rate of 6.9 percent when compared to the other amino acids. It also promotes mental vigor, muscle coordination and helps calm emotions.

L-Valine was dissolved in two equivalents of NaOH solution at 0 \degree C. To the solution, 1.2 equivalents of acryloyl chloride was added dropwise. The reaction was stirred at room temperature for 1 hour, and the mixture was neutralized with concentrated HCl to pH ~ 3. The precipitated product was filtered with suction and the melting point was determined to be 99-101 \degree C (literature 100 \degree C)\textsuperscript{177}. The formed \textit{N}-acryloyl valine 57 was then reacted with the racemic alcohol 17 to afford the lactam 58 in 79 \% yield (Scheme 5.7 and 5.8). The structure of amino acid attached lactam 58 was corroborated by \textit{1}H NMR (Figure 5.7).

Scheme 5.7: Synthesis of \textit{N}-acryloyl L-valine 57.
Scheme 5.8: Coupling of 57 with lactam 17.

\[
\begin{align*}
\text{EDCI, DMAP} & \\
\text{Dry CH}_2\text{Cl}_2, \text{r.t} & \\
79\% & \\
\end{align*}
\]

Figure 5.7: $^1$H NMR analysis of lactam 58.

5.3.4 N-Acryloyl Proline (59)

Proline is also one of the twenty proteinogenic units which are used in living organisms as the building blocks of proteins. The other nineteen amino acids consist of all primary amines, while only proline has a secondary amine. The side chain binding to the nitrogen prevents rotation that provides proline with unique conformational constraints.$^{178}$

Proline is a relatively non-polar amino acid, and since it does not have hydrogen on the amide group, proline can not act as a hydrogen bond donor. Proline can act as a structural disruptor for $\alpha$ helices, and as a turning point in $\beta$ sheets. Multiple prolines and/or hydroxyprolines in a row can create a proline helix which is the predominant structure in collagen.

L-Proline is a non-essential, neutral, genetically coded amino acid. It is extremely important for the proper functioning of joints and tendons; also helps maintain and strengthen heart muscles.$^{179}$
To prepare \(N\)-acryloyl proline (59), a mixture of L-proline and NaOH (2 equivalents) solution was dissolved in chilled water. To the solution, 1.2 equivalents of acryloyl chloride was added dropwise under cooling at 0°C. The reaction was stirred at room temperature for 1 hr, and the mixture was neutralized with concentrated HCl to pH ~ 3. The precipitated product was filtered with suction and the melting point was found to be 109-110°C (Scheme 5.9).

Scheme 5.9: Synthesis of \(N\)-acryloyl L-proline 59.

With the \(N\)-acryloyl proline 59 in hand, the coupling reaction with racemic \(\beta\)-lactam 17 was performed under EDC/DMAP conditions to afford lactam 60 in 84% yield (Scheme 5.10). The structure of amino acid attached lactam 60 was corroborated by \(^1\)H NMR (Figure 5.8).

Scheme 5.10: Coupling of 59 with lactam 17.
5.3.5 N-Acryloyl Methionine (61)

Methionine is one of the essential amino acids and building blocks of proteins. It cannot be produced by the body, and must be provided by the diet. It supplies sulfur and other compounds required by the body for normal metabolism and growth. Methionine also has many functions such as (1) prevents disorders of the hair, skin and nails;\(^\text{180}\) (2) helps lower cholesterol levels by increasing the liver's production of lecithin;\(^\text{181}\) (3) reduces liver fat and protects the kidneys; (4) serves as a natural chelating agent for heavy metals;\(^\text{182}\) (5) regulates the formation of ammonia and creates ammonia-free urine which reduces bladder irritation and (6) influences hair follicles and promotes hair growth.\(^\text{183}\)

Some research demonstrated that people with AIDS had low levels of methionine and this may explain some aspects of the disease process.\(^\text{184}\) A preliminary study has suggested that methionine may improve memory recall in people with AIDS-related nervous system degeneration.\(^\text{185}\) Other preliminary studies have suggested that methionine may help treat some symptoms of Parkinson’s disease.\(^\text{186}\)

A mixture of L-methionine and NaOH (2 equivalents) was dissolved in chilled water. To the solution, 1.2 equivalents of acryloyl chloride was added dropwise under cooling at 0 °C. The reaction was then stirred at room temperature for 1 hr, and the mixture was neutralized with concentrated HCl. In this case, only a small amount of precipitate was formed after being acidified with concentrated HCl to pH ~ 3. Thus, the resulting solution was extracted with ethyl acetate three times and dried over MgSO\(_4\) and the solvent was evaporated to afford a solid with a melting point of 113-115 °C (Scheme 5.11).
Scheme 5.11: Synthesis of $N$-acryloyl $L$-methionine 61.

The coupling reaction between racemic C$_3$-hydroxy methylthio $\beta$-lactam 17 and acryloyl- $L$-methionine 61 was performed under EDC/DMAP conditions to afford lactam 62 in 78 % yield (Scheme 5.12). The structure of amino acid attached lactam 62 was corroborated by $^1$H NMR (Figure 5.9).

Scheme 5.12: Coupling of 61 with lactam 17.
5.3.6 \textit{N}-Acryloyl Threonine Derivative (64)

Threonine is an alcohol-containing amino acid that can not be produced by metabolism and must be taken from the diet as well. This amino acid plays an important role along with glycine and serine in porphyrin metabolism. Excessive use of threonine can cause the formation of too much urea and consequently ammonia toxicity in your body. To be used effectively, threonine requires vitamin B6, magnesium, and niacin.

Threonine is an important part of many proteins in the body and is necessary for the formation of tooth enamel and elastin and collagen which both are needed for healthy skin and wound healing. It is also an important amino acid for the nervous system. There are relatively high levels of threonine in the central nervous system. It has been used as a supplement to help alleviate anxiety and some cases of depression. The role of threonine in the functioning of the nervous system is highlighted by the body's increased demand for this amino acid during times of stress.

Direct amidation of \textit{l}-threonine with acryloyl chloride failed to afford the desired protected amino acid 64 (Scheme 5.13). Thus, its methyl ester derivative, compound 63, was formed with SOCl$_2$ refluxing in methanol (Scheme 5.14). The threonine methyl ester was then dissolved in dichloromethane and 1.2 equivalents of triethylamine was added. The solution was chilled and acryloyl chloride (1.1 equiv) was added dropwise. The cooling solution was warmed to room temperature and stirred overnight. The solvents were removed in vacuum and the residues were dissolved in ethyl acetate. The crystals (triethylammonium hydrochloride) were filtered out and the solution was washed three times with 1 M NaHSO$_4$ and 5% of NaHCO$_3$ and once with brine. The organic layer was dried over MgSO$_4$ and concentrated to give acryloyl-threonine methyl ester 64.
Scheme 5.13: First attempt at synthesizing of N-acryloyl threonine.

Scheme 5.14: Another route for the synthesis of N-acryloyl threonine 64.

To prepare threonine-linked lactam 65, the racemic C₃-hydroxy methylthio β-lactam 17 was first converted to an acid by reacting with succinic anhydride. The unstable intermediate was not isolated but instead reacted directly with the amino acid 64 under EDC/DMAP conditions to afford lactam 65 in 69% yield (Scheme 5.15).

5.4 Emulsion Polymerization of Acryloyl Amino Acid-Linked Nanoparticles

5.4.1 Procedure and Formulation
As mentioned earlier, polyacrylamides are normally synthesized by free-radical chain polymerization. Herein, polyacrylamides of N-acryloyl amino acids or their esters, onto which a β-lactam antibiotic is attached, were generated via emulsion polymerization. The general procedure employed for this system involved dissolving the water-insoluble drug in a mixture of liquid acrylates, i.e. butyl acrylate and styrene, at 70°C, and the mixture was then pre-emulsified in purified water containing 3% w/w of sodium dodecylsulfate, a surfactant, with rapid stirring. The resulting homogenous solution of micelles was then treated with potassium persulfate (1% w/w), a radical initiator, to induce free radical polymerization. Scheme 5.16 illustrates a general procedure for preparation of drug-conjugated polyacrylamide nanoparticles. Detailed reaction formulation and reaction conditions are shown in Figure 5.10.

Scheme 5.16: Preparation of polyacrylamide nanoparticles (NP 9, 10, 11 and 12).

![Scheme 5.16](image)

<table>
<thead>
<tr>
<th>Formulation and Rxn Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>components</td>
</tr>
<tr>
<td>β-lactam 54/56/58/60</td>
</tr>
<tr>
<td>Butyl acrylate</td>
</tr>
<tr>
<td>Styrene</td>
</tr>
<tr>
<td>Surfactant</td>
</tr>
<tr>
<td>Radical initiator</td>
</tr>
<tr>
<td>Water</td>
</tr>
<tr>
<td>Rxn Temp</td>
</tr>
</tbody>
</table>

NP 9/10/11/12

Figure 5.10: Formulations used for the preparation of polyacrylamide nanoparticles NP 9-12.

5.4.2 Physical Properties of Polyacrylamide Nanoparticles

1H NMR spectra for a dry and stretchy film obtained by coalescing the nanoparticle emulsions of co-poly (butyl acrylate-styrene-drug 56) showed that the olefinic protons of the acrylamide in the range of 5.8-6.2 ppm did not show up in the spectrum. That indicated that all of the monomeric lactam acrylamide, butyl acrylate and styrene were converted to polymeric particles.
Dynamic light scattering data was acquired on a UPA 150 Honeywell MicroTrac at the University of Florida Particle Engineering Research Center. One drop of the concentrated emulsion (20% solid content) was placed in a 10 mL well filled with nanopure water. Analysis was performed for 3 runs of 180 seconds per run per sample.

Zeta potential value of each emulsion was obtained on a Brookhaven ZetaPALS. The emulsion solution was diluted to 1.5% (~ pH = 7) of solid content using nanopure water for analysis and 2 × 10 runs per sample was performed. The dispersant viscosity of the emulsion solution was 0.8872cP, and the dielectric constant of medium was 78.55. Table 5.1 summarizes the particle size and zeta potential value for each polymer. The average particle size was uniform 35-40 nm in diameter.

Table 5.1: Comparison of average particle size (nm) and surface charge (-mV) of each polyacrylamide nanoparticle.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Linkage in monomer</th>
<th>Dynamic light scattering (nm)</th>
<th>Zeta potential (-mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NP 9</td>
<td>Glycine</td>
<td>34.6</td>
<td>-57.02 ± 3.11</td>
</tr>
<tr>
<td>NP 10</td>
<td>Alanine</td>
<td>36.9</td>
<td>-66.45 ± 3.37</td>
</tr>
<tr>
<td>NP 11</td>
<td>Valine</td>
<td>35.6</td>
<td>-61.42 ± 0.41</td>
</tr>
<tr>
<td>NP 12</td>
<td>Proline</td>
<td>39.1</td>
<td>ND</td>
</tr>
</tbody>
</table>

5.5 Biological Activities against *S. aureus* and MRSA

All media and supplies were purchased from Fisher Scientific. All bacteria analyzed were purchased from Hardy Diagnostics, (ATCC sources), or were clinical isolates obtained from Lakeland Regional Medical Center.

The antibacterial activities for both drug monomer and its polymer were determined by the standard Kirby-Bauer well diffusion testing and agar dilution minimal inhibitory concentration (MIC) assay. For Kirby-Bauer testing, the wells were filled with 20 µL of antimicrobial solution or nanoparticle emulsion and the plates were then incubated for 24 hours at 37°C. Zones of inhibition were determined for each well using a millimeter scale. For MIC assay, the antimicrobial and polyacrylamide nanoparticle emulsion concentrations analyzed were determined by the standard NCCLS protocol M7-A5. The concentrations of the emulsions analyzed were based on the drug content incorporated in the emulsions, and these concentrations were placed in a pre-determined well of a 24 well plate. Muller Hinton agar was added to each well in liquid form to produce a total well volume of 1.5 mL.

5.5.1 Monomer Activities against *S. aureus* and MRSA

Comparison of the observed zone of inhibition (measured in mm) of all monomers 54-62 against *S. aureus* (ATCC 25923) and a strain of MRSA (ATCC 43300) is illustrated in Figure 5.11. The MIC values are presented below each sample as well.
Figure 5.11: Comparison of bioactivities of acrylated monomers 54, 56, 58, 60 and 62.

The amino acid-containing acrylamide lactam monomers showed some correlation between the structural features and biological activity. Zones of inhibition showed a trend that with lengthening or branching the R group, bioactivities decreased. In the case of glycine-derived lactam 54, the higher activity may be attributed to its simple structure; however, lactam 62, having a fairly long side chain with a terminal –SCH3 group, exhibited the lowest bioactivities.

The same trend was also found in the MICs profiles of these compounds, 32 µg/mL is the concentration for glycine lactam 54, and 64 µg/mL for lactams 56 and 58 with amino acids bearing a methyl or isopropyl group. Lactams 60 and 62, each having a ring or longer chain, MICs are around 128 µg/mL. Thus, increasing lipophilicity in the amino acid side chain leads to a lower degree of biological activity.

5.5.2 Polymer Activities against *S. aureus* and MRSA

In the present study, polyanhydrides molecules based on amino acids-linked β-lactam drugs were prepared from each of the five acrylated amino acid monomers above, and tested for anti-MRSA activity. Comparison of the MICs for each polymer system is shown in Figure 5.12. Experimental data showed none of the polymers tested in the Kirby Bauer assay expressed significant activity against *S. aureus* and MRSA, indicating that these polymers may not be able to diffuse through the agar at a sufficient concentration to inhibit bacteria growth. However, the MICs of the nanoparticles illustrated a slight increase of bioactivity against MRSA. For example, for nanoparticle containing valine-attached lactam 58, its MICs increased to 32 µg/mL compared to its monomer 58 having MICs of 64 µg/mL. Thus, the polyanhydride nanoparticle containing drug did not destroy antibacterial activity.
Since both acryloyl amino acid-linked monomer and nanoparticles show good bioactivities against MRSA, and it will be interesting to investigate the potent activities against other bacteria, such as *Bacillus*, or cancer cell line. Prior work on screening of β-lactams with cancer cell shows that *N*-methylthio β-lactam 1 has unique anticancer properties against human leukemic and solid tumor cell lines. In Jurkat T (leukemia) cells, the lactam 1 was found to inhibit cellular mitosis. Further in vitro screening on Jurkat cells with another β-lactam 56, also showed that the acryloyl-alanine-attached lactam 56 exhibited the similar potent anticancer activity as lactam 1. With this promising preliminary data, five other acryloyl-amino acid attached β-lactams 54, 58, 60, 62 along with three other β-lactams, 65-68 were screened for anticancer properties (Figure 5.13). Lactam 25 was investigated further in an animal model as mentioned in chapter two. Lactam 67 is another long chain derivative similar to compound 65, and lactam 68 has two hydroxyl groups on the lactam which increases the water solubility of the lactam.

Figure 5.12: Comparison of bioactivities of polyacrylamide nanoparticles versus their monomers.

Figure 5.13: Compounds subjected to anticancer screening in Dr. Q. Ping Dou’s laboratory (Wayne State University).
Compound 67 bears a similar long chain at the C₃ position of the lactam. The synthesis of 67 is shown in Scheme 5.17. The synthesis started with methylation of L-methionine with SOCl₂ refluxing in methanol for 3 hours. The resulting methyl ester 66 was then reacted with the unstable succinic ester of the lactam to afford 67 in 72% yield.

Scheme 5.17: Synthesis of lactam 67.

The preparation of lactam 68 is illustrated in Scheme 5.18. The PMP group of lactam 47 was deprotected with CAN to give lactam 69. N-Methylthiolation of 69 with the usual reagent (14) gave methylthio lactam 70. Hydrolysis of both acetate groups of 70 using two equivalents of KOH afforded the final compound, dihydroxy lactam 68.

Scheme 5.18: Synthesis of di-hydroxy lactam 68.

Conditions: a) ceric ammonium nitrate, CH₃CN, water, 0 °C; b) N-methylthiophthalimide 14, Hunig’s base, dry CH₂Cl₂, refluxing; c) K₂CO₃, MeOH, 0 °C.
For the anticancer assays, trypan blue was added to Jurkat cells treated with the lactams to visualize percentage of cell death at 25 µg/mL and 50 µg/mL drug concentrations (Figure 5.14). β-Lactam 1 was used as a positive control.

![Percent Cell Death after 24 Hours](image)

Figure 5.14: Trypan blue staining of Jurkat cells with the lactams after 24 hours of treatment.

At 24 hours it is noted that lactam 60 was the only lactam from this group that was more potent than 1. Lactams 65 and 67, two lactams with longer R groups, were the only other lactams with similar activity to 1. Figure 5.15 demonstrates the percentage of cell death after 48 hours of treatment with the lactams.

![Percent Cell Death after 48 Hours](image)

Figure 5.15: Trypan blue staining of the same Jurkat cells after 48 hours of treatment with the lactams.

It is observed that all lactams induce a lower percentage of cell death after 48 hours versus 24 hours. The trend of lactam efficacy seems the same as with 24 hours. Lactam 60 appears to be the most potent; however 54, a lactam with a shorter R group, appears to have a similar efficacy to 1. Those two lactams with longer R groups, seem to have diminished efficacy after 48 hours.
The following screening was done on human leukemia (HL-60) cells shown in Figure 5.16 and Figure 5.17. A different cell line was used to see if the effects of the lactams would be the same, as well as to see which cell line responded to the drug effectively. HL 60 was treated with the lactams to visualize percentage of cell death at 25 µg/mL, 50 µg/mL and 75 µg/mL drug concentrations. As illustrated, cells failed to respond effectively to beta lactam treatment. LLL, which is Z-Leu-Leu-Leu-CHO or MG132, a tripeptidyl proteasome inhibitor, was used as a positive control along with lactam 1 throughout the beta lactam experiments.

Figure 5.16: β-Lactam treatment of HL-60 cells after 24 hrs.

Figure 5.17: β-Lactam treatment of HL-60 cells after 48 hours.
In summary, with respect to Jurkat cell treatment, lacams 60, 65 and 67 were most effective at inducing cell death. It could be assumed from this that increased length of the R group aids in drug function. Overall, the combination of a long chain R group and possibly the addition of a ring structure as a substituent act to increase drug efficacy.

Acryloyl glycine β-lactam 54 appeared to be more effective in the treatment of HL-60 cells, with 60 still having high efficacy, and 65 and 67 inducing about the same percent cell death. Using this data it is hard to determine if the length of the R group has an effect, however, the side chain ring structure does seem to increase drug efficacy.

In conclusion, several acryloyl amino acid-linked monomers show good activities against both Jurkat cell and human leukemia (HL-60), and the polyacrylamide nanoparticles prepared from these monomers have been submitted for anticancer study.

5.6 Conclusions and Future Work

Nano-sized polymer-based delivery systems have received significant interest all over the world, particularly for anticancer agents. Our development in drug delivery using polyacrylate/polyacrylamide nanoparticles has resulted in a new vehicle for antibacterial applications. Several essential features of our system are (1) the straightforward strategy for preparation of functional drug monomers and polymer frameworks in aqueous media using emulsion polymerization, (2) ability to generate small (30-60 nm) and uniform nanoparticles with narrow size distribution, and (3) the nanoparticle substance exhibits better antibacterial activity against S. aureus and MRSA than its monomer. Additionally, the nondegradable and nontoxic nature of polyacrylate/polyacrylamide polymers would ensure that the vehicle itself would not be toxic.

To help understand the mode of action of these nano-sized polymeric antibiotics, or nanobiotics, investigations are being conducted. In the future, longer amino acid chain linkers may be used, such as tri-amino acid, Pro-Hyp-Gly (Hyp = hydroxyproline), which is found most prominently in native collagen, and peptides composed solely of these trimers give rise to the most stable triple helices.188

Another method being developed in our laboratory to track the nanoparticles is to label the polymer using fluorescent markers. As mentioned in chapter two, fluorescence has proved to be a versatile tool for numerous applications, including the molecular interactions in life science. Moreover, the concept of Forster Resonance Energy Transfer (FRET) is especially a promising method. A suitable FRET pair, with an appropriate fluorescence wavelength, could provide information on the mechanism. Ideally, a fluorescent moiety which functions as a donor will be installed onto the lactam monomer and another fluorescent pair will be installed onto a comonomer as an acceptor. Theoretically, the donor will overlap with the absorption wavelength of the other, the acceptor. In other words, the fluorescence of the donor would be quenched by the acceptor. Therefore, as the donor and acceptor are separated, the quenching effect exponentially decreases and the donor “lights up” as its fluorescence is released. This may let us know the path of the nanoparticles through the bacteria cell membrane and into the cell.

However, there is no literature yet on fluorescence-active polymeric nanoparticles. Therefore, it may open a new area in biological diagnostics if fluorescence-active nanoparticles are successfully prepared and applied appropriately to detection of specific biological molecules, such as proteins or microbes acting as water dispersed biological sensors or biological imaging agents. There remains a considerable amount of room for further exploration on the design and properties of polyacrylate/polyacrylamide nanoparticles, both in the drug delivery/drug discovery areas and new biomaterials. The research described in this thesis may provide a foundation for building upon existing methodologies in each of these areas.
CHAPTER 6 MATERIALS AND METHODS

6.1 Synthetic Procedures

All reagents were purchased from Sigma-Aldrich Chemical Company and used without further purification. Solvents were obtained from Fisher Scientific Company. Thin layer chromatography (TLC) was carried out using EM Reagent plates with a fluorescence indicator (SiO₂-60, F-254). Products were purified by flash chromatography using J.T. Baker flash chromatography silica gel (40 µm). ¹³C NMR spectra were proton broad-band decoupled.

6.1.1 Preparation of N-Anisylimines (6)

Aldehyde (1 mmol), anisidine (1 mmol), and 1 mg of camphorsulfonic acid (cat.) were dissolved in dichloromethane and stirred at room temperature for 1 hr. TLC showed the disappearance of the starting materials and the solvent was then removed in vacuo and the imine was recrystallized from methanol.

\[
\begin{align*}
\text{5a: } & R=H \\
\text{5b: } & R=\text{Cl}
\end{align*}
\]

\[
\begin{align*}
\text{6a: } & R=H, \quad 93\% \\
\text{6b: } & R=\text{Cl}, \quad 95\%
\end{align*}
\]

6.1.2 Preparation of Acid Chlorides

Methoxyacetyl chloride: To a 250 ml flame dried round bottom flask containing methoxyacetic acid (31.38 g, 0.35 mol) was added thionyl chloride (41.4 g, 0.35 mol) dropwise while stirring for 0.5 hr at 0°C. The ice bath was removed and the mixture reacted for 12 hr at 30°C. The pale yellow solution was then distilled to provide pure acid chloride (bp 112-113°C).
Acetoxyacetyl chloride (7): To a 250 ml round bottom flask containing glycolic acid (10 g, 0.13 mol) was added acetyl chloride (20 g, 0.26 mol) dropwise at 0°C. The ice bath was removed and the resulting slush dissolved within 30 min after the acetyl chloride addition. After 1 hr, a white precipitate forms. Benzene (50 ml) was added and the reaction stirred for an additional 12 hr. The solid was then filtered and washed with benzene and ice cold methanol to give pure product 26 in 80% yield (mp 62-63°C). Afterwards, thionyl chloride (31.8 g, 0.24 mol) was added dropwise at 0°C to a 250 ml flask containing acid 26 (24.6 g, 0.24 mol) while stirring. The ice bath was then removed and the resulting slush was stirred overnight at 30°C. After 12 hr, the liquid was distilled in vacuo to give the acetoxyacetyl chloride in 60% yield from glycolic acid.

AcOCl

6.1.3 Preparation of N-4-Anisyl Azetidin-2-ones 8

Imine (1 mmol) and N,N-ethyldiisopropylamine (5 mmol) or triethylamine (5 mmol) were added to a 250 ml round bottom flask containing dry dichloromethane (50 ml per g of imine) and the mixture was chilled in an ice bath to 0 °C. Acid chloride (1.2 eq. 6 mol) in dry methylene chloride (50 ml per g acid) was added dropwise (5 ml per min) to the stirring solution. Following addition of the acid chloride, the flask was removed from the ice bath and warmed to r.t. The reaction was monitored by thin layer chromatography. After water extraction twice, the organic layer was dried with magnesium sulfate, filtered, and the crude material was concentrated in vacuo. Diethyl ether or methanol was then added and the solution chilled to precipitate the β-lactam. When silica gel chromatography was required for purification, separation was achieved with ethyl acetate and hexane mixtures (1:1).

**cis-3-Acetoxy-N-(4-methoxyphenyl)-4-phenylazetidin-2-one (8a):** white solid, mp 153-155 °C. 1H NMR (250 MHz, CDCl3) δ 7.34-7.30 (5H, m), 7.28 (2H, d, J = 8.9 Hz), 6.79 (2H, d, J = 8.9 Hz), 5.92 (1H, d, J = 4.8 Hz), 5.33 (1H, d, J = 4.8 Hz), 3.74 (3H, s), 1.66 (3H, s); 13C NMR (63 MHz, CDCl3) δ 169.1, 161.2, 156.5, 132.2, 130.2, 128.7, 128.4, 127.8, 118.7, 61.3, 55.3, 19.7.

**cis-3-Acetoxy-N-(4-chlorophenyl)-4-phenylazetidin-2-one (8b):** white solid, mp 130-132°C. 1H NMR (250 MHz, CDCl3) δ 7.43 (1H, d, J = 8.9 Hz), 7.32-7.23 (5H, m), 6.83 (2H, d, J = 8.9 Hz), 6.16 (1H, d, J = 5.0 Hz), 5.78 (1H, d, J = 5.0 Hz), 3.76 (3H, s), 1.76 (3H, s); 13C NMR (63 MHz, CDCl3) δ 168.6, 161.3, 156.6, 133.8, 130.1, 129.9, 129.7, 128.6, 126.7, 118.5, 114.4, 75.4, 58.1, 55.3, 19.8.
6.1.4 Hydrolysis of N-Aryl Protected β-Lactam 8

To a solution of 8b (1.23 g, 3.56 mmol) in 30 mL of acetone was added a solution of KOH (200 mg, 3.56 mmol) in 10 mL of MeOH at 0°C. The hydrolysis was complete after the addition of KOH/MeOH as indicated by TLC. The reaction was quenched by adding an equal volume of water upon which the product precipitated out of solution. The product was filtered and dried to give 1.07 g of the product in 99% yield.

\[ \text{OCH}_3 \]

cis-3-Hydroxy-N-(4-methoxyphenyl)-4-phenylazetidin-2-one (9a): white solid, mp 183-184°C. \(^1^H\) NMR (250 MHz, CDCl\(_3\)) \(\delta\) 7.48 (1H, d, \(J = 7.5\) Hz), 7.33-7.22 (5H, m), 6.84 (2H, d, \(J = 8.9\) Hz), 5.63 (1H, d, \(J = 5.1\) Hz), 5.33 (1H, d, \(J = 5.1\) Hz), 4.88 (1H, brs), 3.78 (3H, s); \(^{13}\)C NMR (63 MHz, DMSO-d\(_6\)) \(\delta\) 166.6, 156.1, 133.1, 132.9, 130.9, 129.7, 129.6, 128.9, 127.3, 118.6, 114.9, 77.2, 60.0, 55.6.

cis-4-(2-Chlorophenyl)-3-hydroxy-N-(4-methoxyphenyl)azetidin-2-one (9b): white solid, mp 193-194°C. \(^1^H\) NMR (250 MHz, CDCl\(_3\)) \(\delta\) 7.48 (1H, d, \(J = 7.5\) Hz), 7.33-7.22 (5H, m), 6.84 (2H, d, \(J = 8.9\) Hz), 5.63 (1H, d, \(J = 5.1\) Hz), 5.33 (1H, d, \(J = 5.1\) Hz), 4.88 (1H, brs), 3.78 (3H, s); \(^{13}\)C NMR (63 MHz, DMSO-d\(_6\)) \(\delta\) 166.6, 156.1, 133.1, 132.9, 130.9, 129.7, 129.6, 128.9, 127.3, 118.6, 114.9, 77.2, 60.0, 55.6.

6.1.5 Substitution of Sulfonate at C\(_3\) Lactam

\[ \text{OCH}_3 \]

Synthesis of cis-N-(4-Methoxyphenyl)-3-methylsulfonyl-4-phenylazetidin-2-one (10b): Compound 9a (269 mg, 1.00 mmol) was dissolved in dry CH\(_2\)Cl\(_2\) (15 mL), and 60 mg (1.50 mmol) of NaH (60% in mineral oil, unwashed) was added. After stirring for 30 min at room temperature the resulting solution was then added dropwise methanesulfonyl chloride (115 mg, 1.00 mmol). The resulting solution was stirred at rt for 30 min. The solution was washed with brine (3 x 15 mL). The organic layer was dried with anhydrous MgSO\(_4\), filtered, and evaporated, and the residue was washed with cold MeOH to give 267 mg of 10b as a white solid in 93% yield, mp 158-160°C. \(^1^H\) NMR (250 MHz, CDCl\(_3\)) \(\delta\) 7.48 (2H, d, \(J = 7.8\) Hz), 7.39-7.26 (7H, m), 6.85 (2H, d, \(J = 8.7\) Hz), 5.94 (1H, d, \(J = 5.1\) Hz), 5.82 (1H, d, \(J = 5.1\) Hz), 3.75 (3H, s), 3.02 (3H, s); \(^{13}\)C NMR (63 MHz, CDCl\(_3\)) \(\delta\) 163.2, 157.3, 132.5, 131.8, 130.2, 129.8, 129.3, 129.1, 128.5, 128.1, 119.4, 114.9, 102.1, 79.8, 61.9, 55.9, 39.2.
**cis-N-(4-Methoxyphenyl)-4-phenyl-3-(4-toluenesulfonyl)azetidin-2-one (10a):** White solid, mp 148-151°C, 75% yield. $^1$H NMR (250 MHz, CDCl$_3$) $\delta$ 7.46 (2H, d, $J$ = 7.9 Hz), 7.36-7.28 (8H, m), 6.77 (2H, d, $J$ = 8.5 Hz), 5.78 (1H, d, $J$ = 4.9 Hz), 5.28 (1H, d, $J$ = 4.9 Hz), 3.73 (3H, s), 2.43 (3H, s); $^{13}$C NMR (63 MHz, CDCl$_3$) $\delta$ 161.2, 157.6, 147.2, 138.8, 133.4, 131.9, 130.2 (2C), 129.3, 128.5, 128.2, 127.8 (2C), 127.4 (2C), 119.1 (2C), 79.6, 58.1, 55.8, 22.1.

**cis-Benzenesulfonyl-N-(4-methoxyphenyl)-4-phenylazetidin-2-one (10c):** White solid, mp 162-165°C, 84% yield. $^1$H NMR (250 MHz, CDCl$_3$) $\delta$ 8.05 (2H, d, $J$ = 4.0 Hz), 7.75-7.60 (5H, m), 7.50-7.18 (5H, m), 6.79 (2H, d, $J$ = 8.6 Hz), 5.90 (1H, d, $J$ = 5.0 Hz), 5.77 (1H, d, $J$ = 5.0 Hz), 3.74 (3H, s); $^{13}$C NMR (63 MHz, CDCl$_3$) $\delta$ 160.7, 157.6, 136.2, 134.5, 133.2, 132.1, 129.6, 129.2, 128.5, 128.2, 127.8 (2C), 127.1 (2C), 119.3 (2C), 114.8 (2C), 79.6, 62.1, 55.8.

### 6.1.6 N-Dearylation of N-(4-Methoxyphenyl) Substituted Lactams

To a solution of lactam **10b** (0.347 g, 1.00 mmol) in CH$_3$CN (10 mL) at 0°C was added dropwise a solution of ceric ammonium nitrate (1.64 g, 3.00 mmol) in 14.9 mL of water within 20 mins. The reaction was stirred for another 15 min and then diluted with EtOAc (30 mL). The resulting solution was washed with water (30 mL) extracting with EtOAc (4 × 30 mL). The combined organic layer was then washed with 5% NaHCO$_3$ (30 mL), extract the aqueous layer with EtOAc (30 mL) twice. The combined organic layer was washed with 10% NaHSO$_3$ (2 × 30 mL) till the aqueous layer became clear. Finally organic was washed with 5% NaHCO$_3$ (30 mL) and brine (30 mL). The organic layer was dried with anhydrous MgSO$_4$, filtered, and evaporated, and the residue was chromatographed on silica gel (1:2 EtOAc/hexanes) to give 0.216 g of **11b** as yellow oil in 90% yield. $^1$H NMR (250 MHz, CDCl$_3$) $\delta$ 7.36-7.19 (5H, m), 6.58 (1H, brs), 5.71 (1H, d, $J$ = 4.9 Hz), 4.99 (1H, d, $J$ = 4.9 Hz), 2.68 (3H, s); $^{13}$C NMR (63 MHz, CDCl$_3$) $\delta$ 164.7, 134.2, 129.6, 129.1, 128.7, 128.5, 128.3, 128.2, 126.6 (2C), 125.1 (2C), 81.6, 58.5.

**cis-4-Phenyl-3-(4-toluenesulfonyl)azetidin-2-one (11a):** Yellow oil, 85% yield. $^1$H NMR (250 MHz, CDCl$_3$) $\delta$ 7.45-7.18 (9H, m), 6.41 (1H, brs), 5.69 (1H, d, $J$ = 2.4 Hz), 4.95 (1H, d, $J$ = 4.7 Hz), 2.41 (3H, s); $^{13}$C NMR (63 MHz, CDCl$_3$) $\delta$ 164.3, 136.1, 134.6, 134.4, 130.0, 129.3, 129.0, 128.5, 128.3, 128.2, 126.6 (2C), 125.1 (2C), 81.6, 58.5.

**cis-Benzenesulfonyl-4-phenylazetidin-2-one (11c):** Yellow oil, 88% yield. $^1$H NMR (250 MHz, CDCl$_3$) $\delta$ 8.05 (1H, d, $J$ = 8.0 Hz), 7.78-7.63 (6H, m), 7.48-7.20 (3H, m), 6.42 (1H, brs), 5.83 (1H, d, $J$ = 4.8 Hz), 5.41 (1H, d, $J$ = 4.8 Hz); $^{13}$C NMR (63 MHz, CDCl$_3$) $\delta$ 164.2, 136.0, 134.5, 134.3, 129.6, 129.2, 128.9, 128.5, 128.2, 128.1, 127.3, 127.1, 116.5, 81.5, 58.4.

---

116
6.1.7 Procedure for the $N$-Methylthiolation of Lactams 3

\[
\begin{align*}
\text{11} & \quad \text{11a: } R_1 = \text{Me} \\
\text{11b: } R_1 = \text{Me} \\
\text{11c: } R_1 = \text{Ph} \\
\text{11} & \quad \text{3a: } R_1 = \text{Me} \\
\text{3b: } R_1 = \text{Me} \\
\text{3c: } R_1 = \text{Ph} \\
\end{align*}
\]

Compound 11b (0.240 g, 1.00 mmol) was dissolved in dry CH$_2$Cl$_2$ (8 mL), and the solution of $N$-methylthiophthalimide (0.194 g, 1.00 mmol) in dry CH$_2$Cl$_2$ (4 mL) was added. To the resulting solution was then added triethylamine (3 drops). The reaction was refluxed for 2 h and then diluted with CH$_2$Cl$_2$ (20 mL). The resulting solution was washed with 1% aqueous KOH (2 x 20 mL). The organic layer was dried with anhydrous MgSO$_4$, filtered, and evaporated, and the residue was chromatographed (1:3 EtOAc/hexanes) to give 206 mg of 3b as a yellow solid in 64 % yield. $^1$H NMR (250 MHz, CDCl$_3$) \( \delta \) 7.47-7.26 (5H, m), 5.91 (1H, d, \( J = 5.3 \) Hz), 5.54 (1H, d, \( J = 5.3 \) Hz), 2.96 (3H, s), 2.53 (3H, s); $^{13}$C NMR (63 MHz) \( \delta \) 165.7, 133.2, 129.4, 129.0, 128.7, 127.4, 126.0, 79.9, 60.9, 38.0, 20.9.

cis-$N$-(Methylthio)-4-phenyl-3-(4-toluene-sulfonyl)azetidin-2-one (3a): Yellow solid, mp 85-87°C, 74% yield. $^1$H NMR (250 MHz, CDCl$_3$) \( \delta \) 7.41-7.26 (5H, m), 7.19-7.15 (4H, m), 5.74 (1H, d, \( J = 5.0 \) Hz), 4.88 (1H, d, \( J = 5.0 \) Hz), 2.40 (3H, s), 2.35 (3H, s); $^{13}$C NMR (63 MHz, CDCl$_3$) \( \delta \) 166.4, 145.3, 132.4, 132.0, 129.8, 129.2, 129.0, 128.5, 127.8 (2C), 126.8 (2C), 80.6, 66.0, 22.1, 21.7.

cis-3-Benzenesulfonyl-$N$-methylthio-4-phenylazetidin-2-one (3c): Yellow oil, 72% yield. $^1$H NMR (250 MHz, CDCl$_3$) \( \delta \) 7.64-7.57 (3H, m), 7.45-7.39 (2H, m), 7.30-7.21 (5H, m), 5.85 (1H, d, \( J = 5.1 \) Hz), 5.46 (1H, d, \( J = 5.1 \) Hz), 2.43 (3H, s); $^{13}$C NMR (63 MHz, CDCl$_3$) \( \delta \) 165.2, 134.5, 133.5, 133.2, 129.2, 128.9, 128.7, 128.3, 128.0, 127.8, 127.6, 125.8, 80.0, 60.8, 20.8.

6.1.8 Preparation of Sulfur-Transfer Reagents: $N$-methylthiophthalimide 14

In a mixture of pyridine (30 mL) and acetonitrile (38 mL) containing disulfide (41 mmol) and phthalimide (41 mmol) at 0 °C, bromine (1.2 eq. 49 mmol in 49 mL of acetonitrile) was added dropwise at 0°C while stirring within 1 hr. After another 1 hr of stirring, water (150 mL) was added to the solution over 30 mins and allowed to stand for 1 hr to precipitate the product from the solution. The compound was filtered off and recrystallized in MeOH to give the product 14 in 80 % yield as a white solid, mp 176-177 °C.
6.1.9 Fluorescent lactam 18

\[
\begin{align*}
\text{cis-4-(2-Chlorophenyl)-3-dansyl-N-(4-methoxyphenyl)-4-(2-chlorophenyl)azetidin-2-one (10d):} \\
\text{Yellow solid, 55% yield. }^1\text{H NMR (250 MHz, CDCl}_3\text{) }\delta\text{ 7.64-7.57 (3H, m), 7.45-7.39 (2H, m), 7.30-7.21 (5H, m), 5.85 (1H, d, } J = 5.1 \text{ Hz), 5.46 (1H, d, } J = 5.1 \text{ Hz), 2.43 (3H, s), 1.29 (s, 6H); } ^{13}\text{C NMR (63 MHz, CDCl}_3\text{) }\delta\text{ 165.2, 134.5, 133.5, 133.2, 129.2, 128.9, 128.7, 128.2, 128.0, 127.8, 127.6, 126.7, 125.8, 80.0, 60.8.}
\end{align*}
\]

\[
\begin{align*}
\text{cis-4-(2-Chlorophenyl)-3-hydroxy-N-methylthio-azetidin-2-one (17):} \\
\text{Yellow solid, 84% yield. }^1\text{H NMR (250 MHz, CDCl}_3\text{) }\delta\text{ 7.30-7.21 (5H, m), 5.26 (1H, d, } J = 5.2 \text{ Hz), 5.20 (1H, d, } J = 5.2 \text{ Hz), 2.42 (3H, s); } ^{13}\text{C NMR (63 MHz, CDCl}_3\text{) }\delta\text{ 175.2, 168.2, 135.5, 129.2, 128.9, 128.5, 127.9, 126.7, 80.0, 60.8, 22.0.}
\end{align*}
\]
**cis-4-(2-Chlorophenyl)-3-dansyl-N-methylthio-azetidin-2-one (18):** Yellow solid, 55% yield. $^1$H NMR (250 MHz, CDCl$_3$) $\delta$ 7.64-7.57 (3H, m), 7.45-7.39 (2H, m), 7.30-7.21 (5H, m), 5.85 (1H, d, $J = 5.1$ Hz), 5.46 (1H, d, $J = 5.1$ Hz), 2.43 (3H, s), 1.29 (s, 6H); $^{13}$C NMR (63 MHz, CDCl$_3$) $\delta$ 165.2, 134.5, 133.5, 133.2, 129.2, 128.9, 128.7, 128.2, 128.0, 127.8, 127.6, 126.7, 125.8, 80.0, 60.8, 22.1.

### 6.1.10 Amino-Substituted Lactam

![Diagram of Amino-Substituted Lactam](attachment:image.png)

**Conditions:** a.) P$_2$O$_5$, DMSO; b.) RR'NH, NaBH(OAc)$_3$, AcOH, ClCH$_2$CH$_2$Cl; c.) (NH$_4$)$_2$Ce(NO$_3$)$_6$, MeCN-H$_2$O, 0 °C; d.) N-methylthiophthalimide, triethylamine, CH$_2$Cl$_2$, reflux.

**Synthesis of trans-4-(2-Chlorophenyl)-3-cyclopentylamino-N-(4-methoxyphenyl)azetidin-2-one (21a):** Cyclopentylamine (35.6 mg, 0.33 mmol) and compound 20 (100 mg, 0.33 mmol) were mixed in ClCH$_2$CH$_2$Cl (1.32 mL) and then treated with sodium triacetoxyborohydride (98.6 mg, 0.465 mmol) and AcOH (19.8 mg, 0.33 mmol). The mixture was stirred at rt under a N$_2$ atmosphere for 1 h until the reactants were consumed completely. The reaction mixture was quenched by adding 1 N NaOH, and the product was extracted with ether. The ether extract was washed with brine and dried with MgSO$_4$. The solvent was evaporated to give 21a in 88% yield.

**trans-4-(2-Chlorophenyl)-3-cyclopentylamino-N-(4-methoxyphenyl)azetidin-2-one (21a):** $^1$H NMR (250 MHz, CDCl$_3$) $\delta$ 7.30-7.19 (4H, m), 6.90 (2H, d, $J = 8.8$ Hz), 6.69 (2H, d, $J = 8.8$ Hz), 4.97 (2H, s), 3.78 (1H, brs), 3.69 (3H, s), 1.71 (4H, m); $^{13}$C NMR (63 MHz, CDCl$_3$) $\delta$ 165.8, 157.9, 140.2, 133.2, 130.5, 130.4, 129.5, 129.2, 127.6, 127.4, 119.5, 114.7, 55.4, 50.0, 48.4, 42.8, 33.3, 33.2, 27.9, 27.8.

**trans-3-Benzylamino-4-(2-chlorophenyl)-N-(4-methoxyphenyl)azetidin-2-one (21b):** $^1$H NMR (250 MHz, CDCl$_3$) $\delta$ 7.27-7.12 (9H, m), 6.93 (1H, d, $J = 8.8$ Hz), 6.70 (1H, d, $J = 8.8$ Hz), 4.96 (2H, s), 4.23 (2H, d, $J = 6.0$ Hz), 3.69 (3H, s); $^{13}$C NMR (63 MHz, CDCl$_3$) $\delta$ 164.0, 161.7, 159.4, 137.9, 134.3, 134.1, 133.9, 133.8, 130.4, 129.4, 129.0, 128.4, 128.2, 127.9, 127.4, 127.1, 119.0, 114.6, 55.7, 53.4, 51.7, 43.5.
trans-4-(2-Chlorophenyl)-3-diethylamino-N-(4-methoxyphenyl)azetidin-2-one (21c): \(^1\)H NMR (250 MHz, CDCl\(_3\)) \(\delta 7.31-7.10\) (4H, m), 6.65 (2H, d, \(J = 8.8\) Hz), 5.00 (2H, s), 3.67 (3H, s), 3.18 (2H, q, \(J = 7.0\) Hz), 3.09 (2H, q, \(J = 7.1\) Hz), 1.14 (3H, t, \(J = 7.0\) Hz), 0.60 (3H, d, \(J = 7.1\) Hz); \(^{13}\)C NMR (63 MHz, CDCl\(_3\)) \(\delta 165.9, 164.2, 160.7, 134.2, 132.2, 131.1, 129.7, 129.4, 127.5, 114.5, 55.8, 49.3, 42.3, 37.8, 14.0, 12.2.

trans-3-(2-Chlorophenyl)-3-diisobutylamino- N-(4-methoxyphenyl)azetidin-2-one (21d): \(^1\)H NMR (250 MHz, CDCl\(_3\)) \(\delta 7.31-7.20\) (4H, m), 6.96 (2H, d, \(J = 8.8\) Hz), 6.65 (2H, d, \(J = 8.8\) Hz), 4.98 (2H, s), 3.68 (3H, s), 2.90 (4H, t, \(J = 6.6\) Hz), 1.90 (1H, m), 1.45 (1H, m), 0.76 (6H, d, \(J = 6.6\) Hz), 0.35 (6H, d, \(J = 6.6\) Hz); \(^{13}\)C NMR (63 MHz, CDCl\(_3\)) \(\delta 163.1, 156.2, 136.9, 135.2, 134.1, 130.2, 129.4, 127.5, 122.3, 122.2, 114.2, 114.0, 71.9, 60.0, 56.2, 55.0, 41.6, 28.3, 26.8, 20.6, 20.5, 20.3, 20.2.

Procedure for the N-Dearylation of N-(4-Methoxyphenyl)substituted Lactams (22a): To a solution of lactam 21a (0.375 g, 1.00 mmol) in CH\(_3\)CN (2.2 mL) at rt was added dropwise a solution of ceric ammonium nitrate (1.64 g, 3.00 mmol) in 1.5 mL of water. The reaction was stirred for 5 min and then diluted with EtOAc (20 mL). The resulting solution was washed sequentially with water (20 mL), 5% NaHCO\(_3\) (2 x 20 mL), 5% NaHSO\(_3\) (2 x 20 mL) and brine (20 mL). The organic layer was dried with anhydrous MgSO\(_4\), filtered, and evaporated, and the residue was chromatographed on silica gel (1:2 EtOAc/hexanes) to give 0.224 g of 22a as yellow oil in 85% yield.

trans-4-(2-Chlorophenyl)-3-propylaminoazetidin-2-one (22a): \(^1\)H NMR (250 MHz, CDCl\(_3\)) \(\delta 7.88\) (1H, brs), 7.31- 7.12 (4H, m), 4.73 (1H, d, \(J = 6.5\) Hz), 4.49 (1H, d, \(J = 6.4\) Hz), 3.83 (1H, brs), 1.84 (4H, m), 1.43 (4H, m); \(^{13}\)C NMR (63 MHz, CDCl\(_3\)) \(\delta 179.5, 140.4, 133.2, 130.4, 129.5, 129.2, 127.6, 55.4, 42.7, 42.5, 33.3, 33.2, 27.9, 27.6.

trans-3-Benzylamino-4-(2-chlorophenyl)azetidin-2-one (22b): clear oil, 91% yield. \(^1\)H NMR (250 MHz, CDCl\(_3\)) \(\delta 7.78\) (1H, brs), 7.33-7.16 (9H, m), 4.53 (1H, d, \(J = 6.3\) Hz), 4.42 (1H, d, \(J = 6.1\) Hz); \(^{13}\)C NMR (63 MHz, CDCl\(_3\)) \(\delta 187.6, 160.0, 137.0, 134.7, 130.4, 130.3, 130.1, 130.0, 129.7, 129.6, 129.2, 128.3, 127.6, 55.7, 51.8, 43.6.

trans-4-(2-Chlorophenyl)-3-diethylaminoazetidin-2-one (22c): clear oil, 84% yield. \(^1\)H NMR (250 MHz, CDCl\(_3\)) \(\delta 7.62\) (1H, brs), 7.31-7.20 (4H, m), 4.75 (1H, d, \(J = 6.5\) Hz), 4.49 (1H, d, \(J = 6.4\) Hz), 3.83 (1H, brs), 1.84 (4H, m), 1.43 (4H, m), \(^{13}\)C NMR (63 MHz, CDCl\(_3\)) \(\delta 165.9, 134.2, 132.2, 131.1, 129.7, 129.4, 127.5, 55.8, 49.3, 42.3, 37.8, 14.0, 12.2.

trans-4-(2-Chlorophenyl)-3-diisobutylamino-azetidin-2-one (22d): clear oil, 87 % yield. \(^1\)H NMR (250 MHz, CDCl\(_3\)) \(\delta 7.31-7.20\) (4H, m), 6.96 (2H, d, \(J = 8.8\) Hz), 6.65 (2H, d, \(J = 8.8\) Hz), 4.98 (2H, s), 3.68 (3H, s), 2.90 (4H, t, \(J = 6.6\) Hz), 1.90 (1H, m), 1.45 (1H, m), 0.76 (6H, d, \(J = 6.6\) Hz), 0.35 (6H, d, \(J = 6.6\) Hz); \(^{13}\)C NMR (63 MHz, CDCl\(_3\)) \(\delta 163.1, 136.9, 135.2, 134.1, 130.2, 129.4, 127.5, 71.9, 56.2, 55.0, 41.6, 28.3, 26.8, 20.6, 20.5, 20.3, 20.2.

Procedure for the synthesis of N-methylthio lactams (19a): To a solution of lactam 21a (0.035 g, 0.133 mmol) in CH\(_3\)CN (2.2 mL) at rt was added dropwise a solution of ceric ammonium nitrate (1.64 g, 3.00 mmol) in 1.5 mL of water. The reaction was stirred for 5 min and then diluted with EtOAc (20 mL). The resulting solution was washed sequentially with water (20 mL), 5% NaHCO\(_3\) (2 x 20 mL), 5% NaHSO\(_3\) (2 x 20 mL) and brine (20 mL). The organic layer was dried with anhydrous MgSO\(_4\), filtered, and evaporated, and the residue was chromatographed on silica gel (1:2 EtOAc/hexanes) to give 0.224 g of 22a as yellow oil in 85% yield.

trans-4-(2-Chlorophenyl)-3-propylamin oazetidin-2-one (22a): \(^1\)H NMR (250 MHz, CDCl\(_3\)) \(\delta 7.88\) (1H, brs), 7.31-7.12 (4H, m), 4.73 (1H, d, \(J = 6.5\) Hz), 4.49 (1H, d, \(J = 6.4\) Hz), 3.83 (1H, brs), 1.84 (4H, m), 1.43 (4H, m), \(^{13}\)C NMR (63 MHz, CDCl\(_3\)) \(\delta 179.5, 140.4, 133.2, 130.4, 129.5, 129.2, 127.6, 42.5, 42.2, 33.3, 33.2, 27.9, 27.6.

Procedure for the N-Dearylation of N-(4-Methoxyphenyl)substituted Lactams (22a): To a solution of lactam 21a (0.035 g, 0.133 mmol) in benzene was added N-methylthiophthalimide (0.025 g, 0.133 mmol) and 1 drop of triethylamine. The mixture was refluxed overnight and washed with 1% KOH. The organic layer was dried over Na\(_2\)SO\(_4\) and the solvent was removed under reduced pressure. The crude material was purified by column chromatography (1:4 EtOAc/hexanes) to yield 0.028 g of 22a as yellow oil in 68% yield.
trans-3-Benzylamino-N-methylthioazetidin-2-one (19b): clear oil, 69 % yield. $^1$H NMR (250 MHz, CDCl$_3$) $\delta$ 7.25-7.05 (6H, m), 6.61 (3H, m), 4.53 (1H, d, $J = 6.3$ Hz), 4.42 (1H, d, $J = 6.1$ Hz), 2.41 (3H, s); $^{13}$C NMR (63 MHz, CDCl$_3$) $\delta$ 187.5, 160.0, 134.7, 130.4, 130.3, 130.1, 130.0, 129.7, 129.6, 129.2, 128.3, 127.6, 55.7, 51.8, 43.6, 19.4.

trans-3-Diethylamino-N-methylthioazetidin-2-one (19c): clear oil, 70 % yield. $^1$H NMR (250 MHz, CDCl$_3$) $\delta$ 7.62 (1H, brs), 7.28-7.19 (4H, m), 4.75 (1H, d, $J = 5.2$ Hz), 4.49 (1H, d, $J = 5.2$ Hz), 3.18 (2H, q, $J = 7.1$ Hz), 3.09 (2H, q, $J = 7.2$ Hz), 2.41 (3H, s); $^{13}$C NMR (63 MHz, CDCl$_3$) $\delta$ 175.2, 134.5, 129.2, 128.9, 128.7, 128.2, 127.8, 60.8, 54.2, 48.6, 26.2, 25.8, 20.8, 11.4, 11.3.

trans-3-Diisobutylamino-N-methylthioazetidin-2-one (19d): $^1$H NMR (250 MHz, CDCl$_3$) $\delta$ 7.28-7.13 (4H, m), 4.48 (2H, d, $J = 6.2$ Hz), 3.59 (2H, d, $J = 7.5$ Hz), 3.13 (2H, d, $J = 7.5$ Hz), 1.99 (1H, m), 1.96 (1H, m), 0.81 (12H, m); $^{13}$C NMR (63 MHz, CDCl$_3$) $\delta$ 173.1, 140.2, 136.9, 130.2, 129.4, 127.5, 127.2, 49.9, 48.2, 41.6, 41.3, 39.2, 27.3, 26.8, 23.5, 23.4, 20.2.

6.1.11 Synthesis of Azido Lactam 19e

$$\begin{align*}
\text{trans-3-Azido-4-(2-chlorophenyl)-N-(4-methoxyphenyl)azetidin-2-one (23):} \quad & \text{To a solution of $\beta$-lactam 9b (795 mg, 2.5 mmol) in 20 mL of dry CH$_2$Cl$_2$ was added NaH (60\% suspension in mineral oil, 125 mg, 5 mmol) and the mixture was stirred for 15 min. Methanesulfonyl chloride (342 mg, 3 mmol) was then added dropwise to the solution, and the resulting solution was stirred at rt for 30 min. The solution was washed with brine (3×25 mL), and the organic layer was dried with anhydrous MgSO$_4$, filtered, and evaporated, and the residue was washed with cold MeOH to give 830 mg as a white solid in 82\% yield. To the solution of above compound (405 mg, 1 mmol) in dry DMF was added NaN$_3$ (195 mg, 3 mmol) and the resulting solution was heated to 80$^\circ$C for 24 hr. After cooling to rt, the solution was concentrated under vacuum. The crude compound was dissolved in EtOAc and washed with water (3×20 mL), and the organic layer was dried with MgSO$_4$, filtered, and evaporated to give 23 in 81\% yield. $^1$H NMR (250 MHz, CDCl$_3$) $\delta$ 7.24-7.10 (4H, m), 6.80-6.74 (2H, m), 5.24 (1H, d, $J = 1.8$ Hz), 4.44 (1H, d, $J = 1.8$ Hz), 3.70 (3H, s); $^{13}$C NMR (63 MHz, CDCl$_3$) $\delta$ 164.2, 156.2, 142.5, 133.5, 132.2, 128.9, 128.7, 128.2, 127.9, 126.8, 121.9, 121.5, 114.2, 113.8, 60.8, 52.2.

trans-3-Azido-4-(2-chlorophenyl)azetidin-2-one (24): $^1$H NMR (250 MHz, CDCl$_3$) $\delta$ 7.38-7.19 (4H, m), 6.20 (1H, brs), 4.90 (1H, d, $J = 1.8$ Hz), 4.33 (1H, d, $J = 1.8$ Hz); $^{13}$C NMR (63 MHz, CDCl$_3$) $\delta$ 172.1, 142.3, 132.2, 128.4, 128.2, 128.0, 126.7, 64.2, 44.3.

trans-3-Azido-4-(2-chlorophenyl)-N-methylthio-azetidin-2-one (19e): $^1$H NMR (250 MHz, CDCl$_3$) $\delta$
cis-3-(2-tert-Butoxycarbonylaminopropionic-ester)-4-(2-chlorophenyl)-N-methylthio-azetidin-2-one (25): \(^1\)H NMR (250 MHz, CDCl\(_3\)) \(\delta\) 7.35-7.19 (4H, m), 5.74 (1H, d, \(J = 1.8\) Hz), 4.95 (1H, d, \(J = 1.8\) Hz), 2.37 (3H, s); \(^{13}\)C NMR (63 MHz, CDCl\(_3\)) \(\delta\) 168.2, 142.5, 133.5, 128.9, 128.7, 128.2, 128.0, 60.8, 52.2, 20.8.

6.1.12 Synthesis of Boc-Protected Lactam 25

\[
\begin{align*}
\text{17} \quad &\xrightarrow{26, \text{ EDCI, DMAP, CH}_2\text{Cl}_2} \quad \text{25} \\
\text{COOH} \quad &\xrightarrow{\text{Boc}_2\text{O, NaOH}} \quad \text{COOH} \\
\text{NHBoc} \quad &\xrightarrow{\text{26, EDCI, DMAP, CH}_2\text{Cl}_2} \quad \text{25}
\end{align*}
\]

\textit{cis-3-(2-tert-Butoxycarbonylaminopropionic-ester)-4-(2-chlorophenyl)-N-methylthio-azetidin-2-one (25)}: \(^1\)H NMR (250 MHz, CDCl\(_3\)) \(\delta\) 7.35-7.19 (4H, m), 5.74 (1H, d, \(J = 1.8\) Hz), 4.95 (1H, d, \(J = 1.8\) Hz), 2.37 (3H, s); \(^{13}\)C NMR (63 MHz, CDCl\(_3\)) \(\delta\) 168.2, 142.5, 133.5, 128.9, 128.7, 128.2, 128.0, 60.8, 52.2, 22.8.

6.1.13 Racemic Lactam 27

\[
\begin{align*}
\text{a.} \quad &\text{Acryloyl chloride, NaH, CH}_2\text{Cl}_2; \quad \text{b.} \quad \text{Ceric ammonium nitrate, CH}_3\text{CN, H}_2\text{O, 0 \degree C}; \quad \text{c.} \quad \text{N}-\text{methylthio phthalimide, CH}_2\text{Cl}_2, \text{Hunig's base, reflux.}
\end{align*}
\]

\textit{Synthesis of cis-3-Acryloyloxy-4-(2-chlorophenyl)-N-(4-methoxyphenyl)azetidin-2-one (30):} To a solution of \(\beta\)-lactam 9b (2.94 g, 9.7 mmol) in 15 mL of dry CH\(_2\)Cl\(_2\) was added NaH (60 % suspension in mineral oil, 582 mg, 14.55 mmol) and the mixture was stirred for 15 min. Acryloyl chloride (1.05 g, 11.6 mmol) was then added dropwise. The mixture was stirred at r.t for 30 min until the TLC indicating the disappeararance of the starting material. The reaction was washed with water twice and combined organic layers were dried with MgSO\(_4\). The crude material was purified by column chromatography on silica gel (CH\(_2\)Cl\(_2\)) to give 2.94 g of 30 in 85% as colorless oil. \(^1\)H NMR (250 MHz, CDCl\(_3\)) \(\delta\) 7.45-7.22 (6H, m), 6.88 (2H, d, \(J = 8.8\) Hz), 6.31 (1H, d, \(J = 4.8\) Hz), 6.11 (1H, dd, \(J = 4.8, 15.2\) Hz), 5.85 (1H, dd, \(J = 4.9, 15.4\) Hz), 5.81 (1H, d, \(J = 5.0\) Hz), 5.71 (1H, dd, \(J = 4.8, 15.2\) Hz), 3.72 (3H, s). \(^{13}\)C NMR (400 MHz, CDCl\(_3\)) \(\delta\) 163.9, 161.6, 159.9, 132.5, 130.5, 130.3, 130.0, 128.8, 127.8, 127.0, 126.7, 119.0, 118.9, 114.7, 114.4, 58.5, 55.6, 30.0.

\textit{Procedure for Synthesis of cis-3-Acryloyloxy-4-(2-chlorophenyl)azetidin-2-one (31):} To a solution of 30 (0.25 g, 0.70 mmol) in 7.5 mL of CH\(_3\)CN at 0 \degree C was added a solution of ceric ammonium nitrate (1.15 g, 2.10 mmol) dissolved in 10.4 mL of water dropwise within 20 min. The reaction was
allowed to stir at 0 °C for another 20 min, and then was quenched by the addition of 25 mL of water and extracted with EtOAc (4 × 25 mL). The combined organic layers were washed with 25 mL of 5% of NaHCO₃, 10% of NaHSO₃ (twice) and brine, and then dried over MgSO₄. The solvent was removed under reduced pressure to yield, and the crude product was purified by column chromatography (1:1 EtOAc/hexanes) to give 0.16 g of 31 in 90% as a yellowish oil. ¹H NMR (400 MHz, CDCl₃) δ 7.41-7.22 (4H, m), 6.59 (1H, bs), 6.21 (1H, d, J = 5.0 Hz), 6.08 (1H, dd, J = 2.4, 10.8 Hz), 6.04 (1H, dd, J = 2.4, 10.0 Hz), 5.80 (1H, dd, J = 2.4, 10.8 Hz), 5.39 (1H, d, J = 4.8 Hz). ¹³C NMR (400 MHz, CDCl₃) δ 164.8, 159.9, 132.5, 130.5, 128.8, 127.8, 127.0, 119.0, 118.9, 114.7, 58.5, 55.6.

Procedure for synthesis of cis-3-Acryloyloxy-4-(2-chlorophenyl)-N-methylthioazetidin-2-one (27): To a solution of 31 (414.5 mg, 1.65 mmol) in dry CH₂Cl₂ was added N-methylthiophthalimide 14 (287 mg, 1.48 mmol) and Hunig’s base (640 mg, 4.95 mmol). The mixture was refluxed overnight and washed with 1% of KOH. The organic layer was dried over MgSO₄. The solvent was removed under reduced pressure to yield, and the crude product was purified by column chromatography (1:1 EtOAc/hexanes) to give 400 mg of 27 in 82 % as white solid. ¹H NMR (400 MHz, CDCl₃) δ 7.31-7.20 (4H, m), 6.15 (1H, d, J = 3.6 Hz), 5.98 (1H, dd, J = 2.4, 9.2 Hz), 5.62 (1H, dd, J = 2.4, 9.6 Hz), 5.47 (1H, d, J = 3.2 Hz), 2.45 (3H, s). ¹³C NMR (250 MHz, CDCl₃) δ 168.5, 163.6, 132.8, 132.5, 130.7, 130.0, 129.7, 128.8, 126.9, 126.8, 62.4, 55.4, 22.1.

6.1.14 Enantiopure Acrylate Lactams (+)-27 and (-)-27

(-)-(3S,4R)-4-(2-Chlorophenyl)-3-hydroxy-N-(4-methoxyphenyl)azetidin-2-one (28): white solid, mp 208 °C; ¹H NMR (250 MHz, CDCl₃) δ 7.30-7.24 (6H, m), 6.82 (2H, q, J = 3.0 Hz), 5.60 (1H, d, J = 5.6 Hz), 5.31 (1H, d, J = 4.4 Hz), 3.75 (3H, s).

(+)-(3R,4S)-3-Acetoxy-4-(2-chlorophenyl)-N-(4-methoxyphenyl)azetidin-2-one (29): white solid. ¹H NMR (250 MHz, CDCl₃) δ 7.37-7.18 (6H, m), 6.74 (2H, d, J = 8.7 Hz), 6.08 (2H, d, J = 4.8 Hz), 5.71 (1H, d, J = 4.6 Hz), 3.68 (3H, s), 1.68 (3H, s).
(−)-(3S,4R)-3-Acryloyloxy-4-(2-chlorophenyl)-N-(4-methoxyphenyl)azetidin-2-one (32): white solid, 98% yield. ¹H NMR (250 MHz, CDCl₃) 8 7.34-7.19 (6H, m), 6.76 (2H, d, J = 5.0 Hz), 6.20 (1H, dd, J = 5.0 Hz), 6.03 (1H, dd, J = 5.0, 15.4 Hz), 5.76 (1H, d, J = 4.9 Hz), 5.71 (1H, dd, J = 4.6, 15.2 Hz), 5.60 (1H, d, J = 4.97 Hz), 3.70 (3H, s).

(−)-(3S,4R)-3-Acryloyloxy-4-(2-chlorophenyl)azetidin-2-one (33): yellow oil, 91% yield. ¹H NMR (250 MHz, CDCl₃) 8 7.29-7.19 (4H, m), 6.36 (1H, bs), 6.20 (1H, dd, J = 4.8 Hz), 6.01 (1H, dd, J = 5.6 Hz), 5.73 (1H, d, J = 4.9 Hz), 5.66 (1H, dd, J = 5.2 Hz), 5.38 (1H, d, J = 4.8 Hz).

(−)-(3S,4R)-3-Acryloyloxy-4-(2-chlorophenyl)-N-methylthio)azetidin-2-one ((−)-27): light yellow solid, 78% yield. ¹H NMR (250 MHz, CDCl₃) 8 7.29-7.19 (4H, m), 6.12 (1H, dd, J = 4.8, 15.0 Hz), 6.00 (1H, dd, J = 5.6, 15.2 Hz), 5.73 (1H, d, J = 4.9 Hz), 5.64 (1H, dd, J = 5.2, 15.2 Hz), 5.43 (1H, d, J = 4.8 Hz), 2.43 (3H, s).
(+)-(3R,4S)-4-(2-Chlorophenyl)-3-hydroxy-N-(4-methoxyphenyl)azetidin-2-one (34): white solid, mp 208 °C; $^1$H NMR (250 MHz, CDCl$_3$) $\delta$ 7.30-7.24 (6H, m), 6.82 (2H, q, $J$ = 3.0 Hz), 5.60 (1H, d, $J$ = 5.6 Hz), 5.31 (1H, d, $J$ = 4.4 Hz), 3.75 (3H, s).

(+)-(3R,4S)-3-Acryloyloxy-4-(2-chlorophenyl)-N-(4-methoxyphenyl)azetidin-2-one (35): To the solution of lactam (50 mg) in 5 ml dry methylene chloride was added NaH (12 mg, 1.5 eq.). After stirring for 15 mins, the solution became light yellow clear and acryloyl chloride (14.85 mg, 1.1 eq.) was added to the resulting solution. The solution turned into clear and TLC showed the whole consumption of the starting material after another 10-min stirring. The product was purified by column using methylene chloride to get rid of the mineral oil. White solid, 98 % yield. $^1$H NMR (CDCl$_3$) $\delta$ 7.34-7.17 (6H, m), 6.73 (2H, d, $J$ = 8.8 Hz), 6.2 (1H, dd, $J$ = 5.0 Hz), 6.00 (1H, dd, $J$ = 5.0 Hz), 5.74 (1H, d, $J$ = 4.9 Hz), 5.62 (1H, dd, $J$ = 4.9, 15.4 Hz), 4.99 (1H, d, $J$ = 4.9 Hz), 3.68 (3H, s).

(+)-(3R,4S)-3-Acryloyloxy-4-(2-chlorophenyl)-N-(methylthio)azetidin-2-one (+ 27): White solid, 82 % yield. $^1$H NMR (CDCl$_3$) $\delta$ 7.29-7.19 (4H, m), 6.12 (1H, dd, $J$ = 4.8, 15.2 Hz), 5.97 (1H, dd, $J$ = 5.0, 15.2 Hz), 5.73 (1H, d, $J$ = 4.9 Hz), 5.62 (1H, dd, $J$ = 5.2 Hz), 5.45 (1H, d, $J$ = 4.8 Hz), 2.43 (3H, s).

6.1.15 C$_4$-Acrylate Lactam 37
Synthesis of cis-4-Acetoxyphenyl-N-(4-methoxyphenyl)imine (42): To a solution of p-anisidine (1.08 g, 8.81 mmol) in 15 mL of CH₂Cl₂ was added p-hydroxybenzaldehyde (1.07 g, 8.81 mmol), and a catalytic amount of (+)-camphorsulfonic acid. The resultant mixture was stirred until TLC indicated the disappearance of starting materials. The resulting compound was used for the next step without further purification. To the solution of 4-hydroxyphenyl-N-(4-methoxyphenyl)-imine (1 eq.) was added 2 eq. of triethylamine and acetyl chloride (1.5 eq.) was then added dropwise to the solution. The resulting solution was stirred at r.t. for 15 mins, and then washed with H₂O (2 × 20 mL). The organic layer was dried with anhydrous MgSO₄, filtered, and evaporated to afford 42 as a light yellow solid in 92% yield, mp 95-96 °C. 

1H NMR (250 MHz, CDCl₃) δ 8.39 (1H, s), 7.84 (2H, d, J = 8.5 Hz), 7.20-7.11(4H, m), 6.86 (2H, d, J = 8.8 Hz), 3.77(3H, s), 2.26 (3H, s). 13C NMR (400 MHz, CDCl₃) δ 168.2, 161.2, 159.0, 157.3, 144.4, 129.9(2C), 128.8, 122.4, 122.2 (2C), 121.7, 114.6 (2C), 55.7, 21.4.

cis-4-(4-propenoyloxyphenyl)-3-(methoxy)azetidin-2-one (46): white solid, 78 % yield; mp 94-96 °C; 
1H NMR (250 MHz, CDCl₃) δ 7.33 (2H, d, J = 8.4 Hz), 7.09 (2H, d, J = 8.4 Hz), 6.55 (1H, d, J = 17.3 Hz), 6.26 (1H, dd, J = 17.3, 10.4 Hz), 6.00 (1H, d, J = 10.4 Hz), 4.78 (1H, d, J = 4.5 Hz), 4.68-4.66 (1H, m), 3.11(3H, s); 13C NMR (63 MHz, CDCl₃): δ 168.0, 164.5, 150.5, 133.2, 132.9, 128.8, 127.7, 121.5, 116.0, 86.6, 65.5, 22.0.

cis-4-(4-propenoyloxyphenyl)-3-methoxy-1-(methylthio)azetidin-2-one (37): white solid; 89 % yield; mp 87-88 °C; 
1H NMR (250 MHz, CDCl₃): δ 7.29 (2H, d, J = 8.4 Hz), 6.50 (1H, d, J = 17.3 Hz), 6.21 (1H, dd, J = 10.3, 17.1 Hz), 5.92 (1H, d, J = 10.4 Hz), 4.75 (1H, d, J = 4.8 Hz), 4.69 (1H, d, J = 4.8 Hz), 3.05 (3H, s), 2.26 (3H, s); 13C NMR (63 MHz, CDCl₃): δ 170.3, 164.2, 150.9, 132.9, 131.1, 129.9, 127.7, 121.4, 86.5, 65.5, 58.3, 22.0.

6.1.16 Diacrylate Lactam 38
Synthesis of cis-3-Acetoxy-4-(4-acetoxynphenyl)-N-(4-methoxyphenyl)azetidin-2-one (47): Imine 43 (3.99 g, 14.8 mmol) was dissolved in 40 mL of freshly distilled CH₂Cl₂. The solution was cooled to approximately 5°C in an ice bath. Triethylamine (3 eq., 4.15 g, 44.0 mmol) was added followed by methoxycetyl chloride (1.3 eq., 2.73 g, 19.2 mmol) dissolved in 5 mL of CH₂Cl₂. The reaction mixture was stirred until no further change in TLC was observed for 3 hr. The solvent was removed under reduced pressure and the crude material was purified by washing with ice cold MeOH and cold ether. The product was isolated in 65% yield, 3.49 g of 47, as a white solid, mp 143-144 °C. ¹H NMR (250 MHz, CDCl₃) δ 7.32-7.27 (4H, m), 7.04 (2H, d, J = 8.3 Hz), 6.88 (2H, d, J = 6.4 Hz), 5.99 (1H, d, J = 4.8 Hz), 5.29 (1H, d, J = 4.6 Hz), 3.71 (3H, s), 2.22 (3H, s), 1.62 (3H, s). ¹³C NMR (400 MHz, CDCl₃) δ 169.5, 169.3, 161.3, 156.9, 151.2, 139.4, 130.3, 130.0, 129.2, 129.1, 121.9 (2C), 121.2, 119.0, 114.7 (2C), 61.1, 55.6, 21.3, 20.0.

Synthesis of cis-3-Hydroxy-4-(4-hydroxyphenyl)-N-(4-methoxyphenyl)azetidin-2-one (48): To a solution of compound 47 (0.86 g, 2.32 mmol) in cold acetone (12 mL) was added a solution of KOH (0.26 g, 4.64 mmol) in 3 mL of MeOH dropwise. The hydrolysis was complete after the addition of KOH/MeOH as indicated by TLC. The product precipitated out of the solution after stirring for 5 mins, and filtered and dried to give a white solid in 92% yield. mp 193-194 °C. ¹H NMR (400 MHz, CDCl₃) δ 7.23-7.18 (4H, m), 6.88 (2H, d, J = 6.4 Hz), 5.99 (1H, d, J = 4.8 Hz), 5.29 (1H, d, J = 4.6 Hz), 3.71 (3H, s), 2.22 (3H, s), 1.62 (3H, s). ¹³C NMR (400 MHz, CDCl₃) δ 161.8, 133.7, 129.2, 129.1, 121.2, 121.1, 119.5, 119.0, 118.4, 114.7(2C), 80.9, 66.4, 59.0.

Synthesis of cis-3-Acryloyl-4-(4-acryloylphenyl)-N-(4-methoxyphenyl)azetidin-2-one (49): To a solution of 48 (0.31 g, 1.09 mmol) in 10 mL of CH₂Cl₂ was added triethylamine (0.22 g, 2.20 mmol). Acryloyl chloride (0.19 g, 2.20 mmol) was added dropwise to the solution at 0 °C. The resulting solution was stirred at r.t. for 15 mins, and then washed with H₂O (2 × 10 mL). The organic layer was dried with anhydrous MgSO₄, filtered, and evaporated. The crude material was purified by recrystallization in CH₂Cl₂ to give a white solid in 93 % yield, mp 118-119 °C. ¹H NMR (400 MHz, CDCl₃) δ 7.27-7.19 (4H, m), 7.03 (2H, d, J = 8.4 Hz), 6.75 (2H, d, J = 8.8 Hz), 6.51 (1H, dd, J = 2.4, 8.8 Hz), 6.21 (1H, dd, J = 2.4, 7.6 Hz), 6.07 (1H, dd, J = 2.6, 10.0 Hz), 5.97 (1H, d, J = 4.8 Hz), 5.94 (1H, dd, J = 2.2, 10.4 Hz), 5.32 (1H, d, J = 4.8 Hz), 3.73 (3H, s). ¹³C NMR (400 MHz, CDCl₃) δ 164.8, 162.9, 161.7, 157.6, 151.4, 140.2, 133.1, 132.8, 130.3, 130.0, 129.0, 127.9, 127.7, 126.5, 121.9(2C), 119.1, 114.7, 61.2, 55.7.
Synthesis of *cis*-3-Acryloyl-4-(4-acryloylphenyl)azetidin-2-one (50): To a solution of 49 (82.0 mg, 0.21 mmol) in 3 mL of CH$_3$CN was added a solution of ceric ammonium nitrate (342 mg, 0.62 mmol) dissolved in 3.1 mL of water. The reaction was allowed to stir for 15 min. The solution was extracted with EtOAc (3 x 20 mL) and washed with 10 mL of water, 5% NaHCO$_3$, 10% NaHSO$_3$ and brine, and then dried over MgSO$_4$. The solvent was removed under reduced pressure to yield, and the crude product was purified by column chromatography (1:1 EtOAc/hexanes) to 50 as a white solid in 84% yield. $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 7.27 (2H, d, $J$ = 8.4 Hz), 7.20 (2H, d, $J$ = 8.0 Hz), 6.52 (1H, d, $J$ = 17.2 Hz), 6.34 (1H, bs), 6.25 (1H, dd, $J$ = 2.4, 10.4 Hz), 6.02 (1H, d, $J$ = 4.4 Hz), 5.74 (1H, dd, $J$ = 3.6, 10.4 Hz), 5.36 (1H, dd, $J$ = 2.2, 9.4 Hz), 5.00 (1H, d, $J$ = 4.8 Hz). $^{13}$C NMR (400 MHz, CDCl$_3$) $\delta$ 165.7, 164.6, 164.3, 150.9, 133.1, 132.8, 132.4, 130.3, 130.0, 128.8, 127.9, 126.6, 121.7, 78.3, 57.6.

Synthesis of *cis*-3-Acryloyl-4-(4-acryloylphenyl)-N-methylthioazetidin-2-one (38): Compound 50 (0.039 g, 0.10 mmol) was dissolved in dry CH$_2$Cl$_2$ (5 mL), and the solution of N-methylthiophthalimide (0.019 g, 0.10 mmol) was added. To the resulting solution was then added triethylamine (0.016 mL, 0.10 mmol). The reaction was refluxed for overnight and then diluted with CH$_2$Cl$_2$ (20 mL). The resulting solution was washed with 1% aqueous KOH (2 x 10 mL). The organic layer was dried with anhydrous MgSO$_4$, filtered, and evaporated to give 28 mg of 38 as a white solid in 72% yield. $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 7.36 (2H, d, $J$ = 8.4 Hz), 7.15 (1H, d, $J$ = 8.4 Hz), 6.61 (1H, dd, $J$ = 10.4, 6.8 Hz), 6.00 (1H, dd, $J$ = 10.4, 6.6 Hz), 4.80 (1H, d, $J$ = 4.8 Hz), 4.75 (1H, d, $J$ = 5.2 Hz), 3.15 (3H, s), 2.34 (3H, s). $^{13}$C NMR (400 MHz, CDCl$_3$) $\delta$ 168.2, 164.6, 164.0, 151.4, 135.4, 133.1, 130.3, 130.2, 129.8, 127.9, 127.7, 126.4, 121.8(2C), 78.1, 65.5, 22.3.

6.1.17 C$_3$-Long Chain Lactam 39

![Diagram of the synthesis of C$_3$-Long Chain Lactam 39]
Synthesis of cis-3-Acryloyloxy ethyl succinate-4-(2-chlorophenyl)-N-(4-methoxyphenyl) azetidin-2-one (51): To the solution of hydroxyl lactam 9b (497.8 mg, 1.64 mmol) in 5 mL of CH₂Cl₂ was added mono-2-(acryloyloxy)ethyl succinate (345.9 mg, 1.6 mmol), EDC (306.7 mg, 1.6 mmol) and DMAP (196 mg, 1.6 mmol) in an ice bath. Then the solution was warmed to r.t. and stir for overnight. The crude material was purified by column chromatography using EtOAc/Hexanes (1:1) to give 618.1 mg of 51 in 77% yield as a colorless oil. ¹H NMR (400 MHz, CDCl₃) δ 7.27-7.21 (6H, m), 6.81 (2H, d, J = 8.8 Hz), 6.39 (1H, dd, J = 1.2, 10.8 Hz), 6.18 (1H, d, J = 4.8 Hz), 6.04 (1H, dd, J = 1.2, 10.4 Hz), 5.78 (1H, d, J = 4.8 Hz), 4.32 (4H, q, J = 5.2 Hz), 3.74 (3H, s), 2.42-2.39 (1H, m), 2.33 (2H, t, J = 6.8 Hz), 2.22-2.18 (1H, m). ¹³C NMR (400 MHz, CDCl₃) δ 170.8, 169.9, 166.3, 161.8, 157.2, 134.1, 131.7, 130.1, 130.0, 129.6, 129.4, 128.2(2C), 128.1, 127.1, 126.9, 118.9, 114.7, 62.6, 62.3, 60.0, 58.4, 55.7, 28.7, 28.6.

Synthesis of cis-3-Acryloyloxy ethyl succinate-4-(2-chlorophenyl)azetidin-2-one (52): To a solution of 51 (0.31 g, 0.62 mmol) in 3 mL of CH₃CN was added a solution of ceric ammonium nitrate (1.00 g, 1.85 mmol) dissolved in a minimum amount of water. The reaction was allowed to stir for 5 min. The solution was extracted with EtOAc (3 x 20 mL) and washed with 30 mL of 5% NaHSO₃, 5% NaHCO₃, and brine, and then dried over Na₂SO₄. The solvent was removed under reduced pressure to yield, and the crude product was purified by column chromatography (1:1 EtOAc/hexanes) to give 52 as yellow oil in 86% yield. ¹H NMR (400 MHz, CDCl₃) δ 7.33-7.24 (4H, m), 6.70 (1H, bs), 6.39 (1H, dd, J = 2.4, 10.4 Hz), 6.15 (1H, d, J = 3.2 Hz), 6.12 (1H, dd, J = 2.2, 10.8 Hz), 5.83 (1H, dd, J = 2.4, 10.4 Hz), 5.36 (1H, d, J = 4.8 Hz), 4.32 (2H, t, J = 4.4 Hz), 4.24 (2H, t, J = 2.8 Hz), 2.42-2.36 (1H, m), 2.32 (2H, t, J = 6.4 Hz), 2.24-2.20 (1H, m). ¹³C NMR (400 MHz, CDCl₃) δ 170.8, 169.9, 166.3, 161.8, 157.2, 134.1, 131.7, 130.1, 129.4, 128.2(2C), 128.1, 127.1, 126.9, 118.9, 114.7, 62.6, 62.3, 60.0, 58.4, 55.7, 28.7, 28.6.

Synthesis of cis-3-Acryloyloxy ethyl succinate-4-(2-chlorophenyl) -N-methylthioazetidin-2-one (39): Compound 52 (0.039 g, 0.10 mmol) was dissolved in dry CH₂Cl₂ (5 mL), and the solution of N-methylthiophthalimide (0.019 g, 0.10 mmol) was added. To the resulting solution was then added Hunig’s base (0.038 g, 0.30 mmol). The reaction was refluxed for overnight and then diluted with CH₂Cl₂ (20 mL). The resulting solution was washed with 1% aqueous KOH (2 x 10 mL). The organic layer was dried with anhydrous MgSO₄, filtered, and evaporated, and the residue was chromatographed (1:2 EtOAc/hexanes) to give 28 mg of 39 as yellow oil in 72% yield. ¹H NMR (400 MHz, CDCl₃) δ 7.41-7.19 (4H, m), 6.35 (1H, dd, J = 2.2, 9.6 Hz), 6.11 (1H, d, J = 4.4 Hz), 6.02 (1H, dd, J = 2.4, 10.8 Hz), 5.73 (1H, dd, J = 2.4, 10.8 Hz), 5.42 (1H, d, J = 4.9 Hz), 4.28 (2H, t, J = 3.6 Hz), 4.24 (2H, t, J = 2.8 Hz), 2.44 (3H, s), 2.36-2.30 (4H, m). ¹³C NMR (400 MHz, CDCl₃) δ 171.4, 169.9, 168.3, 166.0, 134.6, 131.6, 130.6, 130.3, 130.2, 130.1, 128.9, 128.1, 126.9, 62.6, 62.2, 28.6, 28.5, 22.1.

6.1.18 Glycine-Attached Lactam 54
**6.1.19 Alanine-Attached Lactam 56**

\[ \text{N-Acryloyl-L-alanine (55):} \quad ^1H \text{ NMR (250 MHz, CDCl}_3) \ \delta \ 6.4 \ (1H, dd, J = 4.4, 15.2 Hz), 6.1 \ (1H, dd, J = 4.0, 15.2 Hz), 5.7 \ (1H, dd, J = 4.4, 15.2 Hz), 4.6 \ (1H, q, J = 4.8 Hz), 1.4 \ (3H, d, J = 3.2 Hz) \]

\[ \text{cis-3-(2-Acryloylaminopropionic ester)-4-(2-chlorophenyl)-N-methylthiazetidin-2-one (56):} \quad \text{White solid.} \quad ^{13}C \text{ NMR (400 MHz, CDCl}_3) \ \delta \ 178.0, 176.3, 166.7, 142.8, 130.8, 129.1, 128.7, 127.6, 125.9, 82.3, 48.6, 51.4, 16.6. \]

**6.1.20 Valine-Attached Lactam 58**
N-Acryloyl-L-valine (57): $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 6.3 (1H, dd, $J = 2.0$, 9.6 Hz), 6.1 (1H, dd, $J = 2.0$, 9.6 Hz), 5.6 (1H, dd, $J = 1.6$, 9.6 Hz), 4.6 (1H, q, $J = 4.8$ Hz), 2.1 (1H, q, $J = 5.6$ Hz), 0.9 (6H, dd, $J = 3.2$, 7.2 Hz).

cis-3-(2-Acryloylamino-3-methyl-butyric ester)-4-(2-chlorophenyl)-N-methylthioazetidin-2-one (58): White solid. $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 7.16-7.10 (4H, m), 6.68 (1H, bs), 6.02 (1H, dd, $J = 5.0$, 11.2 Hz), 5.83 (1H, d, $J = 5.2$ Hz), 5.78 (1H, dd, $J = 5.2$, 9.6 Hz), 5.76 (1H, dd, $J = 4.7$, 9.6 Hz), 5.31 (1H, d, $J = 5.0$ Hz), 4.62 (1H, d, $J = 4.8$ Hz), 2.30 (2H, m), 2.04 (3H, m). $^{13}$C NMR (400 MHz, CDCl$_3$) $\delta$ 177.2, 176.0, 166.7, 142.8, 130.8, 129.1, 128.7, 127.6, 125.9, 82.3, 61.7, 48.6, 51.4, 27.2, 16.6 (2C).

6.1.21 Proline-Attached Lactam 58

Acryloyl-L-proline (59): White solid. mp 109-110 °C. $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 6.43 (2H, m), 5.78 (1H, m), 4.62 (1H, d, $J = 4.8$ Hz), 3.69 (1H, m), 3.58 (1H, m), 3.58 (1H, m), 2.30 (2H, m), 2.04 (3H, m).

cis-3-(1-Acryloyl-pyrrolidine-2-carboxylic ester)-4-(2-chlorophenyl)-N-methylthioazetidin-2-one (60): White solid. $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 7.16-7.10 (4H, m), 6.68 (1H, bs), 6.02 (1H, dd, $J = 5.0$, 11.2 Hz), 5.83 (1H, d, $J = 5.2$ Hz), 5.78 (1H, dd, $J = 5.2$, 9.6 Hz), 5.76 (1H, dd, $J = 4.7$, 9.6 Hz), 5.31 (1H, d, $J = 5.0$ Hz), 4.62 (1H, d, $J = 4.8$ Hz), 2.30 (2H, m), 2.04 (4H, m). $^{13}$C NMR (400 MHz, CDCl$_3$) $\delta$ 176.0, 174.8, 166.7, 142.8, 130.8, 129.1, 128.7, 127.6, 125.9, 82.3, 58.1, 50.5, 42.6, 27.2, 22.8, 20.4, 16.6.
6.1.22 Methionine-Attached Lactam 62

\[
\begin{align*}
\text{N-Acryloyl-\text{\textgamma L}}\text{-methionine (61):} & \quad ^1H \text{ NMR (400 MHz, CDCl}_3) \delta 6.96 (1H, bs), 6.28 (1H, dd, } J = 2.0, 10.4 \text{ Hz), 6.12 (1H, dd, } J = 2.0, 10.0 \text{ Hz), 5.65 (1H, dd, } J = 1.6, 10.4 \text{ Hz), 4.71 (1H, q, } J = 4.8 \text{ Hz), 2.50 (1H, t, } J = 7.2 \text{ Hz), 2.18 (1H, t, } J = 5.6 \text{ Hz), 2.02 (3H, s).} \\
\text{cis-3-(2-Acryloylamino-4-methylsulfanyl-butyricester)-4-(2-chlorophenyl)-N-methylthioazetidin-2-one (62):} & \quad \text{White solid.} \quad ^1H \text{ NMR (400 MHz, CDCl}_3) \delta 7.16-7.10 (6H, m), 6.88 (2H), 6.24 (1H, dd, } J = 3.0, 9.6 \text{ Hz), 6.04 (1H, dd, } J = 3.0, 10.4 \text{ Hz), 5.87 (1H, dd, } J = 2.8, 10.4 \text{ Hz), 5.76 (1H, d, } J = 5.0 \text{ Hz), 5.45 (1H, d, } J = 5.2 \text{ Hz), 4.54 (1H, q, } J = 4.0, 9.2 \text{ Hz), 2.48 (2H, t, } J = 7.2 \text{ Hz), 2.38 (3H, s), 2.11 (2H, t, } J = 5.6 \text{ Hz), 2.03 (3H, s).} \quad 13C \text{ NMR (400 MHz, CDCl}_3) \delta 177.0, 174.2, 166.7, 142.8, 130.8, 129.1, 128.7, 127.6, 125.9, 82.3, 58.1, 53.3, 33.2, 16.9, 9.8. 
\end{align*}
\]

6.1.23 Threonine-Attached Lactam 65
**N-Acryl-L-threonine 64:** White solid, mp 83-85°C, 84.1%. $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 6.31 (1H, dd, $J$ = 1.2, 10.4 Hz), 6.19 (1H, dd, $J$ = 1.2, 10.0 Hz), 5.71 (1H, dd, $J$ = 1.2, 10.4 Hz), 4.60 (1H, dd, $J$ = 2.0, 8.8 Hz), 4.30 (1H, m), 3.77 (3H, s), 2.20 (1H, bs), 1.22 (3H, d, $J$ = 6.8 Hz).

**cis-4-(2-Chlorophenyl)-N-methylthio-3-(succinic acid-2-acryloylamino-2-methoxycarbonyl-1-methyl-ethyl ester)azetidin-2-one (65):** White solid. $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 7.43-7.23 (4H, m), 6.31 (1H, dd, $J$ = 1.2, 10.4 Hz), 6.19 (1H, dd, $J$ = 1.2, 10.0 Hz), 6.02 (1H, d, $J$ = 4.8 Hz), 5.71 (1H, dd, $J$ = 1.2, 10.4 Hz), 5.38 (1H, d, $J$ = 4.8 Hz), 4.60 (1H, dd, $J$ = 2.0, 8.8 Hz), 4.30 (1H, m), 3.77 (3H, s), 2.20 (1H, bs), 1.22 (3H, d, $J$ = 6.8 Hz). $^{13}$C NMR (400 MHz, CDCl$_3$) $\delta$ 176.0, 175.4, 172.2, 166.7, 142.8, 130.8, 129.1, 128.7, 127.6, 130.0, 125.9, 82.3, 59.1, 50.4, 33.2, 29.0, 28.3, 14.0.

### 6.1.24 Methionine-Attached Lactam 67

**66**
cis-4-(2-Chlorophenyl)-1-(methylthio)-3-(methyloxy-4-oxobutanamido)-N-(methylthio) azetidin-2-one (67): White solid. $^1$H NMR (400 MHz, CDCl$_3$) δ 7.43-7.23 (4H, m), 6.02 (1H, d, $J = 4.8$ Hz), 5.71 (1H, dd, $J = 1.2$, 10.4 Hz), 5.38 (1H, d, $J = 4.8$ Hz), 4.22 (1H, m), 3.77 (3H, s), 2.52 (4H, m), 3.27 (1H, dd, $J = 2.0$, 8.8 Hz), 2.20 (3H, s); $^{13}$C NMR (400 MHz, CDCl$_3$) δ 176.0, 175.4, 172.2, 166.7, 142.8, 130.8, 129.1, 128.7, 127.6, 130.9, 125.9, 82.3, 59.1, 50.8, 50.4, 33.2, 29.4, 28.9, 16.8.

6.1.25 Dihydroxy Lactam 68

cis-3-Acetox-4-(4-acetoxyphenyl)-N-methylthioazetidin-2-one (69): White solid. $^1$H NMR (400 MHz, CDCl$_3$) δ 7.26-7.20 (2H, m), 7.02-7.00 (2H, m), 5.77 (1H, d, $J = 2.4$ Hz), 4.94 (1H, d, $J = 2.4$Hz), 2.22 (3H, s), 1.63 (6H, s).

cis-3-hydroxy-4-(4-hydroxyphenyl)-N-methylthioazetidin-2-one (68): White solid. $^1$H NMR (400 MHz, CDCl$_3$) δ 7.26-7.20 (2H, m), 7.02-7.00 (2H, m), 5.77 (1H, d, $J = 2.4$ Hz), 4.94 (1H, d, $J = 2.4$Hz), 2.28 (3H, s).

6.2 Emulsion Polymerization

6.2.1 General procedure for preparation of emulsion polymerization

The general emulsion polymerization procedure employed for this system involves dissolving a water-insoluble acrylated drug in a liquid acrylate, i.e. ethyl acrylate, at 70°C, and the mixture was then pre-emulsified in purified water containing 3% w/w of sodium dodecylsulfate, a surfactant, with rapid stirring. The resulting homogenous solution of micelles was then treated with potassium persulfate (1% w/w), a radical initiator, to induce free radical polymerization.
6.2.2 Formulation of Emulsion Polymerization

1. Formulation of emulsion polymerization of NP-4a

<table>
<thead>
<tr>
<th>components</th>
<th>amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drug 27</td>
<td>300 mg</td>
</tr>
<tr>
<td>Ethyl acrylate</td>
<td>1000 mg</td>
</tr>
<tr>
<td>Surfactant</td>
<td>30 mg</td>
</tr>
<tr>
<td>Radical initiator</td>
<td>10 mg</td>
</tr>
<tr>
<td>Water</td>
<td>4 ml</td>
</tr>
<tr>
<td>Rxn Temp</td>
<td>70 °C</td>
</tr>
</tbody>
</table>

2. Formulation for emulsion polymerization of NP 4b-NP 12.

<table>
<thead>
<tr>
<th>components</th>
<th>amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drug monomer</td>
<td>10 mg</td>
</tr>
<tr>
<td>Butyl acrylate</td>
<td>700 mg</td>
</tr>
<tr>
<td>Styrene</td>
<td>300 mg</td>
</tr>
<tr>
<td>Surfactant</td>
<td>30 mg</td>
</tr>
<tr>
<td>Radical initiator</td>
<td>10 mg</td>
</tr>
<tr>
<td>Water</td>
<td>4 ml</td>
</tr>
<tr>
<td>Rxn Temp</td>
<td>70 °C</td>
</tr>
</tbody>
</table>

6.3 Physical Properties Analysis

6.3.1 Particle size determination

Dynamic light scattering data was acquired on a UPA Honeywell MicroTrac at the University of Florida Particle Engineering Research Center. One drop of concentrated emulsion solution was placed in a 10 mL well filled with nanopure water. Analysis was performed for 3 runs of 180 seconds per run per sample.
6.3.2 Zeta Potential Analysis

Zeta potential value of each emulsion was obtained on a Brookhaven ZetaPALS. The emulsion solution was diluted to 1.5% (~ pH = 7) of solid content using nanopure water for analysis and 2 × 10 runs per sample was performed. The dispersant viscosity of the emulsion solution is 0.8872cP, and the dielectric constant of the medium is 78.55.

6.3.3 Microscopy

The morphology and the particle size was performed scanning electron microscopy (SEM) in the college of Engineering at University of South Florida. The sample of nanoparticles was prepared by high DI water (3,000-fold) of the emulsion, with evaporation under a N_2-stream prior to coating by gold sputter under high vacuum. The gold-coated nanoparticles were then observed by SEM (Hitach S 800). Transmission electron microscopy (TEM) analysis was performed on a FEI Morgagni 268D Electron Microscope in the biology department at University of South Florida. The initial emulsion solution was diluted down to a 10^{-10} concentration using nanopure water, then the solution was drop cast onto a Formvar-coated copper grid. The water content was evaporated by applying a cool stream of air to the drop, and the grid was subsequently view on the microscope.

6.4 Microbiological Test Procedures

The following bacteria were used for the antimicrobial evaluation of N-thiolated β-lactams: Bacillus anthracis (Sterne strain), Bacillus cereus (ATCC 14579), Bacillus coagulans (USF 546), Bacillus globigii (Department of Defense Reagents Program), Bacillus megaterium (ATCC 14581), Bacillus subtilis (ATCC 19569), Bacillus thuringensis (ATCC 10792), Bacteroides fragalis (obtained from Smith-Kline Laboratory), Staphylococcus aureus USF525 (ATCC 25923) Staphylococcus aureus USF652-658 (obtained from Lakeland Regional Medical Center, β-lactamase positive).

6.4.1 Antimicrobial Susceptibility Test

Culture preparation: From a freezer stock in tryptic soy broth (Difco Laboratories, Detroit, MI) and 20% glycerol, a culture of each organism was grown on tryptic soy agar (TSA) plates (Becton-Dickinson Laboratories, Cockeysville, MD) at 37°C for 24 hours. A 10^8 suspension was then made in sterile phosphate buffered saline (pH 7.2) and swabbed across fresh TSA plates.

Well method: A 10^8 standardized cell count suspension was then made in sterile phosphate buffered saline (pH 7.2) and swabbed across fresh TSA plates. Circular wells (6 mm in diameter) were cut into the inoculated plates and 20 µL of a 1 mg/mL stock solution of the test lactam in dimethylsulfoxide (DMSO) was pipetted into the wells. The plates were incubated for 24 hours at 37°C and the antimicrobial susceptibilities were determined by measuring the zones of growth inhibition around each well.

6.4.2 MIC Determinations

Media preparation: The minimum inhibitory concentrations were determined by the agar plate dilution. The test media were prepared in 24 well plates (Costar 3524, Cambridge, MA) by adding a known concentration of the test drug in DMSO together with a solution of Mueller-Hinton II agar (Becton-Dickinson Laboratories, Cockeysville, MD) for a total volume of 1 ml in each well. Calculations of the overall concentration of antibiotic in the wells were standardized by measuring from a 1mg/ml stock solution of the test drug. At this concentration, the microliter quantity is equivalent to the micrograms in
solution. The amount of agar solution added to the wells was determined by subtracting 1000 µl from the quantity of test drug in each well to give a combined volume of 1 ml. Following preparation of the well plates, the media were allowed to solidify at room temperature for 24 hours before inoculation.

Inoculation: From an 24 hour culture of each organism on tryptic soy agar (TSA) plates (Becton-Dickinson Laboratories, Cockeysville, MD), the staphylococcal strains were grown overnight in 5 ml of tryptic soy broth (Difco Laboratories, Detroit, MI) at 37°C. One microliter of each culture was then applied to the appropriate well of agar and incubated at 37°C overnight. After 24 hr, the MICs were determined by examining the wells for growth.

6.5 Interaction of Nanoparticles with *S. aureus* Cells

6.5.1 Whole cell analysis

*S. aureus* was grown up on TSA plates overnight at 37°C. The bacteria were then carried through a series of dilutions in order to obtain a concentration of $10^2$ CFU/ml in trypticase soy broth (TSB). The cells were then exposed to a $10^{10}$ concentration of control nanoparticles for 30 minutes. Thereafter, the broth was treated with 5 mL of 2.5% gluteraldehyde for 4 hours at 4°C. A solution of $10^2$ CFU/ml *S. aureus* in TSB was also treated with 5 mL of 2.5% gluteraldehyde for 4 hours at 4°C and served as a control for the experiment. The broth suspension was then drop cast onto a formvar-coated copper grid and was allowed to dry at room temperature under a cool stream of air. The control cells and cells treated with control nanoparticles were then observed on the FEI Morgagni 268D transmission electron microscope.

6.5.2 Sectioned cell analysis

*S. aureus* was grown up on TSA plates overnight at 37°C. The bacteria were then carried through a series of dilutions in order to obtain a concentration of $10^7$ CFU/ml in TSB. The diluted samples were then exposed to a concentration of $10^5$ control nanoparticles for 30 minutes and then centrifuged at 10,000 xg for 5 minutes. The pellet was washed twice with PBS, then resuspended in 5 ml of PBS and fixed with 5 ml of 2.5% glutaraldehyde for 4 hours at 4°C. The fixed cells were centrifuged at 10,000 xg for 5 minutes and then resuspended in PBS supplemented with 0.1M sucrose. The cells were then centrifuged and resuspended twice in PBS at 10,000 xg for 5 minutes. The cells were then centrifuged again at 10,000 xg for 5 minutes then the pellet was embedded in agar for easier handling. The agar blocks were then washed with PBS and postfixed in 2% osmium tetroxide for 1 hour at room temperature. The cells were then washed twice with PBS then once with 0.9% saline and stained with 1.5% uranyl acetate. The cell-containing agar blocks were then carried through a series of dehydrations using graded ethanol. The cells are then infiltrated and embedded in Spurr’s Plastic and ultrathin sections are cut using a Sorvall MT-2B ultramicrotome and placed on copper mesh grids. The grids are then examined on the FEI Morgagni 268D Electron Microscope.
CHAPTER 7  $^1$H and $^{13}$C NMR SPECTRA

Spectrum 7.1: $^1$H NMR (250 MHz, CDCl$_3$) (6b)

Spectrum 7.2: $^{13}$C NMR (63 MHz, CDCl$_3$) (6b)
Spectrum 7.3: $^1$H NMR (250 MHz, CDCl$_3$) (6a)

Spectrum 7.4: $^1$H NMR (250 MHz, CDCl$_3$) (8a)
Spectrum 7.5: $^1$H NMR (250 MHz, CDCl$_3$) (9a)

Spectrum 7.6: $^1$H NMR (250 MHz, CDCl$_3$) (10a)
Spectrum 7.7: $^1$H NMR (250 MHz, CDCl$_3$) (11a)

Spectrum 7.8: $^1$H NMR (250 MHz, CDCl$_3$) (8b)
Spectrum 7.9: $^1$H NMR (250 MHz, CDCl$_3$) (3a)

Spectrum 7.10: $^{13}$C NMR (63 MHz, CDCl$_3$) (3a)
Spectrum 7.11: $^1$H NMR (250 MHz, CDCl$_3$) (10b)

Spectrum 7.12: $^{13}$C NMR (63 MHz, CDCl$_3$) (10b)
Spectrum 7.13: $^1$H NMR (250 MHz, CDCl$_3$) (3b)

Spectrum 7.14: $^{13}$C NMR (63 MHz, CDCl$_3$) (3b)
Spectrum 7.15: $^1$H NMR (250 MHz, CDCl$_3$) (10c)

Spectrum 7.16: $^{13}$C NMR (63 MHz, CDCl$_3$) (10c)
Spectrum 7.17: $^1$H NMR (250 MHz, CDCl$_3$) (3c)

Spectrum 7.18: $^{13}$C NMR (63 MHz, CDCl$_3$) (3c)
Spectrum 7.19: $^1$H NMR (250 MHz, CDCl$_3$) (15)

Spectrum 7.20: $^1$H NMR (100 MHz, CDCl$_3$) (16)
Spectrum 7.21: $^1$H NMR (250 MHz, CDCl$_3$) (17)

Spectrum 7.22: $^1$H NMR (250 MHz, CDCl$_3$) (17)
Spectrum 7.23: $^1$H NMR (250 MHz, CDCl$_3$) (10d)

Spectrum 7.24: $^{13}$C NMR (63 MHz, CDCl$_3$) (10d)
Spectrum 7.25: $^1$H NMR (250 MHz, CDCl$_3$) (18)

Spectrum 7.26: $^1$H NMR (250 MHz, CDCl$_3$) (21a)
Spectrum 7.27: $^1$H NMR (250 MHz, CDCl$_3$) (20)

Spectrum 7.28: $^{13}$C NMR (63 MHz, CDCl$_3$) (20)
Spectrum 7.29: $^1$H NMR (250 MHz, CDCl$_3$) (22a)

Spectrum 7.30: $^1$H NMR (250 MHz, CDCl$_3$) (19b)
Spectrum 7.31: $^1$H NMR (250 MHz, CDCl$_3$) (21b)

Spectrum 7.32: $^{13}$C NMR (63 MHz, CDCl$_3$) (21b)
Spectrum 7.33: $^1$H NMR (250 MHz, CDCl$_3$) (22b)

Spectrum 7.34: $^{13}$C NMR (63 MHz, CDCl$_3$) (22b)
Spectrum 7.35: $^1$H NMR (250 MHz, CDCl$_3$) (21c)

Spectrum 7.36: $^{13}$C NMR (63 MHz, CDCl$_3$) (21c)
Spectrum 7.37: $^1$H NMR (250 MHz, CDCl$_3$) (22c)

Spectrum 7.38: $^1$H NMR (250 MHz, CDCl$_3$) (19c)
Spectrum 7.39: $^1$H NMR (250 MHz, CDCl$_3$) (21d)

Spectrum 7.40: $^1$H NMR (250 MHz, CDCl$_3$) (19d)
Spectrum 7.41: $^1$H NMR (250 MHz, CDCl$_3$) (22d)

Spectrum 7.42: $^{13}$C NMR (63 MHz, CDCl$_3$) (22d)
Spectrum 7.43: $^1$H NMR (250 MHz, CDCl$_3$) (23)

Spectrum 7.44: $^1$H NMR (250 MHz, CDCl$_3$) (19e)
Spectrum 7.45: $^{13}$C NMR (100 MHz, CDCl$_3$) (30)

Spectrum 7.46: $^1$H NMR (400 MHz, CDCl$_3$) (31)
Spectrum 7.47: $^1$H NMR (400 MHz, CDCl$_3$) (27)

Spectrum 7.48: $^{13}$C NMR (100 MHz, CDCl$_3$) (27)
Spectrum 7.49: $^1$H NMR (250 MHz, CDCl$_3$) (28, 29)

Spectrum 7.50: $^1$H NMR (400 MHz, CDCl$_3$) (34)
Spectrum 7.51: $^1$H NMR (250 MHz, CDCl$_3$) (29)

Spectrum 7.52: $^1$H NMR (400 MHz, CDCl$_3$) (32)
Spectrum 7.53: $^1$H NMR (400 MHz, CDCl$_3$) (33)

Spectrum 7.54: $^1$H NMR (250 MHz, CDCl$_3$) (25)
Spectrum 7.55: $^1$H NMR (250 MHz, CDCl$_3$) (42)

Spectrum 7.56: $^{13}$C NMR (100 MHz, CDCl$_3$) (42)
Spectrum 7.57: $^1$H NMR (400 MHz, CDCl$_3$) (44)

Spectrum 7.58: $^1$H NMR (400 MHz, CDCl$_3$) (45)
Spectrum 7.59: $^1$H NMR (400 MHz, CDCl$_3$) (47)

Spectrum 7.60: $^{13}$C NMR (100 MHz, CDCl$_3$) (47)
Spectrum 7.61: $^{13}$C NMR (100 MHz, CDCl$_3$) (48)

Spectrum 7.62: $^1$H NMR (250 MHz, CDCl$_3$) (14)
Spectrum 7.63: $^1$H NMR (400 MHz, CDCl$_3$) (49)

Spectrum 7.64: $^{13}$C NMR (100 MHz, CDCl$_3$) (49)
Spectrum 7.65: $^1$H NMR (400 MHz, CDCl$_3$) (50)

[Image of a spectrum showing chemical shifts and peak assignments]

Spectrum 7.66: $^{13}$C NMR (100 MHz, CDCl$_3$) (50)

[Image of a spectrum showing carbon chemical shifts and peak assignments]
Spectrum 7.67: $^{13}$C NMR (100 MHz, CDCl$_3$) (38)

Spectrum 7.68: $^{13}$C NMR (100 MHz, CDCl$_3$) (51)
Spectrum 7.69: $^{13}$C NMR (100 MHz, CDCl$_3$) (39)

Spectrum 7.70: $^1$H NMR (250 MHz, CDCl$_3$) (56)
Spectrum 7.71: $^1$H NMR (400 MHz, CDCl$_3$) (57)

Spectrum 7.72: $^1$H NMR (400 MHz, CDCl$_3$) (NP 6c)
Spectrum 7.73: $^1$H NMR (400 MHz, CDCl$_3$) (NP 7)

Spectrum 7.74: $^1$H NMR (400 MHz, CDCl$_3$) (NP 6b)
REFERENCE

1 Di Modugnu E, Felici A, Curr Opin Anti-Infective Invest Drugs 1999, 1, 26-39.
11 Andreotti D, Biondi S, CurrOpin Anti-Infective Invest Drugs, 2000, 2, 133-139.


Uekama K., Otagiri M. Crit Rev Ther Drug Carrier Syst. 1987, 3(1), 1-40.


ABOUT THE AUTHOR

Yang Wang received her bachelor’s degree in School of Chemical Engineering at Dalian University of Technology, China in 2000. She began research in the synthetic laboratory of Professor Edward Turos from January, 2002. Currently, Yang continues to conduct research on nanoparticle-based delivery of anticancer agents.