

**ANTIMICROBIAL POLYMERS:
PEPTIDE-MIMETIC DESIGN AND MECHANISM OF ACTION**

by

Edmund Francis Palermo

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Macromolecular Science and Engineering)
in The University of Michigan
2011

Doctoral Committee:

Assistant Professor Kenichi Kuroda, Chair
Professor Richard M. Laine
Professor Ayyalusamy Ramamoorthy
Assistant Professor Anne J. McNeil

© Edmund Francis Palermo, 2011

DEDICATION

To Gabrielle C. Todd, for her support, encouragement, and helpful discussions

ACKNOWLEDGEMENTS

The author gratefully acknowledges his parents, Edmund Francis and Diane Palermo, and his sister Maria Palermo, who always placed the highest emphasis on work ethic and education. The author is also grateful to the thesis committee members for their time and helpful comments. Professor Kuroda and members of the Kuroda laboratory deserve acknowledgement for many thoughtful discussions. Many thanks are due to Nonna Hamilton, administrator of the Macromolecular and Engineering Center, for her invaluable assistance.

This dissertation is based on the following publications co-authored by the candidate: Chapter I (Palermo and Kuroda, *Appl. Microbiol. Biotechnol.*, **2010**, 87(5), 1605-1615), Chapter II (Palermo and Kuroda, *Biomacromolecules*, **2009**, 10(6), 1416-1428), Chapter III (Palermo et al., *J. Phys. Chem. B*, **2011**, 115(2), 366-375), and Chapter IV (Palermo et al., *Biomacromolecules* **2009**, 10(11), 3098-3107).

Solid state NMR was performed in the laboratory of Professor Ayyalusamy Ramamoorthy in Chemistry, fluorescence imaging and flow cytometry were done with Chuanwu Xi of the Department of Public Health, and Gregory Caputo and co-workers in Chemistry and Biochemistry at Rowan University performed the bacteria permeabilization studies. The molecular dynamics simulations were done by Dr. Satayavani Vemparala of the Institute of Mathematical Sciences, in India. Professors Masami Kamigaito and Kotaro Satoh of Applied Chemistry at Nagoya University, Japan, supervised the author during the summer of 2010, for the NSF East Asia Summer and Pacific Institutes (EAPSI) fellowship. Professor Eric Krukoni of Biologic and Materials Sciences, School of Dentistry, provided assistance and advice on microbiology, and Professor Robert Davenport of the University of Michigan Hospital generously supplied units of human red blood cells for this work.

TABLE OF CONTENTS

DEDICATION	ii
ACKNOWLEDGEMENTS	iii
LIST OF TABLES	vi
LIST OF FIGURES	vii
LIST OF APPENDICES	xi
LIST OF ABBREVIATIONS	xii
CHAPTER	
I. Introduction and Background: The Convergence of Peptide and Polymer Science toward Novel Antibiotics	1
Motivation and Objectives	1
Host Defense Peptides	2
Synthetic Polymer Biocides	8
Peptide-mimetic Antimicrobial Polymers	9
II. Effect of Cationic Group Structure on Antimicrobial and Hemolytic Activity of Amphiphilic Copolymers	15
Introduction	15
Polymer Design, Synthesis and Characterization	18
Antimicrobial Activity	21
Hemolytic Activity	25
Selectivity	26
Bactericidal Activity vs. pH	28
Hemolytic Activity vs. pH	31
Conclusions	34
III. Role of Cationic Group Structure in Membrane Binding and Disruption by Amphiphilic Copolymers	36
Introduction	36
Dansyl-labeled Polymer Synthesis and Characterization	38
Water-Octanol Partition Coefficients	41
Polymer-Liposome Dissociation Constants	43
Polymer-Induced Liposome Leakage	47
Solid-state NMR	49

Membrane Disruption Mechanism	51
Conclusions	53
IV. Amphiphilic Polymethacrylamides as an Antimicrobial Design Platform	55
Introduction	55
Polymer Synthesis and Characterization	56
Antimicrobial Activity	59
Hemolytic Activity	62
Cytotoxicity	64
Liposome Dye Leakage	67
Conclusions	72
V. Role of Cationic Side Chain Spacer Groups in Activity and Mechanism of Antimicrobial Action by Amphiphilic Copolymers	74
Introduction	74
Polymer Synthesis and Characterization	76
Structure-Activity Relationships	78
Synthesis of Fluorophore-labeled Polymers	81
Fluorescence Imaging of Bacteria Cells	82
Flow Cytometry Analysis	84
<i>E. coli</i> Membrane Permeabilization	86
Mechanism of Osmotic Lysis	88
Molecular Dynamics Simulation	91
Mechanism of Antimicrobial Action	94
Conclusions	95
VI. Conclusions and Future Directions	96
Structure-Activity Relationships	96
Future Directions in Polymer Chemistry	97
Mechanism of Antimicrobial Action	98
Future Mechanistic Work	99
Challenges Remaining	100
APPENDICES	101
REFERENCES	150

LIST OF TABLES

TABLE

I-1	Structural Features and Activity of Antimicrobial Peptides and Polymers	9
II-1	Characterization of methacrylate random copolymers containing different ammonium functional groups. R = methyl	22
II-2	Characterization of methacrylate random copolymers containing different ammonium functional groups. R = butyl	23
III-1	Characterization of random copolymers with dansyl end groups	39
IV-1	Characterization of methacrylamide random copolymers	57
V-1	Characterization of methacrylate random copolymers containing different cationic side chain spacer groups	77
B-1	Characterization of the polymers bearing primary amines and methyl groups	117
B-2	Characterization of the polymers bearing tertiary amines and methyl groups	119
B-3	Characterization of the polymers bearing quaternary ammonium salt groups and methyl groups	120
B-4	Characterization of the polymers bearing primary amines and butyl groups	120
B-5	Characterization of the polymers bearing tertiary amines and butyl groups	121
B-6	Characterization of the polymers bearing quaternary ammonium salt groups and butyl groups	121
B-7	Characterization of the FITC-labeled polymers	132

LIST OF FIGURES

FIGURE

I-1	Sequence and secondary structure of magainin-2, a prototypical host defense peptide	3
I-2	Schematic drawing of the bacteria-selective membrane binding exerted by antimicrobial peptides due to a combination of electrostatic attraction and the hydrophobic effect	5
I-3	Proposed mechanisms of membrane disruption exerted by antimicrobial peptides	6
I-4	Structures of (A) peptides, (B) β -peptides, and (C) peptoids	7
I-5	Polymer disinfectants based on benzyalkonium chlorides	8
I-6	Chemical structures which possess facially amphiphilic (FA) conformations on bacterial membranes	10
I-7	Synthesis of a peptide-mimetic antimicrobial polymer	11
I-8	Peptide-mimetic antimicrobial polymers are obtained by the convergence of peptide and polymer science	13
II-1	Synthesis of amphiphilic random copolymers bearing primary, tertiary, or quaternary ammonium salts	18
II-2	Titration of amphiphilic random copolymers bearing primary, tertiary, or quaternary ammonium salts	19
II-3	Antimicrobial activities (MIC) of random copolymers	24
II-4	Hemolytic activities (HC_{50}) of random copolymers	26
II-5	Selectivity Indices (HC_{50}/MBC) of random copolymers	27
II-6	Bactericidal activity (MBC) as a function of pH and extent of ionization	29
II-7	Hemolysis as a function of pH	32
II-8	Hemolytic activity (HC_{50}) as a function of pH and extent of ionization	33
III-1	Synthesis of amphiphilic random copolymers bearing primary, tertiary, or quaternary ammonium salts and dansyl end groups	39
III-2	Potentiometric titration curves for the representative copolymers	40
III-3	Partition coefficients of each polymer between octanol and aqueous phase	42
III-4	Fluorescence enhancement of dansyl-labeled polymers upon partitioning into the hydrophobic membrane environment	43

III-5	Binding isotherms of polymers to POPC vesicles as a function of pH	44
III-6	Dissociation constants for polymer-POPC vesicle binding monitored by dansyl fluorescence	45
III-7	Partition coefficients for the copolymers in the presence of anionic detergent	46
III-8	Schematic of polymer-induced leakage of entrapped, self-quenching fluorophore from within liposomes	48
III-9	Sulforhodamine B (SRB) leakage from POPC liposomes induced by the copolymers as a function of pH	48
III-10	Experimental ³¹ P chemical shift spectra of mechanically-aligned POPC bilayers with the copolymers incorporated	50
IV-1	Comparison of chemical structures for polymethacrylates and polymethacrylamides	56
IV-2	Synthesis of the amphiphilic methacrylamide random copolymers	56
IV-3	Potentiometric titration data for the cationic homopolymer	58
IV-4	Antimicrobial activities (MIC) of methacrylamide random copolymers	59
IV-5	Hemolysis dose-response curves for the methacrylamide copolymers	62
IV-6	Hemolysis as a function of hydrophobic comonomer content	63
IV-7	Cytotoxicity of the copolymers against HEp-2 cells by the XTT assay	65
IV-8	Cytotoxicity as a function of hydrophobic comonomer content	66
IV-9	Sulforhodamine B (SRB) leakage from liposomes induced by the copolymers as a function of lipid composition	68
IV-10	Kinetics of sulforhodamine B (SRB) leakage from liposomes	70
V-1	Spacer arm design strategy and hypothesis	75
V-2	Generalized synthetic route and chemical structures of the amphiphilic polymethacrylate derivatives with different side chain groups connecting the amines to the polymer backbone	76
V-3	Optimization of the antimicrobial efficacy in methacrylate copolymers	78
V-4	Generalized synthetic route and chemical structures of the amphiphilic polymethacrylate derivatives with different spacer arms and a fluorophore in the polymer end group	81
V-5	Chemical structure of propidium iodide	83
V-6	Fluorescence microscopy images of fluorophore-labeled polymer incubated with live bacteria cells	83
V-7	Flow cytometry histograms of fluorophore-labeled polymer incubated with live bacteria cells	84
V-8	Two-dimensional flow cytometry analysis showing the binding and membrane damage to <i>E. coli</i> cells exerted by polymers	85

V-9	Permeabilization of <i>E. coli</i> outer membrane (OM) and inner membrane (IM) induced by the polymers	87
V-10	Effect of external osmolyte diameter on the MBC of copolymers in phosphate buffered saline	89
V-11	Molecular dynamics simulation	92
B-1	¹ H NMR spectrum of copolymer with primary amines	118
B-2	¹ H NMR spectrum of copolymer with tertiary amines	119
B-3	¹ H NMR spectrum of copolymer with quaternary ammonium salts	121
B-4	¹ H NMR spectrum of dansyl-labeled copolymers	123
B-5	¹ H NMR spectrum of copolymers with varying spacer arms	124
B-6	Base-induced isomerization of the methacrylate containing a primary amine in the side chain	125
B-7	¹ H NMR of the methacrylate monomer	125
B-8	¹ H NMR of the isomerized methacrylamide monomer	126
B-9	¹ H NMR spectrum of methacrylamide copolymer	126
B-10	Calculated mole fractions of hydrophobic comonomer and degree of polymerizations based on interpretation of the ¹ H NMR spectra	127
B-11	MALDI-TOF-MS chromatographs of representative copolymers	129
B-12	MALDI-TOF-MS chromatographs of fluorophore-labeled copolymers	130
B-13	Normalized emission of the dansyl-labeled polymers	131
B-14	Fluorescence intensity versus concentration of the polymers in methanol	131
B-15	Absorbance and Emission spectra of the FITC-labeled polymers	132
B-16	Epifluorescence image of <i>E. coli</i> cells incubated with 10 μM F-E2	133
B-17	Epifluorescence image of <i>E. coli</i> cells incubated with 10 μM F-E4	134
B-18	Epifluorescence image of <i>E. coli</i> cells incubated with 10 μM F-E6	135
B-19	Epifluorescence image of <i>E. coli</i> cells incubated with 10 μM F-Ec6	136
B-20	Epifluorescence image of <i>E. coli</i> cells incubated with 10 μM F-B2	137
B-21	Epifluorescence image of <i>S. aureus</i> cells incubated with 10 μM F-E2	138
B-22	Epifluorescence image of <i>S. aureus</i> cells incubated with 10 μM F-E4	139
B-23	Epifluorescence image of <i>S. aureus</i> cells incubated with 10 μM F-E6	140
B-24	Epifluorescence image of <i>S. aureus</i> cells incubated with 10 μM F-Ec6	141
B-25	Epifluorescence image of <i>S. aureus</i> cells incubated with 10 μM F-B2	142
B-26	Chemical structures of model copolymers	143
B-27	Snapshots of the four systems at the end of ~50 ns simulations	144
B-28	Z-density profiles of various components of lipid-polymer systems averaged over last 10 ns of MD simulation	145

B-29	The distance between center of mass of the hydrophobic and amine groups	146
B-30	The stretching of the polymer is measured as the distance between the ester carbons of first and last monomers	147
B-31	The stretching of the E6 polymer is measured as the distance between the ester carbons of first and last monomers	148
B-32	The radial distribution function of interactions of spacer groups of polymers with charged groups of lipid bilayers	149

LIST OF APPENDICES

APPENDIX

A. Materials and Methods	102
Materials	102
Monomer Synthesis	103
Polymer Synthesis	104
Potentiometric Titration	106
Water-Octanol Partition Coefficients	107
Antimicrobial Activity Assays	108
Hemolytic Activity Assays	110
Liposome-Polymer Binding	111
Liposome Dye Leakage	112
Cell Culture and XTT Assay	113
Fluorescence Imaging	114
Flow Cytometry Analysis	114
<i>E. coli</i> Membrane Permeabilization	115
Molecular Dynamics Simulation	115
B. Polymer Characterization	
Characterization of Copolymers by ¹ H NMR Analysis	117
Characterization of Copolymers by MALDI-TOF-MS	129
Absorbance and Emission of Dye-Labeled Polymers	131
Supplemental Fluorescence Images	133
Supplemental Molecular Dynamics Simulation Data	143

LIST OF ABBREVIATIONS

AEMA	aminoethylmethacrylate
AIBN	azo-bis-isobutyronitrile
AMP	Antimicrobial Peptide
BMA	butylmethacrylate
CL	cardiolipin
CMC	Critical Micelle Concentration
CTAB	cetyltrimethylammonium bromide
DDP	dodecylphosphate
DMAEMA	N,N-dimethylaminoethylmethacrylate
DOPG	1,2-dioleoyl- <i>sn</i> -glycero-3-[phospho- <i>rac</i> -(1-glycerol)]
DP	degree of polymerization
EMA	ethylmethacrylate
FITC	fluorescein isothiocyanate
HC ₅₀	Hemolytic Concentration
HDP	Host Defense Peptide
IC ₅₀	Cytotoxic Concentration
K _D	Dissociation constant
Lysl-DOPG	1,2-dioleoyl- <i>sn</i> -glycero-3-[phospho- <i>rac</i> -(3-lysyl(1-glycerol))]
log <i>P</i>	water-octanol partition coefficient
MALDI-TOF-MS	Matrix-Assisted Laser Desorption Ionization Time of Flight Mass Spectroscopy
MBC	Minimum Bactericidal Concentration
MIC	Minimum Inhibitory Concentration
MMA	methylmethacrylate
MMP	methylmercaptopropionate
MW	molecular weight
NMR	Nuclear Magnetic Resonance
PBS	phosphate buffered saline
PEG	polyethylene glycol
PI	propidium iodide
POPC	1-palmitoyl-2-oleoyl- <i>sn</i> -glycero-3-phosphocholine
POPE	1-palmitoyl-2-oleoyl- <i>sn</i> -glycero-3-phosphoethanolamine
POPG	1-palmitoyl-2-oleoyl- <i>sn</i> -glycero-3-phospho-(1'- <i>syn</i> -glycerol)
QAS	quaternary ammonium salt
RBC	Red Blood Cell
SRB	sulforhodamine B
TFA	trifluoroacetic acid