Novel amphiphilic polyanhydrides for vaccine delivery

by

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To my family, with all my respect, love, and gratitude
TABLE OF CONTENTS

LIST OF FIGURES ........................................................................................................ ix

LIST OF TABLES ........................................................................................................... xvi

LIST OF SCHEMES ....................................................................................................... xvii

LIST OF SYMBOLS AND NOMENCLATURE ........................................................... xviii

ACKNOWLEDGEMENTS .............................................................................................. xxi

ABSTRACT .................................................................................................................. xxv

CHAPTER 1 INTRODUCTION .................................................................................... 1
  1.1 Introduction ........................................................................................................ 1
  1.2 References ........................................................................................................ 5

CHAPTER 2 LITERATURE REVIEW ....................................................................... 7
  2.1 Summary ............................................................................................................ 7
  2.2 Polyanhydrides .................................................................................................. 8
    2.2.1 Introduction ............................................................................................... 8
    2.2.2 Classification .......................................................................................... 11
      2.2.2.1 Aliphatic Polyanhydrides ............................................................... 11
      2.2.2.2 Unsaturated Polyanhydrides ......................................................... 11
      2.2.2.3 Aromatic Polyanhydrides ............................................................... 12
      2.2.2.4 Other Polyanhydride Chemistries ................................................ 12
    2.2.3 Synthesis .................................................................................................... 15
      2.2.3.1 Melt Polycondensation .................................................................. 15
      2.2.3.2 Schotten-Bauman Condensation .................................................. 16
      2.2.3.3 Dehydrative Coupling .................................................................. 17
      2.2.3.4 Ring Opening Polymerization ....................................................... 18
    2.2.4 Characterization ......................................................................................... 19
      2.2.4.1 Chemical Structure and Composition .......................................... 19
      2.2.4.2 Molecular Weight .......................................................................... 20
      2.2.4.3 Thermal Properties ....................................................................... 21
      2.2.4.4 Phase Behavior .............................................................................. 22
      2.2.4.5 Stability .......................................................................................... 23
      2.2.4.6 Degradation and Erosion ............................................................... 24
    2.2.5 Polyanhydride-based Drug Delivery Systems .......................................... 25
      2.2.5.1 Biocompatibility ............................................................................. 25
      2.2.5.2 Drug/Polymer Interactions ............................................................ 26
2.2.5.3 Device Fabrication
2.2.5.4 In Vitro Release
2.2.5.5 In Vivo Delivery

2.3 Protein Stability
2.3.1 Introduction
2.3.2 Chemical Instability
2.3.2.1 Deamidation
2.3.2.2 Hydrolysis
2.3.2.3 Oxidation
2.3.2.4 β-Elimination of Disulfide
2.3.2.5 Disulfide Scrambling
2.3.3 Physical Instability
2.3.3.1 Thermal Instability
2.3.3.2 pH
2.3.3.3 Interfacial Tension
2.3.3.4 Dehydration

2.4 Immune System
2.4.1 Introduction
2.4.2 Antigen Presentation
2.4.3 Immune Response

2.5 Adjuvants
2.5.1 Introduction
2.5.2 Functions of Adjuvants

2.6 Polymer Vaccines
2.6.1 Introduction
2.6.2 Polyesters
2.6.3 Polyanhydrides
2.6.4 Other Polymers
2.6.4.1 Natural-derived
2.6.4.2 Synthetic
2.6.4.3 Polymers in Plasmid DNA Vaccines

2.7 Peptide Antigen-based Cancer Vaccines
2.7.1 Introduction
2.7.2 Antigens for Cancer Therapy
2.7.3 Mucin-1
2.7.3.1 Ex Vivo Characterization
2.7.3.2 In Vivo Responses
2.7.3.3 Influence of Injection Location
2.7.3.4 Clinical Studies
2.7.4 Mucin-4

2.8 Conclusions

2.9 References
CHAPTER 3 RESEARCH OBJECTIVES ................................................................. 90

3.1 Research Objectives ............................................................................... 90
3.2 Specific Goals ......................................................................................... 91

CHAPTER 4 SYNTHESIS AND CHARACTERIZATION OF NOVEL POLYANHYDRIDES WITH TAILORED EROSION MECHANISMS .... 92

4.1 Abstract ................................................................................................... 92
4.2 Introduction ............................................................................................. 93
4.3 Experimental ........................................................................................... 96
   4.3.1 Materials .................................................................................... 96
   4.3.2 Monomer Synthesis ................................................................ 96
   4.3.3 Polymer Synthesis ..................................................................... 97
   4.3.4 Characterization ........................................................................ 98
   4.3.5 Erosion and Degradation Studies .............................................. 99
   4.3.6 CPTEG Monomer Solubility ....................................................... 99
4.4 Results and Discussion ......................................................................... 100
   4.4.1 Structural Characterization ...................................................... 100
   4.4.2 Thermal Characterization ........................................................ 103
   4.4.3 Erosion and Degradation ......................................................... 104
4.5 Conclusions ........................................................................................... 111
4.6 Acknowledgements ............................................................................... 111
4.7 References ............................................................................................ 112

CHAPTER 5 AMPHIPHILIC POLYANHYDRIDES FOR PROTEIN STABILIZATION AND RELEASE .................................................. 115

5.1 Abstract ................................................................................................. 115
5.2 Introduction .......................................................................................... 116
5.3 Materials and Methods .......................................................................... 119
   5.3.1 Materials .................................................................................. 119
   5.3.2 Polymer Synthesis and Characterization ................................. 120
   5.3.3 Protein Incubation in Monomer Solutions ................................ 120
       5.3.3.1 Primary Structure ....................................................... 121
       5.3.3.2 Secondary Structure .................................................. 121
       5.3.3.3 Tertiary Structure ....................................................... 121
       5.3.3.4 Ovalbumin Antigenicity .............................................. 122
       5.3.3.5 Lysozyme Enzymatic Activity ..................................... 122
   5.3.4 Microsphere Fabrication .......................................................... 123
       5.3.4.1 Solid/oil/oil (S/O/O) .................................................... 123
       5.3.4.2 Cryogenic Atomization (CA) ....................................... 123
   5.3.5 Protein Release ....................................................................... 124
       5.3.5.1 Total Protein Encapsulated ........................................ 125
5.4 Results ................................................................................................. 126
CHAPTER 7 EVALUATION OF NON-SPECIFIC IMMUNITY OF POLYANHYDRIDE MICROSPHERES: IMPLICATIONS FOR THE DESIGN OF PANCREATIC CANCER VACCINES

8.1 Introduction .......................................................................................... 208
8.2 Experimental Methods ........................................................................ 210
  8.2.1 Fabrication of CPTEG:CPH Microspheres .................................... 210
  8.2.2 In Vitro Activation of Effector Cells: CTL Assay ........................ 210
  8.2.3 In Vivo Activation of Effector Cells ............................................. 211
    8.2.3.1 51Cr Release Assay .............................................................. 211
    8.2.3.2 Flow Cytometry ................................................................. 212
  8.2.4 In Vivo Evaluation of Non-Specific Tumor Immunity ................ 213
8.3 Results and Discussion ......................................................................... 214
  8.3.1 CTL Assay .................................................................................. 214
  8.3.2 In Vivo Activation of Effector Cells ............................................. 216
    8.3.2.1 51Cr Release Assay .............................................................. 216
    8.3.2.2 Flow Cytometry ................................................................. 217
  8.3.3 In Vivo Evaluation of Non-Specific Tumor Immunity ............... 221
8.4 Conclusions ........................................................................................... 222
8.5 Experimental Design of CPTEG:CPH Cancer Vaccines ............... 223
  8.5.1 Antitumor Immune Response of CPTEG:CPH Microspheres.. 223
  8.5.2 Design of Mucin-1 and Mucin-4 Cancer Vaccine............... 223
    8.5.2.1 Antigen Presentation by DCs................................. 224
    8.5.2.2 In Vivo Studies.................................................. 224
    8.5.2.3 Vaccine Regimen................................................. 225
    8.5.2.4 Antibody Production.......................................... 225
    8.5.2.5 Cell Proliferation and Cytokine Analysis .............. 226
    8.5.2.6 Vaccine Evaluation in Transgenic Model ............... 226
  8.6 Overall Conclusions .................................................... 227
  8.7 References......................................................................... 228

CHAPTER 9 CONCLUSIONS AND FUTURE WORK ........................................... 231

  9.1 Conclusions ........................................................................ 231
  9.2 Future Work ...................................................................... 233
    9.2.1 Adjuvant Capabilities with Model Antigen .................. 234
    9.2.2 Activation of other Immune Cells ......................... 237
    9.2.3 Carbohydrate Attachment..................................... 239
    9.2.4 High Throughput Analysis .................................. 241
    9.2.5 Wound Healing Applications............................... 242
    9.2.6 Acknowledgements ............................................ 244
  9.3 References ......................................................................... 245

VITA ................................................................................................. 246
LIST OF FIGURES

1.1 Conventional drug administration (left) versus controlled drug release (right). .... 2

1.2 Chemical structures of poly(CPH) (top) and poly(CPTEG) (bottom). ................... 5

2.1 Hydrolysis of polyanhydrides. ................................................................................. 10

2.2 Mechanisms of polymer erosion: surface (left) and bulk (right). t₀ and t_f are the times previous and subsequent erosion. .................................................. 10

2.3 Melt condensation polymerization of polyanhydrides. ........................................ 16

2.4 Dendritic cell (DC) activation.................................................................................... 41

2.5 Recognition of antigen and adjuvant by immature DC. An adjuvant may interact directly with DC thru PAMPs (a), or have a ‘depot’ effect to where the antigen is presented over time as the adjuvant is metabolized or erodes (b) .................................................................................................. 47

2.6 Tumor antigen processing and presentation. Mucin-1 enters the cytoplasm where is degraded into small peptide fragments by the proteasomes. These fragments are then transported into the endoplasmic reticulum (ER), where they bind to MHC I molecules and subsequently are presented on the surface for immune system’s recognition. ......................................................................................... 68

4.1 Chemical structures of poly(CPH) (top) and poly(CPTEG) (bottom). The letters (a-i) represent the peaks in the 1H NMR spectra shown in Fig. 4.3 and 4.6 ................................................................. 95

4.2 1H NMR spectrum of CPTEG Diacid................................................................. 101

4.3 1H NMR spectra of (a) poly(CPTEG), (b) 80:20, (c) 50:50, (d) 20:80 CPTEG:CPH copolymers, and (e) poly(CPH). .................................................. 102

4.4 Fractional mass loss from tablets of poly(CPTEG), 50:50 CPTEG:CPH copolymer and poly(CPH). Error bars indicate standard deviation......................................................................................... 105

4.5 Fractional water content in tablets of poly(CPTEG), 50:50 CPTEG:CPH copolymer and poly(CPH). Error bars indicate standard deviation......................................................................................... 107
4.6 $^1H$ NMR spectra of 50:50 CPTEG:CPH copolymer tablets during erosion. ................................................................. 107

4.7 Percent molecular weight loss from eroding tablets of poly(CPTEG), 50:50 CPTEG:CPH copolymer, and poly(CPH). ................................................................. 108

4.8 Saturation concentration data of CPTEG and CPH monomers in aqueous buffer (CPH solubility data from Ref. 31). ................................................................. 109

4.9 SEM micrographs depicting surface morphology of poly(CPTEG), 50:50 CPTEG:CPH copolymer, and poly(CPH) tablets after 7 days (top) and 28 days (bottom) of erosion. .................................................................... 110

5.1 Chemical structures of poly(CPH) (top) and poly(CPTEG) (bottom). .................. 118

5.2 SDS-PAGE of Ova (a) and Lys (b). Lane 1: MW Standard ladder; lane 2: Non-lyophilized protein; lanes 3, 4 protein in CPTEG solution (day 0, 7); lanes 5,6: protein in 50/50 CPTEG/CPH solution (day 0, 7); lanes 7,8 : protein in CPH solution (day 0, 7); lanes 9,10 in gel (a) Ova in phosphate buffer (day 0, 7). .................................................................. 127

5.3 CD spectra of Ova incubated in (a) CPTEG saturated solution and (b) CPH saturated solution ..................................................................................................... 127

5.4 Fluorescence spectra of Ova incubated in (a) CPTEG saturated solution and (b) CPH saturated solution. ......................................................................................... 129

5.5 Protein activity after incubation with CPTEG:CPH degradation products. (a) antigenicity of Ova and (b) enzymatic activity of Lys. Error bars indicate standard deviation of triplicate samples .................................................. 130

5.6 SEM image and particle size distribution of Ova-loaded 10:90 CPTEG:CPH microspheres fabricated by cryogenic atomization (CA). Scale bars represent 20$\mu$m ........................................................................ 131

5.7 Protein released from CPTEG:CPH microspheres. (a) Ova released from 20:80 and 10:90 CA microspheres, (b) Lys released from 20:80 CA and S/O/O microspheres. Error bars represent standard deviation of triplicate samples ................................................................. 132
5.8 Protein activity after release from CPTEG:CPH microspheres.  
(a) antigenicity of Ova after release from 20:80 and 10:90 CA microspheres, (b) enzymatic activity of Lys after release from 20:80 S/O/O and CA microspheres. Error bars indicate standard deviation of triplicate samples. * represents $p$-value < 0.05 as determined by statistical test ................................................................. 135

6.1 Chemical structures of polymers used, from top, left to right: poly(sebacic acid), poly(1,6-bis(p-carboxyphenoxy)hexane), and poly(1,8-bis(p-carboxyphenoxy)-3,6-dioxaocetane). Here n represents the number of repeating monomer units .................................................................................. 146

6.2 Contact angle of polyanhydride films. Error bars represent standard deviations from three experiments. A student-t test was performed at P-value<0.05 (\* = statistically different from CPH and SA homopolymers, \# = statistically different from CPTEG homopolymer .... 156

6.3 50:50 CPH:SA microspheres fabricated by S/O/O (left) and CA (right) methods. Scale bars represent 50 \( \mu \)m............................................................. 157

6.4 20:80 CPTEG:CPH microspheres fabricated by S/O/O (left) and CA (right) methods. Scale bars represent 50 \( \mu \)m............................................................. 157

6.5 Fraction of ova released from poly(SA) and CPH:SA copolymer microspheres using S/O/O (left) and CA (right) fabrication techniques. Error bars indicate standard deviation .................. 158

6.6 Fraction of ova released from poly(CPTEG) and CPTEG:CPH copolymer microspheres using S/O/O (left) and CA (right) fabrication techniques. Error bars indicate standard deviation .................. 159

6.7 SDS-PAGE of ova released from microspheres over two weeks. Lane 1 – protein ladder; lane 2 – ova at pH 10; lane 3 – poly(SA); lane 4 – 20:80 CPH:SA; lane 5 – 50:50 CPH:SA; lane 6 – poly(CPTEG); lane 7 – 20:80 CPTEG:CPH; and lane 8 – 50:50 CPTEG:CPH. .................................................. 161

6.8 Polyclonal western blot of ova released from microspheres over two weeks. Lane 1 – protein ladder; lane 2 – ova at pH 10; lane 3 – poly(SA); lane 4 – 20:80 CPH:SA; lane 5 – 50:50 CPH:SA; lane 6 – poly(CPTEG); lane 7 – 20:80 CPTEG:CPH; and lane 8 – 50:50 CPTEG:CPH. ........................................................................... 162
7.1 Chemical structures of (a) poly(SA), (b) poly(CPH), and (c) poly CPTEG. (m and n represents degree of polymerization) ........................................ 176

7.2 SEM Images of blank microspheres: (a) CPTEG, (b) 50:50 CPTEG:CPH, (c) 20:80 CPTEG:CPH, (d) SA, (e) 20:80 CPH:SA, and (f) 50:50 CPH:SA. Scale bars represents: 5μm in (b),(c),(f); 10μm in (a),(d); 20μm in (e) ................................................................. 185

7.3 Size Distribution of polyanhydride microspheres. Error bars represent standard deviation of two replicates. An average of 800 particles were analyzed ................................................................. 186

7.4 Inverted microscope images of NS BMDCs (left) and BMDCs incubated with 20:80 CPTEG:CPH microspheres (right). White arrows indicate microspheres that appear to be phagocytosed by the BMDCs ................................................................. 187

7.5 Representative flow cytometry histogram of BMDCs stained with anti-CD11c. Positive population was gated with respect isotype control .............. 188

7.6 MHCII Histograms of BMDCs incubated with different stimulation treatments: (a) NS, (b) LPS, (c) CPTEG, (d) 50:50 CPTEG:CPH, (e) 20:80 CPTEG:CPH, (f) 10:90 CPTEG:CPH, (g) SA, (h) 20:80 CPH:SA, and (i) 50:50 CPH:SA. DCs expressing MHCII (open histograms), were gated with respect to the background staining with isotype controls (solid black histograms). Concentration of polyanhydride microspheres was 0.125 mg/mL corresponding to 1:6 microsphere:DC ratio ................................................................. 189

7.7 MHCI histograms of NS BMDCs (solid black), 50:50 CPH:SA-treated BMDCs (tinted gray), and 50:50 CPTEG:CPH-treated BMDCs (dashed line). Concentration of polyanhydride microspheres was 0.125 mg/mL corresponding to 1:6 microsphere:DC ratio ................................................................. 190

7.8 Stimulation trend of polyanhydride microspheres for MHCI surface expression ................................................................. 190

7.9 CD86 surface expression of BMDCs after stimulation with CPTEG:CPH microspheres (0.25 mg/mL, microsphere:DC 1:3). The data is presented as fold increase over NS BMDCs. Error bars represent standard error from 3-4 separate experiments. A student-t test was used for statistical analysis (P-value<0.05). (* = statistically different from NS; +=statistically different from LPS) ................................................................. 192
7.10 CD40 surface expression of BMDCs after stimulation with CPTEG:CPH microspheres (0.25 mg/mL, microsphere:DC 1:3). The data is presented as fold increase over NS BMDCs. Error bars represent standard error from 3-4 separate experiments. A student-t test was used for statistical analysis (P-value<0.05). (* = statistically different from NS; +=statistically different from LPS). ................................. 192

7.11 DC-SIGN CD209 surface expression of BMDCs after stimulation with CPTEG:CPH microspheres (0.25 mg/mL, microsphere:DC 1:3). The data is presented as fold increase over NS BMDCs. Error bars represent standard error from 3-4 separate experiments. A student-t test was used for statistical analysis (P-value<0.05). (* = statistically different from NS; +=statistically different from LPS) ................................. 193

7.12 Cytokines secreted by BMDCs after incubation with stimulation treatments.................................................................................................................. 194

7.13 The biplot of PCA on adjuvant cell marker expression data. Each point corresponds to the projection of an experiment data point on PC1 and PC2. Data of SA system are colored blue, and CPTEG system yellow. Circles are used to show the distribution of data under the same treatment. For convenience, only the compositions of SA and CPTEG are used as circle labels. PC1 and PC2 together explain 95.67% data variance. Increasing amount of SA or CPTEG to CPH increases dissimilarity from untreated cell. CPTEG system has a stronger impact on cell marker expression than SA system. ....................... 196

7.14 Immune activation of DCs. Signal 1: Pathogens can interact with different PAMPs present in the DCs. Pathogens that interact with TLRs are processed by intracellular signaling that lead to DC maturation and induction of inflammatory cytokines. Other receptors, such as C-type lectins recognize specific carbohydrate structures on the pathogens that are subsequently internalized and degraded in the lysosomes to enhance antigen processing and presentation. After DC becomes mature, it migrates to the lymph nodes where the DC-SIGN expression optimizes the initial T cell binding. Signal 2: The antigen presentation by MHC-peptide complexes and the expression of the co-stimulatory molecules (CD86, CD40) seals the immunologic synapse needed for T cell activation. Signal 3: The sustained interactions of DC with T cells enhance the antigen presenting capacity of DCs by maintenance of MHC complexes and upregulation of co-stimulatory molecules............................... 201
8.1 SEM image of blank 20:80 CPTEG:CPH microspheres and CTL assay results of PBMCs incubated with 20:80 CPTEG:CPH microspheres. .......................... 215

8.2 Cytotoxicity of lymphocytes from mice injected with blank CPTEG:CPH microspheres measured by $^{51}$Cr release assay. ........................................ 217

8.3 Flow cytometry histograms of LN cells expressing CD8a from mouse injected with 20:80 (left) and 50:50 CPTEG:CPH microspheres (right). Cells were gated with respect to the corresponding isotype .......... 218

8.4 Analysis of CD8 lymphocytes population. CD62L vs. CD44 representative dot plots of lymphocytes of mice injected with: (a) PBS, (b) 20:80 CPTEG:CPH, and (c) 50:50 CPTEG:CPH microspheres .............. 219

8.5 Flow cytometric results of lymphocytes expressing CD8. Error bars represent standard deviations. N=5; * = statistically different from PBS group (P-value<0.05) ................................................................. 220

8.6 Flow cytometric results of spleenocytes expressing CD8. N=5; Error bars represent standard deviations ................................................................. 220

8.7 Mass of Panc02 tumors induced in C57BL/6 mice injected with blank CPTEG:CPH microspheres. ........................................................................ 222

9.1 Total Ova-specific serum antibodies of immunized mice. Error bars represent standard deviation in a group (N=5). ($
\uparrow$ = statistically different from each other, P-value<0.05). ....................... 234

9.2 Antigen-specific proliferation of lymphocytes isolated from immunized mice. Error bars represent standard deviation within each group................................................................. 236

9.3 Activation of Pre-B cell line 7OZ/3. Flow cytometry histogram of cells incubated with 20:80 CPTEG:CPH microspheres (top). Open histograms represent isotype control. The activation results obtained from flow cytometry were analyzed with respect to the % of positive cells expressing IgM and the corresponding mean fluorescence intensity (MFI) (bottom) ................................................................................. 238

9.4 Coupling reaction for attachment of Lactose and Di-mannose into CPTEG:CPH microspheres. The carboxyl activating agents 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHC) were used for coupling the sugars ... 240
9.5 SEM image of 20:80 CPTEG:CPH microspheres after coupling Lactose to the surface. Scale bar represents 20 μm ........................................ 240

9.6 Linear gradient from 0-28 mg/mL of 50:50 CPTEG:CPH copolymer ................ 241

9.7 Optical photomicrographs of in vitro wound healing assay. HCT116 cells immediately after creating wound (left) and after being incubated for 24 h with uterocalin-loaded 50:50 CPTEG:CPH microspheres (right) .............. 243
**LIST OF TABLES**

2.1 Typical polyanhydrides used for drug delivery applications .................................. 14

2.2 Structure of common polymers used as vaccine adjuvants ................................. 51

4.1 Composition of CPTEG:CPH copolymers calculated from NMR after polymerization .......................................................... 100

4.2 Thermal characterization (* Data from Ref. 12)....................................................... 103

5.1 Effect of the dissolution of polymer degradation products on pH. ......................... 137

6.1 Parameters for S/O/O double emulsion. ............................................................. 150

6.2 Parameters for CA. ................................................................................................. 151

7.1 Parameters used for microsphere fabrication (Lopac et. al) ............................. 181

8.1 Percent of lymphocytes and spleenocytes expressing the surface marker CD8. (N=5 mice) ................................................................. 218
LIST OF SCHEMES

4.1 Synthesis of CPTEG monomer. ................................................................. 97

4.2 Synthesis of poly(CPTEG). ................................................................. 98
# LIST OF SYMBOLS AND NOMENCLATURE

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>AA</td>
<td>Adipic acid</td>
</tr>
<tr>
<td>Ac</td>
<td>Acetylated end group</td>
</tr>
<tr>
<td>ACDA</td>
<td>Acetylenedicarboxylic acid</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid</td>
</tr>
<tr>
<td>BCNU</td>
<td>1,3-bis[2-chloroethyl]-1-nitro-sourea</td>
</tr>
<tr>
<td>BMDCs</td>
<td>Bone marrow dendritic cells</td>
</tr>
<tr>
<td>CA</td>
<td>Cryogenic atomization</td>
</tr>
<tr>
<td>CD</td>
<td>Circular dichroism</td>
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<tr>
<td>CD8, CD40, CD44, CD86</td>
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<tr>
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<tr>
<td>CL</td>
<td>Caprolactone</td>
</tr>
<tr>
<td>CPH</td>
<td>1,6-bis-(p-carboxyphenoxy)hexane</td>
</tr>
<tr>
<td>CPP</td>
<td>1,3-bis(p-carboxyphenoxy)propane</td>
</tr>
<tr>
<td>CPTEG</td>
<td>1,8-bis-(p-carboxyphenoxy)-3,6-dioxaoctane</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T lymphocytes</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>DC-SIGN</td>
<td>Dendritic cell-specific ICAM-3-grabbing non-integrin</td>
</tr>
<tr>
<td>DD</td>
<td>Dodecanedioic anhydride</td>
</tr>
<tr>
<td>DMA</td>
<td>Dynamic mechanical analysis</td>
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<tr>
<td>DMF</td>
<td>Dimethyl formamide</td>
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<tr>
<td>GM-CSF</td>
<td>Granulocyte macrophage colony stimulating factor</td>
</tr>
<tr>
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</tr>
<tr>
<td>ΔH</td>
<td>Heat of fusion</td>
</tr>
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<td>HCT116</td>
<td>Human colorectal carcinoma cells</td>
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HLA  Human leukocyte antigen
1H NMR  Proton nuclear magnetic resonance
IFN-γ  Interferon gamma
IgG  Immunoglobulin G
IgM  Immunoglobulin M
IL  Interleukins
IPA  Isophthalic acid
LAL  Limulus anebocyte lysate
LDH  Lactate dehydrogenase
LN  Lymph node
LPS  Lipopolysaccharide
Lys  Lysozyme
LT  Labile toxin
MART  Melanoma antigen-derived peptides
MFI  Mean fluorescence intensity
MHC  Major histocompatibility complex
MPLA  Monophosphoryl lipid A
MS  Microspheres
MTP  Muramyl tripeptide
Mucin-1.Tg  Mouse transgenic for human Mucin-1
Mw  Weight average molecular weight
η  Intrinsic viscosity
NF-κB  Nuclear factor kappa B
NHC  N-hydroxysuccinimide
NK  Natural killer
NS  Non-stimulated
Ova  Ovalbumin
PAMPs  Pathogen-associated molecular patterns
PB  Phosphate buffer
PBMC  Peripheral blood mononuclear cell
PBS  Phosphate buffer saline
PBST  Phosphate buffer saline containing thimerosal
PCA  Principal component analysis
PE  Phycoerythrin
PEA  Poly(ester-amide)
PEG  Poly(ethylene glycol)
PGA  Poly(glycolic acid)
pI  Isoelectric point
PI  Propidium iodide
PLA  Poly(lactic acid)
PLGA  Poly(lactic-co-glycolic acid)
PNA  p-nitroaniline
PVA  Poly(vinyl alcohol)
pNPp  p-nitrophenyl phosphatase
PVDF  Poly(vinylidene fluoride)
PVM/MA Poly(vinyl methyl ether-alt-maleic anhydride)
SA Sebacic acid
SA*,CPP*, Carboxylic end groups
CPH*,CPTEG* Small-angle x-ray scattering
SAXS Subcutaneous
sc Sodium-dodecyl sulfate-polyacrylamide gel electrophoresis
SDS-PAGE Scanning electron microscopy
SEM Solid/oil/oil
S/O/O Spleen
SP 4,4’-stilbenenedicarboxylic acid
STDA Terephthalic acid
TA Triethylene glycol
TEG Transgenic
Tg Glass transition temperature
Tg Helper T cells
Th Toll-like receptor
TLR Melting temperature
Tm N-trimethyl chitosan chloride
TMC Tumor necrosis factor alpha
TNF-α Target cell
Tr Tetanus toxoid
TT UNICEF United Nations Children’s Fund
UNICEF Ultra-violet light
UV Vapor pressure osmometry
VPO World Health Organization
WHO Water/oil/oil
w/o/o Tetanus toxoid
w/o/w Water/oil/water
wt Antibody
Y Wild type
ACKNOWLEDGEMENTS

It is hard to describe my feelings as I complete my Ph.D. degree at Iowa State University (ISU). My life has significantly changed over the years of pursuing my Ph.D. and I can state with total confidence that this is the best decision I have made in my life. I would like to acknowledge every single person, the ones that encouraged me to apply, my family, my friends and all the fascinating people that I have met throughout these years that made this a great experience that I will never forget. The space is limited and I will do my best to recognize the people that influenced my career from the beginning.

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In February of 2003, when I decided to apply to different universities, I received an e-mail from Nancy Knight, a student recruiter from ISU. I am very thankful to her as she was the first person who told me about the great opportunities offered here for minority students like me. After joining the program in August of 2003, and being the first Puerto Rican to pursue a Ph.D. in Chemical Engineering at ISU, Nancy gave me the opportunity of sharing my personal experience with
students in the University of Puerto Rico. I am pleased to see that more Puerto Ricans are becoming interested in the research done in ISU.

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language, and more importantly being at home with my family and friends and missing all the parties, activities, and common gatherings that now have become a treasure whenever I go back to Puerto Rico. I am a firm believer that every human being comes to Earth with a purpose, whatever their spiritual beliefs are. It is true that I have sacrificed something invaluable in my life, but by doing that I was able to grow and achieve my goal of becoming a scientist to research therapies to improve human health. From now on, my major goal is to become a known scientist in cancer research. With the help of God, in memory of those many people that have died of cancer, I will do my best to fulfill what I think is my personal mission in life.
ABSTRACT

The rapid development of biopharmaceuticals suggests that many future vaccines will involve the delivery of peptide or protein subunits. The overall goal of this work is to design novel vaccine adjuvants based on biodegradable polymers that protect, stabilize, and enhance the immunogenicity of these protein antigens. Polyanhydrides, which are surface erodible polymers, have shown excellent performance as drug carriers. Their hydrophobic nature prevents water penetration into the bulk, thus eliminating water-induced covalent aggregation of proteins. Unfortunately, protein inactivation by non-covalent aggregation may still persist. It has been suggested that the use of carriers containing both hydrophobic and hydrophilic entities may provide a gentler environment for proteins. Hence, the synthesis and characterization of a novel amphiphilic polyanhydride system based on the anhydride monomers 1,6-bis(\(\rho\)-carboxyphenoxy)hexane (CPH) and 1,8-bis(\(\rho\)-carboxyphenoxy)-3,6-dioxaoctane (CPTEG) was carried out. Subsequently, as most vaccines involve the delivery of peptides or protein subunits, protein stabilization in the CPTEG:CPH environment was analyzed. It was demonstrated that CPTEG:CPH system provides a gentle environment for proteins and sustained release from copolymer microspheres is attained. In order to evaluate the adjuvant characteristics of the CPTEG:CPH system, the activation of immune cells incubated with CPTEG:CPH microspheres and the implications for vaccine design were addressed. The promising adjuvant capabilities of CPTEG-content microspheres were evidenced by enhanced maturation of dendritic cells, the most potent antigen
presenting cells of the immune system. Altogether, the studies presented in this thesis provide an excellent foundation for testing the viability of this system as an effective adjuvant for the development of vaccines. Development and application of this technology will facilitate the rational design of vaccines and the ability to appropriately redirect the immune response to develop protective immunity.
1.1 Introduction

Approximately 20 years ago, the first protein drug (recombinant insulin) was approved by the US Food and Drug Administration (FDA). Since then, research in the area of therapeutic protein delivery has increased to the point where around 500 candidate biopharmaceuticals are currently undergoing clinical evaluation and annual research expenditures surpass $30 billion. A study done in 2003 revealed that the largest category under development were vaccines, with 50% aimed at treating or preventing cancer and the rest aimed at treating infectious diseases (i.e., hepatitis and HIV).

Despite the rapid growth of these biopharmaceuticals, there is still a lack of proper administration. Many future vaccines will involve the delivery of peptide or protein subunits, which means that besides enhancing the immune response, the delivery carrier must ensure protein stability. Proteins are amphiphilic structures arranged in three-dimensional patterns that need to be conserved for their natural bioactivity. Typically, therapeutic proteins are unstable in physiological environments and their delicate structures can be easily disrupted. Hence they have short half lives when administered alone and their incorporation in therapeutic regimens is a challenge. There is an urgent need to design appropriate protein carriers that will provide adequate stabilization for therapeutic efficacy. The design of such carriers
involves a fundamental understanding of the interactions between the protein and the carrier, and the carrier chemistry.

For about two decades, the need for suitable materials for the delivery of drugs in a safe and controlled manner has led to the development of numerous biodegradable polymers. Controlled release of a variety of therapeutic drugs has been achieved with the use of biodegradable polymeric devices for the treatment of a wide range of complex diseases such as cancer, Alzheimer, and diabetes. Some of the outstanding characteristics of these carriers that make them attractive for drug delivery applications include: enhanced efficacy, reduced toxicity, and improved patient compliance and convenience when compared to conventional drug administrations (Fig. 1.1). In a controlled drug release regimen, the level of drug is maintained within the desired therapeutic range for longer periods with a single dose. On the other hand, in a conventional drug administration, more doses are needed to obtain the desired therapeutic efficacy and there is a high risk of under/over dosing in the patient.

Figure 1.1. Conventional drug administration (left) versus controlled drug release (right).
Among biodegradable polymers, polyesters and polyanhydrides have shown excellent performance as drug carriers. Biocompatibility studies have shown that these hydrophobic biomaterials degrade into non-mutagenic and non-cytotoxic products\textsuperscript{5-7}. However, the erosion mechanism of each polymer confers special characteristics that in turn affect drug release. Polyesters degrade by bulk erosion, where a significant amount of water enters into the device. Therefore, interactions between drug and water are likely to take place. In contrast, polyanhydrides are surface erodible polymers, where the rate of erosion far exceeds the rate of water penetration. In consequence, the drug is released at the same rate as the polymer degradation and water-drug interactions are minimized. This is particularly relevant when delivering proteins, as these can be easily destabilized by chemical and physical pathways, as discussed below.

Two common physical pathways responsible for protein denaturation are covalent and non-covalent aggregation. As discussed above, the highly hydrophobic nature of polyanhydrides prevents water penetration into the bulk, thus eliminating water-induced covalent aggregation of some proteins. Unfortunately, protein inactivation by non-covalent aggregation may still persist. It has been suggested that the use of carriers containing both hydrophobic and hydrophilic entities may provide a gentler environment for proteins, since they are naturally amphiphilic structures\textsuperscript{8,9}.

In addition to stabilizing protein antigens in a vaccine delivery device, it is critical to enhance the immune response. A physiological immune response begins with the activation of the antigen presenting cell (APC). This is the crucial step of the immune system initiation. Without proper activation of these cells, no response is
generated against a particular antigen. The best APCs responsible to activate helper T cells, killer T cells and B cells are dendritic cells (DCs)\textsuperscript{10}. As most recombinant proteins are poorly immunogenic, a vaccine adjuvant, which does not have a specific antigenic effect, must stabilize the protein antigen while enhancing the activation of DCs.

The overall objective of this research is to design a novel amphiphilic polyanhydride system for the sustained delivery of vaccines, which will provide the dual characteristics of protein stabilization and immune modulation. A rational approach to achieve this goal begins with the synthesis and characterization of the novel polyanhydride system, composed of the anhydride monomers 1,6-bis($p$-carboxyphenoxy)hexane (CPH) and 1,8-bis($p$-carboxyphenoxy)-3,6-dioxaoctane (CPTEG) (Fig. 1.2). Briefly, synthesis, purity, material properties, and erosion mechanisms are analyzed in the context of drug delivery applications in Chapter 4. As most vaccines involve protein sub-units, protein stabilization and release will be demonstrated from CPTEG:CPH amphiphilic carriers in Chapters 5 and 6. The adjuvant characteristics of CPTEG:CPH microspheres are investigated in Chapter 7 by their ability to activate DCs. The understanding gained from these experiments will provide insights for the evaluation of CPTEG:CPH formulations for cancer vaccine delivery using animal models as described in Chapter 8. The last chapter discusses current and future experiments that embrace a variety of potential biomedical applications of this novel amphiphilic system.
Figure 1.2. Chemical structures of poly(CPH) (top) and poly(CPTEG) (bottom).

1.2 References


CHAPTER 2
LITERATURE REVIEW

2.1 Summary

Polyanhydrides are a class of bioerodible polymers that have shown excellent characteristics as drug and protein delivery carriers. The properties of these biomaterials can be tailored to obtain desirable controlled release characteristics. In Section 2.2, the extensive research on these promising biomaterials is discussed. Briefly, the chemical structures and synthesis methods of various polyanhydrides are presented, followed by a discussion of their physical, chemical and thermal properties with potential biomedical applications discussed at the end. Section 2.3 discusses the mechanisms that can alter protein stability. Chemical and physical degradation pathways that can lead to protein denaturation are presented in order to motivate the development of a rational approach when designing new delivery carriers for these fragile macromolecules. Highlights of the immune system are presented in Section 2.4, where antigen presentation and the subsequent immune response are discussed. In section 2.5, the functions of adjuvants and their importance in vaccines are reviewed. The role of the biodegradable polymers that had been investigated as vaccine carriers is discussed in a comprehensive review in Section 2.6. Finally, Section 2.7 introduces the potential of peptide antigen-based cancer vaccines and the promising glycoproteins Mucin-1 and Mucin-4, which are overexpressed in various types of adenocarcinomas are presented.
2.2 Polyanhydrides

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2.2.1 Introduction

The need for suitable materials for the delivery of drugs in a safe and controlled manner has led to the development of numerous biodegradable polymers. Controlled release of a variety of therapeutic agents has been achieved with the use of biodegradable polymeric devices. Research has focused on poly(α-hydroxy acids), poly(orthoesters), and poly(anhydrides). Poly(α-hydroxy acids) (e.g., poly(lactic acid) (PLA), poly(glycolic acid) (PGA), and copolymers) undergo bulk erosion and the drug release kinetics from these carriers is not well defined. On the other hand, poly(orthoesters) and polyanhydrides undergo surface erosion with predictable kinetics. In the case of polyanhydrides, the degradation rates can be tailored to suit specific applications by changing the chemistry.

Polyanhydrides are comprised of monomer units connected by water-labile anhydride bonds. In the presence of water the polymer is cleaved across the anhydride bond into two carboxylic acid groups (Fig 2.1). It is precisely this hydrolytic instability that precluded their use in the textile industry in the 1950’s and led researchers to suggest their potential as drug delivery carriers in the 1980’s. Since then, polyanhydrides have been synthesized with a wide range of chemistries for a
variety of biomedical applications. The promising characteristics of polyanhydrides for biomedical applications rely on the surface erosion mechanism that translates into well-controlled release kinetics, where the drug release rate coincides with the degradation rate of the polymer. In an aqueous environment, the macromolecules at the surface break into smaller chains before water penetrates into the device. Thus, the drug is released as the polymer degrades. In contrast, bulk eroding polymers degrade slowly and water penetrates into the system much faster, having, in consequence, less predictable kinetics as drug is released from the entire matrix. A comparison of surface and bulk erosion mechanisms is shown in Fig. 2.2.

Polyanhydride-based drug delivery devices (Gliadel®) have been approved by the Food and Drug Administration (FDA) for the treatment of brain tumors. This device is a polyanhydride wafer composed of sebacic acid (SA) and 1,3-bis(\(\rho\)-carboxyphenoxy)propane (CPP) (CPP:SA copolymer in 20:80 molar ratio) loaded with the chemotherapeutic agent, carmustine, 1,3-bis[2-chloroethyl]-1-nitro-sourea (BCNU). Other potential applications of CPP:SA copolymers include the release of bethanechol for the treatment of Alzheimer's disease and the controlled release of insulin\(^1\). The treatment of osteomyelitis, which is a bone infection difficult to treat by conventional methods, has been carried out with 20:80 CPP:SA copolymer loaded with gentamicin sulfate\(^1\). Several chemotherapeutic drugs, local anesthetics, anticoagulants, neuroactive drugs, and vaccines have been delivered using polyanhydrides.
This section provides an overview of polyanhydrides and their potential as drug carriers. First, chemical structures and synthesis methods are discussed. This is followed by a discussion of the physical, chemical and thermal properties and the effect of these on the degradation mechanism of polyanhydrides. This is followed by a description of drug release from polyanhydride systems.
2.2.2 Classification

There are three major classes of polyanhydrides: aliphatic, unsaturated, and aromatic. The chemical structures are shown in Table 2.1.

2.2.2.1 Aliphatic Polyanhydrides

The first aliphatic polyanhydride synthesized was from the monomer adipic acid (AA), which is thermally unstable and forms cyclic dimers and polymeric rings when heated at high temperatures. In the 1930’s, the aliphatic polyanhydride most widely used currently in drug delivery applications, poly(sebacic acid) (SA), was synthesized for the first time. Typical properties of aliphatic polyanhydrides include crystallinity, melting temperature range of 50-90°C (increasing with monomer chain length), and solubility in chlorinated hydrocarbons. These degrade and are eliminated from the body within weeks. When copolymerized with aromatic polyanhydrides, the degradation time can be extended to several months as the aromatic composition increases. The most widely studied aliphatic-aromatic copolymer system is based on SA and 1,3-bis(p-carboxyphenoxy)propane (CPP).

2.2.2.2 Unsaturated Polyanhydrides

The development of unsaturated polyanhydrides responded to the necessity of improving the mechanical properties of the polymers for applications such as temporary replacement of bone. Unsaturated polyanhydrides, prepared by melt or solution polymerization, include homopolymers of fumaric acid (FA), acetylenedicarboxylic acid (ACDA), and 4,4’-stilbenedicarboxylic acid (STDA).
The chemical structures of poly(FA) and poly(ACDA) are shown in Table 2.1. These polymers are highly crystalline and insoluble in common organic solvents. The double bonds of these monomers make them suitable for further crosslinking to improve mechanical properties of polyanhydrides. When copolymerized with aliphatic diacids, less crystalline polymers with enhanced solubility in chlorinated solvents result.

2.2.2.3 Aromatic Polyanhydrides

The first aromatic polyanhydrides synthesized were poly(isophthalic acid) (IPA) and poly(terephthalic acid) (TA). A few common aromatic polyanhydrides are shown in Table 2.1. Homopolymers of aromatic diacids are crystalline, insoluble in common organic solvents, and have melting points greater than 100°C. Their hydrophobicity results in a slow degradation rate that can last over a year in some cases. Thus aromatic polyanhydrides are not suitable for drug delivery when used as homopolymers. To overcome their slow degradation rates, they have been copolymerized with aliphatic diacids, i.e., CPP:SA copolymers, and with other aromatic monomers. The copolymers of the aromatic monomers TA and IPA are amorphous, soluble, have a faster degradation, and a melting point below 120°C.

2.2.2.4 Other Polyanhydride Chemistries

Although it is impossible to discuss in detail all the polyanhydrides that have been synthesized, some distinguishable classes are discussed here. Typical examples of novel classes of polyanhydrides include those derived from amino acids
and fatty acids, and those modified by copolymerization with esters and ethers. The polyanhydrides derived from amino acids, including trimellitylimido glycine (shown in Table 2.1), pyromellitylimido alanine, and trimellitylimido L-tyrosine have been copolymerized with aliphatic (SA) and aromatic (CPP and CPH) monomers to obtain enhanced degradation and improved mechanical strength due the presence of the imide bond\(^7\). These polymers have been studied as vaccine carriers\(^8\). Some polyanhydrides have been synthesized from dimer and trimer unsaturated fatty acids, and from nonlinear hydrophobic fatty acid esters such as ricinoleic and maleic acid. Other classes of polyanhydrides include ones copolymerized with esters and ethers, which have been suggested as potential drug carriers in the last decade\(^9,10\).

Uhrich and co-workers recently synthesized novel poly(anhydride-co-ester)s containing salicylic acid in the backbone\(^11-13\). The \textit{in vitro/in vivo} release of salicylic acid (the active form of aspirin) was studied for the treatment of Crohn’s disease and tuberculosis. Copolymers of aliphatic polyanhydrides with \(\epsilon\)-caprolactone, trimethylene carbonate, ethylene glycol\(^14\), and lactic acid have been synthesized. Several modifications of anhydride monomers have been carried out in order to obtain desired characteristics for particular applications\(^15-17\). An example is the incorporation of triethylene glycol (TEG) into an aromatic monomer (CPH) in order to enhance the hydrophilicity of the monomer, resulting in faster degradation rate\(^18\). The resulting polymer (Table 2.1) is poly(1,8-bis(\(\rho\)-carboxyphenoxy)-3,6-dioxaoctane) (CPTEG).
Table 2.1. Typical polyanhydrides used for drug delivery applications

<table>
<thead>
<tr>
<th>Classification</th>
<th>R group</th>
<th>Examples</th>
</tr>
</thead>
</table>
| **Aliphatic polyanhydrides** | $\text{R} \text{O}_x \text{O}_n \text{C}_2\text{H}_x \text{C}_2\text{H}_x \text{C}_2\text{H}_x \text{C}_2\text{O}_x \text{O}_n \text{C}_2\text{O}$ | $x=4$ Adipic anhydride (AA)  
$x=8$ Sebacic anhydride (SA) |
| **Unsaturated polyanhydrides** | $\text{C}=\text{C}$ | Fumaric anhydride (FA)  
Acetylenedicarboxylic anhydride (ACDA) |
| **Aromatic polyanhydrides** | meta: isophthalic anhydride (IPA)  
para: terephthalic anhydride (TA) | $x=1$ bis(p-carboxyphenoxy)methane (CPM)  
$x=3$ 1,3-bis(p-carboxyphenoxy)propane (CPP)  
$x=6$ 1,6-bis(p-carboxyphenoxy)hexane (CPH) |
| **Novel polyanhydrides** | trimellitylimido glycine (TMAgly) | $x=5$ 1,8-bis(p-carboxyphenoxy)-3,6-dioxaoctane (CPTEG) |
2.2.3 Synthesis

The most widely used method to synthesize polyanhydrides is melt condensation polymerization, which results in high molecular weight polymers\textsuperscript{19}. Other methods include Schotten-Baumann condensation, dehydrative coupling, and ring opening polymerization.

2.2.3.1 Melt Polycondensation

The general process for melt polycondensation of polyanhydrides is shown in Figure 2.3. It consists of reacting dicarboxylic acid monomers with an excess of acetic anhydride to form oligomers that are polymerized at high temperature under vacuum. The degree of polymerization is influenced by the monomer purity, the strength of the vacuum, the reaction temperature, and the reaction time. It has been found that for most polyanhydrides, the optimal polymerization temperature is in the range of 170-190\textdegree{}C\textsuperscript{20}. In general, the condensation reaction is conducted for 2-3 hours, as significant depolymerization can occur after heating for longer periods\textsuperscript{21}. With optimal conditions, molecular weights greater than 100,000 can be produced.

The polyanhydrides synthesized by melt condensation have fiber-forming properties in the molten state. They hydrolyze when exposed to air and this degradation is mainly controlled by the composition of the polymer. Homopolymers of aromatic monomers, such as CPH, degrade at a rate that is several orders of magnitude lower than that of homopolymers of aliphatic monomers\textsuperscript{21}. 
Several variations have been made to the melt condensation process. In the case of polymerization with propionic anhydride and butyric anhydride, harsh conditions can be used for the removal of unreacted anhydride due to the high boiling point of both chemicals. A variety of catalysts have been used to polymerize polyanhydrides within 20-60 minutes, but the main disadvantage for biomedical applications is the potential toxicity from catalysts such as cadmium acetate, earth metal oxides, and ZnEt₂·H₂O.

2.2.3.2 Schotten-Bauman Condensation

The Schotten-Bauman condensation produces polyanhydrides with moderate molecular weights by a dehydrochlorination reaction between a diacid chloride and a dicarboxylic acid. The polymerization takes place by reacting the monomers for 1 hour at room temperature, and it can be conducted via solution or interfacial
methods. Solvents that are used in solution polymerization include dichloromethane, chloroform, benzene, and ethyl ether. The degree of polymerization obtained with this method is ~20-30. Lower molecular weight products are obtained for less reactive monomers such as isophthaloyl chloride.

Polymerization conducted in aqueous interfacial systems suffers from hydrolytic decomposition. The decomposition reaction can be minimized when contact with water is avoided. In the case of polymerization in non-aqueous interfacial environments, products with number average molecular weights up to 5,000 can be obtained\textsuperscript{21}. Various aromatic polymers were prepared from the reaction of equimolar amounts of the acid dissolved in aqueous base and the corresponding diacid chloride dissolved in organic solvent. Reaction occurred between dibasic acid in one phase and an acid chloride in the other. Polar solvents for this reaction include dimethylformamide and 1,4-dicyanobutane.

2.2.3.3 Dehydrative Coupling

Another method to synthesize polyanhydrides is by dehydrative coupling of two carboxyl groups. Even though this method produces lower molecular weights (mostly oligomers) compared with the methods described above, it is a single step polymerization where a dicarboxylic acid monomer can be directly converted into the polymer. Moreover, it can be conducted at low temperatures suitable for monomers that cannot resist harsh reaction conditions.

A number of dehydrative agents have been effective in coupling the carboxyl groups. The most effective agents are bis[2-oxo-3-oxazolidinyl]phosphinic chloride,
N-phenylphosphoroamidochloridate, diphenyl chlorophosphate, diethyl phosphorobromidate, dicyclohexylcarbodiimide, chlorosulfonylisocyanate, and 1,4-phenylene diisocyanate\textsuperscript{21}. In general, higher molecular weights were obtained with polar solvents such as dichloromethane and chloroform. The major disadvantage of this method is the problematic isolation and purification of the final products while preventing hydrolytic decomposition.

2.2.3.4 Ring Opening Polymerization

Low molecular weight linear polymers undergo transformations between linear and cyclic forms. When a mixture of low and high molecular weight polymers is subjected to molecular distillation, cyclic monomers and dimers are distilled off and a high molecular weight polymer remains behind. The cyclic molecules are transformed to a polymer that contains large ring structures\textsuperscript{6}.

Another variation of this process is the preparation of adipic acid from cyclic adipic anhydride (oxepane-2,7-dione). The monomer is prepared by the reaction of adipic acid and acetic anhydride followed by catalytic depolymerization under vacuum. Factors that affect this reaction include temperature, reaction time, and, if used, concentration of catalyst. When catalyzed, reaction at 180°C for 30 minutes produced polymers with molecular weights up to 300,000. Uncatalyzed reactions that were carried longer than 2 hours at 180°C yielded low molecular weight polymers\textsuperscript{6}. 
2.2.4 Characterization

In order to understand the properties that make polyanhydrides suitable drug carriers, their chemical, physical, and thermal behavior need to be characterized. This section discusses methods to determine the chemical structure and composition, the molecular weight, the thermal properties, the phase behavior, the stability, and the erosion mechanism of polyanhydrides.

2.2.4.1 Chemical Structure and Composition

The technique most widely used for determining the chemical structure and composition of polyanhydrides is $^1$H NMR spectroscopy. The chemical structure is assigned in accord with the chemical shifts characteristic of aliphatic and aromatic protons. The protons close to electronegative groups, i.e. aromatic groups, absorb at lower frequencies (6.5-8.5 ppm), while aliphatic protons absorb at higher frequencies (1-2ppm) \(^{22}\). $^1$H NMR has also been used to determine the degree of randomness in polyanhydride copolymers\(^ {23}\). By integration of NMR peaks it is possible to determine if a copolymer has a random or block-like structure. Other useful information obtainable from $^1$H NMR spectra includes the conversion of polymerization reactions, the actual composition of the polymer, the polymer molecular weight, and degradation rate.

Fourier transform infrared (FTIR) and Raman spectroscopy have also been used to authenticate polyanhydrides structures. Aliphatic polymers absorb at 1740 and 1810 cm\(^{-1}\), while aromatic polymers absorb at 1720 and 1780 cm\(^{-1}\) \(^{23}\). All the polyanhydrides show methylene bands due to deformation, stretching, rocking, and
twisting. Aside from being used to ascertain polyanhydrides structures, these techniques can be used to determine degradation progress, by monitoring the area of carboxylic acid peak (1770-1675 cm\(^{-1}\)) with respect to the characteristic anhydride peaks over time.

2.2.4.2 Molecular Weight

The molecular weight of polyanhydrides can be determined by gel permeation chromatography (GPC), viscosity measurements, and \(^1\)H NMR spectroscopy. Vapor pressure osmometry (VPO) cannot be used for molecular weight determination, as depolymerization occurs during the experiment. The weight average molecular weight (M\(_w\)) of polyanhydrides ranges from 5,000 to 300,000. Typical polydispersity indexes are in the range of 2 to 15, which increases with molecular weight. GPC determines the molecular weight relative to polystyrene standards. The intrinsic viscosity (\(\eta\)) is proportional to M\(_w\), as shown by the Mark Houwink relationship for CPP:SA copolymer (Eq 1). This relationship was calculated from viscosity experiments and M\(_w\) values from GPC.

\[
\eta_{CHCl_3}^{23^\circC} = 3.88 \times 10^{-7} \times M_w^{0.658}
\]  

An alternative way to estimate the molecular weight of polyanhydrides is by end group analysis from \(^1\)H NMR spectra. The degree of polymerization can be calculated from the ratio of the area of the inner chain protons to the area of terminating groups. The number average degree of polymerization (DP) of CPP:SA copolymers is represented in Eq. 2, where (CPP) and (SA) depict the area of scaled
inner chain protons, (Ac) represent the acetylated end group, and (SA*) and (CPP*) designate the carboxylic terminated polymer chain\textsuperscript{24}.

\[ DP = \frac{2[(CPP) + (SA)]}{[(Ac) + (SA*) + (CPP*)]} \]  \hspace{1cm} (2)

2.2.4.3 Thermal Properties

The thermal transitions of polyanhydrides have been determined from differential scanning calorimetry (DSC). DSC thermal scans provide properties such as glass transition temperature (\(T_g\)), melting temperature (\(T_m\)), and heat of fusion (\(\Delta H\)). It is important to know the values of \(T_g\) and \(T_m\) in the fabrication of drug delivery devices such as tablets and microspheres. While \(T_g\) determines the minimum temperature required for compression molding, \(T_m\) determines the minimum temperature necessary for injection molding or melt compression. A general decreasing trend in \(T_g\)'s has been observed as methylene groups are added into the main chain of an anhydride monomer. As mentioned earlier, aliphatic polyanhydrides melt at temperatures below 100°C and aromatic polyanhydrides have melting points greater than 100°C.

It has been shown that the crystallinity of polymers affects erosion and drug release rates, as crystalline regions erode slower than amorphous ones\textsuperscript{25}. Moreover, highly crystalline polyanhydrides affect device morphology as it creates irregular external surfaces. The crystallinity of polyanhydrides has been determined using X-ray diffraction, DSC, \(^1\)H NMR spectroscopy, and small-angle X-ray scattering (SAXS). It has been demonstrated that homopolymers of aromatic and aliphatic
Diacids are crystalline. When copolymerized, polyanhydrides exhibited a decrease in crystallinity in copolymers of equimolar compositions, i.e. CPP:SA, CPH:SA, and FA:SA copolymers $^{25}$. The $\Delta H$ from DSC thermographs exhibited a decrease as the copolymers approached equimolar compositions. This decrease in crystallinity is representative of the random behavior of the polymer chain, as determined by $^1\text{H}$ NMR spectra. In general, the copolymers rich in one monomer had higher crystallinity.

### 2.2.4.4 Phase Behavior

Polymers blends, which display distinct physical and chemical properties, are used for the design of materials for diverse applications. This variation in properties may lead to microphase separation, which in turns affect drug release, as drugs thermodynamically partition between the phases, depending on their compatibility with the phase $^{26}$. Research has shown that aliphatic, aromatic, and copolymers of anhydrides monomers are miscible and the blends had a single melting temperature that was lower than that of the starting polymers $^6$. On the other hand, polyanhydrides that are partially miscible with poly(orthoesters), poly(hydroxybutyric acids), and low molecular weight poly(esters), with two melting temperatures, was clearly indicative of the phase separation. Blends of polyanhydrides with poly(caprolactone) (poly(CL)) are completely immiscible. Degradation studies in blends of poly(CL) with poly(dodecanedioic anhydride) (poly(DD)) indicated that the anhydride component degraded rapidly and released from the blend, without affecting poly(CL) degradation $^{23}$. Other studies include the characterization of
microphase-separated copolymers of poly(SA) with poly(CPH) or poly(ethylene glycol)\textsuperscript{27, 28}. The phase diagram for the poly(CPH)/poly(SA) blend system has been determined using small angle X-ray scattering (SAXS), optical microscopy, and molecular simulations, while the blends of poly(SA) and PEG were characterized by DSC and IR spectra. The poly(CPH)/poly(SA) system exhibits an upper critical solution temperature behavior.

2.2.4.5 Stability

The stability of polyanhydrides has been studied in solid state and in dry chloroform. Aromatic polyanhydrides such as poly(CPP), poly(CPH), and poly(CPM) maintained their original molecular weight for at least 1 year in solid state upon storage under dry argon or vacuum at 21°C. In contrast, aliphatic polyanhydrides such as poly(SA) have a rapidly degradation rate at the same storage conditions\textsuperscript{1}. Studies performed with GPC revealed that weight average molecular weight tend to decrease rapidly initially, and later a constant stabilized decrease in molecular weight is observed. The decrease in molecular weight was explained by an internal anhydride interchange mechanism resulting in ring formation, as revealed by \textsuperscript{1}H NMR. This mechanism was supported by the fact that the decrease in molecular weight was reversible and heating of the depolymerized polymer at 180°C for 20 min yielded the original high molecular weight polymer\textsuperscript{23}. It is important to mention that polyanhydrides experienced significant weight loss when stored at ambient conditions in which water attacks the anhydride bonds.
The stability of polyanhydrides in solution was studied using chloroform under dry nitrogen atmosphere at 37°C. The aromatic polyanhydrides remained stable under these conditions during a 3 day period, while copolymers with aliphatic SA had a significant molecular weight loss during the same time period. Therefore, polyanhydrides can be processed in solution environment as long as the time is not extended over this period.

\(\gamma\)-irradiation methods have been utilized for sterilization of polyanhydrides. In this technique, aliphatic and aromatic homo- and copolymers were irradiated at 2.5 MRad and the chemical structure as well as the physical properties were found to be the same before and after irradiation. The studies showed that saturated polyanhydrides are stable during \(\gamma\)-irradiation, as a slight increase in molecular weight was observed. Electron paramagnetic resonance (EPR) spectroscopy was used to characterize free radicals in \(\gamma\)-sterilized polyanhydrides. Polymers with high melting temperatures produced the highest yields of room temperature radicals, which in turn transform into less conjugated polyanhydrides that leads to lower molecular weight polymers.

2.2.4.6 Degradation and Erosion

Polymer erosion (i.e., mass loss) is a complex process that is determined by numerous factors that include the molecular weight loss (degradation), the swelling, the dissolution and diffusion of oligomers and monomers, and morphological changes. Polyanhydrides undergo degradation prior to erosion, as a consequence
of the chemical instability of the anhydride bond. Thus, degradation and erosion are limited to the surface, as water does not penetrate into the device\textsuperscript{31}.

Erosion kinetics is complicated when the anhydride monomers of a copolymer system exhibit micro-phase separation that leads to erosion of the different phases at different rates. The erosion of a fast eroding phase may leave intact the slow eroding phase\textsuperscript{32}. At this point, the monomer solubility plays a major role in polyanhydride erosion kinetics, as monomers are accumulated in eroding zones of the matrix and its dissolution will depend on the pH of the microenvironment\textsuperscript{33}. It is known that the saturation concentration of the monomers CPH, SA, and CPTEG is a function of pH and that at a particular pH, the order of solubility of the monomers is CPTEG > SA > CPH, which provides valuable information when describing drug release from polymers containing any of these monomers\textsuperscript{18,32}.

\textbf{2.2.5 Polyanhydride-based Drug Delivery Systems}

\textbf{2.2.5.1 Biocompatibility}

The biocompatibility of implantable polyanhydride disks was studied in the brains of rats, rabbits, monkeys, and eventually in human clinical trials\textsuperscript{34}. Wafers of poly(CPP:SA) and poly(FAD:SA) were implanted in the frontal lobes of rats, rabbits, and monkeys. In all studies the animals receiving the implants showed no behavioral changes or neurological deficits indicating that the polymers were not invoking a systemic or local toxicity. To determine how the body metabolized the poly(CPP:SA) radio-labeled copolymers were implanted in the brains of rats\textsuperscript{20}. Seven days after implantation 40\% of the \textsuperscript{14}C SA-labeled polymer had been excreted as CO\textsubscript{2}, 10\% in
urine, and 2% feces, and 10% still in the implanted device. In the same period only 4% of the $^{14}$C CPP-labeled polymer was excreted by urine and feces.

The biocompatibility of poly(CPP), poly(TA), and copolymers of CPP:SA and CPP:TA implanted in the corneas of rabbits was studied\textsuperscript{35, 36}. Six weeks after implantation, the cornea remained clear and showed no evidence of corneal edema or neovascularization, indicating biocompatibility of the polymer matrix implant.

Subcutaneous implants of 20:80 CPP:SA copolymer were administered in rats at doses of 40 and 120 times the size to be used in humans. The purpose of these experiments was to test the systemic toxicity of the polymers. Eight weeks after implanting the disks the rats were sacrificed and their organs underwent histopathological evaluations. In general there was little to no difference between the organs of the experimental group (receiving the implant) and those of the control group. Again, in all cases the polymers underwent degradation and were found to cause minimal inflammation at the site of implantation. Thus, polyanhydrides are inert and suitable for \textit{in vivo} drug delivery\textsuperscript{37}.

\subsection*{2.2.5.2 Drug/Polymer Interactions}

When selecting polymers as drug delivery carriers, it is necessary to establish whether the polymer will react with the incorporated or the released drug. Three factors need to be considered: the reactivity of the drug, the hydrophobicity of the drug, and the fabrication method. The reactivity of CPP:SA copolymer with the para substituted anilines: \textit{p}-nitroaniline (PNA), \textit{p}-bromoaniline, and \textit{p}-anisidine, and \textit{p}-phenylenediamine was examined\textsuperscript{36}. The model drugs were incorporated into the
polymer matrix using injection and compression molding. When injection molding was used to encapsulate the drugs at 120°C the more reactive drugs (p-bromoaniline, and p-anisidine, and p-phenylenediamine) reacted with the polymer forming amides. However, when the drugs were incorporated into the polymer matrix using compression molding at room temperature the drugs did not react with the polymer during the fabrication process.

The hydrophobicity of the drug can also influence interactions between the drug and the polymer. When hydrophilic dyes (acid orange and brilliant blue) were encapsulated in polyanhydrides the T_m's of the polymers were unchanged. When hydrophobic dyes (p-nitroaniline and methyl red) were encapsulated the T_m's of the polymers changed, indicating an interaction between the polymer and the drug.

2.2.5.3 Device Fabrication

Polyanhydride drug delivery devices have been fabricated as implantable and injectable devices. Implantable devices are fabricated by either compression, melt molding, or by solvent casting. The first step of compression molding is to obtain a fine powder of the drug and the polymer. The powders are physically mixed and placed in a piston mold. The wafer is formed by applying a pressure (typically 30 kpsi) and by heating the sample to a temperature 5-10°C above the T_g of the polymer. One drawback of this method is the uneven distribution of the drug in the polymer, leading to poor reproducibility. The Gliadel® system is a compression molded wafer. To overcome the problem of uneven drug distribution, the drug and
polymer are spray dried together to form microspheres. The microspheres are then compression molded to form the wafer.

The alternative to compression molding is melt compression. This procedure requires the polymer and drug to be heated 10°C above the $T_m$ of the polymer, forming a viscous solution\textsuperscript{1}.\textsuperscript{40}. The solution can then be placed in either a conventional mold under low pressure or it can be injection molded. This fabrication method results in an even drug distribution. However, the elevated temperatures needed to melt the polymer could cause adverse reactions in temperature sensitive drugs, such as proteins.

Solvent casting is done by co-dissolving or suspending the drug in the polymer solution. The solution is then poured into a flat open mold and cooled on dry ice. The resulting film is often fragile. If the drug is not soluble in the polymer it will settle on the bottom of the film leading to an uneven drug distribution\textsuperscript{26, 29}.

To form an injectable drug delivery device the drug is loaded into polymer microspheres. Drug-loaded polyanhydride microspheres have been fabricated using different methods. The most common method is the solvent extraction method; which includes water/oil/water, water/oil/oil or solid/oil/oil (dependent on whether the drug is soluble in the polymer solvent). In the w/o/w method the drug (typically proteins) is dissolved in an aqueous phase and then emulsified with a larger volume of polymer dissolved in an organic, typically methylene chloride. The inner emulsion is then added to a larger volume of water that contains a surfactant, usually PVA, and allowed to stir for several hours in order to extract the solvents. In the case of
w/o/o or s/o/o the outer aqueous PVA phase is replaced with an immiscible organic, i.e. silicon oil. The spheres are typically collected by either centrifugation or filtration.

Microspheres can also be fabricated by the hot-melt procedure, however this method is not ideal for encapsulating temperature sensitive drugs, such as proteins. Spray drying or atomizing the polymer and drug together can also be used to fabricate microspheres. This method requires the use of either a spray dryer or atomization. In the case of the spray dryer the polymer/drug suspension is pumped into the spray drier and as the suspension is sprayed a stream of air causes the polymer spheres to harden. Microspheres are fabricated by atomization by passing the drug/polymer suspension through an atomizing nozzle. As the polymer/drug spheres leave the nozzle they are collected in a bath of liquid nitrogen sitting on top of a frozen layer of ethanol. The liquid nitrogen/ethanol bath is then stored at -80°C for three days. During this time the ethanol slowly thaws and the frozen microspheres fall into it. As the microspheres sit in the ethanol the organic solvent (methylene chloride) slowly diffuses out, leaving solid spheres to be collected by filtration.

2.2.5.4 In Vitro Release

The rate at which an encapsulated drug will be released from a polyanhydride device, either a wafer or a microsphere, is strongly dependent on polymer composition and drug distribution. Other factors that contribute to the release rate of drugs include: fabrication technique, size/shape of the device, and pH of the surrounding media.
The hydrophobicity of a drug influences its distribution within the polymeric device\textsuperscript{26}. PNA and disperse yellow have higher affinities for poly(CPH) and poly(SA) respectively. The two drugs were encapsulated in tablets of poly(CPH), poly(SA), and copolymers of the two to determine if the drugs would partition into the more favorable polymer micro-domain. When the dominant polymer had a low affinity for the drug, a burst effect was seen. In the case of 50:50 CPH:SA copolymer, each drug followed the release of the monomers, indicating that the drug was partitioning into the more favorable domain.

The size of the device may also influence drug distribution and release rate\textsuperscript{42}. Monodisperse microspheres of differing average diameters were studied to determine the influence of the size of the device on the delivery. Smaller diameter microspheres showed a more prolonged release rate of drug than did microspheres that had a large diameter. As the diameter increased the time it took for the microsphere to form by precipitation increased, thus increasing the time for the drug to segregate towards the surface of the microspheres.

As the anhydride bonds in the polymer backbone are hydrolyzed carboxylic acids are formed. The formation of the acidic degradation products reduces the local pH of the eroding device. The diffusion of the acidic degradation products away from the device is expedited when the device is in a basic solution. However, when the device is in an acidic solution the erosion process is slowed significantly\textsuperscript{1, 43}. 
2.2.5.5 In Vivo Delivery

As mentioned previously, the 20:80 CPP:SA copolymer was the first polyanhydride to be clinically tested in humans. The copolymer was used to encapsulate BCNU, a chemotherapeutic drug used to treat glioblastoma multiforme, a fatal form of brain cancer. BCNU was co-dissolved with the polymer and disks were fabricated by compression molding. The preclinical trials in rat, rabbit, dog, and monkey brains demonstrated the effectiveness of the polymer in delivering an active drug that remained localized, minimizing systemic reaction to the drug\textsuperscript{44}. The wafer, once implanted into the brain of the glioblastoma patients, releases the BCNU for \textasciitilde3 weeks\textsuperscript{45}. SEM was used to monitor the erosion of the wafer both \textit{in vitro} and \textit{in vivo}\textsuperscript{46}. It was found that the erosion of the wafer was controlled by diffusion of BCNU and erosion of the polymer. The delivery device was approved in 1996 by the US-FDA for use in conjunction with surgery for patients suffering from recurrent glioblastoma. In 2003 the US-FDA approved the use of the device in newly diagnosed advanced cases of malignant gliomas to be used in conjunction with surgery and radiation.

The use of 20:80 CPP:SA and 18:82 FAD:SA copolymers disks as drug delivery devices for carboplatin, a treatment for glioma, was also investigated in rodents\textsuperscript{47}. The majority of the drug was released in seven days from the CPP:SA copolymer disk and 65% of the drug was released from the FAD:SA copolymer disk in seven days. This method of delivery was more effective than systemic therapy and did not cause systemic toxicity.
A separate polyanhydride system has also been investigated for treatment of osteomyelitis, a bone infection typically caused by bacteria. 50:50 FAD:SA copolymer implants containing gentamicin were tested in the backs of rats, in infected tarsocrural joints of horses, and in humans with infected prosthetic hips or knees. In all cases the local delivery of gentamicin was successful and the systemic exposure to the drug was avoided.

2.3 Protein Stability

2.3.1 Introduction

There are several hundred protein-based drugs that are being currently investigated by the U.S. FDA. In 2003, the estimated annual research expenditures in this area was $30 billion. However, major challenges still exist as a consequence of protein susceptibility to chemical and physical instability, which in consequence may hinder their therapeutic efficacy.

Proteins have complex structures essential for their biological activity. They possess different structural levels that start with the primary structure (amino acid sequence) and can extend to more complex secondary, tertiary and quaternary structures. In general, the hydrophobic groups of the protein are packed within the core and the polar residues that can interact with polar solvents are located in the surface. Instability of peptides involves primarily chemical pathways of degradation, while proteins can degrade by chemical and physical pathways to the same extent. The stabilities of the interactions are so interdependent that disruption of a very limited number of interactions tends to disrupt all of them. This section reviews the
most prominent routes of protein inactivation by chemical and physical pathways and how these can be related with polymeric systems.

2.3.2 Chemical Instability

Chemical instability is produced when a new chemical structure is formed as a result of bond formation or disruption\textsuperscript{53}. Typically this degradation pathway involves the disruption of the primary sequence and the reactivity of the side chains\textsuperscript{54}. The chemical reactions that will be discussed are the most commonly encountered, including deamidation, hydrolysis, oxidation, β-elimination of disulfide, and disulfide scrambling.

2.3.2.1 Deamidation

The deamidation of asparagines and glutamines residues of a protein are the most common chemical pathway that lead to protein degradation, and it can be influenced by endogenous and exogenous factors\textsuperscript{55-58}. Deamidation causes an intramolecular rearrangement to form a five-membered succinimide ring that in turn is susceptible to hydrolysis and racemization\textsuperscript{59}. Among the endogenous factors, the nature of the amino acid residue near the asparagines is the principal cause for deamidation. Important is the fact that certain proteins will not be affected by this factor as their native conformational arrangement does not provide the flexibility to produce the cyclic imide\textsuperscript{53}. On the other hand, the major external factor that influences deamidation of these residues is the pH environment. Under neutral or basic conditions, deamidation proceeds through a five-membered cyclic imide.
formed by intramolecular attack. The rate constants of deamidation in these environments were much faster than in acidic solutions, and maximum stability was observed in the pH range of 3.0-5.0 \(^{60}\). Other external factors that may influence the deamidation of protein residues are elevated temperature, nature of buffer, buffer concentration, moisture and ionic strength. As expected, this chemical pathway can lead to changes in protein conformation and aggregation, detrimental to the protein.

2.3.2.2 Hydrolysis

An acidic environment may cause the spontaneous hydrolysis of the peptide backbone. This reaction is of particular importance when peptides and proteins are loaded into a polymeric system that erodes by bulk erosion mechanism, allowing significant amount of water to enter into the device. Biodegradable polymers that are particularly unstable are the polyesters that degrade into monomeric acidic entities. The amino acid that is most labile to this degradation pathway is the aspartic acid, which hydrolyzes about 100 times faster than other peptide bonds\(^{59}\). Cleavage can occur at either the carbon or nitrogen terminal peptide bonds of aspartate to form a cyclic imide prone to racemization.

2.3.2.3 Oxidation

The amino acid residues most prone to oxidation in the presence of oxidizing agents are serine, cysteine, methionine, histidine, tryptophan, and tyrosine. At mildly acidic conditions in the presence of hydrogen peroxide, methionine is the primary residue predisposed to oxidative degradation. In contrast, the hydroxyl radical reacts
non-selectively with all amino acids. The loss of biological activity will be determined by the location where the oxidation occurs that may disrupt the protein native structure. Besides oxidation by reactive oxygen species, there are other oxidative degradation pathways. Some of these routes includes metal-catalyzed oxidation and auto-oxidation by molecular oxygen in the absence of any catalytic process \(^{53}\).

2.3.2.4 \(\beta\)-Elimination of Disulfide

Disulfide bonds of cysteines are susceptible to \(\beta\)-elimination. Unstable products results from the nucleophilic attack of a hydroxide ion on a carbon atom in a carbon-sulfur bond. The presence of the hydroxide ion catalyzes the \(\beta\)-elimination, so alkaline conditions facilitates this chemical degradation reaction\(^{59}\). The bioavailability of the proteins undergoing \(\beta\)-elimination is likely to be affected as disulfides play a role in maintaining the three-dimensional structure of a protein.

2.3.2.5 Disulfide Scrambling

As mentioned above, disulfide bonds are partially responsible for maintaining the conformational structure of a protein. In the same way, an incorrect formation and position of these bonds may lead to drastic changes in protein function. Adsorption and non-covalent aggregation (precipitation) can also result from disulfide scrambling that can occur at acidic and alkaline environments. The low solubility, low bioactivity, and potential immunogenicity of resulting aggregate are a major concern.
2.3.3 Physical Instability

Physical instability refers to changes in the structural level of proteins that can lead to denaturation, adsorption, and precipitation. The protein loses its ability to retain at least its tertiary structure, that is crucial for biological activity. The factors that promote this instability comprise changes in temperature, pH, and ionic strength, among others. Some proteins unfold by means of the so-called two-state equilibrium process, which involves a thermodynamic equilibrium between a native and denatured state. This unfolded state is characterized by minimal or no bioactivity. Advances in research led to the discovery that unfolding of globular proteins involves equilibrium intermediates called “molten globules”, which are characterized by having a native like secondary structure but lacking the tertiary structure important for their biological function. These intermediates can lead to irreversible denaturation by promoting aggregation.

The forces involved in stabilizing the native state include electrostatic, van der Waals, and hydrophobic interactions. The native structure is stabilized slightly over the denatured one; typically, the Gibbs free energy for the folding equation is only about 5-50 kJ/mol. Therefore, a moderate change in the protein environment can lead to the disruption of the delicate balance of forces involved in stabilizing protein conformation.

Protein unfolding usually leads to the exposure of hydrophobic moieties previously buried in the protein interior. This unfavorable situation can be ameliorated by the association of unfolded molecules via non-covalent interactions to form large molecular weight insoluble aggregates. In addition to aggregating
between them, unfolded proteins may bind to hydrophobic polymeric surfaces and in consequence promote non-covalent aggregation. Unfolding is a reversible process, but aggregation is practically irreversible\textsuperscript{59}. Exogenous factors that cause protein's physical instability that will be discussed are thermal instability, pH, interfacial tension, and dehydration.

2.3.3.1 Thermal Instability

When proteins are exposed to increasing temperature, losses of solubility or bioactivity occurs over a fairly narrow range. These changes may or may not be reversible, depending on the protein nature and the severe conditions of the temperature. As the temperature is increased, a number of bonds in the protein molecule are weakened and in consequence the conformational tertiary structure is disrupted to become more flexible. As these bonds are first weakened, the protein exposes groups that were previously buried to solvent. If heating ceases at this stage the protein should be able to readily refold to the native structure. When heating continues, the hydrogen bonds responsible to maintain the secondary structure of the protein will begin to be disrupted. As these bonds are broken, water can interact with and form new hydrogen bonds with the amide nitrogen and carbonyl oxygen of the peptide bonds. As a result, more hydrophobic groups are exposed to the solvent and an irreversible unfolded state is produced. At temperatures past the 100 °C, hydrothermal degradation escapes biosynthesis\textsuperscript{63}. 
2.3.3.2 pH

The effect of pH in physical instability is unpredictable and highly protein dependent. At the isoelectric point (pI) of the protein, the protein charge is neutral. Proteins tend to be least soluble at pH values near their isoelectric point, where charge repulsions will be at minimum and many proteins will precipitate (aggregated) as a result of the reduced solubility. If the pH is lowered below the pI, usually at acidic pH, some of the protein’s side chain carboxyl groups become protonated and lose their ionic charge. The like charges will repel each other and prevent the protein from aggregating. Nevertheless, the intramolecular repulsion may be so strong that unfolding of the protein can occur as the requirement for flexibility is fulfilled by the compensation of attractive and repulsive interactions. This will have an effect similar to that of mild heat treatment on the protein structure and some hydrophobic groups that were previously buried in the protein interior are exposed. In some cases the unfolding may be extensive enough to expose significant hydrophobic groups and cause irreversible aggregation. It is important to take into consideration that some proteins contain acid labile groups and even relatively mild acid treatment may cause irreversible loss of their function. The effects of high pH are analogous to those of low pH. At basic pH, amino groups become deprotonated, and in consequence large negative charge is conferred to the protein that can lead to unfolding and aggregation. As can be seen both pH extremes lead to conformational changes that may result in protein denaturation.
2.3.3.3 Interfacial Tension

The inherent surface activity of the protein promotes their tendency to concentrate at interfaces. The more important role of the interface lies in its ability to generate or kinetically trap partially unfolded proteins, which in consequence produce aggregates that lead to denaturation\textsuperscript{53}. The common interfaces that lead to protein inactivation are either liquid-air or aqueous-organic interfaces\textsuperscript{54}. In the aqueous-organic interface, the protein comes into contact with a hydrophobic environment, where protein hydrophobic groups gain more flexibility that can lead to unfolding. The flexibility of the tertiary structure of the protein confers the protein the ability to accommodate into the lowest energy configuration possible. The principle of like dissolves like can be applied to this process, as hydrophobic groups that usually lie in the interior will tend to expose to the organic solvent and the hydrophilic groups at the surface will try to hide in order to look for more stable conformational arrangement. This process will continue until random fluctuations in protein structure can no longer yield a configuration of lower free energy. This unfolding is essentially non-reversible because of the large energy barriers.

The same forces are in operation when a protein migrates to a liquid-air interface. Hydrophobic groups tend to associate in the air and the protein unfolds. The presence of shear causes to help unfold the protein and to introduce more air into the solution. Both of these effects can be minimized by keeping the temperature low (to weaken hydrophobic bonds) and by minimizing the interfacial area. If the interface is limited, then only a small amount of protein will be able to denature. The
presence of this denatured protein will serve as a barrier to further denaturation.

2.3.3.4 Dehydration

The dehydration or lyophilization process can result in protein instability if appropriate precautions are not taken. The kinetic approach assumes that some degree of mobility is required for unfolding to occur. Consequently by vitrification of a protein in a glassy amorphous matrix below its glass transition temperature ($T_g$), the kinetic barrier to unfolding will be limiting. The possible changes in conformational structure during lyophilization may or may not be reversible. The irreversibility of the conformational altered proteins in the dehydrated solid may be largely due the competitive process of refolding and aggregation, which occurs during the reconstitution step.
2.4 Immune System

2.4.1 Introduction

A physiological immune response begins with the antigen presenting cell (APC). This is the crucial step of the immune system activation. Without proper activation of these cells, no response is generated against a particular antigen. The best APC responsible to activate helper T cells, killer T cells and B cells are the dendritic cells (DC). Immature DCs are found under the skin and mucous membranes where they sample surrounding for possible pathogens through pathogen-associated molecular patterns (PAMPs) receptors, which play a role in the activation of DCs and influence their subsequent activation. After detecting a pathogen, these cells engulf it through phagocytosis and migrate to lymph nodes where they become mature. Once inside the DC, pathogens are degraded into small fragments that are further expressed at their surface where they can be presented to T cells and B cells (Fig. 2.4). After the specific T cell and B cell becomes activated, these will generate a cascade of events that will lead to attack the disease.

![Figure 2.4. Dendritic cell (DC) activation.](image_url)
2.4.2 Antigen Presentation

There are two antigen presenting pathways of DCs to the Major Histocompatibility Complex (MHC) molecules, whose function is to bind peptide fragments derived from pathogens and display them on the cell surface for T cell recognition\(^66\). One of these pathways is the endogenous, which involves presentation of peptide-MHC I molecules to CD8\(^+\) T cells. Tumor specific CD8\(^+\) T cells activated by DCs presenting tumor antigens can kill tumor cells directly by activating cytotoxic mechanisms (granzymes and perforins) associated with these tumor-specific CD8\(^+\) T cells. On the other hand, the exogenous pathway involves presentation of MHC II molecules to CD4\(^+\) T cells. Subset of activated CD4\(^+\) T cells, known as helper T cells Th1 and Th2, are responsible for cell-mediated and humoral immunity respectively\(^67\). Both CD4\(^+\) and CD8\(^+\) T cells are important in achieving immunological control of tumors and the survival and persistence of CD8\(^+\) T cells as memory cells is regulated by tumor specific CD4\(^+\) T cells\(^68\). Tumor cells do not usually express MHC class II molecules\(^69\), therefore most tumor antigens are not presented to CD4\(^+\) helper T cells, a fact that has to be considered when developing a cancer vaccine. There is a need to find proper immunomodulatory agents that enhance response from CD4\(^+\) helper T cells to ensure their proper activation.

2.4.3 Immune Response

The effector functions of T cells are determined by the array of effector molecules they produce after their activation. This activation can be measured by means of cytokine production. Cytokines are proteins that act as chemical
messengers that regulate the innate and adaptive immune responses as a result of immune recognition. Activated DCs produce the cytokines tumor necrosis factor alpha (TNF-α), which mediates acute inflammation, and a variety of interleukins, such as IL-1, IL-6, IL-8, IL-12, and IL-10. IL-1 induces secretion of IL-2, which induces maturation of the precursors for antigen specific cytotoxic T lymphocytes (CTL) and natural killer (NK) cells, and B cells. NK cell cytotoxicity do not require specific antigen recognition as opposed to CTLs. Th1 responses associate with the release of interferon gamma (IFN-γ), which is an immunoregulatory protein, and tumor necrosis factor (TNF) for immediate anti-tumor effects. A Th2 response associate with release of IL-4, IL-5, and IL-13. Thus the immune response generated after antigen presentation can be elucidated by the proper measurement of these cytokine profiles.
2.5 Adjuvants

2.5.1 Introduction

An adjuvant is an agent that stimulates the immune system, increasing the response to a vaccine, while not having any specific antigenic effect. Adjuvants are immunoenhancing materials that perform three major functions, i) provide a “depot” for the antigen, creating an antigenic reservoir for slow release, ii) facilitate targeting of the antigen to immune cells (APCs) and enhancing phagocytosis, and iii) modulate and enhance the type of immune response induced by the antigen alone (e.g., isotype switching induce Th1 vs. Th2 bias)\textsuperscript{71-73}. Adjuvants may also provide the danger signal the immune system needs in order to respond to the antigen as it would to an active infection\textsuperscript{74}. 

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\textsuperscript{8}Major professor to Maria Torres, corresponding author
2.5.2 Functions of Adjuvants

The first function, providing a depot for the immunogen, is accomplished by entrapping the antigen in a poorly metabolized, non-dissolving or slowly dissolving substance, or otherwise sequestering the antigen to allow for the slow clearance of the antigen from the body. Some of these adjuvants are discussed in more depth in other sections of the review. Aluminum phosphate and aluminum hydroxide, commonly referred to as alum, are the adjuvants most often used in human vaccines and the gel-like matrix of alum creates a slow-release environment for the immunogen. Oil-water emulsions also work by sequestering the antigen and slowly releasing it. The classic oil-in-water emulsion, Incomplete Freund’s adjuvant, is widely used in livestock vaccines, even though it has a tendency to induce granulomas\textsuperscript{75}. It is not used in vaccine formulations for human use because of this tendency. Other mineral oil emulsions, such as Drakeol, Marcol, ISA 206, and ISA 25 from Seppic Montanide are carriers also used in various livestock vaccines\textsuperscript{75}. Recently, MF59, a variation of the biodegradable oil squalene, has proven to be a potent adjuvant with a satisfactory safety record and, thus, is suitable for human use\textsuperscript{75,76}. Virosomes, virus-like particles, ISCOMs, and liposomes all allow for the slow clearance of antigen by incorporating the antigen into small particles composed of stabilized lipids, phospholipids, or proteins. Furthermore, antigen sequestering can be achieved by incorporating the antigen into microspheres composed of polymeric units of a biodegradable material. As the microsphere degrades, the antigen is released. Thus many different carrier formulations provide antigen depots once injected.
The second function of adjuvants is to enhance the immune response by targeting the antigen to immune cells, enhancing phagocytosis, and/or activating the APC. This can be accomplished by properties of the antigen, by a property of the carrier, or by inclusion of immunostimulatory molecules. Pertussis toxin binds with high affinity to epithelial cells, enhancing uptake of the vaccine\(^ {74}\). Other toxins, cholera toxin and *Escherichia coli* heat-labile toxin (LT), bind selectively to M cells of the intestinal tract\(^ {77}\). These cells translocate the vaccine particle across the epithelial barrier to a region rich in lymphocytes\(^ {74,75,78}\). While bacterial toxins such as cholera toxin and *E. coli* LT augment a strong humoral (Th2) immune responses, the response to the anti-toxin may overshadow the response to the conjugate antigen\(^ {74}\). Another bacterial-derived immunostimulant is LPS which is derived from the outer membrane of gram-negative bacteria such as *B. pertussis*. These bacterial products directly interact with the innate immune system via LPS receptors CD14 and TLR-4\(^ {78}\). Human TLRs, when triggered by LPS, stimulate the activation of NF-\(\kappa\)B, a transcriptional activator for the production of pro-inflammatory cytokines\(^ {75}\). Because humans are very sensitive to endotoxins, LPS is generally too toxic for inclusion in many human vaccine preparations and the majority of injectable solutions for medical use are pyrogen free.

These first two mechanisms of immunity are illustrated in Figure 2.5. Some adjuvants may interact directly with TLRs on APC (See Fig 2.5a), and can be derived from pathogens that display highly conserved structures (e.g. PAMPs)\(^ {79}\). As illustrated in figure 2.5a, an adjuvant can interact with the PAMP directly or release
antigen as in the more traditional depot effect. Many adjuvants exhibit a combination of these characteristics.

Immune modulation can be influenced by other characteristics of the adjuvant/delivery system. As mentioned above, an immune response can be categorized as either Th1- or Th2-like. Many different factors can contribute to Th1-Th2 bias of the immune response including route of antigen delivery (intramuscular, subcutaneous, intranasal, oral), antigen dose, duration of antigen presentation, number or frequency of immunizations and inclusion of co-stimulatory molecules (e.g. LPS, exogenous cytokines) with the antigen. Adjuvants can affect all of these factors in different ways, the role of the vaccinologist is the use the correct adjuvant to induce a protective immune response.

In addition, the form (e.g. particulate or soluble) of the antigen, delivery system, and route of delivery can all affect the Th1-Th2 bias of a subsequent
immune response to a vaccine, and the type of immune response (Th1 or Th2) that will be protective varies with the disease in question. Antigen, adjuvants and delivery systems need to be chosen with care to obtain the most protective response. Current licensed vaccines for the most part are lacking in their ability to induce Th1 type immune responses without also generating undesirable toxic side-effects such as the severe inflammation associated with whole-cell pertussis vaccines. While traditional alum-based vaccines initiate T helper type 2 (Th2) response, a T helper type 1 (Th1) response may be more effective for preventing some diseases. Alum is still widely used in veterinary vaccines, but is frequently associated with granulomas in tissues and subsequent carcass losses. Oil-based liposomes are capable of inducing a strong Th1 response, but are also associated with adverse tissue reactivity, granuloma formation, and subsequent carcass loss.

In the United States, two adjuvants are currently approved for use in humans; alum and MF59, a biodegradable plant oil emulsion containing muramyl tripeptide. Highly purified muramyl tripeptide (MTP) is a synthetic component similar to that found in mycobacterial cell walls and MTP retains immunostimulatory properties while eliminating much the toxic effects associated with the whole bacterium. MF59 is used in the H5N1 bird flu vaccine developed by Novartis. MF59 was chosen for dose-sparing effects and is recommended in elderly (>65) including those with underlying chronic conditions such as diabetes.
2.6 Polymer Vaccines

2.6.1 Introduction

Biodegradable polymers have been studied for many years because they show promise for the development of single dose vaccines. Polymeric compounds have the ability to sustain the release of the vaccine antigen by a controlled mechanism over an extended period of time, thus eliminating the need of subsequent doses of vaccines. Other potential advantages of these materials are that immunomodulatory properties (i.e. adjuvanticity) can also be achieved with the proper tailoring of the polymer chemistry. Studies evaluating the use of controlled-release, single dose polymeric vaccines in both laboratory animals and livestock species (i.e., sheep, mini-pigs, cattle, and horses) have shown promise when encapsulating protein antigens.

Biodegradable polymers also offer the advantage that MPLA, CpG DNA motifs or other immunoenhancing molecules can be incorporated to create a pathogen-mimicking solid particle. Polymeric vaccine particles have been shown to induce demonstrable immune responses when administered by several routes including, parenteral (e.g. intramuscularly or subcutaneously), intranasal, or orally.

These materials also have the added advantage over stable (non-degradable) devices (e.g. pumps) in that after administration, there is no need to remove them, therefore eliminating another surgical procedure. Furthermore, most are manufactured from synthetic base compounds, eliminating many potential reactive antigenic or allergenic epitopes that can accompany animal or plant derived materials.
The controlled release of antigens by parenteral administration of polymeric microspheres has been assessed. The release kinetics from the polymeric adjuvant plays a major role. There are two main approaches: “pulsatile” and “continuous” release\textsuperscript{59}. The “pulsatile” release can be obtained from depot or storage formation at the site of injection and the “continuous” release is achieved when the antigen is efficiently presented to antigen presenting cells in a continuous manner. Microspheres greater than 10 μm can act as depots at the site of injection, while the smaller microspheres can be efficiently taken up (phagocytosed) by antigen-presenting cells\textsuperscript{95}. After the antigen presenting cells take up the microspheres, they can present the antigen to immune cells responsible for eliciting an immune response. Thus, the controlled release over prolonged periods may produce an sustained immune response. On the other hand, a pulsatile release is expected to exhibit high levels of antibodies after the second pulse, when memory cells are already available. Despite the advantages of enhancing and modulating immunogenicity using polymeric microspheres as vaccine carriers, to date no vaccines based on polymeric carriers have been approved for human use.

The two most widely studied polymer classes for controlled release vaccines are polyesters\textsuperscript{97-106} and polyanhydrides\textsuperscript{1, 6, 8, 11, 25, 36, 38, 107-113}. Other classes of polymeric compounds have been evaluated and shown to successfully deliver antigen to laboratory animals\textsuperscript{73, 114-126}. Key findings of research done with these polymeric systems as vaccines carriers are discussed below and some of the chemistries are shown in Table 2.2.
Table 2.2. Structure of common polymers used as vaccine adjuvants.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Structure</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polysaccharides</td>
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<td></td>
</tr>
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<td>127</td>
</tr>
<tr>
<td>Chitosan</td>
<td><img src="image" alt="Chitosan structure" /></td>
<td>128, 77</td>
</tr>
<tr>
<td>N-trimethyl chitosan</td>
<td><img src="image" alt="N-trimethyl chitosan structure" /></td>
<td>128</td>
</tr>
<tr>
<td>Polyanhydrides</td>
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<td></td>
</tr>
<tr>
<td>Poly(sebacic acid)</td>
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<td>8, 129, 130</td>
</tr>
<tr>
<td>SA</td>
<td><img src="image" alt="SA structure" /></td>
<td>8, 129, 130</td>
</tr>
<tr>
<td>1,3-bis((p)-carboxyphenoxy)propane CPP</td>
<td><img src="image" alt="CPP structure" /></td>
<td>8, 113</td>
</tr>
<tr>
<td>1,6-bis((p)-carboxyphenoxy)hexane CPH</td>
<td><img src="image" alt="CPH structure" /></td>
<td>90, 129, 130</td>
</tr>
<tr>
<td>1,8-bis((p)-carboxyphenoxy)-3,6-dioxaoctane CPTEG</td>
<td><img src="image" alt="CPTEG structure" /></td>
<td>131-133</td>
</tr>
</tbody>
</table>

[Image of Dextran structure]

[Image of Chitosan structure]

[Image of N-trimethyl chitosan structure]

[Image of Poly(sebacic acid) structure]

[Image of SA structure]

[Image of CPP structure]

[Image of CPH structure]

[Image of CPTEG structure]
Poly(trimellitylimido-L-tyrosine)

Poly(ortho ester)s

Poly(ester-amide)s
Phenylalanine-based PEA
Leucine-based PEA

Polyesters
Poly(lactic acid) LA
Poly(glycolic acid) GA
Poly-ε-caprolactone
2.6.2 Polyesters

Microspheres composed of polyesters have been the most widely studied. Polymers of lactic acid and glycolic acid (e.g. poly(lactide-co-glycolide), PLGA) have been utilized in biomedical applications such as bone pins and dissolvable sutures for many years and recently have proven effective as vaccine delivery vehicles for the induction of protective immunity in laboratory animals\textsuperscript{97-101}. The greatest benefits of PLGA in biodegradable materials is that the degradation products, lactic acid and glycolic acid, are naturally occurring metabolites and are readily absorbed by neighboring cells\textsuperscript{102, 140}. However, as the polyester degrades and the acidic monomers are released, an acidic microenvironment is created. Prolonged exposure to aqueous or acidic environments has been shown to be detrimental to the stability and immunogenicity of proteins, especially the proteins used in recombinant and subunit vaccines, e.g., tetanus toxoid (TT) and diphtheria toxoid\textsuperscript{104, 105}. Some attempts to minimize this acidity have been recently evaluated by incorporating a basic compound like magnesium carbonate (MgCO\textsubscript{3}) into the PLGA microsphere\textsuperscript{106}. However, subsequent analysis indicated that the MgCO\textsubscript{3} does not
significantly improved peptide stability but did enhance the antibody production, acting as a potential adjuvant.

Antigen-loaded PLGA microspheres function as an adjuvant by at least two mechanisms: 1) creating a depot for the antigen in vivo, and 2) enhancing phagocytic uptake of the antigen-loaded particle by APCs\textsuperscript{101}. The uptake of PLGA microspheres by macrophages or DCs has been demonstrated following administration by intraperitoneal or intradermal routes, respectively\textsuperscript{141}. Other immunostimulatory properties of PLGA were observed in studies showing an enhanced cytokine production and proliferation when cells were incubated in vitro with blank PLGA microspheres\textsuperscript{140}. Similarly, oral administration of PLGA nanoparticles containing type II collagen promoted the induction of tolerogenic immune responses that ameliorated arthritis\textsuperscript{142}. The prolonged presence of the nanoparticles in the Peyer’s patches and the induction of elevated TGFβ suggested the differential activation of DCs that modulated the subsequent immune response.

Vaccine formulations based on PLGA, PLA, or PGA variants have been successful in inducing immune responses to a large number of antigens including: \textit{Yersinia pestis} antigens, HIV gp140, \textit{Bordetella pertussis} antigens, measles virus antigen, OVA antigen, TT, diphtheria toxin, type II collagen, malarial antigens, cancer cell antigens, \textit{Escherichia coli} adhesion proteins, \textit{Vibrio cholerae} antigens, influenza virus antigens, hepatitis B viral antigens, and ricin toxoid\textsuperscript{77, 140, 142, 143}. These vaccines have been delivered by a variety of routes including intradermally, intravaginally, intranasally, orally, or parenterally into laboratory animals to induce both serum antibodies, mucosal IgA, cell-mediated responses and facilitated the
induction of secondary immune responses (e.g., isotype switching) as determined when individuals were analyzed up to a year after single immunization\textsuperscript{140, 144}. Many groups have reported the successful induction of immunity following use of a single dose vaccine formulation composed of PLGA microspheres of various compositions\textsuperscript{145, 99, 146-148, 149, 150}. Furthermore, encapsulation of antigens in PLGA microspheres was shown to enhance antigen presentation via MHC I leading to increased activation of antigen specific cytotoxic T cells\textsuperscript{89, 143, 145}. However, most of these studies were conducted in vitro, some investigations included MPLA, a known Th1 immune response activator in the microsphere while others used multiple injection regimens in vivo. There is no consensus opinion, however, as to whether PLGA-based vaccines are more efficacious than current adjuvant systems such as alum. Antibody responses induced in mice and guinea pigs following vaccination with TT-loaded PLGA were greater than those induced by single injection of soluble TT alone or two doses of alum absorbed TT. Additionally, a stronger anamnestic response (higher titer) was observed when individuals that had received the TT-loaded PLGA microparticles were boosted one year later\textsuperscript{144}. On the other hand, Walker et al, observed that encapsulation of TT in PLGA microspheres did not induce serum antibody titers higher than alum-based TT vaccines\textsuperscript{149}. Only small amounts of antigenically active TT were released in the first two days from PLGA microspheres, even though protein continued to be released for up to 11 weeks\textsuperscript{144}. Collectively, evaluation of PLGA studies does not provide strong correlation between release of antigenic peptides, length of in vitro release of peptides, and immune response to those peptides in vivo.
Some studies have suggested that immunization with PLGA microspheres effects immune deviation. Moore et al showed the ability of HIV gp120 protein loaded PLGA microspheres to shift the T cell response from a dominant Th2 or mixed Th1/Th2 to a more dominant Th1 immune response as indicated by the presence of IFNγ producing CD4+ T cells\textsuperscript{151}. In other studies, the Th2-biased hepatitis B core antigen has been formulated with the Th1 immune stimulator MPLA in PLGA nanoparticles to develop a stronger Th1 response\textsuperscript{72}. More recently, a vaccine formulation prepared against malaria and composed of PLGA microspheres and Montanide ISA 720 was shown to induce an antibody response (IgG isotype class switching) characteristic of Th1 response\textsuperscript{152}.

Variations in reported efficacy of PLGA microspheres may be due to dose of antigen, method of encapsulation (e.g. spray drying vs solvent evaporation), route of immunization, and/or the size of the microspheres\textsuperscript{103, 143}. Following primary immunization with small microspheres (10-20 μm), a greater anamnestic response was generated one year later following a low dose booster than that observed in animals initially receiving larger microspheres (>60 μm)\textsuperscript{144}, however, nanoparticles (200-600 nm) were less effective at inducing cell-mediated immune response than microspheres\textsuperscript{143}. This may be because microspheres <10μm in diameter are readily phagocytosed by macrophages and DCs that would enhance antigen processing and presentation\textsuperscript{100, 147, 153-156}. On the other hand, the route of immunization with PLGA microparticles influenced the type of immune response generated. The intraperitoneal route induced Th1 cell-mediated response while the intramuscular route induced a Th2 humoral response\textsuperscript{143}. Even though all the extensive research
done with PLGA as antigen carriers, some with success in animal models, no formulation has been reported to induce a protective immunity in humans\textsuperscript{157}.

\textbf{2.6.3 Polyanhydrides}

Polyanhydrides are a class of surface erodible, biocompatible polymers that have been extensively used as carriers for controlled drug delivery\textsuperscript{1, 6, 11, 25, 38, 107-112, 158-160}. These biodegradable polymers are currently approved by the FDA for use in a variety of biomedical applications and can also be fabricated into protein-loaded microspheres\textsuperscript{107}. Biocompatibility studies have shown that these biomaterials degrade into carboxylic acids, which are non-mutagenic and non-cytotoxic products\textsuperscript{23, 34}. The surface erosion mechanism leads to a controlled release profile with predictable degradation profiles, which can range from days to months, depending on the co-polymer composition\textsuperscript{26, 129}. In addition, studies involving polyanhydride delivery systems for vaccines have shown attractive features such as improved adjuvanticity, antigen stabilization, and enhanced immune responses\textsuperscript{1, 22, 59, 107}.

The main advantage of polyanhydrides over polyesters as antigen carriers is associated with the enhanced protein stability following encapsulation. Studies have shown that polyanhydrides are capable of stabilizing polypeptides and sustaining their release without the inclusion of potentially reactive excipients or stabilizers\textsuperscript{130, 133, 161, 162}. The hydrophobicity and surface erosion characteristics of polyanhydrides prevent water from penetrating to the interior of the microsphere thus preserving the encapsulated antigen in its native state (i.e., increased stability). Furthermore, the
degradation products of polyanhydrides are less acidic than those of polyesters, which may further enhance the stability of encapsulated antigens and reduce tissue reactions to the polymer\textsuperscript{130, 133}. Despite these beneficial characteristics, the use of polyanhydrides for vaccine delivery has not been extensively evaluated.

Recently, \textit{in vivo} studies to evaluate the induction of immune responses following immunization with antigen-loaded microspheres based on the anhydride monomers sebacic acid (SA) and 1,6-bis(p-carboxyphenoxy)hexane (CPH)\textsuperscript{90} were performed. Microspheres encapsulating TT antigen were injected in C3H/HeOuJ mice. These studies demonstrated that TT maintained its immunogenicity and antigenicity following encapsulation. The type of immune response generated, Th1 vs. Th2, was evaluated by antibody isotypes measured by ELISA. It was observed that TT loaded 20:80 CPH:SA microspheres enhanced the immune response after a single dose and indicated a Th2 dominant response. However the 50:50 CPH:SA produced a balanced Th1/Th2 response. Total TT-specific IgG titer remained high regardless of dominant isotype. The preferential enhancement of the Th1 immune response resulting in more balanced immune response (i.e., immune deviation) is a unique and valuable feature of this delivery vehicle that makes it a promising adjuvant candidate for vaccines. Current work is corroborating the immunomodulatory properties of the CPH:SA system with other antigens as well as investigating the adjuvant properties of novel amphiphilic polyanhydride chemistries. Copolymers of CPH and 1,8-bis(p-carboxyphenoxy)-3,6-dioxaoctane (CPTEG), which contain ethylene glycol moieties in the polymer backbone, are promising
candidates for the development of vaccines as it has been shown to provide a conducive environment for protein stabilization\textsuperscript{131-133, 163}.

Anhydride monomers have been copolymerized with other chemistries and their potential as adjuvants have been evaluated. An immunogenic subcellular extract obtained from \textit{Salmonella enteriditis} cells (HE) has been encapsulated in nanoparticles of the copolymer comprised of methyl vinyl ether and maleic anhydride (PVM/MA), best known as Gantrez\textsuperscript{®} polymer\textsuperscript{139}. In this study, 80\% of the Gantrez\textsuperscript{®}-HE immunized mice survived even when the nanoparticle formulation was administered 49 days previous the lethal challenge. As early as 10 days after immunization, a Th1 immune response was demonstrable in these mice as determined by the IgG2a antibody titer in the serum. On the other hand, a dominant Th2 immune response was present at 49 days after immunization (IgG1>IgG2a). Since it is known that a Th1/Th2 balance is required to protect against \textit{Salmonella enteriditis} infection, this copolymer is an ideal candidate for the development of future vaccines. In this regard, blank nanoparticles of Gantrez\textsuperscript{®} administered subcutaneously four weeks prior to challenge induced a level of protection similar to that induced by antigen-loaded nanoparticles or the Rv6 commercial available vaccine against \textit{Salmonella enteriditis} serovar abortusuis\textsuperscript{136}. While the authors did not demonstrate the presence of antigen-specific immunity, this data suggests that the blank nanoparticles were able to induce and sustain sufficient innate immunity to provide non-specific protection against subsequent \textit{Salmonella} infection. In this same study, abortusovis antigen-loaded poly(\varepsilon-caprolactone) microparticles did not induced protection.
In another attempt to design suitable carriers specifically intended for vaccine delivery, Hanes et al. synthesized poly(anhydrides-co-imides) with the adjuvant L-tyrosine incorporated in the polymer backbone. In these studies, a predictable and controlled protein release was observed from microspheres of poly[trimellitylimido-L-tyrosine-co-sebacic acid-co-1,3-bis(carboxyphenoxy)propane] and polymeric implants were well tolerated after subcutaneous implantation in rats. More recent studies demonstrating the suitability of polyanhydrides for use in single dose vaccines involved the design of a core-shelled cylindrical device composed of a biodegradable hydrophobic coating and laminated core of polyanhydrides and polyphosphazenes. Polyanhydrides based on SA were used as isolating layers of the cylinder in order to produce a pulsatile drug release, a mechanism which would minimize doses of vaccines. Even though these polyanhydride systems showed promising characteristics for vaccines design, no further in vivo studies evaluating the characteristics of the proposed adjuvant were validated.

A comparative study between polyanhydrides and polyesters has demonstrated the potential capabilities of polyanhydrides for oral vaccination. Microspheres (0.1-10 μm) composed of fumaric acid (FA) and SA proved to have strong adhesive interactions with the mucosal gastrointestinal lining of rats, as opposed to poly(lactic acid) (LA), which showed minimal uptake. The adhesive interactions are ideal to prolong the biological activity of the delivered antigen or bioavailability of encapsulated drugs. Not surprisingly, plasmid DNA- and anticoagulant drug dicumarol-encapsulated FA:SA microspheres enhanced gene activity and plasma drug levels, respectively, when compared to the controls. In the
same studies, blends of FA and LA were used for insulin delivery and groups that received the formulation were able to regulate glucose levels as opposed to the groups that received insulin only. Even though the biological activity of insulin was preserved, it was the adhesive characteristic of FA the responsible for the efficient delivery.

2.6.4 Other Polymers

2.6.4.1 Natural-derived

Several naturally derived polymeric materials, such as dextran, chitosan, starch, and alginate have been evaluated in laboratory models for use as vaccine adjuvants. In the case of dextran, it has been chemically modified or use in conjunction with other adjuvants in order to improve its immunogenicity. Immunization of cattle with dextran in combination with mineral oil against *Streptococcus bovis* and *Lactobacillus* spp induced the highest serum IgG responses when compared with other adjuvants (i.e. FCA, QuilA, alum), presumably due to the combined effect of both substances\textsuperscript{114}. In studies involving vaccination of cattle against *Mycobacterium tuberculosis*, diethylaminoethyl (DEAE)-dextran induced high levels of IL-2 and low levels of IFN-\(\gamma\), indicating a strong humoral response not desirable for this particular disease\textsuperscript{115}. Interesting results were obtained when a dietary supplementation of Lactobacillus casei with dextran enhances humoral immune responses, and chickens were able to maintain the growth of the bacteria in their intestines and prevent possible infections\textsuperscript{116}. Vaccines that have been evaluated utilizing cross-linked dextran microparticles, containing
conjugated TT induced serum antibody to TT for long periods, eliminating the need of additional booster doses\textsuperscript{127}.

Chitosan, a synthetic cationic polysaccharide from the exoskeleton of insects, can also be formulated into microparticles capable of encapsulating antigen\textsuperscript{128}. Studies with chitosan showed that the immune bias induced by vaccination with antigen containing chitosan microparticles was more dependent on the route of delivery (e. g., intranasal vs. parenteral) than the nature of this adjuvant\textsuperscript{77, 117}. An intranasal delivery of N-trimethyl chitosan chloride (TMC) containing diphtheria toxoid enhanced the immune response when compared with the conventional alum adsorbed vaccine\textsuperscript{128}. This enhancement in nasal vaccination is probably a result of the mucoadhesive properties of chitosan, which enhance penetration across nasal mucosa\textsuperscript{118, 119}. More recent studies with chitosan and TMC establish that chemical variables, such as molecular weight in chitosan and degree of quaternization in TMC influence the magnitude of the immune response after nasal administration\textsuperscript{120}.

Another natural polymer with potential in vaccines is starch, which also has been assessed in mucosal vaccines. Some advantages of starch include its inert properties, proven safety, and commercial availability\textsuperscript{121}. Heritage et al found that human serum albumin delivered on starch microparticles grafted with polydimethylsiloxane stimulated systemic and mucosal immune responses\textsuperscript{122}. Similarly to studies done with chitosan, the route of administration of starch influences the immune response\textsuperscript{123}. Among oral, subcutaneous, and intramuscular administrations, the subcutaneous induced stronger Th2 responses. However, when comparing oral and intramuscular routes, stronger Th2 response was induced after
oral primary administration and a stronger Th1 response after oral booster doses. Although the adjuvant capabilities of starch were proved with success in mice studies, a human vaccine trial was not successful\textsuperscript{166}.

Alginate microparticles offer several advantages for vaccine applications, including good biocompatibility, ease of preparation, and antigen protection during fabrication and administration\textsuperscript{121, 167}. Alginate microparticles have been administered to several animal species (i.e. mice, rabbits, cattle, and chicken)\textsuperscript{167}. The enhancement of the immune response induced in the animals after oral administration with antigen-loaded alginate microparticles shows promise for the development of veterinary vaccines. Nevertheless, in vitro studies show that alginate is not the optimum chemistry to activate human-derived DCs, as it decreases the expression of co-stimulatory molecules and antigen presenting complexes when compared to non-treated cells\textsuperscript{168}. Other in vitro studies that simulated gastric fluid environment showed that alginate microparticles were not able to stabilize live rotavirus vaccines\textsuperscript{169}.

2.6.4.2 Synthetic

Some novel polymer chemistries have been researched to overcome the limitations of available polymers as vaccine carriers. The novel poly(ester-amide) (PEA) copolymers, composed of amino acid residues, diols, and dicarboxylic acids, have been shown to enhance cellular immunity\textsuperscript{124}. Polyamide gives PEA its superior mechanical and thermal properties, while the polyester portion is responsible for its flexibility and hydrolytic susceptibility, allowing PEA to degrade within a reasonable
period of time. It is biodegradable, however, in contrast to polyester and polyanhydrides, PEA degrades by enzymatic cleavage within the body\textsuperscript{135, 170}. Thus, shelf life and handling doesn’t affect its degradation rate and the polymer remains intact until is needed for therapy. PEA has been conjugated with several therapeutics peptides, including human melanoma antigen-derived peptides (MART), a synthetic peptide based on the gp120 protein of HIV, and a MHC\textsubscript{II} restricted T-cell epitope from the influenza A virus hemagglutinin (HA) protein\textsuperscript{124}. In general, the studies evaluating PEA-peptide conjugates demonstrated that cellular immunity, encompassing both MHC\textsubscript{I}- and MHC\textsubscript{II}-restricted T-cell responses, was enhanced.

More recently, in vivo studies in mice have shown that poly(ethylene glycol)-stabilized poly(propylene sulfide) nanoparticles target the APCs directly in the lymph nodes\textsuperscript{137, 138}. In these studies it was found that particles in the size range of 20 to 45nm enter lymphatic vessels and subsequently target DCs in the lymph nodes. The cross-linked polymer system used here degrades into a water soluble polymer under oxidative conditions.

2.6.4.3 Polymers in Plasmid DNA Vaccines

Plasmid DNA vaccines represent a promising alternative against intracellular pathogens. Even though plasmid DNA immunogens have elicited strong cell-mediated responses in small laboratory animals, these have not had success in limited human clinical trials\textsuperscript{125}. Ideal adjuvants will improve the magnitude of plasmid DNA expression, must protect DNA from enzymatic degradation, and must
facilitate the DNA plasmid uptake into cells. Several polymer chemistries have been evaluated in conjunction with DNA vaccines and a thorough discussion of this topic is beyond the scope of this review. In short, microspheres of polylactic, polycarbonates, polystyrene, and poly-(ortho-esters) have been used in DNA vaccination and their administration resulted in enhanced immune responses when compared to naked DNA administrations\textsuperscript{73, 126, 134}. 
2.7 Peptide Antigen-based Cancer Vaccines

2.7.1 Introduction

Currently, there are three standard therapies for cancer treatment, including surgery, chemotherapy, and radiation. Immunotherapy is becoming a fourth promising therapy, with several successful clinical trials currently investigated. Immunotherapy requires the development of complementary strategies that address all the variables involved in immune surveillance. The immune system does not recognize cancer cells as foreign bodies to be eradicated, even though tumor cells display characteristic antigens on their surface. In other words the tumor cells are known to be antigenic but not immunogenic. A possible explanation is that patients with tumor progression have shown a remarkable secretion of immunosuppressive factors and downregulation of various components of the antigen presentation pathway. The immune activation still exists but to a lower extent, where tumor progression rate is slower but not prevented. The goal of the vaccines is to trigger the immune system to attack these malignant cells by means of recognition of these surface antigens and efficiently present them to the cells that are responsible to minimize tumor progression. The focus of this work is on pancreatic cancer, as it is one of the most lethal forms in cancer related diseases.

2.7.2 Antigens for Cancer Therapy

Immunotherapy is becoming the fourth alternative to cancer treatment, with a $6 billion market expected by 2010. These immunotherapy treatments pretend to boost, direct, or restore the patient’s immune system to fight cancer as tumor cells
display specific antigens on their surface. By proper recognition of these markers, an enhanced immune response could be achieved. Two of the pancreatic tumor cell markers that have been well characterized are the glycoproteins Mucin-1 and Mucin-4, which are part of a major group of mucins.

Mucins are a family of large, heavily glycosylated proteins that are expressed by various epithelial tissue. The structural feature common to all mucins is the tandem repeat domain, which comprises identical or highly similar sequences that are rich in serine, threonine and proline residues. These tandem repeats can be differentially O-glycosylated, a characteristic that will determine their structure and function. It has been long suspected that alterations in mucin expression and inappropriate expression of aberrant forms of mucins contribute to the development of cancer by influencing growth, differentiation and immune surveillance\textsuperscript{172}. This aberrant expression in cancer cells is a consequence of the degradation of core proteins during the transformation of the disease\textsuperscript{172}. 
2.7.3 Mucin-1

Among mucins, Mucin-1 is normally produced by epithelial tissue and expressed aberrantly in carcinomas of pancreas, lung, breast, and prostate. The process that leads to over-expression of Mucin-1 in malignant cells is a result of the alterations in the glycosylation pattern with exposure of internal core peptides that are normally masked in normal tissues\textsuperscript{173}. It is precisely this exposure that leads to possible targeting of these peptide epitopes in tumor cells. Fig. 2.6 shows a model of how Mucin-1 is presented on the surface of tumor cells for recognition by the immune system.

Figure 2.6. Tumor antigen processing and presentation. Mucin-1 enters the cytoplasm where is degraded into small peptide fragments by the proteosomes. These fragments are then transported into the endoplasmic reticulum (ER), where they bind to MHC I molecules and subsequently are presented on the surface for immune system’s recognition.

2.7.3.1 Ex vivo Characterization

Extensive research has investigated the characteristics of Mucin-1 in tumor cells. A study done in two pancreatic cell lines, Panc-1 and S2-013, revealed that more than 98% were positive for surface expression of an epitope-tagged form of Mucin-1 \textsuperscript{174}. These results demonstrate for the first time that the heavily O-glycosylated tandem repeat domain of Mucin-1 can simultaneously mediate and
block binding to adhesion molecules with some molecular specificity and further support the hypothesis that Mucin-1 plays a role in the metastatic spread of tumor cells\textsuperscript{175}.

2.7.3.2 In vivo Responses

The antigenicity of Mucin-1 has been widely investigated in animal models. Among all the studies, a remarkable research done in chimpanzees showed that the key to elicit potent immunity to tumor Mucin-1 may be in generating Mucin-1 specific T-helper cell responses and cytotoxic T cell responses by priming Mucin-1 specific CD4\(^+\) T cells and promote Th1 cytokine profile\textsuperscript{176}. These results are in concordance with the hypothesis that a cancer vaccine should prime the activation of MHC II pathway as discussed in the previous section.

Studies in murine models have provided a better understanding of immunogenicity of Mucin-1. A transgenic mice model offers a useful tool to examine how human genetic diseases can be modeled by introducing the same mutation into a mouse or other animal. In 1998, a C57BL/6 mouse transgenic for human Mucin-1 (Mucin-1.Tg) was developed to evaluate Mucin-1 specific tumor immunity. These Mucin-1.Tg mice recognized Mucin-1 as a self protein, and similar to cancer patients, specific responses to this protein should result in autoimmunity. This investigation compared immune responses induced in both, Mucin-1.Tg and wild type (wt) mice, after tumor challenge. Results revealed that wt mice developed protective tumor immunity mediated by Mucin-1 specific CD4\(^+\) T lymphocytes, while Mucin-1.Tg mice were functionally tolerant to Mucin-1 and their tumor growth could
not be controlled\textsuperscript{177}. This implies that by tuning the immune response to actively recognize Mucin-1 as a non-self protein on cancer cells will lead to tumor rejection.

2.7.3.3 Influence of Injection Location

Tumor immunity specific for Mucin-1 was produced in wt mice (C57BL/6) by two different procedures: subcutaneous (sc) immunization with a low dose of the pancreatic cancer cell line Panc02-Mucin-1 and by adoptive transfer of spleen and lymph node cells harvested from wt mice previously immunized sc with Panc02-Mucin-1. Positive results of the two procedures were obtained, which demonstrated that immune responses to Mucin-1 presented at the sc site can be detected and adoptively transferred\textsuperscript{178}. Similarly, another study compared mice challenged with the same cancer cell line (Panc02-Mucin-1) by orthotopic injection into the pancreas and by sc injection\textsuperscript{179}. The immune responses produced by sc injection resulted in rejection of tumors that were subsequently challenged at the pancreatic site. This paper disproved the hypothesis that the sc environment down regulates expression of Mucin-1 in these tumor cells. These studies evidenced that Mucin-1 can be successfully recognized when injected as a vaccine and induce the immune response when is properly presented.

2.7.3.4 Clinical Studies

Several clinical studies have investigated the characteristics of Mucin-1 in pancreatic cancer patients. In one of the immunohistochemical studies, invasive carcinoma with a poor outcome showed a pattern of Mucin-1 positive, whereas
many of the non-invasive tumors with favorable outcome showed a pattern of Mucin-1 negative\textsuperscript{180}. This confirms that Mucin-1 is a molecule typically expressed in malignant tumors, which is in concordance with previous findings on \textit{ex-vivo} and \textit{in-vivo} studies discussed above. Other clinical studies on pancreatic cancer patients showed that tumor antigen specific T cell responses occur regularly during pancreatic cancer disease and lead to the enrichment of tumor cell-reactive memory T cells in the bone marrow\textsuperscript{181}, clearly indicative of the immunogenicity of the disease. These memory T cells have a therapeutic potential as they display a predominant Th1 cytokine profile upon stimulation of DCs and are able to kill autologous tumor cells \textit{ex-vivo}. Several questions to be answered regarding tumor immunity include does the immune system has all the tools to fight cancer, why it becomes tolerant and does not produce the necessary response against the disease. Previous research has reported a dominance of Th2 cytokines in the blood of 41 cancer patients\textsuperscript{181}. The dominance of Th2 cytokines suggests systemic tumor-induced immunosuppression, which potentially inhibit the induction of tumor-reactive T cells and tumor surpass immune surveillance. The ability of polyanhydride-based adjuvants to provide immunomodulatory capabilities makes them promising carriers for cancer vaccines.
2.7.4 Mucin-4

Other mucin that has been envisioned as a potential candidate for tumor vaccine antigen is Mucin-4, which is also a membrane associated mucin. It contains three epidermal growth factors domains that function in receptor-ligand interactions, two regions rich in potential N-glycosylation sites, one hydrophobic transmembrane region, and one short cytoplasmic tail\textsuperscript{172, 182}. Similarly to Mucin-1, Mucin-4 contributes to the regulation of differentiation, proliferation, and metastasis of tumor cells. It is aberrantly expressed in pre-malignant and malignant lesions of the pancreas but not expressed in normal pancreatic epithelial cells, a characteristic that makes this antigen very promising for cancer immunotherapy as healthy tissues will likely remain healthy\textsuperscript{183}. Studies done in nude mice showed that after injecting pancreatic cell lines expressing human Mucin-4, high levels of Mucin-4 were detected in the tumor injected orthotopically but not in the tumor injected subcutaneously nor in the normal pancreas tissue\textsuperscript{182}.

Mucin-4 has also been correlated with poor patient prognosis in other types of cancer such as lung adenocarcinomas\textsuperscript{184}. In this recent work, it has been demonstrated that antibodies against Mucin-4 have been detected in patients with lung cancer, suggesting that this glycoprotein is immunogenic in cancer patients and represent an excellent antigen candidate for the development of future cancer vaccines.
2.8 Conclusions

Polyanhydrides are promising as biomaterials since they possess a unique combination of properties that includes hydrolytically labile backbone, hydrophobic bulk, and chemistry that can be easily combined with other functional groups to design novel materials. These materials are primarily surface-erodible and offer the potential to stabilize protein drugs and sustain release from days to months. The microstructure characteristics of copolymer systems can be exploited to tailor drug release profiles. The versatility of polyanhydride chemistry promises a new class of drug release systems for specific applications. The powerful combination of protein stabilization and immune deviation provided by amphiphilic polyanhydrides offers tantalizing prospects for development of cancer vaccines which is the main goal of this research.
2.9 References


CHAPTER 3
RESEARCH OBJECTIVES

3.1 Research Objectives

The overall goal of this research was to design novel biodegradable adjuvants that can be used a single dose vaccine with the dual functions of protein stabilization and enhanced immune response. In order to accomplish this goal, a fundamental characterization of the novel material system must be performed, as the chemistry and properties of the material affects drug stabilization and release. The importance of preserving conformational epitopes to elicit a protective immune response is essential for vaccine effectiveness. In vitro evaluation of this stability is essential to prove that a new formulation will not be deleterious for the therapeutic value of the protein drug. For this reason, an accurate assessment of the stability of the protein drug during conditions that mimic the in vivo release environment will be analyzed with several model proteins. Using the insights gained, the immunomodulatory capabilities of CPTEG:CPH microsphere formulations will be evaluated for testing the viability of this system as an effective adjuvant for the development of cancer vaccines.
3.2 Specific Goals

The specific goals (SG) of this work are:

**SG1**: Synthesis and characterization of the novel amphiphilic CPTEG:CPH system.

**SG2**: Assessment of protein stabilization and sustained release from CPTEG:CPH system.

**SG3**: *Ex vivo* and *in vivo* evaluation of the immunomodulatory capabilities of CPTEG:CPH microspheres.
CHAPTER 4
SYNTHESIS AND CHARACTERIZATION OF NOVEL POLYANHYDRIDES WITH
TAILORED EROSION MECHANISMS

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4.1 Abstract

We have designed a new synthesis route to create polyanhydrides based on
monomers that contain hydrophilic entities within highly hydrophobic backbones.
The method results in polyanhydrides that can be easily processed into drug-
containing tablets. The synthesis, characterization and erosion studies of
polyanhydride copolymers based on 1,6-bis(p-carboxyphenoxy)hexane (CPH),
which is highly hydrophobic, and 1,8-bis(p-carboxyphenoxy)-3,6-dioxaoctane
(CPTEG), which has hydrophilic oligomeric ethylene glycol segments in the
monomer unit, was performed using a combination of molecular spectroscopy,
thermal analysis, gravimetry, and scanning electron microscopy. The studies demonstrate that by increasing the CPH content in the CPTEG:CPH copolymers, the erosion of the system can be tailored from bulk-eroding to surface-eroding mechanism. These systems have promise as protein carriers.

4.2 Introduction

Polyanhydrides are a class of surface bioerodible polymers that have been extensively used as carriers for controlled drug delivery. The promising characteristics of this class of polymers has led to extensive research on the chemistry, microstructure, and biocompatibility in the last two decades\textsuperscript{1-12}. The biocompatibility studies, both \textit{in vitro} and \textit{in vivo}, have shown that these biomaterials degrade into non-mutagenic and non-cytotoxic products\textsuperscript{13,14}. The surface erosion mechanism exhibited by these polymers leads to a controlled release profile with a predictable hydrolytic degradation, which can range from days to months, depending on the polymer chemistry.

The overall goal of our research is to engineer biomaterials suitable for the stabilization and sustained release of proteins. Currently there are several hundred investigational new protein drugs that have not been approved by the United States Food and Drug Administration (FDA) due the lack of a suitable delivery device\textsuperscript{15}. Finding the appropriate carrier for proteins is a complex task that involves a fundamental understanding of the inactivation mechanisms of the protein, the interactions between the protein and the carrier, and the carrier chemistry.
It has been suggested that the use of carriers containing both hydrophobic and hydrophilic entities may provide a gentler environment for proteins\textsuperscript{16,17}. Previous research has demonstrated that polyanhydrides, which are highly hydrophobic, can prevent covalent aggregation by reducing the water penetration into the core\textsuperscript{18-20}. However, strong hydrophobic interactions between the polymer and the protein may lead to non-covalent aggregation. Thus, our central focus involves the incorporation of hydrophilic entities, i.e. oligomeric ethylene glycol, into the backbone of an aromatic polyanhydride to create a potentially suitable protein carrier\textsuperscript{21}. The choice of ethylene glycol is motivated by its advantageous properties, including the stealth effect provided to various active macromolecules, its biocompatibility, and its low toxicity\textsuperscript{22}. Previous studies have demonstrated that poly(ethylene glycol) is a useful carrier for oligonucleotides and ribozymes when polymerized with an aliphatic polyamine\textsuperscript{23,24}. When copolymerized with bulk eroding poly(lactide-co-glycolide) or poly(lactic acid), polyethylene glycol adds the hydrophilicity necessary for a faster degrading system for delivery of peptides and proteins\textsuperscript{16,17,25}, with the added disadvantage of not preventing potentially deleterious water-protein interactions.

It is important to point out that aliphatic polyanhydrides have been copolymerized with segments of polyethylene glycol\textsuperscript{26} resulting in a fast degrading system that is not desirable for a long-term controlled release application. To overcome this disadvantage, we incorporated oligomeric ethylene glycols (i.e., triethylene and pentaethylene glycol) into the monomer of a hydrophobic anhydride\textsuperscript{21}. These materials were synthesized by halogenation of oligomeric ethylene glycol chains, which were then reacted with $p$-hydroxybenzoic acid to
produce the monomer. The monomer was then polymerized with acetic anhydride and heated under vacuum. The yields of the resulting monomer were low, the procedure was complex, and the resulting polymer did not have desirable characteristics for device fabrication21.

In this paper, we have developed a modified synthesis route, in which the halogenation of the ethylene glycol chain is not necessary. This method results in polymers that are easy to process into pharmaceutical formulations such as tablets and microspheres. This paper focuses on copolymers based on the anhydride monomers 1,6-bis(\(\rho\)-carboxyphenoxy)hexane (CPH) and 1,8-bis(\(\rho\)-carboxyphenoxy)-3,6-dioxaoctane (CPTEG), which contains oligomeric ethylene glycol moieties (the chemical structures are shown in Fig. 4.1). The synthesis, characterization and erosion mechanism of these novel polyanhydrides are discussed.

Figure 4.1. Chemical structures of poly(CPH) (top) and poly(CPTEG) (bottom). The letters (a-i) represent the peaks in the \(^1\)H NMR spectra shown in Fig. 4.3 and 4.6.
4.3 Experimental

4.3.1 Materials

The chemicals 4-hydroxybenzoic acid, 1,6-dibromohexane and tri-ethylene glycol were purchased from Sigma Aldrich (St. Louis, MO); 4-\(p\)-fluorobenzonitrile was purchased from Apollo Scientific (Cheshire, UK); acetic anhydride, methylene chloride, potassium carbonate, petroleum ether, toluene, dimethyl formamide (DMF), sulfuric acid, acetic acid, and acetonitrile were obtained from Fisher Scientific (Fairlawn, NJ). Deuterated chemicals for NMR analysis (chloroform and dimethyl sulfoxide (DMSO)) were purchased from Cambridge Isotope Laboratories (Andover, MA).

4.3.2 Monomer Synthesis

The CPH monomer was synthesized from 4-hydroxybenzoic acid using a procedure that was first developed by Conix \(^{27}\). To synthesize the CPTEG monomer, 45 mL of tri-ethylene glycol, 100mL of toluene, 300 mL of dimethyl formamide and 0.897 mol of potassium carbonate were mixed in a round bottom flask placed in an oil bath at 170°C. The addition of toluene allowed the azeotropic distillation of water from the reaction mixture prior to reaction. Next, 0.684 mol of 4-\(p\)-fluorobenzonitrile was added and allowed to react overnight at 150°C. After cooling, all the solvents were removed using a rotary evaporator. The resulting dinitrile solution was hydrolyzed with a mixture containing equal volumes (50 mL) of water, acetic and sulfuric acid. The reaction was carried out at 160°C under a nitrogen atmosphere. The resulting diacid was precipitated using 1L of deionized water. A white powder
was obtained after successive washes with acetonitrile. A generalized procedure for CPTEG monomer synthesis is presented in Scheme 4.1.


4.3.3 Polymer Synthesis

Due to problems with prepolymer isolation, a new procedure for making poly(CPTEG) and CPTEG:CPH copolymers, starting directly from the diacids, was developed. The synthesis of poly(CPTEG) is shown in Scheme 4.2. In a typical experiment, 2g of the monomer and 100mL of acetic anhydride were added to a round bottom flask and reacted for 30 minutes at 125°C. The acetic anhydride was removed in the rotary evaporator and the resulting viscous liquid was polymerized in an oil bath at 140°C, under vacuum (<0.03 torr) for 90 minutes. The polymer was
isolated by precipitating from methylene chloride into petroleum ether in a 1:10 ratio. The copolymer compositions synthesized were 20:80, 50:50, and 80:20 CPTEG:CPH. Additionally, homopolymers of CPH and CPTEG were synthesized. The CPTEG homopolymer was synthesized by polycondensation of the CPTEG monomer as described above.

\[
\text{Scheme 4.2. Synthesis of poly(CPTEG).}
\]

**4.3.4 Characterization**

The purity of the monomers and polymers was verified using \(^1\)H NMR spectra obtained from a Varian VXR-300 MHz NMR spectrometer and infrared spectra obtained from a Nicolet 6700 FT-IR spectrometer. Number average molecular weights were estimated by end group analysis from \(^1\)H NMR spectra. Perkin Elmer DSC 7 and DMA were used for the thermal characterization. The samples were heated in two cycles from -20 to 110\(^\circ\)C at a rate of 5\(^\circ\)C/min in the DSC. For the DMA analysis, 100 mg tablets were heated from -20 to 100\(^\circ\)C at a rate of 3\(^\circ\)C/min, and a
three point bending test was performed at a frequency of 1 Hz, a dynamic force of 90 mN and a static force of 100 mN. Scanning Electron Microscopy (SEM) (Hitachi S-2460 N) was used to study the surface and the cross section of the polymer tablets during erosion. The dried tablets were coated with gold prior to imaging.

4.3.5 Erosion and Degradation Studies

Tablets of 100 mg of poly(CPTEG), poly(CPH) and 50:50 CPTEG:CPH copolymer were melt compressed for 2 min in a Carver Press (Wabash, IN) at a pressure of 600 psi and at a temperature just above the melting point of the polymer. Then the tablets were placed into 25 mL of phosphate buffer (0.1M, pH 7.4) in an incubator operating at 37°C and 100 rpm. The buffer was replaced daily. At different time intervals, duplicate samples of tablets were taken out of the buffer for further analysis. The water swelling and the mass loss of the tablets were determined by gravimetric analysis. The surface morphology of the tablets was monitored by SEM.

4.3.6 CPTEG Monomer Solubility

To further characterize the erosion mechanism, the solubility of CPTEG monomer was determined by dissolving an excess of diacid in 15 mL of phosphate buffer at different pH values. The pH values were adjusted with 0.1 M NaOH or 0.1 M HCl. The diacid concentration was calculated from UV absorbance (Shimadzu) using Beer’s law at 288 nm.
4.4 Results and Discussion

4.4.1 Structural Characterization

The $^1$H NMR spectrum of CPTEG monomer is shown in Fig. 4.2. The peak designation demonstrates the purity of the diacid produced with the new synthesis route, as no traces of solvents were present in the product. The $^1$H NMR spectra in Fig. 4.3 confirm the successful synthesis of poly(CPTEG), CPTEG:CPH copolymers, and poly(CPH). The peak designations with respect to deuterated chloroform ($\delta=7.26$ ppm) was confirmed based on reported values$^{21,29}$. The aromatic proton peaks of CPTEG and CPH monomers (a-d) have the characteristic chemical shifts in the $\delta=6.8-8.1$ ppm range. The inner chain protons close to the electronegative oxygen atoms in both monomers (e-h) are represented in the expected range of 3.6-4.4 ppm, and the protected protons in the inner chain of CPH (j,k) are represented at $\delta=1.7$ and 1.5 ppm. Finally, the acetylated end groups (i) have a chemical shift at $\delta=2.1$ ppm. The actual composition of the polymers after polymerization was calculated from an end group analysis of the spectra and the results are shown in Table 4.1. There is excellent agreement between the molar feed ratio and the actual compositions of the polymers. This behavior has also been observed for polyanhydrides based on sebacic anhydride (SA), 1,3-bis($p$-carboxyphenoxy)propane (CPP), and CPH$^{29,30}$.

<table>
<thead>
<tr>
<th>Molar feed composition CPTEG:CPH</th>
<th>Actual molar composition CPTEG:CPH</th>
</tr>
</thead>
<tbody>
<tr>
<td>20:80</td>
<td>23:77</td>
</tr>
<tr>
<td>50:50</td>
<td>49:51</td>
</tr>
<tr>
<td>80:20</td>
<td>77:23</td>
</tr>
</tbody>
</table>
Fourier Transform-Infrared (FT-IR) spectroscopy was also used to characterize polyanhydride composition. The IR spectra (not shown) of CPTEG:CPH copolymers had a doublet at 1720 and 1780 cm\(^{-1}\) indicating the presence of a carboxylic anhydride of an aromatic polymer\(^3\). The presence of a peak at \(~2900\ \text{cm}^{-1}\) represented the O-H bond of the carboxylic acid\(^1\) in the IR spectrum of the monomers (not shown).

The number average molecular weight of the polymers was estimated by calculating the degree of polymerization (DP) using the peak area normalized with the protons represented (as shown in Eq. 1). In Eq. 1, the parentheses indicate peak areas of the corresponding species in the \(^1\text{H}\) NMR spectra, the asterisk (*)
represents hydrolyzed chains and Ac represents acetylated chains. The molecular weights of the polymers synthesized ranged from 4,000-14,000 g/mol, which is in the sensitivity range of $^1$H NMR$^{31}$. We note that the sensitivity is high because of the presence of three protons in the end group.

$$DP = \frac{(\text{CPTEG}) + (\text{CPH})}{(\text{Ac}) + (\text{CPTEG}^*) + (\text{CPH}^*)}$$ (1)

Figure 4.3. $^1$H NMR spectra of (a) poly(CPTEG), (b) 80:20, (c) 50:50, (d) 20:80 CPTEG:CPH copolymers, and (e) poly(CPH).
4.4.2 Thermal Characterization

A summary of the thermal properties is shown in Table 4.2. The glass transition temperatures were determined from the DMA studies. The CPTEG homopolymer and all the CPTEG:CPH copolymers have $T_g$s below 20°C and hence are rubbery at room temperature. The rubbery state of these polymers at room temperature is desirable for processing into tablets. The DSC studies for the CPTEG homopolymer and the CPTEG:CPH copolymers did not exhibit any melting peaks, indicating that these polymers are amorphous. In contrast, poly(CPH) melts at 143°C and has a $T_g$ of 47°C \(^\text{12}\), indicating that when copolymerized with CPTEG, the crystal formation is disrupted. This is important since polymer crystallinity affects the erosion mechanism of the polymer, since crystalline regions erode more slowly than the amorphous ones\(^\text{28}\). Thus, it is expected that copolymers rich in CPTEG and the poly(CPTEG) homopolymer would have faster erosion rates and erosion mechanisms that deviate from pure surface erosion.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>$T_g$ (°C)</th>
<th>$T_m$ (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>poly(CPTEG)</td>
<td>9</td>
<td>---</td>
</tr>
<tr>
<td>80:20 CPTEG:CPH</td>
<td>7</td>
<td>---</td>
</tr>
<tr>
<td>50:50 CPTEG:CPH</td>
<td>8</td>
<td>---</td>
</tr>
<tr>
<td>20:80 CPTEG:CPH</td>
<td>18</td>
<td>---</td>
</tr>
<tr>
<td>Poly(CPH)</td>
<td>47</td>
<td>143</td>
</tr>
</tbody>
</table>

Table 4.2. Thermal characterization (* Data from Ref. 12).
4.4.3 Erosion and Degradation

Polymer erosion is a complex process that is determined by numerous factors that include the molecular weight loss (degradation), the swelling, the dissolution and diffusion of oligomers and monomers, and morphological changes\textsuperscript{32,33}. The erosion of poly(CPTEG), poly(CPH), and the 50:50 CPTEG:CPH copolymer was monitored by following mass loss, polymer swelling, molecular weight changes, monomer dissolution, and surface morphology.

The mass loss from the polyanhydride tablets was determined by gravimetry (Fig 4.4). In a period of 28 days, the poly(CPTEG) homopolymer lost \(\sim80\%\) of its total mass, which is attributed to the increased hydrophilicity due the oligomeric ethylene glycol in the aromatic polyanhydride. In the same period, less than 5\% mass was lost in the same period from the highly hydrophobic poly(CPH) homopolymer. The 50:50 CPTEG:CPH copolymer eroded at a rate in between that of the two homopolymers. From these results it can be seen that the polymers exhibit distinct erosion profiles that can be controlled by tailoring copolymer composition. These results are also consistent with the DSC studies, which showed that both poly(CPTEG) and the 50:50 CPTEG:CPH copolymers are amorphous, which would lead to faster erosion rates.
The swelling induced in the polymers due to water uptake may affect the rate of drug release. In surface erodible materials, the drug release is mainly controlled by erosion kinetics\textsuperscript{28}, since no water can penetrate into the system. On the other hand, bulk erodible polymers release drugs as a result of various processes occurring in unison, including erosion kinetics, swelling, and diffusion\textsuperscript{16,25}. The water content present in each sample is shown in Fig 4.5. Even though the mass loss of poly(CPTEG) and 50:50 CPTEG:CPH copolymer was remarkably different (Fig. 4.4), the water content of both polymers followed the same trend. A significant amount of water entered into these tablets, suggesting a transition to a bulk erodible system as the CPTEG content in the copolymer increased. After 14 days, the water content of
poly(CPTEG) exceeded 50%, while in poly(CPH), the water content did not exceed 5%, demonstrating the well known surface erosion mechanism of these hydrophobic polyanhydrides. The water content in the 50:50 CPTEG:CPH copolymer was in an intermediate range between the two homopolymers for the duration of the erosion studies. These data suggest that the erosion of this family of polyanhydrides (i.e., CPTEG-based) can be tuned from “bulk” to “surface” by copolymerizing with hydrophobic CPH.

The degradation of the polymers was studied using $^1$H NMR spectra of eroded tablets in deuterated DMSO ($\delta \sim 2.5$ ppm). Fig. 4.6 shows the degradation progress of 50:50 CPTEG:CPH copolymer from day 0 to day 28. As degradation proceeds, the end group (i) shifted from $\delta = 2.1$ ppm to $\delta = 3.3$ ppm. It is instructive to note the significant decrease of the peak area of the protons corresponding to the polymer backbone (e-k) and the increase in the end group acid peak (i, $\delta \sim 3.3$ ppm) after 28 days of degradation. On day 28, the decreased peaks that still prevail in the spectra correspond to the CPH monomer, clearly indicating that CPH is the last monomer to be released. The molecular weight loss from each polymer composition studied is shown in Fig. 4.7. It is observed that poly(CPTEG) and the 50:50 CPTEG:CPH copolymer display similar rates of molecular weight loss, i.e., 93% for poly(CPTEG) and 85% for the 50:50 CPTEG:CPH copolymer. As expected, the hydrophobic poly(CPH) loses only 8% of its initial molecular weight after 28 days. These studies demonstrate that the added hydrophilicity enhances the degradation rate of the polyanhydride and this is supported by the water penetration data into polymers containing CPTEG (Fig. 4.5).
Figure 4.5. Fractional water content in tablets of poly(CPTEG), 50:50 CPTEG:CPH copolymer and poly(CPH). Error bars indicate standard deviation.

Figure 4.6. $^1$H NMR spectra of 50:50 CPTEG:CPH copolymer tablets during erosion.
Figure 4.7. Percent molecular weight loss from eroding tablets of poly(CPTEG), 50:50 CPTEG:CPH copolymer, and poly(CPH).

The studies indicate that poly(CPTEG) and the 50:50 CPTEG:CPH copolymer have different erosion rates, but similar water swelling and polymer degradation rates. Since erosion is a combination of polymer degradation, water swelling, monomer dissolution, and diffusion\textsuperscript{28,34}, we investigated the solubility of both monomers (Fig. 4.8). The data indicate that the saturation concentration for CPTEG is at least an order of magnitude greater than that of CPH. This difference in monomer solubility may govern the erosion mechanism of the polymer that can be varied from bulk to surface erosion by increasing the CPH content. The solubility data was also used to estimate the logarithmic scales of acidity constants for each diacid, i.e. pKa. These values were estimated by the inflection points of the fitted data. It has been reported that CPH has pKa’s at 3.7 and 6.7\textsuperscript{28}. Our data shows that
CPTEG has pKa's at 5.8 and 8.4, indicating that CPH is a stronger acid. This data is important since the dissolution of the monomers decreases the pH of the microenvironment of an eroding polymer, which in turn limits the monomer solubility, as the dicarboxylic acid monomers become much less soluble at low pH \(^{28}\).

![Graph showing saturation concentration data of CPTEG and CPH monomers in aqueous buffer](image)

Figure 4.8. Saturation concentration data of CPTEG and CPH monomers in aqueous buffer (CPH solubility data from Ref. 31).

Finally, we studied the surface morphology of the tablets during erosion using SEM. The surface morphology of the polymers after 7 and 28 days of exposure to buffer are shown in Fig. 4.9. The poly(CPTEG) images show the homogeneous porosity characteristic of bulk eroding polymers where water entered easily into the tablet\(^{35}\). In contrast, the 50:50 CPTEG:CPH copolymer exhibited a smooth surface with small erosion fronts throughout the tablet. This behavior is typical of systems that exhibit features of both bulk and surface erosion. The surface eroding
poly(CPH) exhibited a smooth surface throughout the 28 day study, consistent with the water penetration studies discussed previously (Fig. 4.5). These results indicate that by varying the CPTEG content in copolymer systems, the erosion mechanism can be tailored from bulk to surface erosion.

Figure 4.9. SEM micrographs depicting surface morphology of poly(CPTEG), 50:50 CPTEG:CPH copolymer, and poly(CPH) tablets after 7 days (top) and 28 days (bottom) of erosion.
4.5 Conclusions

We have synthesized a new class of “bulk eroding” polyanhydrides with tailored amphiphilicity with potential for controlled drug and protein delivery. An improved procedure was developed to synthesize these polymers, which resulted in low-\(T_g\) materials that can be easily processed. When copolymerized with hydrophobic aromatic polyanhydrides, these systems can be tailored from “bulk-eroding” to “surface-eroding”. Such a combined erosion mechanism may prevent protein denaturation by avoiding both hydrophobic non-covalent interactions and covalent aggregation of proteins. We are currently evaluating the potential of these novel biomaterials as protein carriers.

4.6 Acknowledgements

We would like to thank the Whitaker Foundation and the ISU Institute for Combinatorial Discovery for financial support. MPT acknowledges the support of the NSF-Iowa AGEP Fellowship. We also thank Michael Determan for the DMA experiments and Mary Byron, an undergraduate student, who helped with the synthesis.
4.6 References


CHAPTER 5

AMPHIPHILIC POLYANHYDRIDES FOR PROTEIN STABILIZATION AND RELEASE


Maria P. Torres1,2, Amy S. Determan1,3, Gretchen L. Anderson1,4, Balaji Narasimhan1,5, and Surya K. Mallapragada1,5

5.1 Abstract

The overall goal of this research is to design novel amphiphilic biodegradable systems based on polyanhydrides for the stabilization and sustained release of peptides and proteins. Accordingly, copolymers of the anhydrides, 1,6-bis(p-carboxyphenoxy)hexane (CPH) and 1,8-bis(p-carboxyphenoxy)-3,6-dioxaoctane (CPTEG), which is a monomer containing oligomeric ethylene glycol moieties, have been synthesized. Microspheres of different CPTEG:CPH compositions have been fabricated by two non-aqueous methods: solid/oil/oil double emulsion and cryogenic atomization. The ability of this amphiphilic polymeric system to stabilize model proteins (i.e., lysozyme and ovalbumin) was investigated. The structure of both the encapsulated as well as the released protein was monitored using gel electrophoresis, circular dichroism, and fluorescence spectroscopy. It was found that
the CPTEG:CPH system preserves the structural hierarchy of the encapsulated proteins. Activity studies of the released protein indicate the CPTEG:CPH system retains the biological activity of the released protein. These results are promising for future in vivo studies, which involve the design of novel biodegradable polyanhydride carriers for the stabilization and sustained release of therapeutic peptides and proteins.

5.2 Introduction

A recent study revealed that over a quarter of all new drugs approved are biopharmaceuticals with an annual global market surpassing $30 billion. This group includes peptides and proteins intended for therapeutic treatment of a wide range of diseases, including reproductive disorders, blood related diseases, hepatitis B and C, and cancer. Nevertheless, the efficient delivery of these fragile molecules, which exhibit both physical and chemical instability leading to short in vivo half lives, remains a challenge.

The ideal protein carrier must protect the protein from the physiological environment and provide sustained release kinetics ranging from days to months depending on the application. Biodegradable polymeric microspheres have been used successfully in protein delivery. Some of the characteristics of biodegradable carriers that can be manipulated to maintain protein stability include: water swelling, hydrophobicity, and chemical nature of degradation products.

Proteins must be stabilized during device preparation, storage, and administration. Previous research has demonstrated limitations in these stages that
lead to protein inactivation. During device fabrication, the common methods employed for encapsulation expose the protein drug to aqueous/organic interfaces, which are known to be problematic for protein stability\textsuperscript{5, 6}. Protein migrates towards the aqueous dispersing phase and as much as 40\% of protein loaded has been shown to be lost during fabrication\textsuperscript{3, 7}. New attempts to improve protein stability and maximize loading during microsphere fabrication circumvent this problem by using non-aqueous methods, such as solid/oil/oil (S/O/O) double emulsion and cryogenic atomization (CA)\textsuperscript{8-11}. Besides promoting protein stability, these techniques increased the encapsulation efficiency, with efficiencies as high as 85\% in S/O/O and \~100\% in CA, as we reported recently\textsuperscript{10}.

During protein administration, the chemistry of the polymeric carrier must be carefully chosen so as to provide a gentle environment that will maintain the activity of the protein drug. Among biodegradable polymers, bulk-erodible polyesters (e.g., poly(lactic-co-glycolic acid) or PLGA) have been extensively investigated\textsuperscript{12-18}. Deleterious processes occurring during protein release from these polymers have been reported including an acidic microenvironment and strong hydrophobic interactions\textsuperscript{4, 19, 20}. As PLGA degrades, the water content increases, characteristic of bulk erodible systems, and the local acidic environment produced by accumulation of degradation products are significant sources for irreversible physical and chemical inactivation of polypeptides and proteins.

Another class of biodegradable polymers investigated for protein delivery is polyanhydrides, which differ from polyesters in their erosion mechanism. Polyanhydrides exhibit surface erosion, which may prevent covalent aggregation by
reducing water penetration into the device\textsuperscript{21-24}. However, these materials are hydrophobic and strong hydrophobic interactions between the polymer and the protein may lead to non-covalent aggregation.

A promising alternative for the polymers discussed above is the use of amphiphilic carriers for protein stabilization\textsuperscript{25-28}. Correspondingly, we have designed a novel amphiphilic polyanhydride system based on copolymers of the anhydride monomers, 1,6-bis(\(\rho\)-carboxyphenoxy)hexane (CPH) and 1,8-bis(\(\rho\)-carboxyphenoxy)-3,6-dioxaoctane (CPTEG) (Fig. 5.1), which contains oligomeric ethylene glycol\textsuperscript{29, 30}. The incorporation of oligomeric ethylene glycol into the backbone of an aromatic polyanhydride creates the necessary hydrophilicity to create the amphiphilic environment needed for protein stabilization. Moreover, it is due to this amphiphilicity that the erosion mechanism of the CPTEG:CPH system can be tuned from bulk to surface by increasing the CPH content of the copolymer.

\begin{center}
\includegraphics[width=0.5\textwidth]{chemical_structures.png}
\end{center}

\textit{Figure 5.1. Chemical structures of poly(CPH) (top) and poly(CPTEG) (bottom).}

This paper focuses on protein stabilization and sustained release from microspheres based on the CPTEG:CPH system. The proteins chosen for this study are hen egg white lysozyme (Lys) and ovalbumin (Ova). Lys is an acid stabilized protein of 129 amino acids (14 kDa) that has been well studied, and the value of
using it as a model protein for release relies on its similar size to therapeutic
cytokines such as interferons and interleukins\textsuperscript{17}. On the other hand, Ova has been
well studied as a model antigen and is composed of 385 residues (48 kDa). It has a
molten globular or intermediate unstable state in acidic surroundings, which is
deleterious for its activity\textsuperscript{31}. Two non-aqueous methods, S/O/O and CA, were used
to fabricate the microspheres.

5.3 Materials and Methods

5.3.1 Materials

The chemicals needed for monomer synthesis 4-\textit{p}-hydroxybenzoic acid, 1,6-
dibromohexane, 1-methyl-2-pyrrolidinone, and tri-ethylene glycol were purchased
from Sigma Aldrich (St Louis, MO); 4-\textit{p}-fluorobenzonitrile was obtained from Apollo
Scientific (Chesire, UK); potassium carbonate, dimethyl formamide, toluene, sulfuric
acid, acetic acid, and acetonitrile were purchased from Fisher Scientific (Fairlawn,
NJ). The chemicals needed for the polymerization, acetic anhydride, methylene
chloride, and petroleum ether, were purchased from Fisher Scientific (Fairlawn, NJ).
Chicken egg white Ova, hen egg white Lys, monoclonal anti-chicken egg albumin
(clone Ova-14), rabbit anti-chicken egg albumin, alkaline phosphatase conjugated
goat anti-rabbit IgG, fetal calf serum (FCS), Coommasie R-250, Sigma 104
phosphatase substrate, \textit{p}-nitrophenyl phosphatase (\textit{pNPP}) liquid substrate system,
and XTT \textit{in-vitro} toxicology assay kit were purchased from Sigma Aldrich (St Louis,
MO). Ready precast gels (15% acrylamide) and protein molecular weight standards
were purchased from Bio-Rad Laboratories (Hercules, CA). The enzymatic activity of
Lys was determined with the EnzCheck® Lysozyme assay kit from Invitrogen (Carlsbad, CA). Bicinchoninic acid (BCA) protein assay kit and Slide-A-Lyzer dialysis cassettes (10,000 MW cut off membrane) were obtained from Pierce (Rockford, IL).

5.3.2 Polymer Synthesis and Characterization

As we reported recently, CPTEG:CPH copolymers were synthesized by melt polycondensation of CPH and CPTEG diacids\textsuperscript{29}. The purity and degree of polymerization of the polymers was analyzed using \textsuperscript{1}H NMR spectra obtained from a Varian VXR-300 MHz NMR spectrometer.

5.3.3 Protein Incubation in Monomer Solutions

Saturated solutions of CPTEG and CPH diacids in deionized water and phosphate buffer (0.1 M, pH 7.4) were placed in the incubator for two days (37 °C, 100 rpm) and, subsequently, were filtered (0.22 μm). Lyophilized protein (final concentration 250 μg/mL) was added to the respective solutions (CPTEG, 50/50 CPTEG/CPH, CPH, phosphate buffer) and incubated for 1 week (37°C, 100 rpm) prior to structural analysis of the protein. These studies were performed in triplicate and the protein structural analysis described below was done as recently described\textsuperscript{32}. 
5.3.3.1 Primary Structure

The amino acid sequence of each protein before and after incubation was studied with sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. After centrifuging samples (10,000 rpm, 4 ºC, 10 min), 10 μL of each sample was mixed with 10 μL of the reducing buffer (SDS (1% w/v), Tris-HCl (pH 6.8, 0.06 mM), glycerol (3 mM), bromophenol blue (0.01% w/v), and β-mercaptoethanol (0.05% v/v)). The 20 μL solution was heated for 10 minutes at 96 ºC, cooled to room temperature, and loaded into 15% acrylamide pre-cast gel and run for 60 minutes at 130 V. 5 μL of pre-stained low range protein standards were used for reference. After running the gels, these were stained with Coommasie Blue for 30 min and destained overnight.

5.3.3.2 Secondary Structure

Far UV Circular Dichroism (CD) (190-250 nm) was used to monitor the protein secondary structure as recently described32.

5.3.3.3 Tertiary Structure

Fluorescence spectra characteristic of protein residues were used to monitor changes in tertiary structure after incubation in monomer solutions. The emission spectrum (300-500 nm) at an excitation wavelength of 280 nm was analyzed as reported32.
5.3.3.4 Ovalbumin Antigenicity

An enzyme-linked immunosorbent assay (ELISA) was used to study the antigenicity of Ova prior to and after incubation with monomer solution and the protocol followed is described elsewhere\textsuperscript{32}. The epitope availability of incubated samples was obtained by normalizing with protein solutions prior to incubation with the CPTEG:CPH monomers and the antigenicity was reported as relative epitope availability. The assay was also performed for protein released from microspheres (see below).

5.3.3.5 Lysozyme Enzymatic Activity

The enzymatic activity of Lys after incubation with degradation products was evaluated with the EnzChek\textregistered Lysozyme Assay Kit. Lys hydrolyzes linkages of various cell walls of microorganisms. This assay measures the activity in Micrococcus lysodeikticus cell walls, which are previously labeled with fluorescein. The experimental protocol was followed as described by the manufacturer. Triplicates of each sample were averaged. Assays were also performed on samples after release from CPTEG:CPH microspheres (see below). The results were normalized by the activity measured initially (i.e., prior to incubation and release) and reported as fractional lysozyme activity.
5.3.4 Microsphere Fabrication

5.3.4.1 Solid/oil/oil (S/O/O)

Prior to protein encapsulation, the protein was lyophilized as described before\textsuperscript{10}. The S/O/O method was modified from previous reports\textsuperscript{8-10}. Briefly, lyophilized protein (2-3mg) was suspended in a solution of 100 mg of 20:80 CPTEG:CPH copolymer dissolved in 2 mL of methylene chloride to produce the first emulsion. The suspension was obtained by homogenizing the solution at 20,000 rpm for 3 minutes using a Tissue-Tearor\textsuperscript{TM}. The second emulsion was produced after adding a solution of Dow Corning oil 550 (3mL) saturated with methylene chloride (4mL). The mixture was then poured into 200 mL of heptane on ice bath and stirred for 2 h at 300 rpm. Microspheres were collected by filtration and dried under vacuum overnight.

5.3.4.2 Cryogenic Atomization (CA)

As we obtained higher encapsulation efficiencies with CA in previous experiments with unmodified polyanhydrides\textsuperscript{10}, we fabricated microspheres of both 20:80 and 10:90 CPTEG:CPH with this method. The procedure was modified from previously reported studies\textsuperscript{10, 11}. Lyophilized protein (2-3 mg) was suspended in a polymer solution of methylene chloride (7mL for 20:80 CPTEG:CPH and 4mL for 10:90 CPTEG:CPH) at 10,000 rpm for 1 min using a Tissue-Tearor\textsuperscript{TM}. The solution was then pumped with a syringe pump through an 8700-1200 MS ultrasonic atomizing nozzle (Sono Tek Corporation, Milton, NY) into 200 mL of frozen ethanol overlaid with ~100 mL of liquid nitrogen. The parameters used for these experiments
were: 3 mL/min and 1.5 W for 20:80 CPTEG:CPH and 1.5 mL/min and 2.5 W for 10:90 CPTEG:CPH. This procedure was performed at 4°C in order to maintain the temperature below the glass transition temperature of the polymer during pumping. After atomization, the resulting polymer/protein solution was stored at -80°C for three days to allow the methylene chloride to be extracted. The microspheres were then collected by filtration and dried under vacuum overnight.

The microsphere morphology was characterized by SEM. The particle size distribution was obtained from SEM images (250-500x) using a soft imaging system software (analySIS®, Soft Imaging System Corp, Lakewood, CO). An average of 250 particles per image was analyzed.

5.3.5 Protein Release

Microspheres (15 mg) were placed in 1 mL of phosphate buffer (0.1 M, pH 7.4) and incubated at 37°C and 100 rpm. Sodium azide (0.01% w/w) was added to the buffer to prevent microbial contamination. At different time intervals, aliquots of 750 μL of supernatant were collected and replaced with fresh buffer. The aliquots were stored at 4°C and were centrifuged (10,000 rpm for 10 min) prior to BCA analysis. Protein concentration was measured using the BCA analysis from the absorbance at 570nm. At least two replicates of samples in each experiment were evaluated. The experiment was repeated twice, the values were averaged, and the standard deviation was determined.
5.3.5.1 Total Protein Encapsulated

After one month of release, the remaining microspheres were analyzed for residual protein content. Using a recently reported procedure\textsuperscript{10}, the microspheres were suspended in 1 mL of 17 mM of sodium hydroxide and sonicated (Sonics & Materials Inc., Newton, CT). The sample was withdrawn with a syringe, and the vials were washed twice with 1 mL of the same solvent to ensure no residual protein was lost. The 3 mL solution was transferred to a dialysis cassette (Slide-A-Lyzer\textsuperscript{®} 10,000 MWCO, Pierce Biotechnology Inc., Rockford, IL) using the same syringe. The cassettes were placed in 600 mL of 17 mM sodium hydroxide and incubated at 100 rpm and 40°C for 1 week in order to accelerate the degradation of the polymer. This temperature was chosen as it is below the denaturation temperature of lysozyme (81°C) and ovalbumin (71°C)\textsuperscript{34}. After the incubation period, the protein was quantified by BCA analysis. The total protein encapsulated in the microspheres was determined by adding the protein released and the amount remaining in the microspheres. The cumulative release was normalized by this total amount and reported as fractional protein released.
5.4 Results

5.4.1 Protein Stability in the Presence of Degradation Products

5.4.1.1 Primary Structure

The primary structure of Ova and Lys was analyzed with SDS-PAGE (Fig. 5.2). Samples prior to and after incubation were loaded into the gels and were compared to molecular weight standards. Non-lyophilized protein was also loaded to ensure that lyophilization process did not alter the primary structure of native protein. From Fig. 5.2 it can be seen that no structural change occurred in the proteins during lyophilization or incubation as no aggregation or hydrolysis was perceived in the gels. Saturated concentrations of both CPTEG and CPH diacids were not detrimental to the model proteins used in this study, and characteristic bands of Ova at 48 KDa and Lys at 14 kDa were unchanged after 1 week of incubation.

5.4.1.2 Secondary Structure

Circular dichroism (CD) was used to monitor the secondary structure of Lys and Ova during the incubation studies to estimate the type of secondary structure (α-helix vs. β sheet vs. coil) present in the proteins. The CD spectra of Ova (Fig. 5.3) incubated in CPTEG and CPH diacid saturated solutions were identical at 0 and 7 days, showing two minima (208 and 222 nm) that are signatures of α-helices and α-helices + β-sheets. The secondary structure of Lys was also preserved and the characteristic minima were present in the spectra (data not shown).
Figure 5.2. SDS-PAGE of Ova (a) and Lys (b). Lane 1: MW standard ladder; lane 2: Non-lyophilized protein; lanes 3, 4 protein in CPTEG solution (day 0, 7); lanes 5,6: protein in 50/50 CPTEG/CPH solution (day 0, 7); lanes 7,8: protein in CPH solution (day 0, 7); lanes 9,10 in gel (a) Ova in phosphate buffer (day 0, 7).

Figure 5.3. CD spectra of Ova incubated in (a) CPTEG saturated solution and (b) CPH saturated solution.
5.4.1.3 Tertiary Structure

Proteins are usually biologically active only when folded in their native conformations, so understanding their 3-D structures is key to understanding how they function\textsuperscript{36}. The most common exception to two state folding transitions is the occurrence of a stable, partially folded state, known as the molten globule\textsuperscript{36}. The formation of a molten globule state may retain the secondary structure, as it is almost as compact as the fully folded protein, but the tertiary structure is disrupted/unfolded\textsuperscript{37}. It has been reported that this molten globular state can be produced in Ova as a result of harsh environments\textsuperscript{38}. On the other hand, Lys does not have a molten globule structure and unfolds globally by guanidine hydrochloride in the two state type\textsuperscript{36, 39, 40}. With this in mind, the fluorescence spectra were analyzed. Fig. 5.4 shows that in the fluorescence spectra of Ova incubated in CPTEG and CPH saturated solution, the maximum wavelength at days 0 and 7 was in the 336-340 nm range, and suggested that a molten globule state was not formed during the incubation period. Similarly, fluorescence spectra of Lys contained maximum wavelengths in the 343-345 nm range (data not shown). The emission spectrum at the wavelength range of 330-345 nm is characteristic of the tryptophan residues\textsuperscript{41}. No loss of tertiary structure was detected, and it can be concluded that conformational stability of the two model proteins (Ova and Lys) was preserved in the presence of CPTEG and CPH monomers.
Figure 5.4. Fluorescence spectra of Ova incubated in (a) CPTEG saturated solution and (b) CPH saturated solution.

5.4.1.4 Protein Activity

The activity after incubation of the model proteins with CPTEG:CPH degradation products was assessed by measuring the antigenicity of Ova and the enzymatic activity of Lys. The results of the ELISA performed in the Ova samples (Fig. 5.5a) demonstrate that neither CPTEG nor CPH diacid solutions caused a statistically significant change in the antigenicity of Ova after 7 days of incubation. More perturbations were caused when the protein was incubated in phosphate buffer alone, where an increase of ~50% after incubation suggests that protein exposed more epitopes to be recognized and quantified by the assay. The enzymatic activity of lysozyme was also measured (Fig. 5.5b), and as expected, it was maintained during the incubation period, with less than 10% loss in all the solutions analyzed. All these results support our hypothesis that the novel amphiphilic CPTEG:CPH system provides a conducive environment for protein stability.
5.4.2 Microsphere Fabrication

It is desirable to minimize drug particle size during encapsulation in order to minimize the burst effect\textsuperscript{42}. As the size of the protein particles and stability of the dispersion are directly relevant for microsphere performance, the proteins (Ova, Lys) were lyophilized prior to their encapsulation into CPTEG:CPH microspheres.

Microspheres fabricated by S/O/O had a relatively smooth surface of the microspheres prior to and following protein encapsulation (images not shown). The typical size distribution of these microspheres was in the range of 4-60 μm, with the majority of the population in the 10-15 μm range, which is adequate for injectable formulations.
The second method studied was CA, which besides avoiding the deleterious effect of the water/organic interface, maximizes protein encapsulation\textsuperscript{10}. Microspheres of 10:90 and 20:80 CPTEG:CPH were successfully fabricated and images of 10:90 CPTEG:CPH microspheres and particle size distribution are shown in Fig. 5.6. Similar microsphere surface structure was obtained for the 20:80 CPTEG:CPH copolymers, indicating that the slight change in hydrophobicity did not have any effect on the microsphere formation (images not shown). The difference in surface roughness when compared to S/O/O microspheres is due to the difference in solvent extraction rates during the fabrication process. The particle size from cryogenic microspheres resulted in unimodal distributions with diameters in the range of 2-16 μm.

![SEM image and particle size distribution of Ova loaded 10:90 CPTEG:CPH microspheres fabricated by cryogenic atomization. Scale bar represents 20 μm.](image)

5.4.3 Protein Release

The release of Ova and Lys from CPTEG:CPH microspheres is shown in Fig. 5.7. The cumulative protein release was normalized with respect to the total protein encapsulated, which was determined as described before. On average, \sim 100\%
Encapsulation efficiencies were observed for both proteins and fabrication methods studied, which is in agreement with previous work\textsuperscript{10}. A sustained protein release and relatively low initial burst were achieved with all the CPTEG:CPH formulations, which is characteristic of amphiphilic systems where protein is more uniformly distributed. In addition, the initial burst of both CA and S/O/O microspheres were comparable to recently published studies with polyanhydride microspheres composed of 20% CPH\textsuperscript{10}.

![Figure 5.7. Protein released from CPTEG:CPH microspheres. (a) Ova released from 20:80 and 10:90 CA microspheres, (b) Lys released from 20:80 CA and S/O/O microspheres. Error bars represent standard deviation of triplicate samples.](image)

Ova was released from both 20:80 and 10:90 CPTEG:CPH microspheres fabricated by CA to study the effect of copolymer composition on protein release (Fig. 5.7a). The polymer hydrophobicity had the expected effect on protein release,
as protein released faster from 20:80 CPTEG:CPH (67%) than from 10:90 CPTEG:CPH microspheres (54%) in one month.

The effect of microsphere fabrication method on protein release was analyzed from Lys release from 20:80 CPTEG:CPH microspheres fabricated by CA and S/O/O (Fig. 5.7b). As can be seen, there is excellent agreement between the two fabrication methods. During one month of release, 49% and 51% of the total Lys encapsulated were released from CA and S/O/O microspheres, respectively. It is important to note that bursts of less than 10% were observed in these microspheres, suggesting that Lys was homogenously distributed. These results, when compared to the Ova studies described above, demonstrate that protein characteristics influence their distribution and hence the subsequent release. Ova encapsulated in the same polymer formulation (i.e., 20:80 CPTEG:CPH CA microspheres) produced an initial burst of 35%, compared to the 10% obtained with Lys.

5.4.3.1 Protein Activity after Release

The activity of Ova and Lys was analyzed after release from the CPTEG:CPH microspheres similar to the methods used to analyze the proteins incubated in the presence of the degradation products. The antigenicity of Ova released from the CA microsphere formulations (i.e., 20:80 and 10:90 CPTEG:CPH) shown in Fig. 5.8a indicates that the released Ova did not lose significant antigenicity after being released from the two copolymer compositions. Similarly, the activity of Lys was essentially maintained in both 20:80 CPTEG:CPH CA and S/O/O microspheres formulations (Fig. 5.8b). No significant differences were found in the enzymatic
activity of the protein when released from the CA microspheres. On the contrary, Lys activity released from the S/O/O formulation was statistically different (p < 0.05). Possible causes for this difference among the microspheres are the processes involved during fabrication, where different solvents (i.e., methylene chloride, ethanol in CA; methylene chloride, silicon oil, heptane in S/O/O) were involved. The methylene chloride extraction rate in each method was different. In S/O/O, the protein-loaded microsphere formulations were subjected to more stress and organic phases than in CA. These studies suggest that minimizing protein instability during processing and maximizing protein encapsulation are desirable characteristics of the CA method, which make it suitable for protein delivery applications.
Figure 5.8. Protein activity after release from CPTEG:CPH microspheres. (a) antigenicity of Ova after release from 20:80 and 10:90 CA microspheres, (b) enzymatic activity of Lys after release from 20:80 S/O/O and CA microspheres. Error bars indicate standard deviation of triplicate samples. * represents $p$-value < 0.05 as determined by statistical test.
5.5 Discussion

Loss of the folded structure of proteins can be readily followed by observing changes in absorption spectra, CD, fluorescence spectra or in the dimensions of the protein, which generally increase upon denaturation. The interactions are so dependent upon each other that disruption of a very limited number of interactions tends to disrupt all of them. Any structure present in unfolded proteins is local, however, and the global co-operative interactions characteristic of the native state are absent. Thus, it is important to verify that the native structure of the protein is preserved at all structural levels (i.e., primary, secondary, and tertiary structures). The stability of proteins in the presence of degradation products provides invaluable information by mimicking the microenvironment inside the polymeric device during release. After ensuring that no perturbations take place at the structural level, it is equally important to check if the protein maintained its activity. The results obtained in these studies indicate that the two model proteins studied maintained both their structure and activity in the presence of the CPTEG:CPH monomers.

When designing delivery systems for protein drugs, all the processes involved, from fabrication to delivery, which can alter the stability and hinder the therapeutic drug efficacy, must be considered. This paper demonstrates that during the three stages of device fabrication using amphiphilic polyanhydrides, the model proteins Ova and Lys were efficiently stabilized. The stability of the proteins in saturated concentrations of degradation products of CPTEG:CPH was unaltered at all the structural sublevels. In Table 5.1, the pH of the CPTEG and CPH diacid solutions used in these studies is compared to that of the degradation products of
polyesters. It is interesting to note that an acidic microenvironment is produced by the ester monomers even below their saturation concentration. On the other hand, saturated solutions of CPTEG and CPH diacids have pH of 6.5 and 5.5 respectively. Thus, even at saturated monomer concentrations, the microenvironments of the CPTEG:CPH eroding device show little decrease in pH. Not surprisingly, the protein released from CPTEG:CPH microspheres was stable and the activity was essentially unaltered.

Table 5.1. Effect of the dissolution of polymer degradation products on pH.

<table>
<thead>
<tr>
<th>Monomer Solution</th>
<th>Concentration (mM)</th>
<th>Type of monomer</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPTEG</td>
<td>9(saturated)</td>
<td>Anhydride</td>
<td>6.5</td>
</tr>
<tr>
<td>CPH*</td>
<td>1 (saturated)</td>
<td>Anhydride</td>
<td>5.5</td>
</tr>
<tr>
<td>LA*</td>
<td>5</td>
<td>Ester</td>
<td>3.5</td>
</tr>
<tr>
<td>GA*</td>
<td>5</td>
<td>Ester</td>
<td>3.6</td>
</tr>
</tbody>
</table>

* Data was obtained from Reference 32

The model proteins (Ova and Lys) differed in their chemical structure and function, and therefore their mechanisms of instability were dissimilar. The less acidic microenvironments (i.e., CPTEH:CPH) improved Ova stability than at acidic pH, where stronger complexation can result in protein aggregation\textsuperscript{31, 43}. On the other hand, precipitation of albumin is expected when the pH of the aqueous environment approaches the isoelectric point (~4.8). Under these conditions most proteins expose hydrophobic domains which are inherently attractive, a process that will likely occur in degrading environment of polyesters, but not in the CPTEG:CPH system\textsuperscript{43}. In contrast, Lys is a monomeric globular protein that is acid stabilized\textsuperscript{44},
and severe methods such as disulfide scrambling are needed for its partial
denaturation. It is not surprising that the acidic environment of the polyester
degradation products provide a stable environment for this protein.

5.6 Conclusions

This study demonstrates that the amphiphilic CPTEG:CPH system is a
promising protein carrier. Our studies showed that the amphiphilic environment does
not alter protein structure and provides a sustained release profile from
microspheres. These results are promising for future in vivo studies, which involve
the design of novel biodegradable polyanhydride carriers suitable for the stabilization
and sustained release of different therapeutic peptides and proteins.

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5.8 References


CHAPTER 6

EFFECT OF POLYMER CHEMISTRY AND FABRICATION METHOD ON
PROTEIN RELEASE AND STABILITY FROM POLYANHYDRIDE
MICROSPHERES

Reprinted from a paper submitted to Journal of Biomedical Materials Research B:

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6.1 Abstract

The release kinetics and protein stability of ovalbumin-loaded polyanhydrides microspheres with varying chemistries were studied. Polymers based on the anhydride monomers sebacic acid (SA), 1,6-bis(p-carboxyphenoxy)hexane (CPH), and 1,8-bis(p-carboxyphenoxy)-3,6-dioxaoctane (CPTEG) were chosen. Microspheres were fabricated using two non-aqueous methods: a solid/oil/oil emulsion technique and cryogenic atomization. Studies found no significant difference in release kinetics of ovalbumin. Ovalbumin released from microspheres prepared by cryogenic atomization was studied for preservation of primary structure by SDS-PAGE and availability of immunogenic epitopes by western blot. The more hydrophilic polyanhydrides containing CPTEG showed favorable protein stability, preserving both the immunological epitopes and the primary structure.
6.2 Introduction

Biodegradable polymers have been used as carriers for the controlled delivery of drugs and proteins for over two decades. These carriers have the advantages of providing sustained release over long periods of time, well-controlled release profiles, and biocompatibility. The most common biodegradable polymers used in drug delivery applications are polyesters such as poly(D,L-lactide-co-glycolide) (PLGA), polyanhydrides, and poly(orthoesters). A potential draw-back in using the bulk-erodible PLGA for protein delivery is that the water penetration into the bulk is fast and that the degradation products are fairly acidic; for example, a pH of less than 3 for degradation products\(^1\) and a pH of 2 inside a PLGA drug delivery device\(^2\) have been reported. Studies have shown that at these pH values, some proteins can undergo denaturation by unfolding, and in some cases, irreversible aggregation\(^3\). This is problematic for most proteins because a loss in structure is detrimental to function. In comparison, the pH values produced by polyanhydride degradation products are much higher, notably 4.2 for sebacic acid (SA) and 5.5 for 1,6-bis(p-carboxyphenoxy)hexane (CPH)\(^4\). The hydrophobic polyanhydrides help prevent water-induced covalent aggregation of proteins since water penetration into the bulk is negligible; however, non-covalent aggregation due to hydrophobic interactions may result\(^5,6\). This has motivated research to make these materials less hydrophobic\(^7,8\). This was achieved by incorporating oligomeric ethylene glycol units into the backbone of hydrophobic aromatic polyanhydrides, such as poly(CPH), leading to amphiphilic polymers with mixed erosion mechanisms, which result in the reduction of covalent and non-covalent aggregation of proteins\(^7\).
Polyanhydrides have been studied for drug delivery applications since 1983, when Langer and co-workers reported their potential for controlled drug delivery based on their biodegradable properties, and non-toxic and non-mutagenic nature. Due to their ability to erode at the surface, polyanhydrides result in drug release profiles that exhibit a predictable zero-order release rate, making them attractive candidates for drug delivery applications. Degradation of polyanhydrides occurs by base-catalyzed hydrolysis of their anhydride linkages, in the presence of water, to form dicarboxylic acids; their rate of degradation depends upon on the monomer used. SA and CPH tablets, for example, degrade in 54 days and 1 year, respectively. In contrast, 80% of the ethylene glycol containing polyanhydride, poly(1,8-bis(p-carboxyphenoxy)-3,6-dioxaoctane) (poly(CPTEG)), degrades in 28 days. Thus, by combining different anhydride monomers in various ratios, copolymer degradation rates can be tailored for specific applications.

Biodegradable polymers are also preferred for parenteral drug delivery systems as there is no need to remove them following implantation. A size of less than 125 μm is normally preferred for such applications; this size allows for delivery into the tissue by the use of a syringe and needle. Typical methods for microsphere fabrication include hot melt microencapsulation, double emulsion, spray drying, and cryogenic atomization. In particular, previous research has shown that double emulsion methods in which water/organic interfaces are present are potentially detrimental for protein stabilization. Thus, several groups have focused on developing non-aqueous methods for preparing protein-loaded microspheres. Two commonly used non-aqueous techniques for fabricating
microspheres include solid-oil-oil (S/O/O) double emulsion and cryogenic atomization (CA); besides avoiding the water/organic interface, these techniques prevent hydrolysis of the polymer by eliminating water from the process\textsuperscript{33}.

The objective of this work is to systematically study the effects of polymer chemistry and fabrication method on the release kinetics of proteins from polyanhydride microspheres and on the stability of the released protein. Polymer chemistries based on the anhydride monomers SA, CPH, and CPTEG were chosen (Fig. 6.1). Ovalbumin (ova) from chicken egg white was selected as the model protein. Protein-loaded microspheres were fabricated by S/O/O and CA.

![Chemical structures](image)

Figure 6.1. Chemical structures of polymers used, from top, left to right: poly(sebacic acid) (SA), poly(1,6-bis(p-carboxyphenoxy)hexane) (CPH), and poly(1,8-bis(p-carboxyphenoxy)-3,6-dioxaoctane) (CPTEG). Here $n$ represents the number of repeating monomer units.
6.3 Materials and Methods

6.3.1 Materials

Albumin from chicken egg white (ovalbumin/Ova), 1,6-dibromohexane, 4-hydroxybenzoic acid, 1-methyl-2-pyrrolidinone, sebacic acid (99%), monoclonal anti-chicken egg albumin (clone Ova-14), rabbit anti-chicken egg albumin, alkaline phosphatase conjugated goat anti-rabbit IgG, and tri-ethylene glycol were purchased from Sigma-Aldrich (St. Louis, MO). 4-p-fluorobenzonitrile was purchased from Apollo Scientific (Cheshire, UK). Acetic acid, acetic anhydride, acetone, acetonitrile, dimethyl formamide, ethyl ether, heptane, hexane, methylene chloride, petroleum ether, potassium carbonate, sodium hydroxide, sulfuric acid, and toluene were purchased from Fisher Scientific (Fairlaw, NJ). Dialysis cassettes, bicinchoninic acid (BCA) assay reagents, and GelCode blue were purchased from Pierce (Rockford, IL). Low protein molecular weight standards were purchased from BioRad (Hercules, CA). 12% tris-glycine PAGE Duramide Precast Gels were purchased from Lonza Bioscience (Basel, Switzerland). Dow Corning oil, ethanol, and liquid nitrogen were obtained from in-house bulk chemical supplies.

6.3.2 Monomer/polymer Synthesis

To produce the CPH monomer, the method described by Conix\textsuperscript{34} for synthesizing 1,3-bis(\(p\)-carboxyphenoxy)propane was altered, using 1,6-dibromohexane instead of 1,3-dibromopropane. Prepolymers for both CPH and SA were synthesized using a method outlined by Shen et al.\textsuperscript{35}; CPH:SA copolymers of various compositions and poly(SA) were synthesized by melt polycondensation
using a procedure outlined by Kipper and coworkers. The CPTEG monomer and 
CPTEG:CPH copolymers were produced using a technique described by Torres et 
al. The polymers, pre-polymers, and diacids were characterized by $^1$H NMR, using a 
Varian VXR-300 NMR (Palo Alto, CA), to ensure purity; a Waters GPC (Milford, MA) 
was used to measure the polymer molecular weight.

6.3.3 Protein Preparation

Ova obtained from chicken egg white was lyophilized prior to use. Lyophilization occurred by pumping ova (50 mg) in 50 mM ammonium bicarbonate solution (10 mL) over 400 mL of liquid nitrogen. The liquid nitrogen was allowed to boil off, and the remaining protein was placed in a dryer oven overnight; denaturation of freeze-thawed ova at neutral pH has been shown to be highly unlikely.

6.3.4 Contact Angle Measurements

To characterize the relative hydrophobicity of the polymers, contact angle measurements were carried out. Polymers were dissolved in a 2.5 w/v% solution of tetrahydrofuran (for poly(CPTEG) and CPTEG-containing copolymers), or methylene chloride (for poly(CPH), poly(SA), and their copolymers). After filtering solutions with 0.2 μm filters, the solutions were pipetted onto separate round glass cover slides. After the solvent dried, more solution was added until a suitable polymer thickness was obtained. To measure the contact angle, a water droplet was carefully placed on the surface of the polymer film immediately prior to imaging with a CCD camera.
Image J software (NIH, Bethesda, MD) was used to measure the contact angle. The experiment was performed in triplicate. A student-t test ($\alpha=0.05$) was performed with the statistical analysis software JMP® 6 (Cary, NC).

6.3.5 Microsphere Fabrication Methods

Two non-aqueous methods were used to fabricate polyanhydride microspheres: S/O/O double emulsion and CA. As discussed before, previous research has demonstrated that these methods are effective at encapsulating and stabilizing proteins$^4$,$^{20}$.

6.3.5.1 Solid-oil-oil (S/O/O) Double Emulsion

This method was modified from a previously published procedure$^{21}$. Briefly, 100 mg of polymer and 6 mg of ova were dissolved in methylene chloride. A Tissue-Tearor™ homogenizer (Biospec Products Inc., Bartlesville, OK) was used to agitate the solution for one minute. For the second emulsion, Dow Corning oil and methylene chloride were added while the homogenizer was turned down to 10,000 rpm and used for one minute, to allow for thorough mixing during addition. The parameters used for each emulsion step for the different polymer chemistries are shown in Table 6.1. The solution was added drop-wise to a beaker of 200 mL of heptane immersed in an ice bath and stirred at 300 rpm for two hours using a Caframo overhead stirrer (Wiarton, Ontario, Canada). Finally, the microspheres were filtered and placed in a vacuum oven overnight to eliminate any residual heptane.
Table 6.1. Parameters for S/O/O double emulsion

<table>
<thead>
<tr>
<th></th>
<th>Methylene chloride for inner emulsion</th>
<th>Rate for inner emulsion homogenization</th>
<th>Rate for outer emulsion homogenization</th>
</tr>
</thead>
<tbody>
<tr>
<td>20:80 CPTEG:CPH</td>
<td>2 mL</td>
<td>20,000 rpm 3 min</td>
<td>3 mL oil/4 mL MeCl₂ 20,000 rpm 3 min</td>
</tr>
<tr>
<td>Poly(CPTEG) and 50:50 CPTEG:CPH</td>
<td>2 mL</td>
<td>20,000 rpm 3 min</td>
<td>4 mL oil/6 mL MeCl₂ 20,000 rpm 3 min</td>
</tr>
<tr>
<td>Poly(SA)</td>
<td>3 mL</td>
<td>30,000 rpm 1 min</td>
<td>3 mL oil/4 mL MeCl₂ 20,000 rpm 1 min</td>
</tr>
<tr>
<td>20:80 CPH:SA</td>
<td>2 mL</td>
<td>30000 rpm 1 min</td>
<td>3 mL oil/4 mL MeCl₂ 30000 rpm 1 min</td>
</tr>
<tr>
<td>50:50 CPH:SA</td>
<td>2 mL</td>
<td>20000 rpm 1 min</td>
<td>3 mL oil/4 mL MeCl₂ 30000 rpm 1 min</td>
</tr>
</tbody>
</table>

6.3.5.2 Cryogenic Atomization (CA)

CA, which employs an ultrasonic generator to produce a fine mist, was also modified from previously published work. Briefly, 100 mg of each polymer was dissolved in methylene chloride with 6 mg of ova. Using a glass syringe, 20 gauge capillary tube, and programmable syringe pump (KD Scientific, Holliston, MA), the polymer solution was pumped over 200 mL of 200 proof ethanol (frozen by liquid nitrogen), leaving a small layer of liquid nitrogen overlaying the ethanol. The atomizing mist was provided by an ultrasonic atomizing nozzle (SonoTek Corporation, Milton, NY). The beakers were placed in a -80 °C freezer for three days to allow the liquid nitrogen to boil off, the ethanol to thaw, and the methylene chloride to slowly be extracted. Afterwards, the microspheres were filtered and placed in a vacuum oven to dry overnight. Table 6.2 summarizes the operating parameters for this method for each of the polymer chemistries studied.
Table 6.2. Parameters for CA

<table>
<thead>
<tr>
<th></th>
<th>Methylene chloride</th>
<th>Flow rate</th>
<th>Wattage</th>
</tr>
</thead>
<tbody>
<tr>
<td>20:80 CPTEG:CPH, 50:50 CPTEG:CPH, and Poly(CPTEG)</td>
<td>7 mL</td>
<td>3 mL/min</td>
<td>1.5 W</td>
</tr>
<tr>
<td>50:50 CPH:SA</td>
<td>3 mL</td>
<td>1.5 mL/min</td>
<td>2.5 W</td>
</tr>
<tr>
<td>Poly(SA) and 20:80 CPH:SA</td>
<td>3 mL</td>
<td>3 mL/min</td>
<td>1.5 W</td>
</tr>
</tbody>
</table>

6.3.6 Microsphere Characterization

A JEOL 840A scanning electron microscope (SEM) was used to determine relative size and shape of microspheres. Microspheres were smeared onto carbon stubs, coated with 200 Å of gold, and imaged. Size distribution analysis was performed using Image J software (NIH, Bethesda, MD).

6.3.7 Ova Release

Polyanhydride microspheres (10 mg) fabricated by S/O/O or CA were suspended in 1mL of phosphate buffer solution (pH 7.4) with 0.01% sodium azide and placed in an incubator at 37 °C and 100 rpm. The release samples were collected two hours after the initial time point, daily for one week and every other day for 30 days. An aliquot of 750 μL was sampled each time and subsequently replaced with 750 μL of fresh phosphate buffer solution to ensure perfect sink conditions; the samples were also centrifuged before sampling to ensure that no microspheres were removed from the system. In order to quantify the amount of protein released, a bicinchoninic acid (BCA) assay was run on each sample, in duplicate, as described by the manufacturer (Pierce).
After one month of release, the samples were added to 10 kDa molecular weight cut-off dialysis cassettes to determine the amount of protein remaining inside the microspheres. The remaining microspheres were suspended in 3 mL of 17 mM NaOH and sonicated to break up any aggregates. The exposure to a high pH allows for fast degradation of the polymer, since anhydride degradation is base-catalyzed. Each release sample was added to dialysis cassettes and incubated for one week at 40 °C and 100 rpm. A BCA assay was run on each sample in triplicate. The total protein loaded into the microspheres was calculated by adding the protein that was released in one month to the protein extracted from the remaining microspheres. The release data is presented as cumulative fraction of protein released, which is normalized by the total protein loaded into the microspheres.

6.3.8 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Ova-loaded microspheres (5% w/w) were fabricated using CA. 15 mg of these microspheres were added to 1 mL of phosphate buffer solution (pH 7.4), with 0.01% sodium azide, and placed in an incubator at 37 °C and stirred at 100 rpm for 2 hours; 750 μL of solution was removed and replaced with 2.75 mL of 0.01 mM NaOH solution. This was performed to ensure that all analysis was performed on released protein and not on protein adsorbed to the surface of the microsphere. The microspheres were added to 10k molecular weight cut-off dialysis cassettes, placed in 1 L of 0.01 mM NaOH solution, and incubated for 2 weeks at 40 °C and 100 rpm. After two weeks, the solution was removed from the dialysis cassette and centrifuged for 10 minutes at 10,000 rpm to isolate the polymer remaining in the
solution. A BCA assay, performed in triplicate, was used to determine the concentration of ova released from the microspheres.

Using the concentration from the BCA assay, 2 μg of protein from each sample was placed on a rotovap until completely dry. Samples were prepared under reducing conditions by adding 20 μL of 2-mercaptoethanol sample buffer to each sample and placing samples on a heating block at 90 °C for 10 minutes to break up the disulfide linkages. The gels were made in triplicate, to allow for two to be used for western blot analysis (described below). Gels were run at a constant voltage of 120 V until the dye front reached the bottom. The gel set aside for SDS page analysis was rinsed with DI water and placed in gel fixative (50% methanol, 36% DI water, 14% acetic acid) overnight. The next day, the gel was stained with Gelcode blue for a few hours and destained with water overnight; the staining process was repeated to obtain a darker stained gel. The gel was placed between cellophane sheets and dried in a jet drier for 2 hours. This procedure was repeated for pure ova to ensure that the protein structure was preserved under the conditions of the experiment. Therefore, the SDS-PAGE results obtained after release from the microspheres could be related to the stability of the protein released from the different polymer chemistries.
6.3.9 Western Blot

For western blot analysis, the gels were immediately removed after gel electrophoresis, placed between filter paper and a PVDF membrane, and placed back in the electrophoresis chamber for 3 hours at a constant current of 70mA. The membranes were blocked with a casein solution of TBST (tris buffer solution with 0.05% Tween, pH 7.6) and milk powder overnight. The following day, the membranes were rinsed in DI water, placed in a 50 mL centrifuge tube, and 12 μL of primary antibody (anti-ova developed in rabbit) in TBST (1:1000) was added. The membranes were spun for four hours, washed thrice with TBST to remove any unbound antibody, and placed back on the spinner with 12 μL of secondary antibody (anti-rabbit IgG alkaline phosphate developed in goat) in TBST (1:1000). After two hours, the membranes were removed and rinsed thrice with TBST. A colorimetric detection method with napthol phosphate and fast red solution was used to reveal bands. Finally, the membranes were air-dried.
6.4 Results

6.4.1 Contact Angle

In order to assess the hydrophobicities of the various polymers, the contact angles of each of the polymers were measured, as shown in Figure 6.2. As expected, poly(CPTEG), the most hydrophilic and bulk-erodible polymer with a fast degradation profile (within weeks\textsuperscript{7}), has the lowest contact angle (29°) of all the polymer chemistries. As demonstrated by the statistical analysis, poly(CPTEG) was significantly more hydrophilic than all the other polyanhydride chemistries analyzed. In contrast, poly(CPH), which is the most hydrophobic and surface-erodible polymer, and takes years to degrade\textsuperscript{38} has the highest contact angle (60°). As Figure 6.2 demonstrates, an increase in CPH content within the CPTEG:CPH copolymers results in an increase in contact angle, which is consistent with an increase in hydrophobicity. In the surface erodible CPH:SA system, since both CPH and SA are hydrophobic, their copolymers have relatively similar hydrophobicities, as evidenced by contact angles that are statistically indistinguishable from each other.
6.4.2 Release of Ova from Polyanhydride Microspheres

SEM images of 50:50 CPH:SA and 20:80 CPTEG:CPH fabricated by S/O/O and CA methods are shown in Figures 6.3 and 6.4. Size distributions of the CPTEG particles ranged from 4 to 60 μm for S/O/O, with the majority being between 10 and 15 μm in diameter, and 2 to 16 μm in diameter for CA microspheres. For the CPH:SA system, the majority of the microspheres fell in the 6 to 10 μm diameter range for both fabrication methods.
In Figure 6.5 the release profiles of ova from Poly(SA), 20:80 CPH:SA and 50:50 CPH:SA copolymer microspheres fabricated by S/O/O and CA fabrication methods are shown. As can be seen, all these chemistries exhibit near zero-order release kinetics after the initial burst of protein, which is consistent with previous work. Each polyanhydride chemistry exhibited a different release rate consistent with the hydrophobicity of the polymer, but upon comparing the two fabrication methods, this was found to be unrelated to the fabrication method; for example, poly(SA) microspheres fabricated by both S/O/O and CA released 90% of the encapsulated ova protein at the end of thirty days. In the CPH:SA system, as the
polymer hydrophobicity increased, the release rate of the protein decreased. The fabrication method influences the size of the burst. Microspheres produced by the S/O/O technique experienced a smaller initial burst of protein. This is attributed to the interplay between two phenomena: the rate at which the polymer precipitates during microsphere formation, and the rate at which the methylene chloride is extracted into the non-solvent to form the microspheres. In addition, polymer hydrophobicity appears to have an influence on the burst effect, as a greater difference in initial bursts was noted for the less hydrophobic poly(SA) than the respective copolymers.

![Graph showing ova release profiles](image)

**Figure 6.5.** Fraction of ova released from poly(SA) and CPH:SA copolymer microspheres using S/O/O (left) and CA (right) fabrication techniques. Error bars indicate standard deviation.

The ova release profiles from poly(CPTEG), 20:80 CPTEG:CPH and 50:50 CPTEG:CPH copolymer microspheres fabricated by S/O/O and CA methods were also analyzed (Fig. 6.6). Since poly(CPTEG) is bulk-eroding, the protein release kinetics is not directly proportional to the degradation kinetics, but rather depend upon a combination of degradation, water swelling, and diffusion. While one would
expect the protein released from poly(CPTEG) microspheres to have the fastest release profile, the 50:50 CPTEG:CPH copolymer actually releases protein at the same rate (~90% for CA) or slightly faster (90% vs. 80% for S/O/O) than poly(CPTEG). Previous work has shown that even though the mass loss (i.e., erosion) was consistent with the hydrophobicities of poly(CPTEG) and 50:50 CPTEG:CPH copolymer, the water swelling and polymer degradation rates of both chemistries were very similar. Our protein release data is consistent with these observations. On the other hand, the 20:80 CPTEG:CPH microspheres released ova at a slower rate, and both fabrication methods are consistent in their sustained release profiles by releasing ~50% of protein in one month. Once again, the only variation of release kinetics as a result of the fabrication methods is in the initial burst; poly(CPTEG) microspheres demonstrated the largest difference in burst (8% in S/O/O vs. 42% in CA). This is consistent with the observations reported for the CPH:SA system.

Figure 6.6. Fraction of ova released from poly(CPTEG) and CPTEG:CPH copolymer microspheres using S/O/O (left) and CA (right) fabrication techniques. Error bars indicate standard deviation.
6.4.3 SDS-PAGE

Since the release kinetics studies did not show significant differences between the S/O/O and CA microspheres, cryogenic atomized microspheres were used for the protein stability studies due to the ease of scale-up and the simplicity of fabrication. Additionally, in vaccine delivery applications, it is advantageous to have a high initial burst, which results in the induction of a primary immune response.

Ova has a tendency to form moisture-induced covalent aggregates\(^\text{39}\), which is shown by the presence of characteristic bands between 54 and 97 kDa (lane 2), in addition to the normal ova band at 48kDa (Figure 6.7). The ova released from the poly(SA), 20:80 CPH:SA, and 50:50 CPH:SA microspheres (lanes 3, 4, and 5, respectively) show the higher molecular weight band as the non-encapsulated ova (lane 2), but failed to display the bands at 45 kDa. This indicates that the CPH:SA system fails to prevent non-covalent aggregation of the protein, presumably due to hydrophobic interactions; however, considering that no low molecular weight bands are displayed, it can be implied these hydrophobic chemistries did not promote hydrolysis or degradation of the protein. This is consistent with the surface erodible nature of these polymers. However, results obtained with SDS-PAGE will be corroborated with the Western Blot analysis.
The amphiphilic polymers (poly(CPTEG), 20:80 CPTEG:CPH, and 50:50 CPTEG:CPH) all showed normal ova bands at 45 kDa as well as the aggregated state, which are both observed in the non-encapsulated ova. The bands for the unaggregated ova became darker with an increase in CPTEG content. Once again, no low molecular weight bands were present, suggesting these polyanhydride chemistries did not degrade or cause hydrolysis of the protein.

6.4.4 Western Blot

Figure 6.8 shows the western blot analysis conducted on the released protein from each of the fabricated polyanhydride preparations. Again, ova shows strong bands at both an aggregated (54 to 97 kDa) and unaggregated (45 kDa) states. Strong bands are visible for both states, same as the un-encapsulated protein, for
the protein released from each of the CPTEG-containing polymers (lanes 6-8), indicating that the protein epitopes are readily conserved, and that the protein structure is not perturbed. 50:50 CPH:SA preserved the epitopes at the unaggregated ova state, but produced only faint bands for the aggregated protein. Poly(SA), due to the acidic nature of its degradation product, sebacic acid, degrades the protein below detection of the polyclonal western blot; 20:80 CPH:SA also showed a similar effect. After analyzing these results, it can be implied that the absence of bands in the SDS-PAGE of the ova released from these polymers was caused by the severe hydrolysis of the protein into small fragments.

Figure 6.8. Polyclonal western blot of ova released from microspheres over two weeks. Lane 1 – protein ladder; lane 2 – ova at pH 10; lane 3 – poly(SA); lane 4 – 20:80 CPH:SA; lane 5 – 50:50 CPH:SA; lane 6 – poly(CPTEG); lane 7 – 20:80 CPTEG:CPH; and lane 8 – 50:50 CPTEG:CPH.
6.5 Discussion

As expected, the higher the hydrophobicity of the polymer, the slower the degradation rate, as the rate at which water penetrates into the bulk slows in correlation. In regards to the release kinetics of ova, both fabrication methods were consistent. CA is a preferential method of preparing microspheres, due to its ease of scale up and increased encapsulation efficiencies. Burst profiles are correlated with the polymer hydrophobicity, as the most hydrophobic CPH:SA microspheres display the largest bursts regardless of the fabrication method used. This may be attributable to the thermodynamic incompatibility of the protein with hydrophobic copolymers. Therefore, though the actual amount of protein released at the start of the degradation/erosion cycle may vary, the trend of hydrophobicity correlates with the observed burst effect.

When a drug or a protein is incorporated into a microsphere, the drug/protein molecules may be non-uniformly distributed due to thermodynamic incompatibility with the polymer carrier. Therefore, when drug-loaded microspheres are immersed into a solution, the drug that is closer to the surface immediately escapes into the bulk solution, resulting in a large, instantaneous release of the drug. Microspheres fabricated with S/O/O method exhibited different burst characteristics than CA, with higher initial bursts resulting from the cryogenic atomized microspheres. This could be attributed to the differences in polymer precipitation and solvent extraction rate kinetics for each method. For CA, for example, the polymer solution is sprayed into frozen ethanol with an overlaying layer of liquid nitrogen, which hardens the polymer microspheres instantaneously and if the protein was not homogenously distributed in
the polymer solutions it will lead to higher bursts. In addition, the beaker is placed in a -80 °C freezer for three days, over which the methylene chloride is slowly extracted into the ethanol. Due to the slow rate kinetics of the solvent extraction, the protein is also extracted to the surface instead of being evenly dispersed, resulting in a greater burst effect. Microspheres made by the S/O/O method may have more uniformly distributed protein, as the process is conducted in an ice bath, the extraction occurs over 2h, and the solution is poured into the non-solvent rather than controlling the flow rate. Polymer chemistry affected both of these rate kinetics; the more hydrophobic the polyanhydride, the less likely these kinetics had an effect on the initial burst.

As previous studies have shown, hydrophobic polymers affect the stability of the protein\textsuperscript{10}, and the data reported here is consistent with the literature. Overall, these studies indicate that polymer chemistry affects protein stability. The acidity of the SA and 20:80 CPH:SA degradation products affected both the primary structure and recognition of epitopes; only 50:50 CPH:SA fared better at epitope conservation at the unaggregated ova state. This is consistent with previous research which has demonstrated that acidic environments are detrimental to proteins and that lower pH’s produced inside eroding microspheres as a consequence of its degradation products magnify the unfavorable effect\textsuperscript{41-43}. Among the degradation products involved here, SA is the more acidic and, therefore, high SA-containing polymers are expected to be less gentle to proteins. Nevertheless, it is important to mention that the acidity of the degradation product of SA is not as harsh as that caused by the well-studied PLGA\textsuperscript{4}. On the other hand, protein
structure and epitope availability of ova was better maintained in microspheres fabricated using CPTEG regardless of composition or method. This is likely due to the amphiphilic nature of the polymer and less acidic degradation products, which has been shown to be conducive to protein stability\textsuperscript{20, 44}.

These studies are of particular importance when designing protein delivery carriers. As discussed earlier, the polymer chemistry plays an important role that can be beneficial or detrimental for proteins. A balance between hydrophobic and hydrophilic environment (i.e. amphiphilic) is necessary to ensure protein stability, as discussed elsewhere\textsuperscript{44}. Drugs such as insulin have important stability implications; it has been proven to undergo structural changes upon release from encapsulated PLGA microspheres, due to the acidic nature of the polymer as discussed above\textsuperscript{45}. Insulin can also undergo both covalent and noncovalent aggregation when introduced to moisture-rich environments\textsuperscript{46}. Uterocalin, an acute phase protein being investigated for therapeutic use in wound healing applications, has also been theorized to become biologically inactivated upon structural modification\textsuperscript{47, 48}. Since proteins are well structured and ordered, their integrity must not be upset in order for it to function as intended; thus, it is imperative that the delivery device must not cause any disruptions to the structure. This is especially crucial in the areas of vaccination, where multi-epitope vaccines have been proven more effective than their single-epitope counterparts for diseases such as cancer\textsuperscript{49} and AIDS\textsuperscript{50}. By constructing a multi-epitope antigen, antibodies learn to recognize all epitopes, thus becoming more effective. In addition, mutation or evasion of cells is decreased dramatically; mutant epitopes can even be designed and introduced into the protein
structure to avoid such problems\textsuperscript{51}. However, if the polymer delivery vehicle is not capable of preserving the availability of epitopes, the multi-epitope antigen is not able to deliver at its full capacity.

6.6 Conclusion

These studies established the effects of polyanhydride chemistry and microsphere fabrication methods on release kinetics and protein stability. Cryogenically atomized polyanhydride microspheres containing CPTEG demonstrated the best preservation of epitopes and primary structure, thus confirming previous work done on amphiphilic environments being the best suited for ensuring protein stability\textsuperscript{44}.

6.7 Acknowledgements

The authors would like to thank ISU undergraduate students Kristina Staley for help with polymer synthesis and release studies and Andrew Glowacki for help with size distribution analysis. The authors would also like to acknowledge funding from ONR-MURI, the Hunke Fellowship, and NIH.
6.8 References


CHAPTER 7

IN VITRO ACTIVATION OF DENDRITIC CELLS USING POLYANHYDRIDE MICROSPHERES: PROMISING IMPLICATIONS FOR VACCINE DESIGN

A paper to be submitted to Biomaterials, 2008.

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7.1 Introduction

According to the World Health Organization (WHO) 2002 global report, 26% of the total deaths worldwide were due to infectious or parasitic diseases, nearly 14.8 million deaths, compared to 3.4 million due to cancers (malignancies) and 5.2 million to injury (including war violence)¹. In an effort to minimize these casualties, the WHO and the United Nations Children’s Fund (UNICEF) together with other partners developed a Global Immunization Vision and Strategy (GIVS) two years ago. One of the main strategic areas of GIVS is the introduction of new vaccines and technologies against diseases for which no vaccine currently exists. Taken together

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with the fact that the rapid development of biopharmaceuticals suggests that many future vaccines will involve the delivery of peptide or protein subunits that lack a suitable carrier, there is an urgent need to search for viable solutions to design efficacious vaccines and improve public health².

When designing novel vaccine strategies, it is essential to have a detailed understanding of the complex interplay between immune cells of the innate and adaptive immune systems. As the first line of defense, cells of the innate immune system are involved in the recognition of foreign invaders by means of pathogen-associated-molecular patterns (PAMPs)³. Not surprisingly, the most crucial step to activate the immune system involves the activation of antigen presenting cells (APCs). Antigen presenting cells phagocytose pathogens and damaged cells and present antigens derived from these sources to helper T cells. Dendritic cells (DCs) are the only type of APC present in significant numbers in the T cell areas of lymph nodes⁴. Other experimental evidence shows that DCs are the essential APC for naïve T cell initiation and may also be involved in directly presenting MHC class II antigen to B cells and natural killer cells⁴.

Immature DCs are found under the skin and mucous membranes where they sample surroundings for possible pathogens through PAMPs, including toll-like receptors (TLRs) and C-type lectins⁵,⁶. After detecting a pathogen, these cells engulf it through phagocytosis and pinocytosis and migrate to lymph nodes (LN) where they become mature. Once mature, the surface expression of co-stimulatory molecules (i.e., CD80, CD86, CD40) and antigen presenting complexes, such as Major Histocompatibility Complex Class I and Class II (MHC I and MHC II), are upregulated
on the surface of DCs\textsuperscript{7}. Once inside the DC, pathogens are degraded into small
fragments that are further expressed at the surface in the context of MHC molecules,
where they can be presented to T cells and B cells\textsuperscript{3, 8}. Exogenous pathogens are
presented to CD4+ T cells by means of MHC II molecules. Subsets of CD4+ T cells
include the T helper 1 (Th1) and Th2 cells, which are involved in the cell-mediated
and humoral immune pathways, respectively. On the other hand, endogenous
pathogens are presented to CD8+ T cells by means of MHC I complexes on the DC,
which in turn activate cytotoxic T lymphocytes (CTLs). Consecutively, after the
specific T cell and B cell become activated, these cells generate a cascade of events
that lead to attack of the disease.

In addition of presenting antigens to cells of the adaptive immune system,
activated DCs are involved in the polarization of the immune response. Besides the
upregulation of co-stimulatory molecules and antigen presenting complexes,
activated DCs produce cytokines that communicate with the adaptive immune
system. Activated DCs may produce the cytokine tumor necrosis factor alpha
(TNF\alpha), which mediates acute inflammation, and a variety of interleukins, such as IL-
1\beta, IL-6, IL-10, and IL-12. IL-1 induces secretion of IL-2 from T cells, which
enhances proliferation of the precursors for antigen specific CTLs, natural killer (NK)
cells, and helper T cells. Th1 (or cell-mediated) responses are associated with the
release of interferon gamma (IFN\gamma), which is an immunoregulatory protein, and
tumor necrosis factor beta (TNF\beta)\textsuperscript{9}. A Th2 (or humoral) response is associated with
release of IL-4, IL-5, and IL-10 \textsuperscript{10}. 
Adjuvants are added to vaccines to enhance the immune response to the antigen of interest. An adjuvant can be categorized by one of three main functions: 1) acting as a depot for the antigen, slowing antigen clearance from the body as does aluminum-based or mineral oil emulsions, 2) directing the antigen to an APC as is seen with particulate vaccines like liposomes, and 3) activating the APC directly causing a “danger signal” which can be triggered with many bacterial components (i.e., LPS, CpG motifs, lipotechoic acid). Currently, the common adjuvants employed in human vaccines are aluminum-based, which cause some adverse reactions, need multiple doses (i.e., booster shots) to achieve protective immunity, and induce a humoral response with little to no demonstrable T cell response. Biodegradable polymers have shown promise in vaccine design as the controlled release provided by the degrading polymer can modulate the immune response against a particular disease, patient compliance is vastly improved with fewer doses, and both humoral and cellular immune responses have been observed. The controlled release of antigens by parenteral administration of biodegradable polymeric microspheres has been extensively studied. Microspheres greater than 10 μm can act as depots at the site of injection, while smaller microspheres can be efficiently taken up (phagocytosed) by APCs. After the APCs take up the microspheres, they can present the antigen to immune cells responsible for eliciting an immune response.

Polyanhydrides are a class of biodegradable polymers that have shown promise as carriers for controlled drug delivery and have been approved by the FDA for use in humans. Biocompatibility studies have shown the safety of these biomaterials as these degrade into non-mutagenic and non-cytotoxic products.
Another advantage of these polymers is their degradation by a surface erosion mechanism, which leads to a controlled release profile with predictable degradation profiles that can vary from days to months depending on the polymer chemistry. The polyanhydride chemistries used in the present study are based on the aliphatic sebacic acid (SA), the aromatic 1,6-bis(\(p\)-carboxyphenoxy)hexane (CPH), and the novel amphiphilic 1,8-bis(\(p\)-carboxyphenoxy)-3,6-dioxaocetane (CPTEG) (Fig. 7.1).

![Chemical structures of (a) poly(SA), (b) poly(CPH), and (c) poly CPTEG. (m and n represents degree of polymerization).](image)

The biocompatibility of CPH:SA and CPTEG:CPH copolymer libraries has been recently studied using high throughput cell-based screening methods\(^{33}\). CPH:SA and CPTEG:CPH copolymer libraries were incubated with standard myeloma, epithelial, and macrophage cell lines and were found to have no pronounced cytotoxic effect for any polymer composition at concentrations of 2.8 mg/mL and below, which is much higher than that expected in in vivo applications.
Previous work by Kipper et al. has shown that polyanhydrides based on CPH:SA chemistries are suitable vaccine carriers with enhanced adjuvanticity and possess immunomodulatory (i.e., Th1 vs. Th2) capabilities depending on polymer chemistry\textsuperscript{13}. These studies showed that a single dose of CPH:SA microspheres was able to enhance and modulate the induced immune response, depending on the composition of the polymer. While TT loaded 20:80 CPH:SA microspheres indicated a Th2 dominant response in C3H/HeOuJ mice, the 50:50 CPH:SA formulation produced a balanced Th1/Th2 response. Strong Th2 responses have been implicated in development of allergies and hypersensitivity reactions\textsuperscript{34}. The preferential reduction of the Th2 immune response is a unique and valuable characteristic of this delivery vehicle that makes it a promising candidate for vaccines. Although no \textit{in vivo} experiments have been published with the CPTEG:CPH system, this system has shown excellent characteristics for protein stabilization and release\textsuperscript{35, 36}. Preliminary in vivo studies in our laboratories have shown polymer-chemistry mediated immunomodulation in this system.

The purpose of the current study is to obtain a mechanistic understanding of the adjuvanticity and immunomodulatory capabilities of CPH:SA and CPTEG:CPH microspheres and to determine if the polymers act as adjuvants. Since the most potent APCs involved in the immune response are DCs, the \textit{in vitro} evaluation of murine bone marrow derived DC (BMDC) activation after incubation with polyanhydride microspheres was assessed. The surface expression of the antigen presenting complexes MHC I and MHCII, the co-stimulatory molecules CD86 and CD40, and the C-type lectin DC specific ICAM-3 grabbing non-integrin (DC-SIGN)
CD209, which is related to DC migration and the initial DC-T cell binding, was evaluated\textsuperscript{37}. Cytokine secretion from these activated DC was evaluated as a measure of Th1 or Th2 polarization of an immune response. Two different polyanhydride polymer chemistries (CPH:SA and CPTEG:CPH) both with different copolymer compositions were analyzed giving rise to the central hypothesis that surface chemistry plays a major role for DC activation.

### 7.2 Materials and Methods

#### 7.2.1 Materials

The chemicals needed for the synthesis of CPH and CPTEG monomers include: 4-\textit{p}-hydroxybenzoic acid, 1,6-dibromohexane, 1-methyl-2-pyrrolidinone, and triethylene glycol that were purchased from Sigma Aldrich (St Louis, MO); 4-\textit{p}-fluorobenzonitrile was obtained from Apollo Scientific (Cheshire, UK); potassium carbonate, dimethyl formamide, toluene, sulfuric acid, acetic acid, and acetonitrile were purchased from Fisher Scientific (Fairlawn, NJ). Acetic anhydride, methylene chloride, and petroleum ether, were purchased from Fisher Scientific (Fairlawn, NJ). Sebacic acid (99%), \(\beta\)-mercaptoethanol, \textit{E. coli} lipopolysaccharide (LPS) and rat immunoglobulin (IgG) were purchased from Sigma Aldrich (St. Louis, MO). The materials needed for the DC cell culture media include: RPMI 1640, 7.5% sodium bicarbonate, Penicillin-Streptomycin Solution (10,000 UI Penicillin and 10,000 ug/ml Streptomycin), and L-glutamine 200mM Solution, purchased from Mediatech (Herndon, VA); granulocyte macrophage colony stimulating factor (GM-CSF), purchased from PeproTech (Rocky Hill, NJ); and heat inactivated fetal calf serum,
purchased from Valley Biomedical (Winchester, VA). For flow cytometry experiments, the materials needed were: unlabeled mouse IgG, purchased from Pharmingen, Becton Dickinson (Franklin Lakes, NJ); unlabeled CD36/16 FcyR, purchased from Southern Biotech (Birmingham, AL); Purified Hamster IgG Isotype Control clone eBio299Arm, Alexa Fluor® 700 anti-mouse CD11c (clone N418), FITC conjugated anti mouse/rat MHC Class II (I-E^d^) (clone 144-4S), FITC conjugated anti mouse MHC class II (I-A/I-E) (clone M5/114.15.2), PE conjugated anti-mouse MHC Class I (H-2K^d^/H-2D^d^)(clone 34-1-2S), PE/Cy7 anti-mouse CD86 (clone GL-1), allophycocyanin (APC) anti-mouse CD40 (clone 1C10), PE conjugated anti-mouse CIRE (DC-SIGN CD209) (clone 5H10); and corresponding isotypes: Alexa Fluor® 700 conjugated armenian hamster IgG (clone eBio299Arm), FITC conjugated mouse IgG2a κ (clone eBM2a), PE/Cy7 conjugated rat IgG2b (clone KLH/G2b-1-2), APC rat IgG2a κ (clone eBR2a), and PE-conjugated rat IgG2a (clone eBR2a) were all purchased from e-Bioscience (San Diego, CA).

7.2.2 Polymer Synthesis and Characterization

CPH:SA and CPTEG:CPH copolymers were synthesized by melt polycondensation as described previously\textsuperscript{38}. The purity and degree of polymerization of the polymers was analyzed using \textsuperscript{1}H NMR spectra obtained from a Varian VXR-300 MHz NMR spectrometer.
7.2.3 Microsphere Fabrication

Prior to fabricating microspheres, all the glassware and equipment was washed with 70% ethanol to prevent microbial contamination. The procedure used to fabricate microspheres was modified from previously reported studies\textsuperscript{39, 40}. Briefly, polymer dissolved in methylene chloride was pumped with a syringe pump through an 8700-1200 MS ultrasonic atomizing nozzle (Sono Tek Corporation, Milton, NY) into 200 mL of frozen ethanol overlaid with approximately 100 mL of liquid nitrogen. This procedure was performed at 4ºC for 50:50 CPH:SA and CPTEG:CPH microspheres in order to maintain the temperature below the glass transition temperature of the polymers during microsphere preparation. After atomization, the hardened microspheres were stored at -80°C for three days to allow the methylene chloride to be extracted into the ethanol. The microspheres were then collected by filtration and dried under vacuum overnight.

The microsphere morphology was characterized by scanning electron microscopy (SEM) after being covered with gold. The particle size distribution was obtained from SEM images (150-250x) using a soft imaging system software (analySIS\textsuperscript{®}, Soft Imaging System Corp, Lakewood, CO). An average of 800 particles per image was analyzed. The parameters used during fabrication process are shown in Table 7.1.
### Table 7.1: Parameters used for microsphere fabrication (Lopac et. al)

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Methylene Chloride</th>
<th>Flow rate</th>
<th>Wattage ultrasonic nozzle</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPTEG 50:50</td>
<td>7 mL</td>
<td>3 mL/min</td>
<td>1.5 W</td>
</tr>
<tr>
<td>CPTEG:CPH 20:80</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10:90 CPTEG:CPH</td>
<td>4 mL</td>
<td>1.5 mL/min</td>
<td>2.5 W</td>
</tr>
<tr>
<td>50:50 CPH:SA</td>
<td>3 mL</td>
<td>1.5 mL/min</td>
<td>2.5 W</td>
</tr>
<tr>
<td>SA</td>
<td>3 mL</td>
<td>3 mL/min</td>
<td>1.5 W</td>
</tr>
<tr>
<td>20:80 CPH:SA</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

#### 7.2.4 Endotoxin Assay

To ensure that the endotoxin levels of polyanhydride microspheres were under the limit of contamination, a Limulus Amebocyte Lysate (LAL) QCL-1000 (Cambrex, Walkersville, MD) test was used to quantify the bacterial endotoxin levels after microsphere fabrication. Solutions of CPTEG:CPH and CPH:SA microspheres (5 mg/mL) were prepared in endotoxin-free sterile water and placed overnight in a rotator incubated at 37°C. After centrifuging microspheres solution, the LAL test was performed according to manufacturer procedure.

#### 7.2.5 Culture and Stimulation of DCs

Bone marrow derived dendritic cells (BMDCs) were isolated from the femurs and tibia of C3H/HeOuJ mice (ISU Laboratory Animal Resource Facility, Ames, IA) or C56BL/6 (purchased from Harlan Sprague Dawley, Inc, Indianapolis, IN) with a previously developed method. Briefly, after euthanizing mice, the tibia and femur were removed, muscle and other tissue removed, and bone cavities were washed three times with 1mL of culture media. After centrifuging at 200 x g for 10 minutes, the cells were suspended in culture media consisting of RPMI 1640 supplemented
with GM-CSF (10 ng/mL), 1% L-glutamine, 1% penicillin-streptomycin solution, 1% HEPES, 10% heat inactivated fetal bovine serum (FBS). Then cells were placed in 20 x 100 mm Petri dishes (4 x 10^5 cells/mL) and incubated at 37 °C under 5% CO₂ atmosphere. On day 3 and 6, 10ml of additional culture media was added. On day 7, DCs were removed from plates and, after suspending in fresh media, these were transferred to 24-well plates (2.5 x 10^6 cells/well). On day 9, DCs were incubated with the different stimulation treatments. Non-stimulated (NS) DCs and LPS (200 ng/ml) treated DCs were used as negative and positive controls, respectively. After suspending polyanhydride microspheres, these were incubated at concentrations of 0.06, 0.125, or 0.25 mg/mL. These concentrations correlate to approximate microsphere: DC ratios of 1:12, 1:6, and 1:3, respectively. The DC were incubated in the presence of microspheres or stimulants for 48h (37°C, 5% CO₂). Cell viability and morphology was monitored visually with an inverted microscope.

7.2.6 DC Activation

7.2.6.1 Cell Surface Markers

The surface expression of DC markers, co-stimulatory molecules, and antigen presenting complexes was analyzed with flow cytometry. After 48h of incubation with stimulation treatments, DCs were harvested and placed on polystyrene tubes (BD FALCON™, Franklin Lakes, NJ). After centrifuging (1,500 x g, 7min), DCs were resuspended in FACS Buffer (phosphate buffered saline pH 7.2 with 1% FCS and 0.5% Sodium Azide) based Fc blocking solution, containing 0.5 ug/ml purified anti-CD16/CD32(FcγRII/III) (eBioscience) and 100ug/ml Rat IgG, 1% mouse serum
(generated in-house). After blocking DCs for 1h on ice, cells were stained for flow cytometry with CD11c, MHC I, MHCII, CD86, CD40, and DC-SIGN CD209 antibodies. Respective isotype, single color, and unstained controls were included. Propidium iodide (PI) was used for live/death cell gating. The samples were run on a Becton-Dickinson FACSCanto flow cytometer (San Jose, CA) and analyzed with FlowJo (TreeStar Inc, Ashland, OR).

7.2.6.2 Cytokine Release

After incubating the DCs with the respective stimulation treatments for 48 h, 200 μL of cell-free supernatants were collected and stored at -20°C until ready for analysis. Cytokines such as TNF-α, which mediate acute inflammation, and interleukins such as IL-4, IL-6, IL-10, and IL-12(p40) concentration was assayed using Luminex® Multiplex assay (The FlowMetric System, Luminex, Austin, TX) and MasterPlex QT Quantification Software v2.0 (MiraiBio, Alameda CA). Cell-free supernatants were harvested after 72 h of culture and then analyzed for the concentration of TNFa, interferon c (IFNc), IL6, IL4, IL10 and IL12 using a multiplexed flow cytometric assay (The FlowMetric System,Luminex, Austin, Texas, USA). Samples were analyzed in duplicate for three separate experiments.

7.2.7 Statistical Analysis

A student-t test (α=0.05) was performed with the statistical analysis software JMP® 6 (Cary, NC). Flow cytometry data of the BMDCs incubated with the different polyanhydride compositions was compared with the NS and LPS-treated groups.
7.2.8 Principal Component Analysis (PCA)

PCA is a dimension reduction technique, whose goal in this study is to uncover latent features of the data that explain the relationships between polymer chemistry and cell marker expression. By reducing \( N \) original variables to a small number (\( K \)) of the principal components, PCA accomplishes data reduction and facilitates interpretation. The \( K \) principal components are a linear combination of the original \( N \) variables, and make up new axes that represent the directions with maximum variability. An analysis of principal components often reveals relationships that were not previously suspected and thereby allows interpretations that would not ordinarily result \( ^{42,43} \).
7.3 Results

7.3.1 Microsphere Fabrication

After microsphere fabrication by cryogenic atomization, the surface morphology was analyzed with SEM (Fig. 7.2). The images show that poly(CPTEG) and poly(SA) microspheres had a smoother surface than the other compositions which is attributed to processing conditions and differences in polymer properties (e.g., $T_g$). However, when the size distribution was analyzed from SEM images, the majority of the particles of all the polyanhydride chemistries used in this study were under 10 $\mu$m (Fig 7.3). Thus, most of the microspheres were in a size range that is readily phagocytosed by DCs.

Fig. 7.2. SEM Images of blank microspheres: (a) CPTEG, (b) 50:50 CPTEG:CPH, (c) 20:80 CPTEG:CPH, (d) SA, (e) 20:80 CPH:SA, and (f) 50:50 CPH:SA. Scale bars represents: 5 $\mu$m in (b),(c),(f); 10 $\mu$m in (a),(d); 20 $\mu$m in (e).
7.3.2 Endotoxin Assay

To ensure that the subsequent results of DC stimulation with CPTEG:CPH and CPH:SA microspheres are due to the polymers themselves and not a result of their microbial contamination all microsphere formulations were analyzed for endotoxin content using a limulus amebocyte lysate assay. All the polyanhydride microspheres exhibited an endotoxin content of less than 0.1 EU/mL, which is five times lower than the maximum level permitted by the Food and Drug Administration (FDA) for new drugs tested by the LAL test\textsuperscript{44}.

7.3.3 Culture and Stimulation of DCs

During the cell culture of BMDCs, cell viability and morphology was visually assessed with an inverted microscope. After day 6, veiled projections were visible on
most of the non-adherent cells, characteristic dendrites of DCs. On day 11, after the 48h stimulation treatments, polyanhydride microspheres appeared to be readily phagocytosed by the DCs (Fig.7.4). Further assessment is needed to show that polyanhydride microspheres are in fact contained in intracellular compartments (manuscript in preparation).

7.3.4 DC Activation

7.3.4.1 Cell Surface Markers

The surface expression of co-stimulatory molecules was analyzed after gating live (PI negative) cell subpopulations that were CD11c+. The CD11c+ BMDCs in the different experiments represented more than 80% of live cells (Fig.7.5). The flow cytometry histograms of BMDCs expressing MHCII show that all the polyanhydride chemistries enhanced the expression of MHCII in comparison with the untreated group (Fig. 7.6). More than 70% of the BMDCs incubated with the different microsphere compositions, at a concentration of 0.125 mg/mL (1:6 microsphere:DC
ratio) expressed MHCII in contrast to the NS cells, where only 57% were MHCII+. A notable observation from these histograms is that LPS, and most polyanhydrides chemistries, especially the more hydrophobic ones, had two characteristics subpopulations: a broader and taller peak at a dimmer fluorescence and a smaller peak at brighter intensities. This indicates that the cell population was not completely homogenous and there were different levels of maturation. The surface expression of MHCII was also enhanced in the BMDCs incubated with the other concentrations (0.06 and 0.25 mg/mL) in a dose-dependent manner (data not shown).

Fig. 7.5. Representative flow cytometry histogram of BMDCs stained with anti-CD11c. Positive population was gated with respect isotype control.

Although antigen presentation by MHCI usually involves endogenous pathogens derived within the cell, processing of exogenous antigens, especially the particulate ones, for MHCI can occur by MHCI cross-presentation\textsuperscript{45, 46}. The expression of MHCI on DCs was also analyzed after being stimulated with polyanhydride microspheres and the microspheres of the more amphiphilic polymers (i.e. high CPTEG content), enhanced more significantly the expression of MHCI (Fig. 7.7). It can be seen from the histograms shown that 50:50 CPTEG:CPH microspheres enhanced more markedly the expression of MHCI when compared with 50:50 CPH:SA microspheres. As both microsphere compositions had 50% of
the more hydrophobic CPH, it suggests that the increased stimulation of 50:50 CPTEG:CPH was a consequence of the more amphiphilic CPTEG. Nevertheless, no matter the polyanhydride composition, all microspheres enhanced MHCI expression at different stimulation levels, suggesting that there is a role of chemistry for DC activation (data not shown). All the BMDCs incubated with CPTEG:CPH microspheres enhanced the surface expression of MHCI and the stimulation level was directly proportional to the CPTEG content. The CPH:SA microspheres did enhance MHCI expression as well. The general trend for MHCI surface expression on BMDCs after being incubated with polyanhydride microspheres is shown in Figure 7.8.

![MHCII Histograms](image)

**Fig. 7.6.** MHCII Histograms of BMDCs incubated with different stimulation treatments: (a) NS, (b) LPS, (c) CPTEG, (d) 50:50 CPTEG:CPH, (e) 20:80 CPTEG:CPH, (f) 10:90 CPTEG:CPH, (g) SA, (h) 20:80 CPH:SA, and (i) 50:50 CPH:SA. DCs expressing MHCII (open histograms), were gated with respect to the background staining with isotype controls (solid black histograms). Concentration of polyanhydride microspheres was 0.125 mg/mL corresponding to 1:6 microsphere:DC ratio.
Fig. 7.7 MHCI histograms of NS BMDCs (solid black), 50:50 CPH:SA-treated BMDCs (tinted gray), and 50:50 CPTEG:CPH-treated BMDCs (dashed line). Concentration of polyanhydride microspheres was 0.125 mg/mL corresponding to 1:6 microsphere:DC ratio.

Fig. 7.8. Stimulation trend of polyanhydride microspheres for MHCI surface expression


Fig. 7.8. Stimulation trend of polyanhydride microspheres for MHCI surface expression

The surface expression of the co-stimulatory molecule CD86 was also evaluated and clear trends with respect to polymer chemistry were observed again (Fig 7.9). For the CPTEG:CPH system, stimulation was enhanced in the cells incubated with high CPTEG-containing copolymer microspheres, indicative that the amphiphilic environment was more favorable for the activation of BMDCs. Poly CPTEG, 50:50 and 20:80 CPTEG:CPH compositions enhanced the CD86 expression at a level similar to that of LPS-treated cells as indicated by the statistical analysis. The CPH:SA system enhanced the expression of CD86 at a significantly
lower level than the CPTEG:CPH system. However, the trend with the CPH:SA system indicated that an increase in hydrophobicity (i.e. CPH content) was more favorable, and poly(SA) microspheres down regulated the CD86 expression. A possible explanation for the poly(SA) results may be that the degradation of the high SA content polymers produce acidic environments^{47} that could be detrimental to the surrounding cells during the 48 h incubation period.

The magnitude of the fold increase in the surface expression of CD40 of stimulated BMDCs was much higher than that with the other surface molecules analyzed (Fig. 7.10). The LPS-treated BMDCs enhanced the expression of CD40 ~20 fold compared to the expression of the NS group. The stimulation trends in both CPTEG:CPH and CPH:SA systems indicated that a decrease in hydrophobicity increases the expression of this co-stimulatory molecule. The chemistries that improved the CD40 expression most significantly were poly(CPTEG) and 50:50 CPTEG:CPH.
Fig. 7.9. CD86 surface expression of BMDCs after stimulation with CPTEG:CPH microspheres (0.25 mg/mL, microsphere:DC 1:3). The data is presented as fold increase over NS BMDCs. Error bars represent standard error from 3-4 separate experiments. A student-t test was used for statistical analysis (P-value<0.05). (* = statistically different from NS; +=statistically different from LPS)

Fig. 7.10. CD40 surface expression of BMDCs after stimulation with CPTEG:CPH microspheres (0.25 mg/mL, microsphere:DC 1:3). The data is presented as fold increase over NS BMDCs. Error bars represent standard error from 3-4 separate experiments. A student-t test was used for statistical analysis (P-value<0.05). (* = statistically different from NS; +=statistically different from LPS)
The expression of DC-SIGN CD209 was also enhanced with all the polyanhydride chemistries tested (Fig. 7.11). As shown with the other markers studied, the amphiphilic chemistries poly(CPTEG) and 50:50 CPTEG:CPH enhanced the expression more significantly and, in the particular case of DC-SIGN, it was significantly increased when compared to both NS and LPS-treated cells. As has been shown in the past, LPS did not up regulate the expression of DC-SIGN\textsuperscript{37, 48}. In the case of the CPH:SA system, the expression of this surface molecule was significantly enhanced in the 20:80 CPH:SA-treated BMDCs. The surface expression of DC-SIGN is of particular importance, as even though it has been demonstrated that viral pathogens target DC-SIGN for transmission, non-viral pathogens modulate DC maturation through DC-SIGN\textsuperscript{5, 49}.

![Fig. 7.11. DC-SIGN CD209 surface expression of BMDCs after stimulation with CPTEG:CPH microspheres (0.25 mg/mL, microsphere:DC 1:3). The data is presented as fold increase over NS BMDCs. Error bars represent standard error from 3-4 separate experiments. A student-t test was used for statistical analysis (P-value<0.05). (* = statistically different from NS; +=statistically different from LPS)](image-url)
The surface expression of CD86, CD40 and DC-SIGN CD209 were also enhanced in the BMDCs incubated with the other concentrations of polyanhydride microspheres (0.06 and 0.125 mg/mL) in a dose-dependent manner (data not shown).

7.3.4.2 Cytokine Release

It is well known that activated DCs secrete cytokines that act as messengers to the adaptive immune cells. The measurement of the cytokines secreted by stimulated BMDCs support the flow cytometry findings that polyanhydride microspheres are able to activate BMDCs efficiently (Fig 7.12). The specific cytokine profile will determine what immune response is elicited and if the polymer by itself acts as a potential adjuvant to enhance this response. The cytokines that were secreted by the cells incubated with the microspheres were TNFα, IL-6, and IL-12. The cytokines IL-10 and IL-4 were also measured but their levels were below detection with all the polyanhydride chemistries. The secretion of IL-12 and the absence of IL-4 in the cell cultures suggest that polyanhydride chemistries induce a preferential Th1 response, which is required for intracellular pathogens.

Fig. 7.12. Cytokines secreted by BMDCs after incubation with stimulation treatments.
7.3.5 PCA

In this study, cell marker expressions of CD40, DC-SIGN CD209, and CD86 are studied when the BMDCs are incubated with different polymer chemistries. PCA is applied on standardized data and the original data is projected on to a “biplot” (Fig. 7.13). A biplot is a graphical representation of information of both principal components and variables. The first principal component (PC1) explains 62.16% of data variance, and the second principal component (PC2) explains 33.51% of data variance. PC1 and PC2 together account for 95.67% of the data variance, which means PC1 and PC2 could replace the original three variables (CD86, CD40 and CD209) with little loss of information.

We observed that both the CPH:SA and the CPTEG:CPH systems activate DCs. However, the two polymer systems are different from each other in terms of the magnitude of the effect on DC activation. From Fig. 7.13 we can see data points representing cell marker expression when the cells are incubated with polymer (blue and yellow circles) locate clearly differently from the NS data points (red circles). The analysis indicates that as more SA or CPTEG is added to the copolymer, the data move further away from NS, which means more DCs are activated by reducing the hydrophobicity of the polymer (i.e., polymers with higher composition of SA or CPTEG). Additionally, the analysis indicates that the addition of CPTEG has a stronger influence on DC activation than the addition of SA. DCs treated with the CPTEG:CPH system (yellow points) locate further away from NS than DCs treated with CPH:SA system (blue points). The arrows in Fig.7.13 indicate the direction of increasing polymer hydrophilicity. Data points with higher hydrophilicity locate further
away from NS, which means DCs are more activated. The data suggests that 50:50CPTEG:CPH appears to more strongly influence the cell marker expression than poly(CPTEG). This suggests that there may be an optimal amphiphilic chemistry that may maximize DC activation.

Fig. 7.13. The biplot of PCA on adjuvant cell marker expression data. Each point corresponds to the projection of an experiment data point on PC1 and PC2. Data of SA system are colored blue, and CPTEG system yellow. Circles are used to show the distribution of data under the same treatment. For convenience, only the compositions of SA and CPTEG are used as circle labels. PC1 and PC2 together explain 95.67% data variance. Increasing amount of SA or CPTEG to CPH increases dissimilarity from untreated cell. CPTEG system has a stronger impact on cell marker expression than SA system.
7.4 Discussion

There is an immediate need for novel adjuvants to improve vaccine efficacy and at the same time to redirect the immune response against a particular disease. As has been explained before, the design for new adjuvants involves an in-depth understanding of the complex function of the communication between the innate and adaptive immune systems. These studies demonstrated that polyanhydride microspheres are promising candidates as vaccine adjuvants. One important attribute of these biodegradable polymers is that even small amounts of microspheres were capable of enhancing the maturation of murine BMDCs at significant levels. The ratios of polyanhydride microspheres to DCs used in these studies were significantly lower compared to previous studies with polyester microspheres. In these studies, the polyester microspheres were used at ratios that were more than 50 times the ratio used with polyanhydrides in the current study, and in other studies, immune stimulators were added. In spite of these differences, the extent of DC stimulation by the polyanhydride microspheres is superior and they also promote the expression of DC-SIGN CD 209, which suggests that polyanhydride microspheres also promote DC maturation and migration to the LN. The low dosage of the polyanhydride microspheres is a very important characteristic to take into consideration when designing vaccines, as more potent immune responses with minimum amount of adjuvant carriers are desirable.

Overall, the results indicate that an increase in polymer hydrophilicity enhanced the surface expression of MHCII and MHC I complexes and the co-stimulatory molecules CD86, CD40, and DC-SIGN CD209, suggesting that adjuvant
chemistry plays a major role in the activation of DCs. It is very interesting to note that all the surface molecules were enhanced at different levels, which indicates that different polyanhydride chemistries might activate DCs by different pathways. Moreover, it has been recently published that polyanhydride chemistry also affects protein release and stability\(^{36}\). In these studies, the amphiphilic, high CPTEG-containing microspheres enhanced protein stability, which is in accordance with the fact that carriers containing both hydrophobic and hydrophilic entities may provide a gentler environment for proteins\(^{53},^{54}\). Taking together with the results of activation of the BMDCs, high CPTEG-containing polyanhydrides are very promising candidates for the delivery of vaccines. However, both the CPTEG:CPH and CPH:SA chemistries significantly improved the activation of BMDCs (with low doses and especially in comparison with polyesters) and since it is known that a small number of DCs are sufficient to induce strong immune responses\(^{48}\), all the polyanhydride chemistries studied here are potential adjuvants. This activation caused by these biodegradable polymers is in accordance with the “danger signal” model described by Matzinger\(^{55}\). In this model a hydrophobic molecule that is exposed to innate cells is recognized as a “danger signal” and the cells become activated. Since all the polyanhydrides included in these studies are relatively hydrophobic, especially when compared to sugars and lipids, it is more likely that DCs are activated due the hydrophobicity of the polymers and the extent of activation is then further influenced by the individual chemistries. We suggest that an optimal vaccine formulation might consist of a cocktail of microspheres of different compositions, with different release profiles that optimizes antigen stability over longer periods of time and at the same
time enhances the activation and maturation of BMDCs and induces a preferential Th1 immune response.

**Implications for Vaccine Design**

As the mere activation of BMDCs is not an absolute indicator of an effective immune response, the further evaluation of the activation of effector cells is required. In this regard, a model describing the signals required for DCs to activate T cells has been proposed and based on this model, it appears that BMDC activation by polyanhydride microspheres encompasses the major signals for T cell activation (Fig. 7.14)\textsuperscript{48, 56}. The first signal starts when a DC recognizes a pathogen through PAMP receptors such as toll like receptors (TLR) and C-type lectins. Subsequently, these pathogens are degraded within different intracellular compartments after being phagocytosed and different pathways lead to DC activation. This signal mainly involves the antigen-specific stimulation provided by MHC-antigen complexes on the DC surface, which are expressed at higher levels after its maturation. Even though this signal is essential for a specific immune response, it will not induce a strong T cell activation after migrating to the LN without the help of the second signal that involves other accessory molecules expressed by the DCs. The emergence of the B7 co-stimulatory molecules, such as CD86, in DCs after maturation is crucial to activate the clonal expansion of naïve T cells, thus directing the magnitude of the resulting immune response. It has been also proposed that the C-type lectin DC-SIGN has an adhesion role that is essential for the initial DC-T cell interaction in the LN\textsuperscript{37}. Finally, the third activation signal proposed in the model is related to the
maintenance and polarization of the T helper cells that will lead to the selection of the most appropriate effector mechanism. Of particular importance is the expression of the co-stimulatory molecule CD40, as it induces the maintenance of high levels of MHC class II antigens and up regulates the expression of B7 molecules after its binding to the CD40 ligand on T cells. The antigen presenting capacity is enhanced, the response of the T cells is sustained, and the DC survival is prolonged. In addition, the DCs are capable of inducing the polarization of the immune response towards Th1 (cell-mediated) versus Th2 (humoral) pathways as indicated by the cytokine profiles of the activated DCs.

In summary, the polyanhydride microspheres used in this study show that these biodegradable polymers have promising characteristics for the development of vaccine adjuvants. The three major functions of an adjuvant, which are covered by these polymers, include: (1) providing a “depot” or reservoir for the antigen for a slow release; (2) enhancing antigen phagocytosis by APCs; and (3) modulating and enhancing the immune response against the particular antigen alone. All three characteristics are exhibited by polyanhydride microspheres with the added advantage of enhancing protein/antigen stability. Further studies in our laboratories involve the evaluation of antigen presentation by DCs that have been stimulated with polyanhydride microspheres.
Fig. 7.14 Immune activation of DCs. **Signal 1:** Pathogens can interact with different PAMPs present in the DCs. Pathogens that interact with TLRs are processed by intracellular signaling that lead to DC maturation and induction of inflammatory cytokines. Other receptors, such as C-type lectins recognize specific carbohydrate structures on the pathogens that are subsequently internalized and degraded in the lysosomes to enhance antigen processing and presentation. After DC becomes mature, it migrates to the lymph nodes where the DC-SIGN expression optimizes the initial T cell binding. **Signal 2:** The antigen presentation by MHC-peptide complexes and the expression of the co-stimulatory molecules (CD86, CD40) seals the immunologic synapse needed for T cell activation. **Signal 3:** The sustained interactions of DC with T cells enhance the antigen presenting capacity of DCs by maintenance of MHC complexes and upregulation of co-stimulatory molecules.
7.5 Conclusions

Despite the advantages of enhancing and modulating immunogenicity using polymeric microspheres as vaccine carriers, no vaccines based on polymeric carriers have been approved for human use to date. The challenge of overcoming antigen instability, antigen immunogenicity, and the ability to activate APCs, limits the range of materials that can be used for this purpose. The ability of polyanhydride microspheres to activate murine BMDCs in conjunction with their biocompatibility and gentle environment demonstrates that these are promising candidates for the development of vaccines. In addition, the studies described here clearly point out the role of polymer chemistry in APC activation in vitro and lay the foundation for further studies to probe the molecular and cellular mechanisms responsible for this effect.

7.6 Acknowledgements

The authors would like to thank Shawn Rigby for running flow cytometry samples and for his assistance with data analysis, and Andrea Dorn, Mary Byron, and Gretchen Anderson for helping with the BMDC experiments. We would also like to thank the ONR-MURI (NN00014-06-1-1176) award and the ISU Institute for Combinatorial Discovery for financial support. Maria Torres acknowledges support of the NSF-Iowa AGEP Fellowship and the Ruth L. Kirschstein National Service Award (5F31CA126533-02) from the NIH.
### 7.7 References


44. (ed. (FDA), F.a.D.A.) 1-10 (Center for Drug Evaluation and Research, 1987).


CHAPTER 8
EVALUATION OF NON-SPECIFIC IMMUNITY OF POLYANHYDRIDE MICROSPHERES: IMPLICATIONS FOR THE DESIGN OF PANCREATIC CANCER VACCINES

8.1 Introduction

According to the National Cancer Institute, pancreatic cancer is among the top five leading causes of cancer-related deaths in the United States. Essentially all human pancreatic adenocarcinomas are resistant to conventional therapy protocols. Thus, there is an urgent need to search for effective treatments for this lethal disease\(^1\), and the promise of cancer vaccines is leading to the use of immunotherapy as a viable alternative\(^2\). As discussed in Chapter 2, extensive research on tumor markers have led to the discovery of the glycoproteins, Mucin-1 and Mucin-4, as potential pancreatic cancer targets for immunotherapy\(^3\)-\(^{13}\). Humoral and cellular immune responses to Mucin-1 and Mucin-4 have been observed in cancer patients with poor prognosis\(^6\), \(^{14}\). Although there is an aberrant expression of these surface molecules on pancreatic tumors, these are not strongly immunogenic. Thus, an important goal is to design a novel vaccine delivery system that will lead to strong anti-tumor responses against these malignant cells. The potential vaccine will be comprised of two essential components that will function in conjunction: the antigen that is over expressed in the tumor (i.e., Mucin-1 or Mucin-4) and the adjuvant in charge of protecting, stabilizing and delivering this antigen efficiently and at the same time enhancing the immune response against cancerous cells.
In order to understand the potential anti-tumor effects that an adjuvant may have, the evaluation of the immunity of the adjuvant by itself needs to be well understood. This chapter evaluates non-specific anti-tumor immunity conferred by the novel biodegradable amphiphilic system based on the anhydride monomers 1,6-bis(p-carboxyphenoxy)hexane (CPH) and 1,8-bis(p-carboxyphenoxy)-3,6-dioxaoctane (CPTEG). As the results of the previous chapters have shown, microspheres that were fabricated from CPTEG-containing polymers showed excellent characteristics for the design of vaccine adjuvants. The ability of CPTEG:CPH microspheres to stabilize and deliver functional proteins in a controlled and sustained manner has been demonstrated in Chapters 5 and 6. Furthermore, we have shown that CPTEG:CPH microspheres enhance the expression of antigen presenting complexes and T cell co-stimulatory molecules on dendritic cells (DCs), indicating that this novel polyanhydride system has the capability of enhancing the maturation and activation of DCs, which is the crucial step for immune activation (Chapter 7). Although the importance of effective DC activation is highlighted by studies that show few DCs infiltrating tumors and this likely accounts for some of the ability of tumors to escape immune surveillance\(^\text{15}\), the activation of antitumor effector cells needs to be assessed. As explained in Chapter 2, DCs presenting tumor antigens are capable of activating tumor specific CD8\(^+\) T cells, which in turn kill tumor cells directly by activating cytotoxic T lymphocytes (CTLs). The ability of CPTEG:CPH blank microspheres to non-specifically enhance antitumor effector cells in \textit{in vitro} and \textit{in vivo} experiments are analyzed and based on the results obtained,
an experimental approach towards the design of the pancreatic cancer vaccine is described.

8.2 Experimental Methods

8.2.1 Fabrication of CPTEG:CPH Microspheres

As explained in Chapter 4, CPTEG:CPH copolymers were synthesized at various monomers ratios by melt polycondensation\textsuperscript{16}. Endotoxin-free microspheres were fabricated by the non-aqueous method of cryogenic atomization and characterized with scanning electron microscopy (SEM) as previously described (Chapter 7)\textsuperscript{17}.

8.2.2 In Vitro Activation of Effector Cells: CTL Assay

Human peripheral blood mononuclear cells (PBMCs) that express human leukocyte antigen genes of MHCI (i.e., HLA-A2) were isolated by a Ficoll-Hypaque method. Stimulator (γ-irradiated lymphocytes) and responder lymphocytes were incubated in 1:2 ratio with CPTEG:CPH microspheres (0.25 mg/mL). Lymphocytes were fed with media supplemented with rh-IL2 every 3 days. Cells-free supernatants were sampled every 3 days and stored at -80°C for further cytokine analysis. After two weeks, 2 x 10^5 lymphocytes (Effector, E) were incubated for 4 h with 10^4 Panc-1 (Target, Tr) on triplicate wells (96-well V-bottom wells) and the cytotoxicity of T cell lymphocytes (CTL) was evaluated with the non-radioactive cytotoxicity assay CytoTox96\textsuperscript{®} (Promega Corporation, Madison, WI) that quantitatively measures
lactate dehydrogenase (LDH) that is released upon cell lysis. Based on the manufacturer’s protocol, the % cytotoxicity was calculated as:

\[ \% \text{Cytotoxicity} = \left( \frac{\text{Experimental Rel} - \text{Spontaneous E} - \text{Spontaneous Tr Rel}}{\text{Maximum Tr Rel} - \text{Spontaneous Tr Rel}} \right) \times 100 \]

8.2.3 In Vivo Activation of Effector Cells

The non-specific activation of CD8 T cells was evaluated after injecting 20:80 and 50:50 CPTEG:CPH blank microspheres (0.5 mg) into female C57BL/6 mice (N=5) by subcutaneous route. The control group was injected with phosphate buffered saline (PBS). After two weeks, the spleen (SP), the auxiliary, brachial and inguinal lymph nodes (LNs) were removed from each animal after being euthanized with carbon dioxide. LN (lymphocytes) and SP (spleenocytes) single cell suspensions for each animal were prepared with a hand held tissue grinder. After removing remaining tissue and cellular debris with a cell strainer and washing by centrifuging, the cells were counted with a hemocytometer. Trypan blue was added to count viable cells. For the SP suspensions, the red blood cells were lysed prior to counting. Cells were kept on ice until ready for use in the $^{51}$Cr release and flow cytometry assays described below.

8.2.3.1 $^{51}$Cr Release Assay

A $^{51}$Cr release assay was performed with the murine pancreatic cancer cell line Panc02 as the target cells (Tr) and LN and SP suspensions of each individual animal as the effector (E) cells. Briefly, the Tr cells were labeled with Na$_2$$^{51}$CrO$_4$ (300 $\mu$Ci/10$^6$ Tr cells). After 2 h, the labeled Tr cells were washed three times with cold
media and counted. The assay was then performed in 96-well V-bottom plates. The E:Tr ratio used were 50:1, 25:1, and 12.5:1 on triplicate wells. The number of Tr cells was maintained constant in each well \((2 \times 10^3\text{ cells/well})\). Triplicate wells for maximum (detergent solution: 1\%NP40/\%10 acetic acid) and spontaneous (medium only) Tr release were included. After incubating the plate for 5 h \((37°C, 5\%\text{CO}_2)\), 100 \(\mu\text{L}\) of the supernatants were collected and the radioactivity was measured with a 1480 Wallac® Wizard Gamma Counter (Perkin-Elmer Life and Analytical Sciences, Wellesley, MA). The % cytotoxicity was then calculated as:

\[
\%\text{Cytotoxicity} = \left(\frac{\text{Experimental Rel Tr} - \text{Spontaneous Tr Rel}}{\text{Maximum Tr Rel} - \text{Spontaneous Tr Rel}}\right) \times 100
\]

8.2.3.2 Flow Cytometry

For the flow cytometric analysis, 0.5 \(\times 10^6\) cells of the LN and SP suspensions were placed on polystyrene round bottom tubes (BD Falcon™, Franklin Lakes, NJ). The protocol of immunofluorescent staining of cell suspensions of mouse lymphoid tissue from eBioscience (San Diego, CA) was followed with some modifications. All the staining antibodies were also obtained from e-Bioscience and recommended concentrations were used. After centrifuging the already counted LN and SP cells, these were suspended in 100 \(\mu\text{L}\) of blocking buffer (0.5 \(\mu\text{g}\) of anti-CD16/CD32 in FACS buffer) and incubated at 4°C for 30 min. The primary antibodies, fluorescein isothiocyanate (FITC) anti-mouse CD8a (Ly-2), Phycoerythrin (PE) anti-mouse/human CD44 (Pgp-1, Ly24), and PE-Cy7 anti-mouse CD62L were
suspended at the respective concentrations and 50 μL of the staining solution was added to each tube and incubated for 30 min at 4°C protected from light. Control tubes included single color, isotype controls, and unstained cells. After the staining incubation period, the cells were washed three times (FACS buffer, 1% fetal bovine serum (FBS)) and fixed in 2% paraformaldehyde solution until the next day. Samples were run on a Becton Dickinson FACSArria (Franklin Lakes, NJ) cytometer. Data analysis was performed after gating viable cells that expressed CD8. The CD62L vs. CD44 dot plots were used to analyze the CD8 cells that were naïve, in transition from naïve to effector, and effector. A student-t test \( (\alpha=0.05) \) was performed with the statistical analysis software JMP® 6 (Cary, NC).

8.2.4 In Vivo Evaluation Non-Specific Tumor Immunity

On day 0, pancreatic tumor cells (Panc02) \( (7.5 \times 10^5 \text{ cells}) \) were injected in combination with blank CPTEG:CPH microspheres \((0.3 \text{ mg})\) by subcutaneous injection into female C57BL/6 MUC-4 knockout mice \((N=5)\). After three weeks of immunizations mice were sacrificed and tumors were measured and embedded in paraffin for immunohistochemical analysis.
8.3 Results and Discussion

8.3.1 CTL Assay

A CTL assay was performed on PBMCs incubated with blank CPTEG:CPH microspheres in order to evaluate if the adjuvant stimulates the proliferation of CD8\(^+\) effector cells. It was found that the PBMCs stimulated with polyanhydride microspheres induced the lysis of ~70% of the human pancreatic cancer cells Panc-1 (Fig 8.1). On the other hand, the unstimulated cells induced less than 15% cytotoxicity. It is known that Panc-1 expresses antigen by means of the major histocompatibility complex class I (MHCI) and tumor cell lysis is caused by CTLs, which is MHCI-restricted\(^{18}\). It is important to note that the CPTEG:CPH copolymers do not induce cell death at the concentration used in this experiment (0.25 mg/mL) as previous experiments have shown that these polymers are not cytotoxic at concentrations that were over one fold higher\(^{19}\). Thus, the lysis was not caused by the direct contact of the cells with the polymer, instead it was caused by effector cells that were present in the culture. A possible explanation to the increased non-specific proliferation of CTLs of the PMBCs incubated with the polyanhydride microspheres is that monocytes of the PBMCs might have differentiated into DCs that eventually matured with the increased expression of MHCI and T cell co-stimulatory molecules, a process that is enhanced with CPTEG-containing microspheres\(^{17}\). Additionally, even if rh-IL2 was added to the cell culture to induce T cell growth and differentiation, this cytokine can be produced by activated T cells as well\(^{20}\). Thus, if the polyanhydride microspheres induced DC differentiation and activation, it is likely that T cells present in the culture become activated and their
production of rh-IL2 will further increase their proliferation. Unfortunately, the cytokine results were not available. Although the results obtained were non-antigen specific, it has been demonstrated that lymphocytes can become activated non-specifically\textsuperscript{21} and that compounds that induce DC maturation, such as LPS, can induce differentiation of monocytes into DCs\textsuperscript{22}. These are preliminary studies that need to be repeated with more polyanhydride chemistries and the evaluation of the cytokine profiles.

<table>
<thead>
<tr>
<th>Stimulation</th>
<th>% Cytotoxicity against Panc-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatments</td>
<td></td>
</tr>
<tr>
<td>Non-stimulated</td>
<td>11.52%</td>
</tr>
<tr>
<td>20:80 CPTEG:CPH</td>
<td>68.19%</td>
</tr>
</tbody>
</table>

Figure 8.1. SEM image of blank 20:80 CPTEG:CPH microspheres and CTL assay results of PBMCs incubated with 20:80 CPTEG:CPH microspheres
8.3.2 *In Vivo* Activation of Effector Cells

As *in vitro* studies may not be truly reflective of *in vivo* responses, the non-specific activation of anti tumor effector cells by CPTEG:CPH microspheres was evaluated on animal models.

8.3.2.1 $^{51}$Cr Release Assay

In an analogous assay of the *in vitro* CTL assay, the non-specific CTL activation was evaluated after injecting blank CPTEG:CPH microspheres into C57BL/6 mice. The microspheres were injected subcutaneously and after two weeks, the spleenocytes and lymphocytes were evaluated for CTL activity against the murine pancreatic cancer cell line Panc02. Although a different assay was used, both the $^{51}$Cr and LDH release methods have shown to be sensitive for cytotoxicity quantitation. As opposed to the preliminary results shown in the previous section, the CPTEG:CPH microspheres induced less than 3% cytotoxicity against cancer cells obtained from LNs (Fig 8.2) and spleen suspensions (data not shown). These are not unexpected, since the microspheres have the ability to activate DCs at the injection site, but when these mature and migrate to the LNs, in the absence of a specific antigen expression, the naïve cells would not become activated into effector cells.
217

8.3.2.2 Flow Cytometry

The phenotype of the lymphocytes and spleenocytes of the mice injected with PBS and blank CPTEG:CPH microspheres was analyzed with flow cytometry. The surface molecules analyzed were CD44, CD62L and CD8α. As explained earlier, the cells in charge of anti-tumor immune responses are the CTLs, which are activated by CD8⁺ T cells. To analyze the flow cytometric data of the cells isolated from the secondary lymphoid organs, the viable cells of each individual mouse were gated for the surface expression of CD8 (Table 8.1). As noted, there were no significant differences within spleenocytes and lymphocytes across the different formulation groups. It is important to note that percentage of CD8 cells was doubled in the lymphocytes when compared to the spleenocytes, as further analysis is based on these populations. Representative histograms of lymphocytes expressing CD8α are shown in Fig. 8.3.
Table 8.1 Percent of lymphocytes and spleenocytes expressing the surface marker CD8. (N=5 mice)

<table>
<thead>
<tr>
<th>Formulation</th>
<th>% Viable lymphocytes expressing CD8</th>
<th>% Viable spleenocytes expressing CD8</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>21 ± 9</td>
<td>11 ± 1</td>
</tr>
<tr>
<td>20:80 CPTEG:CPH</td>
<td>24 ± 5</td>
<td>12 ± 2</td>
</tr>
<tr>
<td>50:50 CPTEG:CPH</td>
<td>26 ± 1</td>
<td>13 ± 5</td>
</tr>
</tbody>
</table>

Figure 8.3. Flow cytometry histograms of LN cells expressing CD8a from mouse injected with 20:80 (left) and 50:50 CPTEG:CPH microspheres (right). Cells were gated with respect to the corresponding isotype.

In order to evaluate the phenotype of the CD8a cell populations, dot plots of CD62L vs. CD44 of gated CD8a cells from each individual animal were analyzed (Fig. 8.4). It is known that the cell adhesion molecule CD62L is expressed on naïve CD8 T cells and the molecule CD44 is expressed on effector CD8 T cells. Accordingly, the lymphocytes and spleenocytes were classified as naïve CD8 cells (CD62L$^{hi}$, CD44$^{lo}$), cells that were in transition from naïve to effector (CD62L$^{hi}$, CD44$^{hi}$), and effector CD8 cells (CD62$^{lo}$, CD44$^{hi}$).
Figure 8.4. Analysis of CD8 lymphocytes population. CD62L vs. CD44 representative dot plots of lymphocytes of mice injected with: (a) PBS, (b) 20:80 CPTEG:CPH, and (c) 50:50 CPTEG:CPH microspheres.

As shown in Figure 8.5, the majority of the lymphocytes CD8 populations were in the transition from naïve CD8 to effector CD8 T cells. The lymphocytes isolated from the groups that were injected with CPTEG:CPH microspheres showed this transition more significantly than in the group injected with PBS, indicating the potential of polyanhydride microspheres to induce activation of naïve CD8 T cells. The percentage of CD8 lymphocytes that were effector CD8 T cells were lower than naïve CD8 T cells and the differences between the formulation groups were not significant.

The phenotype of the spleenocytes expressing CD8 is shown in Figure 8.6. Whereas it appears that the number of effector CD8 T cells that were induced in the SP was quadrupled from the ones induced in the LN, the results are not as drastic. Taking into account that the overall CD8 cell population in the SP was near half of the overall population when compared to the lymphocytes, the effector CD8 T cells induced in the SP was doubled instead of quadrupled. Nevertheless, the percentage of cells that were classified in transition from naïve to effector CD8 T cells in the LN
was much higher than in the SP. As a clarifying point, the statistical analysis done in all the groups showed no significant differences, and hence no effect can be attributed to the injection of CPTEG:CPH microspheres.

Figure 8.5. Flow cytometric results of lymphocytes expressing CD8. Error bars represent standard deviations. N=5; * = statistically different from PBS group (P-value<0.05).

Figure 8.6. Flow cytometric results of spleenocytes expressing CD8. N=5, Error bars represent standard deviations.
8.3.3 *In Vivo* Evaluation Non-Specific Tumor Immunity

The tumor mass of the mice that received blank CPTEG:CPH microspheres in conjunction with Panc02 cells was weighed (Fig 8.7). Even when the number of tumor cells injected was the same in every animal, the chemistry influenced the tumor growth. The tumor grew more noticeably in the mice injected with 50:50 CPTEG:CPH microspheres than in the ones injected with 20:80 CPTEG:CPH microspheres. These results are surprising as instead of suppressing the proliferation of malignant cells, the CPTEG content stimulated tumor growth. A logical explanation is that, as has been shown before, the high CPTEG-containing copolymers enhanced the activation of DCs and in consequence the production of proinflammatory cytokines such as IL-6 and TNFα was increased\(^{17}\). Additionally, although the vaccine formulations were composed of cancer cells that have the antigenic expression of malignant cells, live cells have still the capability of proliferating. The secretion of proinflammatory cytokines, such as IL-6, has been linked with the development of cancer by the activation of the nuclear factor-kappa B (NF-κB) and thus promoting proliferation of tumor cells instead of apoptosis\(^{23}\). We hypothesize that after the subcutaneous injection of the microspheres and Panc02 cells, the DCs that were resident in the injection site became activated and consequently released the proinflammatory cytokines that prevented the apoptosis of the rapid growing cancerous cells. The immunohistochemical analysis of the tumors is currently being performed.
8.4 Conclusions

These results support our previous findings that the CPTEG:CPH system represents a promise alternative for the delivery of vaccines. Although these are preliminary results, these demonstrate that CPTEG:CPH microspheres enhance the key factors of the innate immune response, indicating adjuvant characteristics for the development of cancer vaccines. Further studies needs to be performed and these will involve the encapsulation of tumor associated antigens (i.e. Mucin-1 and Mucin-4) into CPTEG:CPH microspheres. The experimental approach combines the development of novel biodegradable CPTEG:CPH microspheres with studies that elucidate the induction of appropriate cell-mediated immunity (i.e., antigen-specific T cell responses). The proposed rational experimental design for the development of CPTEG:CPH cancer vaccines is described below.
8.5 Experimental Design of CPTEG:CPH Cancer Vaccines

8.5.1 Antitumor Immune Response of CPTEG:CPH Microspheres

The ability of CPTEG:CPH microspheres to induce an antitumor immune response will be evaluated with microspheres encapsulating whole cell tumor lysates (mixture of tumor antigens). After 2-3 weeks of inducing Panc02 tumors in C57BL/6 mice, the mice will be immunized with tumor antigens-loaded CPTEG:CPH microspheres of various compositions. Injection groups (N=5) will include PBS (negative control), tumor lysate only (positive control), blank CPTEG:CPH microspheres only, tumor lysate-loaded CPTEG:CPH microspheres, and tumor lysate-loaded microspheres plus soluble tumor lysate. After monitoring the tumor size until it becomes unbearable by positive control animals, the mice will be sacrificed. LN and SP suspensions of individual animals will be stimulated in vitro with tumor lysate and the production of CTLs against Panc02 malignant cells will be assessed by $^{51}$Cr release. The phenotype of fresh LN and SP suspensions will be analyzed with flow cytometry to elucidate the activation of natural killer (NK) cells, CTL, and CD4+ effector cells. The immune cells infiltrating the tumors will be investigated by immunohistochemical staining of tumor sections.

8.5.2 Design of Mucin-1 and Mucin-4 Cancer Vaccine

As cancer cells are composed of many antigens that might be expressed in other tissues, the main goal when designing a cancer vaccine is to modulate the response specifically against the malignant cells and not to healthy tissues. As has been explained earlier, the discovery of the aberrant expression of the glycoproteins
Mucin-1 and Mucin-4 in pancreatic cancer provides evidence that anti-tumor immune response is feasible. Experiments are described based on Mucin-1. However, similar experiments with Mucin-4 will be performed.

8.5.2.1 Antigen Presentation by DCs

Blank CPTEG:CPH microspheres enhance the maturation of DCs, the key step in an immune response. In addition to the efficient activation of the DCs, these must be able to present the antigen, in this case Mucin-1 or Mucin-4, in the context of MHC I and/or MHC II in order to effectively initiate a specific immune response. Antigen presentation will be evaluated by assessing the ability of primed T cells to secrete cytokines and to proliferate following incubation in the presence of DCs previously incubated with Mucin-1 loaded microspheres. Culture supernatants will be assayed for the presence of cytokines (IL-2, IL-4, IL-10, and IFNγ) using a fluorescent bead assay.

8.5.2.2 In Vivo Studies

A major challenge in cancer immunotherapy is the development of vaccine formulations that can elicit a cell-mediated immune response in the face of immunological tolerance to the tumor antigen. The success of a cancer vaccine is dictated by the magnitude of the immune response induced, type of immunity generated, and how long this will persist after administration. The studies described above will help determine which CPTEG:CPH composition(s) possess the characteristics of an ideal adjuvant. Mucin-1 loaded microspheres of the
CPTEG:CPH compositions that enhanced activation of DCs will be evaluated on animal models to identify formulations that facilitate the induction of anti-tumor immunity.

8.5.2.3 Vaccine Regimen

Injection formulations will include blank microspheres, Mucin-1 loaded microspheres, Mucin-1 alone, and a mixture of free and antigen-loaded microspheres. Groups of 5 mice will be used for each formulation. C57BL/6 wild type (wt) mice will be immunized subcutaneously (sc) at the base of the tail with 0.5 mg of the corresponding formulation, based on previous work\textsuperscript{29}. The serum will be analyzed prior to and after immunization twice a week for two months. Controls of the experiment will be mice injected with blank microspheres, PBS, and Mucin-1 peptide alone.

8.5.2.4 Antibody Production

The induction of Th1 and Th2 responses will be determined by isotype distribution in the serum using an anti-Mucin-1 antibody. The antibody production will be measured by ELISA, and the production of the immunoglobulin IgG3 will be an indication of Th1, while immunoglobulin IgG1 will be an indicator of a Th2 immune response. Because Mucin-1 peptides are known to be highly immunogenic in wt mice, high titer antibody responses are expected if Mucin-1 is efficiently presented by CPTEG:CPH microspheres.
8.5.2.5 Cell Proliferation and Cytokine Analysis

After two months of the vaccine injection, mice will be euthanized and cytokine production will be tested in cells derived from their lymph nodes (LN). The supernatant fluid will be analyzed for the presence of cytokines (e.g., IFNγ, IL-2, IL-10, and IL-4) using a fluorescent bead assay.

8.5.2.6 Vaccine Evaluation in Transgenic Model

The studies in this section (8.5.2) evaluate the ability of CPTEG:CPH microspheres to induce an immune response to Mucin-1. The vaccine formulations with the best outcomes based on the experiments proposed in this section and the previous evaluation of antitumor responses (8.5.1) will be tested in transgenic mice and the cellular mechanisms of immune response and therapeutic value will be investigated. The real success of the vaccine formulation must be evaluated in the responses generated in a Mucin-1 transgenic animal model, which express Mucin-1 as a self antigen, in the same way as in most cancer patients developing autoimmunity. Recently, human Mucin-1 transgenic (Tg) mice have become available, which enables the testing of immunotherapeutic strategies within the context of tolerance and autoimmunity31.
8.6 Overall Conclusions

The results and experiments outlined in this chapter provide a rational approach for the design of a cancer vaccine based on novel amphiphilic polyanhydrides. It is expected that the encapsulation into and sustained release of stable Mucin-1 and Mucin-4 from CPTEG:CPH microspheres will provide superior adjuvanticity against pancreatic cancer and will provide insights for a broad spectrum of applications in other vaccine-based therapies for intra-cellular pathogens.
8.7 References


8. Tempeo, R.M. et al. CD4+ Lymphocytes Provide MUC1-Specific Tumor Immunity In Vivo that is Undetectable In Vitro and is Absent in MUC1 Transgenic Mice. *J. Immunology* 161, 5500-5506 (1998).


CHAPTER 9
CONCLUSIONS AND FUTURE WORK

9.1 Conclusions

Cancer vaccines are still in the investigational phase and none have been licensed by the FDA for human use. The powerful combination of protein stabilization and immune deviation provided by the amphiphilic CPTEG:CPH polyanhydride system make it a promising prospect for development of cancer vaccines. The synthesis and characterization of the properties of these biomaterials, which can be tailored to obtain desirable controlled release and amphiphilic behavior, have been addressed in this thesis (Chapter 4) to accomplish the first logical step in the design of a novel delivery device. Subsequently, as most vaccines involve the delivery of peptides or protein subunits, protein stabilization in the CPTEG:CPH environment was analyzed in Chapters 5 and 6. It was demonstrated that CPTEG:CPH system does provide a gentle environment for proteins and a sustained release from copolymer microspheres is attained. In order to evaluate the adjuvant characteristics of CPTEG:CPH system, the activation of immune cells incubated with CPTEG:CPH microspheres and the implications for vaccine design were addressed in Chapter 7. The promising adjuvant capabilities of CPTEG-content microspheres were evidenced by an enhanced maturation of the most potent antigen presenting cells of the immune system. Altogether, the studies presented in these four chapters provide an excellent foundation for testing the viability of this system as an effective adjuvant for the development of cancer vaccines. Finally, the non-specific anti-tumor immunity of CPTEG:CPH microspheres was investigated
and a rational approach for the design of a single dose cancer vaccine is discussed in Chapter 8. Although the main focus of this thesis is to design novel adjuvants that can be used in a cancer vaccine, the potential of the amphiphilic CPTEG:CPH system for other biomedical applications is described below.
9.2 Future Work

The promising characteristics of the novel amphiphilic polyanhydride system based on the anhydride monomers CPH and CPTEG have led to its use in a broad range of biomedical applications. The main advantages of these polymers are that by increasing the CPTEG content in the copolymer, it is ensured that: (1) proteins are stabilized while being released in a controlled and predictable manner and (2) the immune cells of the innate immune system are activated, thus inducing a strong immune response. This system has tremendous potential in vaccine design as well as in other therapeutic applications. The adjuvant capabilities of CPTEG:CPH are supported by experiments performed with a model antigen and the activation of other antigen presenting cells besides dendritic cells (DCs). The surface modification of CPTEG:CPH microspheres, the evaluation of the CPTEG:CPH biocompatibility by high throughput experiments, and the potential functions for wound healing applications are addressed.
9.2.1 Adjuvant Capabilities with Model Antigen

In order to determine the capability of CPTEG:CPH microspheres to enhance an antigen-specific immune response, BALB/c mice were injected with CD4⁺ T cells from ovalbumin (Ova) transgenic mice (DO11.10 T cells) 24 h before immunization. The immunization groups injected subcutaneously included: saline, lipopolysaccharide (LPS), Ova alone, 20:80 CPTEG:CPH blank microspheres (MS), LPS + Ova, and MS + Ova. After two weeks, the animals were euthanized by CO₂ asphyxiation. The serum was analyzed for Ova-specific antibodies with an enzyme-linked immunosorbent assay (ELISA) and the mice immunized with MS enhanced Ova-specific antibody production when compared to Ova alone (Fig 9.1).

![Figure 9.1](image)

Figure 9.1. Total Ova-specific serum antibodies of immunized mice. Error bars represent standard deviation in a group (N=5). († = statistically different from each other, P-value<0.05).

An *in vitro* antigen-specific proliferation assay was performed after harvesting the inguinal, auxiliary, and brachial lymph nodes from each immunized mouse. After 72 h of *in vitro* re-stimulation, the proliferation of T cells specific to Ova were measured by the incorporation of methyl-[³H]-thymidine. As shown in Figure 9.2, the
amphiphilic CPTEG:CPH system enhanced T cell proliferation compared to Ova alone and at similar level to the LPS group, which is toxic for human applications. These studies demonstrate the ability of polyanhydrides to enhance the activation of CD4\(^+\) T cells which can be attributed to its promising adjuvant characteristics. The ability of CPTEG:CPH microspheres to enhance antigen presentation to CD4\(^+\) T cells provides evidence that T helper (Th) cells are activated. Further experiments are needed to determine if the polyanhydride microspheres induce Th1 (cell mediated) vs. Th2 (humoral) pathways. From the \textit{in vitro} activation of DCs\(^1\), we hypothesize that CPTEG:CPH system has the ability to modulate the immune response towards Th1, which is needed to destroy intracellular pathogens. As mentioned earlier, current adjuvants approved for human use induce humoral immune responses against extracellular pathogens\(^2\). Therefore, the need for new adjuvants to elicit immune responses against intracellular pathogens such as viruses, certain bacteria and cancer, positions the CPTEG:CPH system as a viable alternative for treatments of these diseases.

The activation of CD4\(^+\) T cells also has implications for the design of cancer vaccines as it has been demonstrated that both CD4\(^+\) and CD8\(^+\) T cells are crucial for anti-tumor immunity\(^3\). In order to have a thorough understanding of anti-tumor immunity of a cancer vaccine, the experiments outlined on Chapter 8 must include the evaluation of the activation of both CD4\(^+\) and CD8\(^+\) effector cells.
Figure 9.2. Antigen-specific proliferation of lymphocytes isolated from immunized mice. Error bars represent standard deviation within each group.
9.2.2 Activation of other Immune Cells

The professional antigen presenting cells (APCs) internalize antigen, and after intracellular degradation present antigen fragments to be recognizable to T cells by means of the context of Major Histocompatibility class I (MHC I) and class II (MHC II). There are three main types of professional APCs and these are B cells, macrophages, and the most potent APCs, the dendritic cells (DCs). It has been shown that CPTEG:CPH microspheres enhance the activation of DCs\(^1\). To study the ability of CPTEG:CPH microspheres to activate other professional APCs, the activation of B cells was assessed. Pre-B cells (7OZ/3) were incubated with CPTEG:CPH microspheres and their activation was measured by flow cytometry (Fig. 9.3). It is known that pre-B cells develop into immature B lymphocytes that express IgM at the surface after being mature\(^4\). As can be seen from the Figure 9.3, the polyanhydride microspheres enhanced the surface expression of IgM in the 7OZ/3 to a similar extent as the positive control cells, which were incubated with LPS. When LPS and blank CPTEG:CPH microspheres were incubated together, these induce a synergistic effect on the Pre-B cells and induced IgM expression to a higher extent.
An interesting finding about the activation of 7OZ/3 in the past concluded that the hydrophilic head group of the LPS was critical for their activation⁵. We hypothesize that CPTEG content in the copolymer was the main inducer for the pre-B cell activation in the studies that are presented here, and this is in agreement with the activation of DCs by CPTEG:CPH microspheres as well. Therefore, the activation of immune cells can be targeted by adjuvants with appropriate chemistries.

Figure 9.3. Activation of Pre-B cell line 7OZ/3. Flow cytometry histogram of cells incubated with 20:80 CPTEG:CPH microspheres (top). Open histograms represent isotype control. The activation results obtained from flow cytometry were analyzed with respect to the % of positive cells expressing IgM and the corresponding mean fluorescence intensity (MFI) (bottom).
9.2.3 Carbohydrate Attachment

As has been discussed in previous chapters, the most important APCs are the highly specialized DCs, which expresses a repertoire of pattern recognition receptors, including toll-like receptors (TLRs) and C-type lectins that can recognize pathogens and modulate the immune response against them. In particular, C-type lectins recognize specific carbohydrate structures that are present on the cell-wall of pathogens or in self glycoproteins\(^6\). These receptors mediate key processes in an immune response such as cellular signaling, cell adhesion, and migration. We propose that the attachment of carbohydrates, such as lactose and di-mannose, to the surface of CPTEG:CPH microspheres will enhance their uptake by DCs. Currently both sugars have been incorporated into the microspheres by a coupling reaction (Fig. 9.4). The percentage of carboxylic acid groups that were present in the surface of the microspheres was estimated from an end group analysis from proton nuclear magnetic resonance (\(^1\)H NMR) spectra of the polymers and the surface area of the microspheres. Then the conditions for the coupling reaction were determined and the Kaiser test was used for the determination of coupling efficiency\(^7\).
Figure 9.4. Coupling reaction for attachment of Lactose and Di-mannose into CPTEG:CPH microspheres. The carboxyl activating agents 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHC) were used for coupling the sugars.

A scanning electron microscopy (SEM) image of 20:80 CPTEG:CPH microspheres after lactose coupling is shown in Fig. 9.5. To ensure that majority of microspheres were under 10 μm for efficient phagocytosis by DCs, the size distribution was determined and it was found that 86% of the microspheres were below this size. Future experiments could involve the evaluation of DC activation of carbohydrate-modified CPTEG:CPH microspheres.

Figure 9.5. SEM image of 20:80 CPTEG:CPH microspheres after coupling Lactose to the surface. Scale bar represents 20 μm.
9.2.4 High Throughput Analysis

The biocompatibility of CPTEG:CPH copolymer libraries has been recently studied using high throughput cell-based screening methods. A rapid prototyping technique was used to synthesize a multi-well substrate for high throughput synthesis of polymer libraries. The CPTEG:CPH copolymer libraries were incubated with standard myeloma, epithelial, and macrophage cell lines and were found to have no pronounced cytotoxic effect for any polymer composition at concentrations of 2.8 mg/mL and below, which is much higher than that expected in in vivo applications (Fig 9.6). Future analysis can evaluate immune activation by cytokine secretion of DCs incubated with CPTEG:CPH libraries to discover optimum chemistries with desirable adjuvant capabilities.

![Graph showing linear gradient from 0-28 mg/mL of 50:50 CPTEG:CPH copolymer.](image)

Figure 9.6. Linear gradient from 0-28 mg/mL of 50:50 CPTEG:CPH copolymer.
9.2.5 Wound Healing Applications

The amphiphilic CPTEG:CPH system provides a gentle environment for protein delivery and stabilization\textsuperscript{9, 10}. The model proteins incubated with degradation products and after being released from microspheres maintain their structural and biological integrity. Uterocalin is a protein that is hypothesized to expedite wound healing and it is known that this protein loses its biological activity if the structure is altered\textsuperscript{11, 12}. The biological activity of uterocalin after release from CPTEG:CPH microspheres is currently being investigated with an \textit{in vitro} cell migration assay. A wound line was induced on human colorectal carcinoma cells (HCT116) and after 24 h of incubating with uterocalin-loaded 50:50 CPTEG:CPH microspheres, the cells that migrated were counted (Fig. 9.7). As can be seen from the images, the protein released from polyanhydride microspheres increased the rate of migration, indicating its functional activity. This preliminary study supports the hypothesis that CPTEG:CPH system provides a conducive environment for proteins and this is the first time it has been demonstrated with a protein with therapeutic value. Future studies can focus on \textit{in vivo} experiments that investigate the wound healing properties of uterocalin-loaded CPTEG:CPH microspheres by studying the infiltration of inflammatory cells into an induced open wound and tissue regeneration after treatment with polyanhydride microspheres.
Altogether, the preliminary results shown here clearly support our previous findings of the CPTEG:CPH system, substantiating our hypothesis of the potential of these novel polyanhydrides for biomedical applications. The combination of biocompatibility, the ability to stabilize proteins, the enhanced activation of immune cells and the enhanced immune response confers a wide range of applications with high therapeutic value.
9.2.6 Acknowledgements

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9.3 References


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