Combinatorial design and development of biomaterials for use as drug delivery devices and immune adjuvants

Latrisha Kay Petersen
Iowa State University

Follow this and additional works at: http://lib.dr.iastate.edu/etd
Part of the Biological Engineering Commons, and the Chemical Engineering Commons

Recommended Citation
Petersen, Latrisha Kay, "Combinatorial design and development of biomaterials for use as drug delivery devices and immune adjuvants" (2011). Graduate Theses and Dissertations. 11988.
http://lib.dr.iastate.edu/etd/11988

This Dissertation is brought to you for free and open access by the Graduate College at Iowa State University Digital Repository. It has been accepted for inclusion in Graduate Theses and Dissertations by an authorized administrator of Iowa State University Digital Repository. For more information, please contact digirep@iastate.edu.
Combinatorial design and development of biomaterials
for use as drug delivery devices and immune adjuvants

by

Latrisha Kay Petersen

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of
DOCTOR OF PHILOSOPHY

Major: Chemical Engineering

Program of Study Committee:
Balaji Narasimhan, Major Professor
Michael J. Wannemuehler
Jennifer O’Donnell
Krishna Rajan
Ian Schneider

Iowa State University
Ames, Iowa
2011

Copyright© Latrisha Kay Petersen, 2011. All rights reserved.
# Table of Contents

List of Figures ........................................................................................................................ ix  
List of Tables .......................................................................................................................... xviii  
Acknowledgments ................................................................................................................ xix  
Abstract .................................................................................................................................. xxiii  

## Chapter 1: Introduction ........................................................................................................1

1.1 Introduction .......................................................................................................................1  
1.2 References .........................................................................................................................5  
1.3 List of Figures .....................................................................................................................9  

## Chapter 2: Literature Review ...............................................................................................10

2.1 Summary ..........................................................................................................................10  
2.2 Polymeric Biomaterials: Drug Delivery Applications ......................................................11  
   2.2.1 Introduction .............................................................................................................11  
   2.2.2 Acid-Catalyzed Polymers .......................................................................................13  
   2.2.3 Polyesters .............................................................................................................14  
   2.2.4 Polyethers .............................................................................................................16  
   2.2.5 Polyanhydrides .................................................................................................17  
2.3 Polymeric Biomaterials: Vaccine Adjuvants ....................................................................21  
   2.3.1 Introduction ...........................................................................................................21  
   2.3.2 Acid-Catalyzed Polymer Adjuvants ........................................................................25  
   2.3.3 Polyester Adjuvants ............................................................................................27  
   2.3.4 Polyether Adjuvants ............................................................................................30
2.3.5 Polyanhydride Adjuvants ........................................................................................................................................31

2.4 Combinatorial Design of Biomaterials for Drug Delivery: Opportunities and Challenges ..........................................................................................................................................................34

2.4.1 Introduction ..........................................................................................................................................................34

2.4.2 Combinatorial Biomaterial Library Fabrication and Characterization ..........................................................................................37

2.4.3 High Throughput Cell-based Screening ..............................................................................................................41

2.4.4 Combinatorial Drug Release .............................................................................................................................46

2.4.5 Expert Opinion ....................................................................................................................................................46

2.4.6 Conclusions ..........................................................................................................................................................48

2.5 References .................................................................................................................................................................49

2.6 List of Figures .............................................................................................................................................................65

2.7 List of Tables ..............................................................................................................................................................71

Chapter 3: Research Objectives ........................................................................................................................................72

Chapter 4: A Novel, High Throughput Method to Study In Vitro Protein Release from Polymer Nanospheres ..................................................................................................................................................74

4.1 Abstract .................................................................................................................................................................75

4.2 Introduction ............................................................................................................................................................75

4.3 Materials and Methods ...........................................................................................................................................78

4.4 Results and Discussion .............................................................................................................................................82

4.5 Conclusions ...........................................................................................................................................................86

4.6 Acknowledgements ..................................................................................................................................................87

4.7 References .............................................................................................................................................................87

4.8 List of Figures ..........................................................................................................................................................91
Chapter 5: High Throughput Analysis of Protein Stability in Polyanhydride Nanoparticles

5.1 Abstract
5.2 Introduction
5.3 Materials and Methods
5.4 Results and Discussion
5.5 Conclusions
5.6 Acknowledgements
5.7 References
5.8 List of Figures
5.9 List of Tables

Chapter 6: Amphiphilic Polyanhydride Nanoparticles Stabilize Bacillus Anthracis Protective Antigen

6.1 Abstract
6.2 Introduction
6.3 Materials and Methods
6.4 Results
6.5 Discussion
6.6 Conclusions
6.7 Acknowledgements
6.8 References
6.9 List of Figures
Chapter 7: High Throughput Cell-based Screening of Biodegradable Polyanhydride Libraries

7.1 Abstract ............................................................................................................................ 155
7.2 Introduction ...................................................................................................................... 156
7.3 Materials and Methods ..................................................................................................... 159
7.4 Results .............................................................................................................................. 166
7.5 Discussion ........................................................................................................................ 172
7.6 Conclusions .................................................................................................................... 173
7.7 Acknowledgements ........................................................................................................ 174
7.8 References ..................................................................................................................... 175
7.9 List of Figures .................................................................................................................. 178
7.10 List of Tables .................................................................................................................. 185

Chapter 8: The Simultaneous Effect of Polymer Chemistry and Device Geometry on the In Vitro Activation of Murine Dendritic Cells

8.1 Abstract ............................................................................................................................ 188
8.2 Introduction ...................................................................................................................... 188
8.3 Materials and Methods ..................................................................................................... 192
8.4 Results .............................................................................................................................. 200
8.5 Discussion ........................................................................................................................ 204
8.6 Conclusions .................................................................................................................... 208
8.7 Acknowledgements ........................................................................................................ 208
8.8 References ..................................................................................................................... 209
Chapter 9: Amphiphilic Polyanhydride Films Promote Neural Stem Cell Adhesion and Differentiation

9.1 Abstract ............................................................................................................................ 225

9.2 Introduction ...................................................................................................................... 225

9.3 Materials and Methods ..................................................................................................... 228

9.4 Results .............................................................................................................................. 233

9.5 Discussion ........................................................................................................................ 236

9.6 Conclusions ...................................................................................................................... 239

9.7 Acknowledgements .......................................................................................................... 240

9.8 References ........................................................................................................................ 240

9.9 List of Figures .................................................................................................................. 243

9.10 Supporting Information .................................................................................................. 248

Chapter 10: Amphiphilic Polyanhydride Nanoparticle Adjuvants Activate Innate Immune Responses in a Pathogen-Mimicking Manner

10.1 Abstract .......................................................................................................................... 251

10.2 Introduction .................................................................................................................... 251

10.3 Materials and Methods .................................................................................................. 253

10.4 Results ............................................................................................................................ 258

10.5 Discussion ...................................................................................................................... 261

10.6 Conclusions .................................................................................................................... 266

10.7 Acknowledgements ........................................................................................................ 267
Chapter 11: High Throughput Evaluation of In Vivo Biodistribution of Polyanhydride Nanoparticles

11.1 Abstract
11.2 Introduction
11.3 Materials and Methods
11.4 Results
11.5 Discussion
11.6 Conclusions
11.7 Acknowledgements
11.8 References

Chapter 12: Conclusions

Chapter 13: Ongoing and Future Work

13.1 CPTEG:CPH Films: Applications in Drug Delivery and Tissue Engineering
13.2 Polyanhydride Particle shape and Size Dependency on Cytotoxicity and Activation of Primary Murine Dendritic Cells
13.3 CPTEG:CPH Nanoparticle Adjuvants for the Single-dose Vaccination Against Anthrax, Pneumonia, and Influenza
13.4 References ................................................................................................................................................316
13.5 List of Figures ........................................................................................................................................319
List of Figures

Figure 1.1: The chemical structure of poly(CPH) .................................................................9
Figure 1.2: The chemical structure of poly(SA) .................................................................9
Figure 1.3: The chemical structure of poly(CPTEG) ..........................................................9

Figure 2.2.1: Polyester chemical structures: a) PGA, b) PLA, c) PLGA, and d) PCL ..........65
Figure 2.2.2: a) Bulk vs. b) surface erosion mechanisms .................................................65
Figure 2.2.3: Acid-catalyzed polymer chemical structures: a) polyacetal, b) polyketal, and c) polyorthoester .................................................................66
Figure 2.2.4: Polyanhydride chemical structures: a) poly(CPH), b) poly(SA), and c) poly(CPTEG) .................................................................66
Figure 2.2.5: Polyether chemical structure: a) poly(ethylene glycol) and b) poly(propylene glycol) .................................................................67

Figure 2.3.1: Induction of cellular vs. humoral immune response upon antigen presentation by a DC .................................................................67
Figure 2.4.1: The combinatorial methodology .................................................................68
Figure 2.4.2: A discrete polyanhydride library polyanhydrides, increasing in SA composition from right to left and front to back .................................................................68
Figure 2.4.3: Mole percent of CPH varying along a discrete composition gradient library as determined in high throughput by FTIR microscopy .................................................................69
Figure 2.4.4: Correlation between mole percent of CPH and TNF-α production .............69
Figure 2.4.5: Fraction of dye released from a compositionally varying polyanhydride library of CPH:SA copolymers .................................................................70
**Figure 4.1:** a) Schematic of modifications made to horizontal pair of wells in the 96-well polypropylene release plate, and b) actual fluorescence images of the 96-well plate while performing the TRBSA release studies from CPH:SA films ....................................................91

**Figure 4.2:** SEM images of TRBSA-loaded polyanhydride nanospheres: A) poly(SA) and B) 50:50 CPH:SA ..............................................................................................................91

**Figure 4.3:** Cumulative mass fraction of BSA released from combinatorially fabricated CPH:SA nanospheres for one month at pH 7.3 as detected by the micro-BCA assay ..........92

**Figure 4.4:** Cumulative mass fraction of TRBSA released from combinatorially fabricated CPH:SA nanospheres for one month at pH 7.3 as detected by the high throughput fluorescence assay .................................................................................................92

**Figure 4.5:** Cumulative mass fraction of TRBSA released from combinatorially fabricated CPH:SA nanospheres for one month at pH 6.0 as detected by the high throughput fluorescence assay .................................................................................................93

**Figure 4.6:** Cumulative mass fraction of TRBSA released from combinatorially fabricated CPH:SA nanospheres for one month at pH 4.3 as detected by the high throughput fluorescence assay .................................................................................................93

**Figure 4.7:** Cumulative mass fraction of TRBSA released from combinatorially fabricated CPH:SA films for one month at pH 7.3 as detected by the high throughput fluorescence assay .................................................................................................94

**Figure 5.1:** Chemical structures of: a) SA; b) CPH; and c) CPTEG; d) polyanhydride hydrolysis mechanism............................................................................................................117

**Figure 5.2:** Schematic describing the high throughput methodology for studying protein stability with polymer nanoparticle libraries from the initial synthesis/fabrication to
storage over time at variable temperatures to the release and subsequent level of stability as affected by pH, temperature, and polymer chemistry.

**Figure 5.3:** SEM images of a) 50:50 CPTEG:CPH and b) 50:50 CPH:SA nanoparticles...

**Figure 5.4:** Antigenicity of BSA following encapsulation and release from CPH:SA and CPTEG:CPH nanoparticles and after exposure to the nanoparticle fabrication conditions (NFC).

**Figure 5.5:** Shelf life study of BSA encapsulated into a library of nanoparticles poly(SA), 25:75 CPH:SA, 50:50 CPH:SA, 75:35 CPH:SA, poly(CPH), 60:40 CPTEG:CPH, 50:50 CPTEG:CPH, 40:60 CPTEG:CPH, 30:70 CPTEG:CPH, 20:80 CPTEG:CPH, and 10:90 CPTEG:CPH) at three different temperatures, 4, 25, and 40 °C.

**Figure 5.6:** Antigenicity of BSA following a seven day incubation in a CPH:SA monomer library.

**Figure 5.7:** Primary structure of BSA using SDS PAGE following a seven day incubation in a CPH:SA monomer library.

**Figure 5.8:** Secondary structure of BSA using FTIR following a seven day incubation in a CPH:SA monomer library to determine α-helix and β-sheet content.

**Figure 6.1:** PA is released from polyanhydride nanoparticles in a chemistry dependent manner with 20:80 CPH:SA and 50:50 CPTEG:CPH releasing the fastest.

**Figure 6.2:** Mice immunized with PA released from 50:50 and 20:80 CPTEG:CPH, adjuvanted with alum, developed the highest antibody titers, the most avid antibody response, and highest neutralizing antibody titers.

**Figure 6.3:** CPTEG:CPH nanoparticle chemistries release actively and antigenically intact PA: A) residual antigenicity and B) residual activity.
**Figure 6.4:** 50:50 CPTEG:CPH best preserves the structural integrity of PA while CPH:SA nanoparticle chemistries cause a loss in primary, secondary and tertiary structural integrity .................................................................150

**Figure 6.5:** PA-loaded polyanhydride nanoparticles are capable of releasing fully functional PA in a dose and time dependent manner dictated by polymer release kinetics and stability of PA ...........................................................................................................151

**Figure 6.6:** PA-loaded polyanhydride nanoparticles are capable of preserving the activity of PA for 2 months when stored at 40, 25, 4, and -20 °C ...........................................................................................................152

**Supplementary Figure 6.1:** PA activity is sensitive to high temperature when stored adsorbed to alum A) or in PBS buffer B) over incremental time points (7, 14, 28, and 58 days) ...........................................................................................................153

**Figure 7.1:** Chemical structures of a) poly(SA); b) poly(CPH); and c) poly(CPTEG) ......178

**Figure 7.2:** Schematic of photolithographic design of discrete thiolene-based multi-well substrates ..................................................................................................................................................179

**Figure 7.3:** Proton NMR spectrum for combinatorially synthesized 50:50 CPH:SA copolymer showing chemical shifts ........................................................................................................................................179

**Figure 7.4:** Proton NMR spectrum for combinatorially synthesized 50:50 CPTEG:CPH copolymer showing chemical shifts ........................................................................................................................................180

**Figure 7.5:** Representative FTIR spectrum of a 60:40 CPH:SA copolymer with characteristic absorbances of 1605 cm⁻¹ for the CPH aromatic peak and 1810 cm⁻¹ for the SA aliphatic peak ........................................................................................................................................180

**Figure 7.6:** Comparison of predicted and experimentally measured composition in a 5x4 discrete library ...........................................................................................................................................181
Figure 7.7: Cell (Sp2/0 mouse myeloma) viability as a function of 50:50 CPH:SA (left) and CPTEG:CPH (right) concentration .................................................................181

Figure 7.8: Effect of CPH:SA copolymer composition on cytotoxicity of Sp2/0 myeloma cells ...............................................................................................................182

Figure 7.9: Effect of CPTEG:CPH copolymer composition on cytotoxicity of Sp2/0 myeloma cells ........................................................................................................183

Figure 7.10: Cytokine secretion from J774 macrophages incubated in multi-well substrates containing 2.8 mg/mL of discrete compositions of CPH:SA was measured by capture immunoassay ......................................................................................................184

Figure 8.1: High throughput FTIR analysis of the polymer chemistry (% CPH) varying across the CPH:SA film library ...........................................................................212


Figure 8.3: Analysis of MHC II expression by C57BL/6 DCs with histograms comparing between treatments in light grey and the isotype non-specific control in dark grey .................................................................214

Figure 8.4: Analysis of CD40 expression by C57BL/6 DCs with histograms comparing between treatments in light grey and the isotype non-specific control in dark grey ..........................................................................................215

Figure 8.5: Analysis of CD86 expression by C57BL/6 DCs with histograms comparing between treatments in light grey and the isotype non-specific control in dark grey .........................................................................................216
Figure 8.6: Analysis of CD209 expression by C57BL/6 DCs with histograms comparing between treatments in light grey and the isotype non-specific control in dark grey.

Figure 8.7: Production of IL-6 and IL-12p40 by C57BL/6 DCs upon stimulation with multiplexed CPH:SA libraries.

Figure 8.8: PCA biplot of CPH:SA nanosphere stimulation of all the cell surface markers, CD209, CD40, CD86 and MHC II and both cytokines IL-6 and IL-12p40.

Figure 8.9: PCA biplot comparing the polyanhydride nanosphere and film systems showing inverse correlations with clusters in opposite quadrants.

Figure 8.10: PCA biplot of CPH:SA film stimulation of all cell surface markers, CD209, CD40, CD86 and MHC II and both cytokines IL-6 and IL-12p40.

Figure 9.1: Fractional release of laminin from CPTEG:CPH copolymer film libraries.

Figure 9.2: Biocompatibility and immune stimulation capabilities of CPTEG:CPH polymer films: A) viability, B) cytokine production and C) cell marker expression.

Figure 9.3: Percentage of hNPCs displaying neuronal (MAP2B and TUJ1) or glial (GFAP) differentiation. The CPTEG-rich films best enhanced hNPC differentiation.

Figure 9.4: Representative epifluorescent images of hNPCs stained for DAPI, MAP2B, TUJ1, and GFAP, after incubation with the treatment groups.

Figure 9.5: CPH-rich chemistries resulted in the most double positive hNPC populations for MAP2B + TUJ1, MAP2B + GFAP, TUJ1 + GFAP, and GFAP + TUJ1.

Supplemental Data Figure 9.1: CPH-rich films best enhanced hNPC differentiation. Subcellular immunolabeling for A) MAP2B and B) TUJ1.
**Supplemental Data Figure 9.2:** CPTEG-rich films best promoted the expression of total double positive hNPCs for TUJ1 + GFAP and GFAP + MAP2B ..........................................................249

**Figure 10.1:** SA- and CPTEG- rich polyanhydride nanoparticles are internalized by dendritic cells ........................................................................................................................................270

**Figure 10.2:** Nanoparticle internalization by DCs is important for CD40 expression but not CD86 or MHC II expression .........................................................................................................................271

**Figure 10.3:** Nanoparticle internalization by DCs is important for cytokine production and is dependent upon nanoparticle chemistry. ..................................................................................................................272

**Figure 10.4:** LPS or 50:50 CPTEG:CPH nanoparticle treatment of DCs revealed similar populations of double positive DCs while different double positive DC populations were generated following treatment with poly(SA) nanoparticles .................................................................................................................................273

**Figure 10.5:** Identification of –OH end groups, oxygen content, alkyl ether, and hydrophobicity as important pathogen-mimicking structural descriptors ............................................................................................................................274

**Supplementary Figure 10.1:** CPTEG- and SA-rich nanoparticles synthesized combinatorially activate DCs similarly to conventionally fabricated nanoparticles ..........276

**Supplementary Figure 10.2:** Cytokine production is induced by nanoparticles co-incubated with DCs in a chemistry-dependent manner .................................................................................................................................278

**Figure 11.1:** Representative mouse images showing differential persistence at the injection site of nanoparticles administered via three routes over a two week period ..........300

**Figure 11.2:** Nanoparticles administered via three routes persisted at the injection site longest when administered IM; however, IN particles rapidly disseminated throughout the body ..................................................................................................................................................................................300

**Figure 11.3:** Nanoparticles were retained in the IM injection site tissue longer than at
the other two sites ..................................................................................................................301

**Figure 11.4:** Representative mouse images indicating that IN administration of two nanoparticle chemistries, 50:50 and 20:80 CPH:SA or 50:50 and 20:80 CPTEG:CPH, resulted in rapid dispersion of the particles throughout the body within the first 24 hours after administration ................................................................................................................302

**Figure 11.5:** Chemistry played an important role in nanoparticle distribution throughout the body ..................................................................................................................................303

**Figure 11.6:** Even though no longer visible in the whole mouse images, all nanoparticle chemistries administered IN were targeted to the lungs and retained there for at least 14 days ........................................................................................................................................304

**Supplementary Figure 11.1:** Administration of nanoparticles via three different routes simultaneously to the same mouse resulted in similar nanoparticle distribution patterns as compared to administration of nanoparticles via each route independently. ....................305

**Supplementary Figure 11.2:** Representative 14 day organ images corresponding to Figure 11.3 separated by filter channel which indicate that IN administered nanoparticles are easily observed in the lung tissue while IM and SC administered nanoparticles are easily observed in the injection site tissue ...........................306

**Supplementary Figure 11.3:** Each mouse administered nanoparticles of two different chemistries results in similar nanoparticle distribution compared to separate mice administered nanoparticles of each chemistry independently. ..............................................307

**Supplementary Figure 11.4:** Representative 14 day organ images corresponding to Figure 11.6 separated by filter channel which indicate that IN administered nanoparticles are easily observed in the lung tissue even though they are no longer
detectable in the whole mouse images.................................................................308

**Figure 13.1:** Images of A) porous and B) electro-spun polymer networks and C) nerve regeneration conduits .................................................................................................................................................................................319

**Figure 13.2:** SEM image of 20 μm monodisperse poly(SA) particles ........................................319

**Figure 13.3:** Total IgG anti-PspA concentrations from the serum of mice immunized with blank 50:50 CPTEG:CPH nanoparticles (blank nano), 1 μg soluble PspA + 2 μg encapsulated PspA into 50:50 CPTEG:CPH nanoparticles (soluble PspA + PspA nano), 1 μg PspA conjugated to alum (PspA + alum), and saline ..................................................................................................................320
List of Tables

Table 2.4.1: Polymeric biomaterials and the various high throughput screening methods that have been developed to study these materials ..........................................................71

Table 4.1: Molecular weight analysis of CPH:SA copolymer film libraries using GPC and $^1$H NMR .................................................................................................................................94

Table 5.1: Amide I region of BSA peak assignment, position, and area .........................121

Table 5.2: Tertiary structure of BSA with fluorescence spectroscopy, following a 7 day incubation in a CPH:SA monomer library, as determined by % change in peak intensity and shift in peak position from 340 nm .............................................................................122

Table 7.1 Number average molecular weight and polydispersity index of combinatorially synthesized CPH:SA copolymers obtained by GPC .................................................................185

Table 7.2 Number average molecular weight of combinatorially synthesized 50:50 CPTEG:CPH copolymers obtained by $^1$H NMR .............................................................................................185

Table 7.3 Summary of linear fit model to data from compositional cytotoxicity experiments ........................................................................................................................................186

Table 8.1: Molecular weight analysis of the CPH:SA polymer film library using GPC and $^1$H NMR .................................................................................................................................222

Table 8.2: Statistical analysis of cell surface marker expression and cytokine production corresponding to Figures 8.3-8.7 .......................................................................................................223

Table 11.1: Thermal properties and contact angles of polyanhydrides ..........................304
Acknowledgments

In completing my Ph.D. in Chemical Engineering at Iowa State University I have many people to thank who have helped me reach this high achievement. I need to give a special thanks to all of those who have mentored, advised, taught, trained, supported, and encouraged me along the path of my Ph.D. Through the support of those at home and at work I have been able to attain this great accomplishment in my life.

I would like to start by thanking the most important people to me, my family. My parents have been a constant foundation of support and encouragement throughout my entire life. They always made sure that academics were a high priority to me, which has enabled me to reach this remarkable success. My brother has also been a great supporter; to him it must have seemed that his crazy older sister wanted to stay in college all her life. Spending 4 of my 9 years at Iowa State University with Nick was a wonderful experience, as we were able to motivate and encourage each other along our college careers. I also need to give a special thanks to a very important person in my life, Keenan. As we have both ventured along this path to attain our Ph.D.s in chemical engineering, we have been continual supporters, listeners, and motivators for each other. Keenan has been there through the sleepless nights, research frustrations, and high anxiety times with his constant encouragement, continual love, and support. Thank you for being there for me.

I am very grateful to my major professor, Dr. Balaji Narasimhan. I could not have asked for a better mentor, motivator, and teacher. His guidance and support through my career as an
undergraduate and graduate student were key to my success and professional development. By hiring me on as an undergraduate in his lab, I was able to explore a field of research that I quickly found that I had passion for. His constant motivation and encouragement continued on into graduate school where I was able to reach this high achievement. Thank you for your guidance and support through all of these years. I am very honored to have worked for you and am fortunate to have had such a wonderful research experience.

I thank my program of study committee members, Dr. Jen O’Donnell, Dr. Ian Schneider, Dr. Krishna Rajan, and Dr. Mike Wannemuehler, for guidance and research suggestions as well as being flexible when I encountered difficulties in scheduling my final defense. Another faculty member that I would like to thank is Dr. Monica Lamm for her continual support and advising in my development as a young professional through my undergraduate and graduate careers.

My research project would not have been possible without the extensive collaborations across Iowa State University. Thank you to all of those who taught, collaborated, and facilitated my interdisciplinary research work, making my thesis exciting and successful. Dr. Wannemuehler and his group, Dr. Jenny Wilson-Welder, Dr. Amanda Ramer-Tait, Yashdeep Phanse, and Lucas Huntimer for teaching me immunology and collaborating on an endless number of projects. Mike, Jenny, and Amanda have all been wonderful mentors to me and I am very grateful for the knowledge and ideas they have shared to enable this strong collaboration between our groups. I need to thank Yash and Lucas for their support and dedication with our collaborative anthrax and in vivo imaging work. Dr. Marit Nilsen-
Hamilton and Lee Bendickson for teaching me cell biology and collaborating on the lipocalin-2 drug delivery work. Dr. Don Sakaguchi and Dr. Jisun Oh for teaching me neural cell biology, fluorescent microscopy, and for helping to initiate some of the exciting tissue engineering applications of polyanhydrides. Dr. Krishna Rajan and his group, Dr. Scott Broderick, Dr. Chang Sun Kong, and Xue Li for their collaboration on the informatics aspect of our vaccine adjuvants. Dr. Bryan Bellaire for his advice on microscopy and bacteriology and collaboration on numerous projects.

Furthermore, I would like to thank all of the previous and current graduate students that I have worked with in the Narasimhan lab that have been wonderful research colleagues and friends. Dr. Maria Torres taught me a great deal of the basic polymer science concepts and introduced me to many of those techniques in our lab. She set a wonderful example as a successful graduate student and was a great mentor to me in my first years doing research at Iowa State. Jon Thorstenson and Senja Lopac were also great teachers in the lab during those early research years. Upon entering graduate school, Brenda Carrillo-Conde and I started in this lab at the same time and she has been a wonderful research partner as we have grown together over these graduate school years both personally and professionally. Brenda, Kathleen Ross, Bret Ulery and I have worked together very closely over the past year(s) and I want to thank them for their support and help as fellow graduate students. I would also like to thank the more recent graduate students in our group, Shannon Haughney, Julia Vela Ramirez, Ana Chavez Santoscoy, and Feng Jia for the wonderful discussions I have had with them and the opportunity to teach and work with them. Several undergraduate researchers, Chelsea Sackett, Andrew Adler, Christian Thormos, Ashley Yeager, and Katie Walz, have
also played a significant role in helping with experiments and data analysis. Thank you all for your contributions, support, and teachings, which have made my graduate school experience wonderful.

Lastly I would like to acknowledge the financial support from the US Department of Defense –Office of Naval Research MURI Program (NN00014-06-1-1176).
Abstract

There are several challenges associated with current strategies for drug and vaccine delivery. These include the need for multiple-dose administrations, which can hinder patience compliance, the requirements for specific storage conditions due to the fragile structure of protein-based molecules, and the need for additional excipients to enhance protein stability or adjuvant the immune response. This work has focused on the development of a high throughput, combinatorial approach to optimize degradable polymeric biomaterials, specifically polyanhydrides, to overcome these challenges associated with drug and vaccine delivery. We have developed high throughput techniques to rapidly fabricate polymer film and nanoparticle libraries to carry out detailed investigations of protein/biomaterial, cell/biomaterial, and host/biomaterial interactions. By developing and employing a highly sensitive fluorescence-based assay we rapidly identified that protein release kinetics are dictated by polymer chemistry, pH, and hydrophobicity, and thus can be tailored for the specific application to potentially eliminate the need for multiple-dose treatments. Further investigation of protein/biomaterial interactions identified polymer chemistry, pH, hydrophobicity, and temperature to be integral factors controlling protein stability during fabrication of the delivery device, storage, and delivery. Amphiphilic polymer chemistries were specifically identified to preserve the structure of both robust and fragile proteins from device fabrication to release. Our investigations of cell/biomaterial interactions revealed that all nanoparticle and polymer film chemistries studied were non-toxic at concentrations expected for human use. Furthermore, cellular activation studies were carried out with antigen presenting cells co-incubated with the polymer libraries which indicated that polymer
films do not possess immune stimulating properties; however, the nanoparticles do, in a chemistry dependent manner. Combining these insights with informatics analysis, we discovered the molecular basis of the “pathogen-mimicking” behavior of amphiphilic polyanhydride nanoparticles. Specific molecular descriptors that were identified for this pathogen-mimicking behavior include alkyl ethers, % hydroxyl end groups, backbone oxygen content, and hydrophobicity. These findings demonstrated the stealth properties of polyanhydride films for tissue engineering and the pathogen-mimicking adjuvant properties of the nanoparticles for vaccine delivery. Finally, host/biomaterial interactions were studied, which indicated that polymer chemistry and administration route affect nanoparticle biodistribution and mucoadhesio. Amphiphilic nanoparticles were identified to reside longest at parenteral administration routes and adhere best to mucosal surfaces. These results point to their ability to provide a long-term antigen depot in vivo. In summary, the studies described in this thesis have created a rational design paradigm for materials selection and optimization for use as drug delivery vehicles and vaccine adjuvants, which will overcome the challenges associated with administration frequency, protein instability, and insufficient immune stimulation.
Chapter 1: Introduction

1.1 Introduction

Biodegradable polymers have the advantage of delivering biological molecules at controlled rates, thereby limiting the need for repeated treatments\(^1\text{-}^{10}\). This has beneficial implications in both vaccine and drug delivery, where multiple treatments are usually required to achieve the desired outcome. In many cases, several doses are necessary to gain full protection against pathogens such as *Bacillus anthracis*, hepatitis B virus, etc\(^{11,12}\). Many conventional methods include frequent inoculations with the antigen or administration of the therapeutic drug to achieve protection against the pathogen or disease treatment. When these administrations are performed via injections, they are often painful and lead to poor patient compliance\(^{13,14}\). Additionally, concerning vaccines, it is suggested that the best way to achieve vaccine efficacy is by vaccinating through the same route that the pathogen uses to infect the host\(^{15}\). Thus, vaccines and therapeutics need to be tailored to use the best adjuvant, administration route, treatment dose, and exposure or controlled release of the antigen/drug. There is a need for controlled delivery systems for vaccines and therapeutic drugs that can meet the desired specifications for disease prevention and treatment.

Current advancements in medicine have led to the development of protein-based drugs for cancer, bacterial, viral and autoimmune diseases. The poster child of these drugs is recombinant insulin for diabetic patients, which was approved by the United States FDA (Food and Drug Administration) approximately 27 years ago\(^{16}\). Many other protein-based biomolecules have also emerged as vaccine antigen candidates (H1N1: influenza, rPA:
anthrax, rF1V: pneumonic plague, TT: tetanus, IPV: polio, etc) and as therapeutic drugs (anti-toxins: animal venom, penicillin: bacterial infection, insulin: diabetes, IL-2: metastatic cancer, IFN-γ: chronic granulomatous, γ-globulin: x-linked agammaglobulinemia, etc.) [17]. However, many of these proteins are fragile and sensitive to structural instability upon storage and administration. The effect of storage on drug stability is even more pronounced in developing countries with limited resources for the necessary storage conditions. To overcome these challenges, polymer-based drug carriers have emerged as frontrunners. Some of these carriers are unique in that they can be fabricated into different device geometries (e.g., films for tissue engineering scaffolds [18, 19], implant coatings [20-24], drug eluting stents [25, 26], microparticles for injectable localized drug delivery [2-7, 9, 27-30], or nanoparticles for inhalable or injectable systemic drug delivery [8, 21, 22, 31-41]) to best fit the desired application of disease treatment. Additionally, many of these polymeric drug carriers are capable of providing protein stabilizing environments as well as controlled release kinetics to both preserve the drug functionality as well as deliver it in a manner to best fit disease treatment [3, 4, 6-9, 37]. Their role in drug delivery has shown great promise due to their biocompatibility and highly tunable behavior resulting in control over release kinetics [42], immune modulation [5, 22, 30, 35, 38, 40], protein stability, and responsiveness to environmental changes such as pH. The primary goal with these polymeric devices is to eliminate multi-dose administration as well as preserve protein-based drugs in less favorable environments leading to a more viable method for drug and vaccine administration.

Current research in this field has focused on versatile groups of polymeric biomaterials with a broad range of properties including: acid-catalyzed polymers (polyacetals, polyketals and
polyorthoesters), polyesters, polyethers, and polyanhydrides. Many of these biomaterials have demonstrated the ability to successfully deliver therapeutic drug-based therapeutics. For example, several polyesters have FDA approval for human use and are commercially available, making them ideal for research in drug delivery. Polyanhydrides have shown many benefits in their ability to stabilize proteins, control the rate of drug release, as well as enhance/modulate the immune response to vaccine antigens \[^{[3, 4, 6-9, 22, 29, 35-38, 40, 43]}\].

Polyanhydrides composed of 1-6 bis\((p\text{-carboxyphenoxy})\)hexane (CPH) (Figure 1.1), sebacic acid (SA) (Figure 1.2) and 1,8-bis-(\(p\text{-carboxyphenoxy})\)-3,6-dioxaoctane (CPTEG) (Figure 1.3) have been studied extensively in a conventional format for use as drug delivery devices. The Gliadel\textsuperscript{®} wafer, a U.S. FDA approved polyanhydride based drug delivery system, has been used in the treatment of brain tumors \[^{[44]}\]. Their history of positive impact for human use \[^{[45]}\] along with their chemical diversity makes polyanhydrides ideal candidates for protein and vaccine delivery. Today, many preferred drugs and antigens are proteins, but they are more susceptible to various forms of deactivation due to their chemical and physical environments \[^{[3, 37, 46]}\]. To ensure protein stability and functionality, it is necessary to provide stable microenvironments for these protein-based drugs during storage, encapsulation and in vivo delivery. Due to their versatile chemistries and degradation rates, polyanhydrides are excellent candidates for protein stabilization and delivery.

The rational design and optimization of polymeric biomaterials is achievable by tailoring their properties and experimental conditions to ensure their function for the specific application. This is typically carried out through careful and time-consuming “conventional” (i.e., one experiment-at-a-time) methods to study the effect of multiple parameters with each
parameter differentially affecting the experimental outcome. For example, these parameters
could include polymer properties (e.g., chemistry, degradation mechanisms and kinetics,
glass transition temperature, melting temperature, surface molecules, etc.), experimental
conditions (e.g., temperature, buffers, pH), protein/polymer interactions (e.g., release
kinetics, stability, etc.), cell/polymer interactions (e.g., cytotoxicity, proliferation, adhesion,
etc.) and host/polymer interactions (e.g., biodistribution, residence time, mucoadhesion, etc.).
Often this trial-and-error process, which involves testing one variable at a time, can become
expensive, time-consuming, and potentially non-reproducible. The rational design of
polymeric biomaterials is a multi-variant problem in which efficient methods enabling
numerous variables to be tested simultaneously are needed to rapidly advance their
development for drug and vaccine delivery applications [47, 48].

The combinatorial approach to discovery was initially propelled into research by the
pharmaceutical industry [49]. Difficulties in efficiently evaluating complex combinations of
proteins, biomolecules, and biological components directed the focus on combinatorial
techniques that have led to the development of biological assays and high throughput
automated sampling and data quantification (e.g., auto samplers and plate readers). More
recently, the combinatorial approach has been applied to biomaterials development focusing
on parallel synthesis and characterization, and optimization of the properties best suited for
specific biomedical applications [8, 21, 22, 36, 38, 43, 48, 50-54]. The design of biomaterials for drug
delivery involves complex interplay between the material, the drug/antigen, the processing
conditions, and the in vivo/in vitro conditions into which the material is applied or delivered.
Optimizing these variables to administer a drug or an antigen at a desired rate to a specific
organ, or to deliver an antigen to a specific cell type to elicit the desired immune response are daunting challenges that could significantly benefit by the use of combinatorial techniques. This methodology also plays a central role in the subsequent biological screening of cellular and host interactions with biomaterials. The versatility of the combinatorial methodology makes it amenable to numerous applications and processes allowing for accelerated discovery, optimization, and reduced experimental and user variability. However, the central role of the combinatorial method is to function as a screening tool for the rapid identification of “hot spots” or areas of interest in a multi-variant parameter space. The identified key areas must then be further investigated and validated through conventional experimentation. In summary, the generality of the technique paves the path for rational design and development of biomaterials for specific applications in drug/vaccine delivery. With this in mind, the overall goal of the research described in this thesis is the rational design and development of polyanhydrides as drug and vaccine delivery devices using combinatorial and high throughput methodologies.

1.2 References

1.3 List of Figures

Figure 1.1: The chemical structure of poly(CPH)

Figure 1.2: The chemical structure of poly(SA)

Figure 1.3: The chemical structure of poly(CPTEG)
Chapter 2: Literature Review

2.1 Summary

Current research in the field of polymeric biomaterials for drug and vaccine delivery has focused on versatile groups of degradable materials including: acid-catalyzed polymers (polyacetals, polyketals and polyorthoesters), polyesters, polyethers, and polyanhydrides. These biomaterials have been used as therapeutic delivery vehicles, vaccine adjuvants, tissue engineering constructs, drug eluting stents, and multi-component implants. Due to their non-toxic, biocompatible, and bioresorbable properties, these biodegradable polymers are excellent candidates for use as protein-based drug delivery devices. Additionally, they have tunable parameters which can be optimized to meet treatment requirements, including tailored release kinetics, protein stabilization, and immune modulation. Section 2.2 gives a brief overview of polymeric biomaterials and their use in drug delivery applications. Section 2.3 demonstrates the ability of polymers to differentially modulate the immune system based upon polymer chemistry, particle size and particle shape. As a result of the large parameter space to be evaluated and optimized to study these protein/polymer and cell/polymer interactions, there is a need for a more rapid, multi-parametric approach to be integrated into the design and development of these biomaterials. These approaches are discussed in Section 2.4, which provides an overview of combinatorial approaches used to design and discover biomaterials and the need for these studies to more efficiently develop therapeutic and prophylactic medical treatments.
2.2 Polymeric Biomaterials: Drug Delivery Applications

2.2.1 Introduction

Polymeric biomaterials have been studied for many decades. Initial applications of these materials originated in the textile industry in the 1910’s. Polyanhydrides and polyesters demonstrated robust mechanical properties upon being spun into fibers and were initially intended for use in the fabrication of textiles and clothing. However, these degradable polymers were soon deemed unusable for such applications. Steps were taken to make these polymers more durable and long-lasting by incorporating carboxylic rings into their backbone \[^{1-3}\]. While these modified materials showed slower rates of degradation these materials were found to be less stable and therefore their use as fibers in the textile industry was short lived.

In the 1960’s, researchers discovered alternative uses for these biomaterials in biomedical applications for drug delivery. Poly(glycolic acid) (PGA) (Figure 2.2.1), a member of the polyester family, was employed for use as surgical sutures and biomedical implants \[^{4-6}\]. While this class of polyesters is biocompatible and non-toxic and has demonstrated much success in the field of biomaterials, their use in protein delivery has some limitations. Commonly used copolymers of PGA and poly(lactic acid) (PLA) (Figure 2.2.1) have non-tunable protein release kinetics, which results in either rapid or very slow protein release \[^{7-17}\]. Effective drug delivery is demonstrated by controlled release kinetics which is difficult to achieve with polyesters. In addition, they undergo bulk erosion which results in diffusion-controlled protein release, as shown in Figure 2.2.2 \[^{7}\]. These polymers also degrade into highly acidic degradation products (pH ~3.5-3.6) which can lead to acid-induced hydrolysis.
of encapsulated therapeutic protein-based drugs intended for drug delivery \cite{18-24}.

These limitations prompted further design and development of biomaterials with alternative degradation mechanisms. In the early 1980’s two classes of polymeric biomaterials were developed, acid-catalyzed polymers (Figure 2.2.3) and polyanhydrides (Figure 2.2.4) \cite{25-30}. These polymeric systems undergo surface erosion which enables protein release kinetics to mimic the polymer degradation, as shown in Figure 2.2.4. In the case of polyanhydrides, the degradation rates can be tailored to suit specific applications by changing the chemistry \cite{30-41}. This method of controlled protein release has been used in a wide range of applications including the treatment of Alzheimer disease and several forms of cancer as well as vaccine delivery of both DNA and protein based treatments \cite{34, 42-47}.

Additional polymeric biomaterials which also emerged as drug delivery devices in the early 1980’s include polyethers (Figure 2.2.5), which have demonstrated use in a wide range of applications due to their high drug loading capabilities and easy administration \cite{48-51}. However, the stability of this polymeric device in water degradation poses challenges and is achieved through enzymatic means, oxidation, or device disassociation and extraction \cite{51}.

In the following sections, a brief overview of polymeric biomaterials and their applications in drug delivery will be discussed Section 2.2.2 deals with acid-catalyzed polymers (polyacetals, polyketals and polyorthoesters), Section 2.2.3 with polyesters, Section 2.2.4 with polyanhydrides, and Section 2.2.5 with polyethers.
2.2.2 Acid-Catalyzed Polymers

Acid-catalyzed polymers emerged in the field of biomaterials for use in drug delivery in the last thirty years [25-29]. The need for surface eroding polymers led to the discovery of several versatile acid-catalyzed polymers including polyacetals, polyketals and polyorthoesters, as shown in Figure 2.2.3.

While non-degradable polyacetals (e.g., Delrin®) have been used in the medical field for many years [52-56], only recently have degradable polyacetals and polyketals been developed for applications in drug delivery and tissue engineering [51, 57]. They have been investigated in this field in the form of thermoresponsive gels [58], microparticles [59, 60] and poly-functionalized polymers with specialized structures [61, 62], all of which have differential protein release profiles based upon release environment pH [18, 19, 51, 63]. Due to their responsiveness to pH they have been used as tumor targeting vehicles, enabling site-specific drug release at the more acidic tumor location [18, 19, 51, 58, 63, 64]. Polyacetal microparticles with polyethylene glycol (PEG) incorporated into their backbone were able to provide prolonged, sustained drug delivery as a result of the increased hydrophilicity of the microparticle device inhibiting cellular uptake. They also have been shown to have minimal effects on local pH environments, limiting their overall effect on inflammation and immune aggravation. Thus, these materials demonstrated biologically inert properties important for drug delivery applications in tissue engineering and administration of local therapeutics in which immune activation is not desired [51]. Additionally, polyacetals have been utilized as therapeutic delivery agents by incorporating anti-cancer drugs, non-steroidal oestrogen diethylstilboestrol (DES), into the polyacetal backbone, thus enabling DES release upon
polymer degradation $^{[51, 58]}$.

Another group of acid-catalyzed polymers similar to polyacetals and polyketals are polyorthoesters. They have been fabricated into microparticles and medical implants but their applications in drug delivery demonstrate a much broader range of therapeutics capabilities including the delivery of cancer chemotherapeutics, periodontal therapeutics, anti-inflammatory agents, intraocular therapeutics, anesthetics, and DNA $^{[65-74]}$. While polyorthoesters lack the ability to provide a long-term sustained drug release, researchers have synthesized polyorthoester-amines, which are capable of providing a more sustained drug release profile $^{[75]}$. This rapidly advancing field of acid-catalyzed polymeric biomaterials is enabling the optimization of drug delivery vehicles and tissue engineering constructs with biologically inert properties $^{[51]}$.

### 2.2.3 Polyesters

The most widely investigated degradable polymeric biomaterials are polyesters, specifically those based on copolymers of lactic and glycolic acids (PLA, PGA, and PLGA) and poly($\varepsilon$-caprolactone) (PCL) (Figure 2.2.1) with wide ranging applications in drug delivery and tissue engineering constructs $^{[10, 12-17, 20-23, 51, 71, 76-107]}$. A significant amount of research has been carried out in the past several decades to investigate these materials for biomedical applications. They have favorable properties including their biocompatibility and low cytotoxicity which is a result of their degradation into cellular metabolites, readily taken up by surrounding cells $^{[51, 108, 109]}$. 
PGA is a hydrophilic polymer with a highly crystalline structure and has been most commonly used in the medical field as resorbable sutures \cite{51,110}. Upon degradation it undergoes bulk erosion (Figure 2.2.2) in which it rapidly loses most of its mechanical properties and results in a burst release of an encapsulated drug \cite{7,110}. To compensate for this structural loss often times PGA is copolymerized with the hydrophobic semi-crystalline poly(lactic acid) (PLA) resulting in PLGA copolymers \cite{17,22,51,77,84-86,90,93,96,99-101,103,105,107,111,112}.

PCL, which is FDA approved for applications in drug delivery and biodegradable sutures, is another member of the polyester family which is semi-crystalline in structure, similar to PLA \cite{51}. However, PCL has a much slower degradation profile ranging from one to three years \textit{in vivo} \cite{113-117}. Due to this prolonged degradation rate it has been utilized in blends or copolymers to expedite the degradation rate \cite{51,77,118-127}.

While all polyesters undergo bulk erosion due to the stability of their ester bonds, controlled drug delivery becomes a difficult task due to several limitations. Despite the incorporation of lactic acid (from 50 to 100%) into the PLGA copolymer, they possess a very limited range of release kinetics \cite{8-10}. Additionally, the ingress of water into the bulk of the polymer network exposes encapsulated protein to low microenvironment pH levels and allows for significant residence time with the surrounding water \cite{20,22,128}. Both mechanisms have demonstrated to be detrimental factors in preserving protein stability \cite{20,22,89,90,128}. To compensate for these problems, basic compounds such as Mg(OH)$_2$ and MgCO$_3$ have been incorporated into the polyester particles or devices and have demonstrated a less acidic release environment upon
protein release; however, additional components can complicate the delivery of fragile therapeutic drugs by affecting the release profiles, biocompatibility, and in vivo side effects [93, 129]. These limitations demonstrate significant hurdles for the use of polyesters in drug delivery.

2.2.4 Polyethers

Polyethers have been investigated for drug delivery applications. The most common polyethers are poly(ethylene glycol) (PEG) and poly(propylene glycol) (PPG) (Figure 2.2.5). They have been used in conjunction with several of the other polymeric biomaterials discussed in this chapter. The incorporation of PEG into the backbone of many of these materials (e.g., polyanhydrides, PCL, and acid-catalyzed polymers) allows for a more amphiphilic environment and in many cases alters the degradation rate, (e.g., reduces the year-long degradation profile of PCL) [77, 78, 121, 130, 131]. Additional studies have demonstrated the ability of PEG to oppose interactions with the host by acting as a phagocytosis and cellular adhesion inhibitor [132, 133]. This can be used as a coating for polymeric micro- and nanoparticles intended for drug delivery in which cellular adhesion or phagocytosis is not desirable.

An additional polyether commonly used in drug delivery applications is the triblock copolymer ([PEG]n-[PPG]m-[PEG]n) known as Pluronic®. This copolymer has unique micelle forming properties, via self assembly in water, due to the hydrophilic PEG and hydrophobic PPG, enabling the formation of nanometer sized particles [134]. In addition, the amphiphilicity of this copolymer enables for high drug loading, on the order of 30%, of
hydrophobic molecules and allows for many cell/polymer interactions including hydrophobic surfaces and cellular membranes \[48\].

Despite the unique properties and significant advantages of the polyether system, there are hurdles which must be overcome. Specifically, polyethers do not undergo water induced hydrolysis because of their hydrolytically stable ether bond and therefore require the use of alternate components (i.e. enzymes) for degradation or surgical extraction \[51\]. In addition, the slow in vivo degradation of high molecular weight polyethers like PEG, can lead to accumulation and subsequent immune activation. By minimizing the polymer molecular weight and utilizing non-invasive degradation mechanisms, this polymeric biomaterial holds much promise for future applications in drug delivery.

### 2.2.5 Polyanhydrides

Polyanhydrides are a class of versatile biomaterials and can often be grouped into three primary classes: 1) aliphatic, 2) unsaturated and fatty-acid derived and 3) aromatic polyanhydrides \[31\]. Polyanhydrides have been studied extensively for use as drug and delivery devices and vaccine adjuvants \[32-37, 39, 40, 135-150\]. Modulation of the stability and release of an encapsulated protein drug is a critically important advantage of these polymeric devices and is credited to the unique chemistry and tunable release kinetics, with the ability to mimic the polymer degradation in an aqueous environment \[7\]. Polyanhydrides have been shown to degrade by surface, bulk, and combined erosion (Figure 2.2.2) mechanisms via hydrolysis into acidic monomers over a period of years to weeks to days \[31, 37, 136\]. These degradation times are directly related to the release rates of encapsulated proteins and can be
modulated by synthesizing copolymers with various combinations of monomers as dictated by application. These polymers also possess important protein stabilization properties, which is a significant problem with other drug delivery devices (i.e. polyesters). They are biocompatible (at concentrations up to 5000 μg/mL in vivo) as they degrade into non-toxic, non-mutagenic degradation products capable of being metabolized intracellularly \[46, 139, 151\].

In 1996, the U.S. FDA approved the use a polyanhydride drug delivery system, the Gliadel™ wafer, for the treatment of brain tumors. The wafer is composed of poly((1,3-bis(p-carboxyphenoxy)propane) and (sebacic acid)) (CPP:SA) in a 20:80 molar ratio encapsulating a chemotherapeutic agent, 1,3-bis[2-chlororthyl]-1-nitro-sourea (BCNU), otherwise known as carmustine \[42\].

The most common aliphatic polyanhydrides include poly(SA) and poly(adipic acid) (AA), which are semi-crystalline polymers that exhibit surface erosion \[31\]. Poly(SA) was first synthesized in 1987 and is often used in coordination with other polyanhydrides \[30\]. Some unsaturated and fatty acid derived polyanhydrides include poly(fumaric acid) (FA) and acetylenedicarboxylic acid (ACDA) \[31\]. They contain significant mechanical strength and when copolymerized together with SA, they are able to increase the bioavailability of orally delivered proteins. Finally the third class is a group of aromatic polyanhydrides, which encompasses poly(isophthalic acid) (IPA), poly(terephthalic acid) (TA), 1-6 bis(p-carboxyphenoxy)hexane (CPH) and 1,8-bis-(p-carboxyphenoxy)-3,6-dioxaoctane (CPTEG) \[31\]. With the exception of CPTEG, the degradation of these hydrophobic, surface eroding polymers can exceed one year and are therefore often copolymerized with more rapidly eroding aliphatic polyanhydrides.
Polyanhydrides based on CPP, CPH, SA, and CPTEG have been well studied (Figure 2.2.4). These polyanhydrides have highly tunable properties when polymerized together as copolymers (CPH:SA or CPTEG:CPH) allowing for tailored release kinetics, protein stability, and immunomodulatory capabilities \[32-36, 39, 139, 142, 143, 145-149\]. Narasimhan and colleagues have extensively studied these polyanhydrides and have demonstrated their excellent characteristics for use as drug and protein carriers. They have demonstrated the ability of these polyanhydrides to be fabricated into multiple device geometries including films, nanoparticles, microparticles, and tablets \[32-36, 39, 40, 46, 47, 136, 139, 142-149, 151-158\]. This allows for their use in a broad range of drug delivery applications including injectable microparticle delivery, inhalable/injectable nanoparticle delivery, implantable tablets, drug eluting stents, multi-component implants, and tissue engineering constructs.

As previously mentioned, polyanhydrides can undergo surface, bulk, or a combination of degradation mechanisms, with CPTEG undergoing bulk and CPH and SA undergoing surface erosion \[38, 39\]. However, these monomers can be copolymerized together to allow for tunable protein release kinetics. As to be expected, the less hydrophobic copolymers (CPTEG and SA rich) tend to degrade more quickly than more hydrophobic copolymers (CPH rich). CPTEG-rich systems tend to exhibit an initial burst of protein whereas the CPH and SA-rich systems demonstrate a much smaller initial burst \[33-36, 39, 147\]. This is also observed with high protein loading. This polymer degradation and subsequent protein release have been well documented in the literature for several proteins, including bovine serum albumin (BSA), ovalbumin, tetanus toxoid, the protective antigen for anthrax, lipocalin-2, and the recombinant F1-V antigen \[32, 34-36, 39, 143, 144\]. Release kinetics between proteins can differ
based upon their affinity for the homopolymer or microphase separated regions of the copolymer \cite{37,38}. It has been reported by Carrillo-Conde et al. that antigens for pneumonic plague, F1–V fusion protein and the F1_{B2T1}–V10, release differently from 50:50 CPTEG:CPH nanoparticles, which is likely a result of differential affinity for the copolymer \cite{32}.

In addition to protein release kinetics it has been reported that protein stability is differentially affected by the polyanhydride copolymer chemistry \cite{32-35,39,142-144,146}. Specifically, CPTEG:CPH copolymers are of key interest because their amphiphilic properties have been shown to provide an excellent environment for protein stabilization, securing both protein structure and function \cite{32-35,39,146}. The structure of the CPTEG monomer differs from that of the CPH monomer in that it has oligomeric ethylene glycols in its backbone. This is what creates the more amphiphilic environment favorable for encapsulating proteins. SA-rich copolymers have been demonstrated to provide a much less favorable protein environment due to their degradation into monomers resulting in an acidic microenvironment, which is harmful to the function of many protein-based drugs \cite{33,35,142,143,146,159}.

While some polyanhydrides possess limitations (e.g., SA-rich materials lead to protein hydrolysis), many of these problems can be overcome due to the availability of a large library of polyanhydrides to copolymerize together allowing for optimization of the desirable polymer system. This class of biomaterials has demonstrated success in terms of biocompatibility, protein stability, and tunable protein release kinetics; additionally, they
have also shown the unique ability to modulate the immune system based upon copolymer chemistry, which will be discussed in the next Section.

2.3 Polymeric Biomaterials: Vaccine Adjuvants

2.3.1 Introduction

Vaccination is one of the most successful medical interventions for eliminating infectious diseases in the past 200 years [160]. It is estimated that for every $1 spent on vaccination, another $5-10 is saved for what would have been spent on disease treatment [161]. The effectiveness of vaccination has been demonstrated through the world-wide eradication of human small pox virus and the elimination of the polio virus in the US, Europe, and the Western Pacific [162].

Despite the benefits and effectiveness of many vaccines available today, infectious disease still remains a leading cause of death worldwide [163, 164]. It is estimated by the World Health Organization that approximately 14% of childhood deaths are caused by vaccine preventable diseases [165]. Problems associated with vaccine administration and disease prevention are often associated with the need for multi-dose administration, poor patient compliance, poor immunogenicity of vaccine antigens, and rapidly evolving/mutating pathogens [166, 167]. Full protection from a pathogen through vaccination often requires multiple doses to be administered incrementally. Often times, the immune system will produce a response to the initial vaccine dose which involves antigen presenting cells and many other naïve immune cells while later vaccine doses are able to promote the long term memory required for full protection. It has been reported that in developing countries that as much as 70% of vaccine
patients drop out after the first administration and are therefore not fully protected\cite{13}. In addition, many current diseases lack FDA approved vaccines because available vaccine antigens for those diseases are not highly immunogenic and are not able to result in the development of protective immunity; however, vaccines remain the most cost effective method to combat infectious disease\cite{168}.

These problems associated with preventive vaccine protection could be addressed with the use of controlled delivery devices and vaccine adjuvants. Controlled delivery devices such as polymeric biomaterials could allow for tunable antigen release, which is able to mimic the multi-dose regimen with a single injection of the soluble antigen plus antigen encapsulated into the polymeric delivery device\cite{46,169}. Additionally, the prolonged residence time of these materials \textit{in vivo} would provide continual antigen (specific-stimulation) exposure to antigen presenting cells (APCs) to fully activate adaptive and innate immune responses. This has been investigated with a host of polymers including acid-catalyzed polymers (polyketals and polyorthoesters, polyesters (PLA, PGA, PLGA, poly(ester-amide) (PEA), and PCL), polyethers (PEG), and polyanhydrides (SA, CPTEG, and CPH))\cite{14,46,47,58,88,170-176}. In addition to their function as vaccine/drug delivery devices, as discussed in Section 2.2, they have also been investigated as vaccine adjuvants\cite{46,47,79,80,86-88,94,103,105,139,145,147-149,158,170,177,178}. An adjuvant is an agent capable of stimulating the immune system without having any inherent antigenic properties\cite{171,179,180}. Such agents are able to encapsulate the desired antigen and deliver it to immune cells while enhancing and modulating the immune response over what the antigen could achieve when administered alone\cite{171,178,181-183}. In addition, adjuvant agents could be encapsulated into polymeric biomaterials to synergistically enhance
the immune response \cite{87, 180}. Currently there are several polymeric adjuvants under investigation for use in vaccination; however, only PLGA microparticles are currently undergoing clinical trials for human use \cite{184}. In addition, there is a wide range of non-polymeric adjuvants (e.g., cytokines, virosomes, oil emulsions, alum, MPLA) in current clinical trials but only one (alum) is approved for human use in the U.S. \cite{171, 180, 184, 185}.

When designing vaccine adjuvants to enhance a specific immune response, it is important to understand the multi-faceted aspects of both the innate and adaptive immune responses and the different branches that stem from these responses (Th1, Th2, Th17, Th0). Innate and adaptive immune systems work in concert to make up the body’s defense mechanism against infectious disease \cite{186}. The innate system is the first line of defense and is responsible for recognizing foreign pathogens or agents. Cells from the innate system would the primary target for vaccine adjuvants since they are the initial responders to pathogenic antigens and determine the robustness of the primary response to the antigen. Much of the foreign body recognition of these cells is triggered through interaction of pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) with pathogen-associated molecular patters (PAMPs) \cite{187}. These PAMPs are comprised of bacterial components such as lipopolysaccharide (LPS) which is found on the surface of gram-negative bacteria \cite{188}. Depending upon the effectiveness of the innate immune response, engagement of the adaptive immune response is conditional.

Dendritic cells (DCs) are primary APCs and play key roles in both innate and adaptive immunity \cite{189}. DCs can be found in all body tissues and are often in an immature state.
allowing for antigen sampling through phagocytosis, endocytosis, micropinocytosis, or receptor-mediated uptake (mannose, complement, or Fc receptors)\(^{[190]}\). There are several subsets of DCs which include but are not limited to Langerhans’, interstitial, myeloid (DC1), or lymphoid (DC2)\(^{[191]}\). Each subset plays a different role in the immune system from antigen processing and presentation to the induction of tolerance (the act of identifying self-antigens which should not be responded upon by the immune system)\(^{[191]}\). As the DCs undergo activation upon encountering a foreign antigen they process the antigen, undergo a maturation process, and then migrate to the draining lymph nodes. The DCs will internalize the foreign antigen and present it on its surface via major histocompatibility complexes (MHC) I or II depending upon the location of the foreign pathogen (MHC I: endogenous pathogen, MHC II: exogenous pathogen)\(^{[179, 192]}\). Additional co-stimulatory molecules (CD80, CD86 and CD40) will be expressed on the cell surface for interaction with naïve T cells, either CD4+ or CD8+. Interactions with each T cell type are dependent upon the cell surface molecule by which the antigen is presented (MHC I or MHC II)\(^{[193, 194]}\). Upon processing of an endogenous pathogen the DC will present the pathogen via MHC I cell surface molecules which results in the maturation and activation of cytotoxic CD8+ T cells\(^{[193]}\). This is an important component of cell-mediated immunity because activation of CD8+ T cells enables them to directly kill infected cells. Conversely, when exogenous antigen is taken up by the DCs and presented via the MHC II cell surface molecule, CD4+ T cells are stimulated. The CD4+ T cell response encompasses both cell-mediated as well as humoral immunity. There are many CD4+ T cell subtypes including Th1, Th2, Th17, and Treg cells\(^{[195-197]}\). Upon activation by the MHC II-antigen complex, a Th2 immune response is characterized by the production of IL-4, IL-5, IL-10, and IL-13, as well as the secretion of
IgG1 and IgE, resulting in a humoral response. Th1 immune responses are dictated by interaction with the MHC II-antigen complex and production of INF-γ and TNF-β as well as secretion of IgG2a which leads to a cell-mediated response characterized by the activation of macrophages. This network of immune responses is illustrated in Figure 2.3.1. Less is known about Th17 responses; however, they are considered to be pro-inflammatory and can be characterized by the production of IL-17. Treg cells are T cells responsible for regulating the immune system and controlling over-stimulation through production of cytokines such as IL-10.

As discussed before, the immune system is a complex network of pathways; however, when designing vaccine adjuvants, it is important to understand the basic directions the immune system can take upon encountering foreign pathogens to enable the design of these materials to better mimicking the natural infection. The following sections will investigate the use of polymeric vaccine adjuvants acid-catalyzed polymers (polyketals and polyorthoesters (Section 2.3.2), polyesters (PLA, PGA, PLGA, PEA and PCL) (Section 2.3.3), polyethers (PEG) (Section 2.3.4), and polyanhydrides (SA, CPTEG, and CPH) (Section 2.3.5).

### 2.3.2 Acid-Catalyzed Polymer Adjuvants

The use of acid-catalyzed polymer adjuvants has recently gained interest in the field of vaccinology. These surface eroding polymers are amendable for use as vaccine delivery devices because they are capable of encapsulating and releasing encapsulated antigens. In addition, they can be fabricated into particles enabling non-invasive administration. Two groups of acid-catalyzed polymers, polyketals and polyorthoesters, have demonstrated
promising vaccine adjuvant properties to enable an enhanced Th1 modulated immune response.

Recently, polyketals were investigated for their effect upon DCs and the subsequent priming of IFN-γ producing CD8+ T cells \cite{199}. The polyketals were administered in the form of microspheres and encapsulated a hydrophobic complex, the antigen, ovalbumin, and a TLR3 agonist, double-stranded RNA analog poly(inosinic acid)–poly(cytidylic acid) (poly(I:C)), both of which were ion-paired to cetyltrimethylammonium bromide (CTAB). The results demonstrated the ability of the antigen-loaded polyketal microparticles to induce a higher percentage of CD8+ T cells and promote more production of TNF-α and IL-2 than the antigen complex administered alone \cite{199}.

The use of DNA vaccines is another vaccine strategy employed in current vaccinology studies because these vaccines are able to elicit both cell-mediated (CD8+ and CD4+ Th1) and humoral (CD4+ Th2) immune responses \cite{200}. DNA vaccines are not effective when administered alone due to the rapid degradation of DNA \textit{in vivo}. Thus, polyorthoesters have been recently studied for use in the delivery of DNA to protect the vaccine from degradation. It has been demonstrated that polyorthoesters have the ability to increase the cell-mediated immune response through activation of CD69+ CD8+ T cells as well as the humoral immune response through enhanced antibody production both before and after antigenic challenge \cite{200}. To better demonstrate this combined immune response activated by the delivery of DNA vaccines through polyorthoesters, pre-immunized mice were challenged with tumors expressing a specific antigenic epitope and demonstrated restricted tumor growth compared
to mice immunized with the DNA vaccine alone or with non-immunized mice \cite{200}.

Acid-catalyzed polymers are under current investigation as polymer adjuvants and vaccine delivery vehicles and have demonstrated much promise due to their non-acidic and non-toxic degradation products enabling antigen preservation and cellular viability. Polyketals also exhibited the ability to shift the immune response to cell-mediated immunity through the activation of CD8+ T cells. These materials have proven to be viable vehicles for the development of efficacious vaccine delivery should be further investigated for such applications.

2.3.3 Polyester Adjuvants

Polyesters have been investigated for use in vaccine delivery and adjuvant properties to enhance immune stimulation as well as modulate the immune response. They have encompassed several different vaccine strategies including antigen-loaded particles, DNA vaccines, as well as particle-based vaccines employing additional adjuvant agents (e.g. MPLA, CpG) \cite{86-88}. However, due to their limited range of release kinetics and acidic monomer degradation products, antigen release and stability have been of primary concern. Despite these limitations several polyesters have been investigated in depth for use in vaccine delivery and administration including PLGA, PLA, PGA, PCL and PEA \cite{15, 79-81, 83, 86-88, 92, 94, 100, 102, 103, 105, 129, 170, 177, 178, 201-210}.

PLA, PGA and copolymers of the lactic and glycolic acid subunits (PLGA) have been studied extensively for use in vaccine applications for the past 15 years\cite{15, 79-81, 83, 86-88, 92, 94, 100, 102, 103, 105, 129, 170, 177, 178, 201-210}.
They are well characterized and FDA approved for drug delivery applications and thus are likely candidates as vaccine adjuvants. Much research has been carried out to examine the adjuvant properties of PLGA microparticles on dendritic cells in vitro [79-81, 83, 88, 94, 103, 105, 170, 207]. Studies have shown that PLGA microparticles can enhance the expression of the cell surface marker CD86 and the production of cytokines [105, 177]. Additional studies have shown that PLGA microspheres encapsulating antigens have demonstrated the ability to enhance antigen presentation via MHC I resulting in an increased activation of antigen specific CD8+ T cells; however, some of these studies included MPLA which is known to have a Th1 bias [80, 86, 88].

To provide a better basis for human vaccination, several studies have also been carried out in vivo with antigen encapsulated into PLGA, PLA, and PGL microspheres to successfully produce an immune response towards *Vibrio cholera* antigens, *Y. pestis* antigens, *B. pertussis* antigens, measles virus antigen, influenza virus antigens, ovalbumin antigen, diphtheria toxin, type II collagen, malarial antigens, tetanus toxoid (TT), ricin toxoid cancer targeted antigens, *E. Coli* adhesion proteins, hepatitis B viral antigens, and HIV gp140 [177, 198, 205, 208, 209, 211-215]. These immune responses were characterized by induction of mucosal IgA, serum antibodies, antibody isotype switching, and cell-mediated responses. Numerous studies have been carried out investigating the encapsulation and release of TT from PLGA microparticles. Some studies demonstrate the ability of TT loaded microparticles to elevate antibody titers over that of soluble antigen as well as antigen + alum [205]. Contrasting results were found with TT loaded microparticles, which did not demonstrate elevated antibody titers over that of TT + alum [209]. In addition, PLGA microspheres have been investigated to
modulate the *in vivo* immune response and have proven successfully in several cases\(^{[178, 206, 216]}\). When encapsulating the HIV protein, gp 120, PLGA microspheres were shown to induce a shift from a Th2 dominant, to a combined Th1/Th2, to a Th1 dominant immune response\(^{[216]}\). Other research has suggested that upon encapsulation of the Th2-strong hepatitis B antigen into PLGA microparticles + MPLA, a shift to Th1 immunity is observed\(^{[178]}\). It is important to note that the adjuvant, MPLA, alone demonstrates a Th1 dominant response. Moreover, an additional study for vaccination against malaria provided evidence of the ability of Montanide ISA-720-loaded PLGA microparticles to induce a Th1 immune response as evidence by IgG isotype class switching\(^{[206]}\). While microspheres based on PLA, PGL, and PGLA have shown much success in the development of vaccine adjuvants and vaccine carriers, there still remains contradicting evidence which lead to inconclusive results for the efficacy of vaccine adjuvants based upon these polymers.

In addition to the lactic and glycolic acid based polyester vaccine adjuvants, PCL has been investigated for applications in this field. Recent studies demonstrate the difference between micro and nano sized particles for efficacious vaccination\(^{[204]}\). Antigen loaded PCL was fabricated into microparticles and nanoparticles and administered for immunization to mice. Upon infectious agent challenge, mice immunized with nanoparticles demonstrated significant protection when compared to the microparticle- and soluble antigen-vaccinated and non-vaccinated controls. Although, additional agents were required for the stabilization of the antigen in the PCL particles, this study confirms the capabilities of PCL as a vaccine delivery device as well as the importance of particle size for induction of the necessary immune response\(^{[204]}\).
Finally, PEA, which is a unique biodegradable polymer that degrades via enzymatic degradation \cite{202,203} has been studied as a vaccine adjuvant. It is mechanically robust due to its amide components, yet its ester bonds allow for flexibility and hydrolytic vulnerability. Due to its degradation mechanism, PEA has unlimited shelf life and storage capabilities and is only triggered for degradation upon administration. This polymer has been conjugated with multiple antigenic peptides for vaccine applications against human melanoma, HIV, and influenza A. The responses demonstrated a significant shift to cell-mediated immunity through MHC I and MHC II driven T cell responses \cite{202,203}.

As described in this Section, there are numerous areas of research on the use of polyesters as vaccine adjuvants and vaccine delivery devices. They have limitations, yet their commercial availability and acceptance in the field of biomaterials has driven researchers to overcome these hurdles to produce vaccine delivery vehicles. From the most common PLGA to the less known PEA, polyesters have and will continue to be a primary focus of research for drug and vaccine delivery.

### 2.3.4 Polyether Adjuvants

Polyethers have been studied to a limited degree as vaccine adjuvants and delivery vehicles with more widespread use in drug delivery. The only polyether reported for use in such applications is PEG. As is the case for many drug delivery applications employing PEG, studies in vaccine delivery have also incorporated other polymers in conjunction with PEG. Recently, poly(propylene sulfide) (PPS) nanoparticles were fabricated in combination with PEG in varying sizes \cite{217,218}. The PPS was the core of the nanoparticle with the PEG
providing a stabilizing corona. These blank particles were administered \textit{in vivo} and found to readily traffic to the draining lymph node at which point there were taken up by approximately \textasciitilde{}40-50\% of the antigen presenting cells. Even after 4 days post injection the particles appeared to reside in lymph node APC and actually as the time increased the number of particle containing APCs increased, indicating the infiltration of more cells to the nanoparticle site [217, 218]. While these particles appear to be possible candidates for vaccine and immunotherapy applications targeting DCs in the lymph node, further studies need to be carried out to assess their viability as drug/vaccine adjuvants or delivery vehicles.

2.3.5 Polyanhydride Adjuvants

Polyanhydrides are a class of biomaterials that have demonstrate much promise as vaccine adjuvants and vaccine delivery vehicles. They possess important properties for vaccine delivery including protein stabilization, controlled release, and biocompatibility. In addition, they are capable of modulating the immune response [46, 139, 145, 147-149, 154]. This versatile class of vaccine adjuvants includes poly(fumaric acid) (FA), poly(methyl vinyl ether-co-maleic anhydride) (MVE-co-MA) and copolymers based on CPTEG:CPH and CPH:SA [46, 139, 145, 147-149, 219, 220].

FA copolymerized with SA has demonstrated efficacy in oral vaccination. This copolymer was fabricated into microspheres and administered orally to rats [219]. The microspheres demonstrated strong adhesive interactions with the mucosal layer of the gastrointestinal tract. These prolonged interactions with the mucosal layer would provide extended antigen release necessary for protective immunity. Additional studies encapsulating plasmid DNA and the
anti-coagulant, dicumarol, in support of the previous work, demonstrated enhanced gene activity as well as plasma drug levels. These results indicate the potential viability for use of poly(FA-co-SA) in vaccine delivery applications [219].

Pолyanhydrides based upon poly(SA) and CPTEG:CPH and CPH:SA copolymers have shown much promise as vaccine adjuvants and drug delivery. Significant in vitro work has indicated that these polyanhydrides when fabricated into nanoparticles are able to modulate the immune response by influencing cell marker expression and cytokine production of DCs [139, 145, 147, 149]. Cell surface marker expression of CD40, MHC I, MHC II, and CD209 was controlled by polymer hydrophobicity. Expression of these markers increased as the polymer chemistry shifted from CPH-rich to SA- and CPTEG-rich polymer chemistries (i.e. decrease in hydrophobicity) [139, 145, 147, 149]. Additionally, the production of cytokines, IL-6 and IL-12p40, were also controlled by polymer chemistry. Production of both cytokines was enhanced upon incubation with SA-rich or CPTEG-rich copolymer chemistries [139, 145, 147, 149]. Additional work has been carried out to investigate the effect of polyanhydride microparticles on immune activation of DCs. Again, the expression of cell surface markers and production of cytokines was enhanced over the non stimulated control [148]. Due to the abundance of –COOH terminated end groups, these nanoparticles can also be surface modified to target specific cellular receptors and immune pathways to modulate the immune response [145]. Furthermore, recent work has demonstrated that amphiphilic polyanhydride nanoparticles, based on 50:50 CPTEG:CPH, possess many properties similar to LPS and bacteria which explained their similarity in internalization and DC activation [145, 147, 149]. However, while influencing DC behavior in a pathogen-mimicking manner, 50:50
CPTEG:CPH nanoparticles do not induce the toxicity and inflammatory response of bacteria/LPS [46, 47, 139, 147, 149, 151, 180] and therefore, provide a novel strategy for vaccination in a non-toxic, pathogen-mimicking approach. In vivo work investigating the use of polyanhydride microparticles as vaccine adjuvants has demonstrated the ability of CPH:SA particles to encapsulate and delivery TT antigen while modulating the immune response [46]. When antigen was delivered alone or in a 20:80 CPH:SA copolymer a Th2 dominant response was induced; however, the delivery of TT encapsulated into 50:50 CPH:SA microparticles resulted in a shift from the Th2 dominant response to a balanced Th1/Th2 response [46]. More recently, long-term protection against a lethal challenge of Y. pestis was achieved with a single-dose intranasal vaccine composed of antigen-encapsulated 50:50 CPTEG:CPH nanoparticles [47]. This group of polyanhydrides has demonstrated much promise for use as vaccine adjuvants and continues to be the focus of further in vivo studies for vaccine protection.

An additional polyanhydride copolymer based upon poly(methyl vinyl ether-co-maleic anhydride) (MVE-co-MA), Gantraz ®, has investigated the ability to protect against Salmonella enteritidis infection via vaccine strategies [220]. In these studies an immunogenic subcellular extract obtained from heat-killed S. enteritidis cells (HE) was loaded into (MVE-co-MA) nanoparticles and used to immunize mice. When challenged 49 days post-immunization approximately 80% of the mice which received the nanoparticle vaccine survived the infection. While serum antibody titers resemble a Th1 dominant response in the initial stages after immunization, later stages reveal a Th2 dominant response, indicating the
importance of a balanced combination of the two immune responses for disease prevention [220].

Polyanhydrides encompass a wide range of promising characteristics for use in vaccine delivery and administration. They are able to encapsulate, stabilize and provide a depot for a vaccine antigen, they are able to be administered via any injection route, and they have been shown to enhance and modulate the immune response, dictated by polymer chemistry and hydrophobicity. When fine-tuned, this polymer system can provide ideal properties to enhance vaccine efficacy as needed based upon limitations with vaccine antigens.

2.4 Combinatorial Design of Biomaterials for Drug Delivery: Opportunities and Challenges

A review article published in Expert Opinion on Drug Delivery 2008, 5(8), 1-10
Latrisha K. Petersen and Balaji Narasimhan

2.4.1 Introduction
Polymeric biomaterials are used in a wide range of biomedical applications from bio-absorbable prostheses [221] and drug delivery [37, 222-224] to tissue scaffolding [225] and sutures. Their role in drug delivery has emerged with great success due to their biocompatibility and highly tunable behavior that results in control over release kinetics [37, 143], immune modulation [46], protein stability [39, 142, 143], and responsiveness to environmental changes such as pH [226]. The rational design of key polymer properties and controlled experimental conditions is essential to fulfill their promise in these applications. This is typically carried
out through careful, time-consuming conventional (i.e., one experiment-at-a-time) design and
development with multiple varying parameters, including polymer properties (e.g.,
chemistry, degradation mechanisms and kinetics, glass transition temperature, melting
temperature), experimental conditions (e.g., temperature, buffers, pH, ionic strength), and
differentialness with polymer/cell interactions (e.g., cytotoxicity, proliferation, adhesion) with each variable
differentially affecting the resulting experimental outcomes. Often this trial-and-error
process, with testing one variable at a time, can become an expensive and non-reproducible
proposition, in which experiments take a considerable amount of time. This is a multi-variant
problem and efficient methods enabling numerous variables to be tested simultaneously are
needed to advance the development of biomaterials for applications in drug delivery and
tissue engineering\textsuperscript{[227]}.

The combinatorial approach to discovery was initially propelled into the spotlight by the
pharmaceutical industry\textsuperscript{[221, 228]}. Difficulty in efficiently evaluating complex combinations of
proteins, biomolecules, and biological components directed the focus on combinatorial
techniques including biological assays and high throughput automated sampling and data
quantification (i.e., auto samplers and plate readers). The combinatorial approach has been
applied to biomaterials and drug delivery development and has focused on the synthesis,
characterization, and optimization of the biomaterial properties best suited for specific
applications in drug delivery. Drug delivery system design is a multivariate problem with
interplay between the biomaterial, the drug, the processing conditions, and the \textit{in vivo}
conditions into which the drug is delivered. Optimizing these variables to administer the drug
at a specific rate to a specific organ or tissue is a daunting challenge that could benefit by the
use of combinatorial methods. This methodology also plays a central role in the subsequent biological screening of cellular interactions with these biomaterials. The versatility of the combinatorial methodology makes it amenable to a number of applications and processes allowing for accelerated discovery, optimization, and reduced experiment-to-experiment variability. It must be borne in mind that the combinatorial method is a screening tool and enables the rapid identification of “hot spots” or areas of interest in a large search space and must be validated by conventional experimentation.

The various steps in the combinatorial process are shown in Figure 2.4.1, beginning with experimental design. The primary goal of this step is to design a process which focuses on a limited parameter space by employing previous knowledge, thus resulting in the most efficient method for collecting useful data. The experimental design is then implemented to rationally design and synthesize either a discrete or continuous combinatorial library, in which properties are varied systematically in one or more directions. Next, high throughput characterization techniques are utilized to investigate the structure-property relationships within the parameter space of the library, which minimizes experimental variability. The large data sets obtained through these analyses are then validated by informatics and statistical methods, which provide insight into the design and development for further experiments. The key findings and results discovered through the combinatorial approach are then validated by conventional techniques, allowing for an accelerated approach to the design and optimization of biomaterials for drug delivery. Throughout the process, there is feedback built into the system to enable optimization at every step.
The keystone of this process for biomaterial design begins with the creation of a single, multi-dimensional library \[229\]. This has been made possible by major advancements in lithography and robotics, enabling highly expedited deposition and synthesis of these libraries \[230\]. The libraries are amenable to high throughput characterization employing methods such as Fourier transform infrared spectroscopy (FTIR), optical microscopy, and gel permeation chromatography (GPC). This parallel characterization results in the rapid evaluation of properties such as phase behavior, contact angle, and glass transition temperature, all of which play a critical role in the design of specific biomaterials as drug carriers.

The main goal of the combinatorial approach in drug delivery is to explore a vast array of biomaterials in a single high throughput experiment which covers a large parameter space and allows for parallel screening. This reduces experiment to experiment inconsistency and provides a large database of information for employing informatics to identify hot spots \[231\]. This review provides a discussion of the various combinatorial methods that have been developed to synthesize and characterize biomaterials, the cell-based high throughput screening methods to characterize their interactions with cells, and combinatorial drug delivery \[232-234\]. The article concludes with the authors’ opinion on the challenges and opportunities provided by the combinatorial approach in the discovery and development of rapid and optimal drug delivery systems based on polymeric biomaterials.

### 2.4.2 Combinatorial Biomaterial Library Fabrication and Characterization

The combinatorial approach has been widely applied to synthesize libraries of polymeric
biomaterials as summarized in Table 2.4.1. Often, polymerization reaction conditions and
duration cannot be varied, so making the process combinatorial involves synthesizing
multiple polymers simultaneously while varying properties such as composition or molecular
weight. Many polymeric biomaterials syntheses have been reported using a parallel approach
[231]. Polymer libraries of poly(dimethylsiloxane) (PDMS) and poly(ε-caprolactone—block-
dimethylsiloxane-block-ε-caprolactone) triblock copolymers were synthesized by Ekin and
Webster with the use of combinatorial experimentation in which novel PDMS oligomers
were synthesized by reacting ethylene carbonate with 3-aminopropyl terminated PDMS
oligomers [235]. A secondary reaction of these oligomers with ε-caprolactone resulted in the
triblock copolymers. Polyanhydrides based on 1-6 bis(p-carboxyphenoxy)hexane (CPH) and
sebacic acid (SA) have been synthesized using a rapid microwave polymerization technique
described by Vogel et al [236]. A high throughput deposition and polymerization method of
fabricating discrete polyanhydride libraries based on rapid prototyping and thiolene
photopolymerization has been described by Vogel et al. [221]. In this method, thiolene-based
multi-wells are used to robotically deposit anhydride monomers and the monomer library is
then subjected to melt polycondensation under vacuum to result in a library of
polyanhydrides. Langer and co-workers have developed a method for synthesizing a library
of poly(β-aminoesters) via the addition of bifunctional amines to bisacrylamides [237, 238].
Brocchini and co-workers have synthesized polyester libraries derived from serinol,
producing a 16-member library by polymerizing four N-substituted serinol-diol monomers
with four commercially available diacids [239].

The fabrication of both continuous and discrete polymer libraries has been reported by
Narasimhan with polyanhydrides \cite{36, 139, 146, 147, 153, 154, 221, 240} and by Meredith with polystyrene (PS) and poly(vinyl methyl ether) (PVME) \cite{241}. Figure 2.4.2 shows an example of a robotically deposited discrete polyanhydride library with a composition gradient. Such libraries have been used to study polymer blend phase behavior annealed over a temperature gradient stage by optical microscopy \cite{221, 242}. The polyanhydride phase behavior from the combinatorial experiments exhibited upper critical solution temperature behavior \cite{221}, which is consistent with the conventionally obtained phase diagram by Kipper et al \cite{243}. Meredith and co-workers reported a PS/PVME phase diagram with lower critical solution temperature behavior \cite{241}. The polymer phase behavior plays a significant role in drug release as it controls the drug distribution within the system, which influences the rate of drug release \cite{37, 38}. Such rapid analysis of phase behavior will provide a basis for the rational optimization and design of porous three-dimensional polymer scaffolds for tissue engineering and drug delivery.

Other polymer properties have been measured at high throughput by Kohn and co-workers \cite{244}. Polyarylate libraries were characterized by GPC, differential scanning calorimetry (DSC) and contact angle measurements. The glass transition temperature ($T_g$) determined from DSC was found to decrease in correlation with the presence of oxygen moieties in the polymer backbone, while the water contact angle was found to decrease with increasing polymer chain length \cite{244}. Knowledge of such properties and their trends within the polymer library are important for the fabrication of drug delivery devices such as tablets and micro- and nano-spheres.
High throughput characterization techniques such as FTIR, GPC, and optical microscopy allow for the rapid screening of polymer libraries. GPC and FTIR employ automated sampling which enables parallel and rapid validation of molecular weight and polydispersity (GPC), and chemical composition, drug interactions, and molecular weight (all with FTIR). FTIR microscopy has been used as a high throughput technique to characterize the linear variation of composition in polymer libraries as shown in Figure 2.4.3 [154, 240]. Optical microscopy has been utilized to observe phase behavior when such linearly varying libraries are annealed along a temperature gradient in a direction that is orthogonal to the composition gradient [242]. In addition to the methods described above, there are a number of studies in the literature on combinatorial synthesis and screening of biomaterials [245-250], including 3D gradients of polymer tissue scaffolds [251-255]. These accelerated methods are amenable to numerous other applications involving property determination and characterization of polymer systems.

Understanding the relationship between important biomaterial properties (e.g., chemistry, T_g, T_m, phase behavior, and drug/polymer interactions) and drug release is paramount for the design and optimization of drug delivery vehicles. For example, Kipper et al. have shown that the phase behavior of biodegradable polymer blends, specifically the length scale of the microphase separation of polyanhydride copolymers, significantly affects the thermodynamic partitioning of drugs in these systems, thus affecting their release kinetics [256]. When the solubility of the drug or protein within a copolymer phase is exceeded, the drug or protein is forced to disperse into less favorable regions, thus resulting in a rapid initial release or burst of the protein [256]. This is also important for the design of multi-drug releasing polymer
systems and degradable polymer systems for protein stabilization \cite{39,142}. Properties such as the T\textsubscript{g} and T\textsubscript{m} are important because they dictate processibility of the materials. For example, the low T\textsubscript{g} of certain polyanhydride copolymers controls the conditions under which microspheres of these copolymers are fabricated \cite{136,156}. Likewise the crystallinity of the biomaterial also affects the processibility and degradation kinetics and hence the release rates of drugs from these materials \cite{256}.

### 2.4.3 High Throughput Cell-Based Screening

Combinatorial approaches have led to the development of biological assays and high throughput plate readers allowing for rapid and parallel data acquisition and quantification \cite{228}. When these high throughput methods are integrated with multi-dimensional polymer libraries, interactions of the polymeric carriers with drugs or cells can be assessed at high throughput. These include, but are not limited to, polymer chemistry effects on cell proliferation, adhesion, transfection, differentiation, cytotoxicity, and immune modulation. Understanding and optimizing these processes can enable the development and optimization of protein therapies, tissue engineering scaffolds, and vaccine delivery systems for localized treatments that target specific organs or tissues.

Cytotoxicity is an important factor to consider when designing polymeric biomaterials for \textit{in vivo} applications. The concentration and composition of polymeric biomaterials often regulate cellular functionality and viability. High throughput methods have been developed to ascertain the cytotoxic effect of biocompatible polyanhydrides based on CPH, SA, and 1,8-bis(\(p\)-carboxyphenoxy)3,6-dioxaocante (CPTEG) on different cells \cite{139,153,154}. The initial
libraries studied were based on a gradient of 25 linearly varying polymer concentrations, which were fabricated using the multi-well system described before \cite{154}. These libraries were screened at high throughput using an \textit{in vitro} cytotoxicity assay (i.e., the MTT assay) and demonstrated that concentrations of the CPH:SA copolymer system less than 14 mg/mL and of the CPTEG:CPH system less than 16 mg/mL resulted in total viability in Sp2/O myeloma cells when compared to the control groups. The average \textit{in vivo} concentration for human applications is 0.5 mg/mL \cite{46}, thus demonstrating the viability of these copolymers as drug carriers. Further, 25-member polyanhydride libraries with a compositional gradient were synthesized and screened for cytotoxicity. It was observed that there were no resulting compositions of either copolymer systems found to be toxic to any of the cell lines \cite{154}.

Other role-specific biomaterials include transfection vectors, a group of specialized polymers studied by Langer and co-workers that collapse on DNA and transfect it into the cell \cite{237,238}. This is a key process for controlling many intracellular functions by targeting the cell nucleus and has potential for use in cancer therapies. The commercially available transfection vector, polyethylene amine, has been found to be problematic due to its inability to target and kill specific tumor cells. It was toxic to healthy cells resulting in tissue damage and wounding. Langer and colleagues used high throughput techniques based on a library of poly(β-aminoesters) to address the cytotoxicity issue, which revealed a few specific cationic polymers that were not cytotoxic. These polymers were further studied \textit{in vivo} and found to eliminate the toxic effect on healthy tissue while reducing the tumor size by 40\% \cite{257}. Combinatorial cell-based transfection screening has also been used for the design of polymer libraries by synthesizing diacrylates with primary or secondary amines. The findings of the
cell uptake studies emphasize the importance of the size of the polymer/DNA transfection complex and the polymer to DNA (N/P) ratio. Hydrophobicity was also shown to play a significant role in the transfection process. The histidine components of the polymer allowed for up to 4 to 8 times better transfection efficiencies than commercially available products. In further studies, this library synthesis process was automated, permitting the study of 1000 reactions per day, creating a 2350 multi-component library \cite{257,258}. This library was used to show that transfection was controlled by polymer molecular mass and end groups \cite{257-259}.

Combinatorial methods provide an ideal platform to investigate cell-biomaterial surface interactions. This enables a rapid means for measuring and screening cell behavior including proliferation, migration, and cell attachment. Kohn and co-workers used a library of biodegradable carbonates to study the relationship between cell proliferation and polymer properties including hydrophobicity and molecular weight \cite{244,260}. It was demonstrated that the cells grew as a function of oxygen incorporation in the polymer backbone \cite{244}. Further studies exploring the relationship between the hydrophobicity of polyarylates and cell proliferation found a significant trend relating the cell growth to decreasing hydrophobicity of the non-oxygen containing polymers \cite{260}. Similar results were reported correlating cell proliferation with the oxygen content of the polymer \cite{231}.

Combinatorial methods can also be used to explore alternate polymer systems for improved cell attachment. Brocchini et al. synthesized a library of polyesters in the presence of N-substituted serinols which added another dimension to the library by allowing for the addition of functional groups to the side chains of the polymers \cite{239}. It was shown that hexyl
side chains on the polyesters inhibited cell attachment. Similar studies with poly(ethylene glycol) (PEG) libraries were carried out by Langer and co-workers in which a polymer blend array of 3456 spots was screened for cell attachment and growth \[261\]. The screening results indicate that very little cell adhesion occurred until the blend approached 30 wt% poly(l-lactide-co-glycolide) (PLGA). This is attributed to phase separation in the copolymers, preventing cellular adhesion in the PEG-rich sections, which have minimal attachment area \[239\].

Cell adhesion and proliferation are important aspects in the design of tissue engineered scaffolds. Meredith et al. have studied the relationships between cell behavior, adhesion and proliferation, and polymer library temperature and composition \[127\]. They created a continuous combinatorial library varying between different compositions of PLGA and poly(\(\varepsilon\)-caprolactone) (PCL) which was annealed over a temperature gradient to vary the microstructure and roughness. There were significant correlations between cell adhesion and the average surface roughness of the amorphous PLGA and the crystalline PCL under normal cell conditions at 37°C. The authors reported that the cell adhesion was at its highest at the high temperature annealed polymer regions and for the mid to high PCL regions on the polymer library. They also investigated the effect of protein adsorption on the polymer library \[127\]. Increasing hydrophobicity of the carbon-rich regions of the library was found to increase protein adsorption. Although protein adsorption was found to be independent of cell adhesion early on in the experiment, the prolonged studies demonstrated that the highest amount of protein adsorption correlated to the regions of high cell attachment (i.e., mid to high PCL regions) \[127\].
Combinatorial techniques have been developed to identify the effect of stem cell differentiation on libraries containing di and triacrylates mixed with a photoinitiator on a poly(hydroxyethyl methacrylate)-based coating \[^{259}\]. The authors report that nearly all compositions provided the necessary framework for cell adhesion and growth, but when retinoic acid was added to the cell growth medium, the monomer composition appeared to differentially regulate these cellular functions. This demonstrates the ability to control cellular behavior thus leading to better optimization of tissue scaffolds in an efficient high throughput approach.

Polymer chemistry is hypothesized to play a significant role in immune modulation, which is a key factor for the design and optimization of polymers as adjuvants. A high throughput technique based on cytokine analysis has been developed to study the effect of polymer chemistry on the immune response \[^{154}\]. Using a CPH:SA polyanhydride library and a macrophage cell line, a correlation was identified between adjuvant hydrophobicity (i.e., increasing CPH content) and TNF-\(\alpha\) production (Figure 2.4.4) and between adjuvant hydrophilicity (i.e., increasing SA content) and IL-6 production. The production of TNF-\(\alpha\) typically leads to inflammation and endothelial activation whereas IL-6 can induce fever, T and B cell growth and differentiation, and production of acute phase proteins \[^{179}\]. Based upon the cytokines released from antigen presenting cells such as macrophages or dendritic cells the adaptive immune response can be modulated between a T helper 1 (TH1) cell-mediated response and a T helper 2 (TH2) humoral response. The TH1 pathway activates macrophages and cytotoxic T cells which are directed to eliminate the invading foreign body and is effective at neutralizing intra-cellular pathogens; the TH2 pathway activates B cells...
leading to antibody production and long term cellular memory and is effective at dealing with extra-cellular pathogens. Tuning the immune response is important for the optimal design of vaccine delivery systems and their ability to target and activate the necessary intracellular processes for successful prophylactic treatment. With the numerous applications for polymers as adjuvants in vaccine delivery devices, knowledge of how the polymer adjuvant modulates immune response pathways (i.e., cellular vs. humoral) is vital for the rational design and optimization of these devices.

2.4.4 Combinatorial Drug Release

Vogel et al. designed a high throughput method to study drug release from a linearly varying polyanhydride library based on CPH and SA [40]. The previously discussed rapid prototyping technique for multi-compositional library synthesis and rapid characterization of polymer properties was modified to study drug release. A non-reactive ultraviolet dye, ethidium bromide bisacrylamide, was encapsulated in the polymer libraries upon combinatorial polymer synthesis and released from the libraries as they were incubated in buffer. The results produced clear trends between the rate of dye released and the degradation characteristics of the polymer carriers. The higher the percent of the faster degrading component, SA, the more rapid was the dye release (Figure 2.4.5) [40]. These results were consistent with conventionally obtained dye release profiles from this polymer system [37].

2.4.5 Expert Opinion

The above examples clearly demonstrate that combinatorial methods have been successfully applied to screen biomaterials for applications in drug delivery and tissue engineering.
However, many challenges and obstacles remain in the path to further the design of biomaterials for drug delivery and tissue engineering. The literature in this area mainly focuses on discrete and continuous polymer libraries, which are typically films and their correlations to cellular behavior which is applicable to areas such as tissue engineering. However, the majority of drug delivery systems employ injectable devices. This emphasizes the need for the development of new combinatorial techniques to fabricate micro- and nano-particles for drug delivery. The development of such a technique would significantly accelerate discovery by fabricating particles of multiple chemistries simultaneously as well as decrease batch-to-batch variability commonly associated within the conventionally synthesized polymer particles, which employ techniques such as double emulsion and spray drying [144, 156, 262, 263].

The challenges in library fabrication lend to the difficulties encountered with screening of intra-cellular processes such as trafficking and transfection. The intracellular processes are often difficult to track due to lack of space and resolution in a typical combinatorial library, which only allows for a restricted population of cells. The behavior of cells can be affected by numerous external variables that can hinder the actual objective of the experiment itself. Screening of these processes can be expensive and difficult to apply to combinatorial libraries, hampering their ability to provide a large library of data. Thus, new methods are required in which large populations of cells can be screened at high throughput for intra-cellular information and processes. Additionally, there are limitations on developing combinatorial methods for drug release since these methods rely upon following a tag (e.g., fluorescent) that needs to be incorporated into the drug of interest.
The implementation of high throughput techniques will lead to the generation of large libraries of data. Due to the major changes in information generation and composition, there is a need for highly developed statistical analysis techniques to address the multi-dimensional error analysis and refined informatics tools to process the enormous libraries of data. These statistical and informatics methods will become an invaluable resource to efficiently mine and analyze large data sets, better focusing the library parameter space on key areas of interest. The development of such methods will require close collaborations between researchers in the areas of computer science, biomaterials, and bioinformatics.

In combining the multiple aspects of combinatorial discovery for biomaterials design, it is clear that there is a lack of formalized educational programs that meld analytical methods, molecular biology, biomaterials, chemistry, computer programming etc. The multi-faceted disciplines related to this field present many hurdles for newcomers to this area, requiring a significant amount of time and effort to master the cross-cutting ideas in combinatorial science. So there is an urgent need to develop novel educational programs that provide both a strong scientific grounding as well as a balanced exposure to the various techniques in this area.

2.4.6 Conclusions

Combinatorial and high throughput approaches to design and optimize biomaterials for drug delivery will become increasingly important as molecular structures become more complex, more variables are thrust into system design, and processes become more expensive [231]. In this brief review, we have discussed initial efforts which demonstrate the viability and
effectiveness of this approach and its ability to accelerate discovery, provide new insights, and reduce time and cost. We have discussed several applications covering the vast array of possibilities for this approach to the rapid discovery and design of biomaterials. High throughput techniques have been employed for processes ranging from biomaterials synthesis and characterization to the downstream development of parallel methods for drug release and cell screening. With the large array of parameters affecting many design problems in drug delivery and tissue engineering, this technique can be an invaluable resource for rapid design and optimization of biomaterials. However, this method is also fraught with numerous obstacles and eliminating these will have to be a primary goal in the next decade if combinatorial methods are to be used routinely for biomaterials discovery and design.

2.5 References

65. Shih C, Higuchi T, Himmelstein KJ. Drug delivery from catalysed erodible polymeric
55


115. Woodward SC, Brewer PS, Moatamed F, Schindler A, Pitt CG. The intracellular
132. Lacasse FX, Filion MC, Phillips NC, Escher E, McMullen JN, Hildgen P. Influence of surface properties at biodegradable microsphere surfaces: effects on plasma protein


204. Estevan M, Irache JM, Grillo MJ, Blasco JM, Gamazo C. Encapsulation of antigenic


2.6 List of Figures

Figure 2.2.1: Polyester chemical structures: a) PGA, b) PLA, c) PLGA, and d) PCL

Figure 2.2.2: a) Bulk and b) surface erosion mechanisms.
Figure 2.2.3: Acid-catalyzed polymer chemical structures: a) polyacetal, b) polyketal, and c) polyorthoester.

Figure 2.2.4: Polyanhydride chemical structures: a) poly(CPH), b) poly(SA), and c) poly(CPTEG).
Figure 2.2.5: Polyether chemical structure: a) poly(ethylene glycol) and b) poly(propylene glycol).

Figure 2.3.1: Induction of cellular vs. humoral immune response upon antigen presentation by a DC.
Figure 2.4.1: The combinatorial methodology.

Figure 2.4.2: A discrete polyanhydride library polyanhydrides, increasing in SA composition from right to left and front to back (CPH is designated by blue and by SA yellow)\textsuperscript{[154]}. 
Figure 2.4.3: Mole percent of CPH varying along a discrete composition gradient library as determined in high throughput by FTIR microscopy \[154\].

Figure 2.4.4: Correlation between mole percent of CPH and TNF-\(\alpha\) production \[154\].
Figure 2.4.5: Fraction of dye released from a compositionally varying polyanhydride library of CPH:SA copolymers. The SA content increases incrementally from the bottom (50%) to top curve (100%) \cite{40}.
## 2.7 List of Tables

Table 2.4.1: Polymeric biomaterials and the various high throughput screening methods that have been developed to study these materials.

<table>
<thead>
<tr>
<th>Polymeric biomaterial</th>
<th>High throughput screening</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polyanhydrides</td>
<td>Cytotoxicity</td>
<td>[40, 154, 221, 236]</td>
</tr>
<tr>
<td></td>
<td>Blend phase behavior</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Immune activation</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Drug delivery</td>
<td></td>
</tr>
<tr>
<td>PEG and polyesters</td>
<td>Cell adhesion and growth</td>
<td>[239, 261]</td>
</tr>
<tr>
<td>Aminoesters</td>
<td>Transfection</td>
<td>[237, 260]</td>
</tr>
<tr>
<td>Poly(ethylene glycol)-400 diacrylate (PEG4000DA) and acryloyl-poly(ethylene glycol)-RGDS (Acr-PEG-RGDS)</td>
<td>Cell adhesion</td>
<td>[245]</td>
</tr>
<tr>
<td>Polyarylates</td>
<td>Cell proliferation</td>
<td>[244, 257, 260]</td>
</tr>
<tr>
<td>PLGA and poly(β-caprolactone) (PCL)</td>
<td>Blend phase behavior</td>
<td>[127, 241, 242, 246]</td>
</tr>
<tr>
<td>PVME and PS</td>
<td>Cytotoxicity</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cell adhesion, growth, aggregation, and protein production</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cell alkaline phosphatase expression</td>
<td></td>
</tr>
<tr>
<td>Plasma polymerized hexane (ppHex) and plasma polymerized allyl amine (ppAAm)</td>
<td>Cell adhesion and growth</td>
<td>[247, 255]</td>
</tr>
<tr>
<td></td>
<td>3D polymer scaffold synthesis</td>
<td></td>
</tr>
<tr>
<td>Poly(dimethylsiloxane) (PDMS) and poly(ε-caprolactone—block-dimethylsiloxane-block-ε-caprolactone)</td>
<td>Synthesis</td>
<td>[229]</td>
</tr>
<tr>
<td></td>
<td>Structural characterization (NMR, DSC, FTIR, and GPC)</td>
<td></td>
</tr>
<tr>
<td>Poly(dichlorodimethylsilane)</td>
<td>Cell adhesion</td>
<td>[248]</td>
</tr>
<tr>
<td>Poly(2-hydroxyethyl methacrylate)</td>
<td>Cell adhesion</td>
<td>[249]</td>
</tr>
<tr>
<td>Poly(L-lactic acid) (PLLA)</td>
<td>Cell proliferation</td>
<td>[250, 251, 253, 264]</td>
</tr>
<tr>
<td>poly(D,L-lactic acid) (PDLLA)</td>
<td>3D polymer scaffold synthesis</td>
<td></td>
</tr>
<tr>
<td>Poly(lactic-co-glycolic acid) (PLGA)</td>
<td>Bone growth</td>
<td></td>
</tr>
<tr>
<td>Polyglycolide and poly(hydroxyacetic acid)</td>
<td>3D polymer scaffold synthesis</td>
<td>[252]</td>
</tr>
<tr>
<td>Collagen-glycosaminoglycan (CG)</td>
<td>3D polymer scaffold synthesis</td>
<td>[254]</td>
</tr>
</tbody>
</table>
Chapter 3: Research Objectives

The overall goal of this research is to develop and utilize high throughput approaches to expedite the design and optimization of degradable polymeric biomaterials for applications in drug and vaccine delivery. The combinatorial approach allows for multiple variables to be investigated in parallel to better understand protein/polymer, cell/polymer, and host/polymer interactions. Informatics analysis were applied to identify underlying trends associated with polymer chemistry and to identify material properties influencing these interactions.

To carry out this research a host of multi-sample substrates (including wells, vials, and tubes) were fabricated and/or modified and used in conjunction with a robotic apparatus controlled by a third party software to enable the rapid synthesis of polymer libraries which were then translated to the fabrication of film and nanoparticle libraries. Upon construction of these libraries, they were characterized by $^1$H NMR, GPC, and FTIR and investigated for protein/polymer interactions, including protein release kinetics and protein stability. The release kinetics were rapidly investigated as a function of polymer chemistry, pH, and device geometry by utilizing a novel, fluorescent based high throughput assay. Protein stability was assessed upon fabrication, storage (time and temperature dependent), and release for all chemistries investigated. These methodologies were translated to study cell/polymer interactions. Several polymer chemistries in both film and nanoparticle geometries were examined for their effect on cellular toxicity (using both cell lines and primary cells), adhesion, differentiation, and immune activation. Additionally, informatics analysis enabled the identification of immune activation ‘hot spots’ which indicated that poly(SA) and 50:50
CPTEG:CPH nanoparticles best enhanced immune activation and that aliphatic carbons, hydroxyl end groups, and hydrophobicity were the properties responsible for this activation. These high throughput methodologies were further employed to investigate polymer/host interactions to study the dependence of chemistry and administration route on nanoparticle biodistribution, mucoadhesion, and depot effects in an \textit{in vivo} mouse model. The combinatorial approach has helped identify the effect of polymer chemistry on protein release kinetics, protein stability, cellular toxicity, cellular adhesion, cellular differentiation, cellular immune activation, and \textit{in vivo} biodistribution and mucoadhesion. These approaches will aid in more efficient and effective design of polyanhydrides for use as drug delivery devices and vaccine adjuvants.

The specific goals (SGs) of this research are as follows:

SG1: Combinatorial polymer synthesis and film and nanoparticle fabrication

SG2: Combinatorial investigation of protein/polymer interactions

SG3: \textit{In vitro} combinatorial examination of cell/polymer interactions

SG4: \textit{In vivo} combinatorial examination of host/polymer interactions
Chapter 4: A Novel, High Throughput Method to Study In vitro Protein Release from Polymer Nanospheres


L. K. Petersen, C. K. Sackett, and B. Narasimhan*

Keywords: Combinatorial, high throughput, polyanhydrides, protein delivery, nanospheres

Department of Chemical and Biological Engineering, Iowa State University, 2035 Sweeney Hall, Ames, IA 50011-2230
*To whom all correspondence should be addressed
4.1 Abstract

Controlled delivery of therapeutic protein drugs using biodegradable polymer carriers is a desired characteristic that enables effective, application-specific therapy and treatment. Previous studies have focused on protein delivery from polymers using conventional “one-sample-at-a-time” techniques, which are time-consuming and costly. In addition, many therapeutic proteins are in limited supply and are expensive, so it is desirable to reduce sample size for design and development of delivery devices. We have developed a rapid, high throughput technique based on a highly sensitive fluorescence-based assay to detect and quantify protein released from polyanhydrides while utilizing relatively small amounts of protein (~40 μg). These studies focused on the release of a model protein, Texas Red conjugated bovine serum albumin, from polyanhydride copolymers based on sebacic acid (SA) and 1,6-bis(p-carboxyphenoxy)hexane (CPH). The protein release profiles were assessed simultaneously to investigate the effect of polymer device geometry (nanospheres vs. films), polymer chemistry, and pH of the release medium. The results indicated that the nanosphere geometry, SA-rich chemistries, and neutral pH release medium led to a more rapid release of the protein compared to the film geometry, CPH-rich chemistries, and acidic pH release medium, respectively. This high throughput fluorescence-based method can be readily extended to study release kinetics for other proteins and polymer systems.

4.2 Introduction

The delivery of protein-based drugs (such as vaccine antigens, therapeutic proteins, and growth factors) using biodegradable polymeric devices has become an extensively explored area of innovation and research over the past decade [1-5]. Often, the dual tasks of providing
an environment capable of maintaining the functionality of the protein drug and releasing it in a sustained manner are challenging. In addition, the assays available for protein release kinetics are limited in that they lack sensitivity and are resource consuming. Many current protein release systems are carried out in a conventional ‘one-sample-at-a-time’ format and require several milligrams of protein for adequate detection and quantification.

Several degradable polymeric biomaterials have shown much potential as protein carriers including polyesters, polyorthoesters, and polyanhydrides [3, 6-13]. Polyester-based systems undergo bulk erosion allowing significant water ingress and increased microenvironment acidity, which may affect both the stability of the protein and its release kinetics. Polyanhydrides, which are generally more hydrophobic, undergo surface erosion through hydrolytic degradation, which makes the erosion more controllable, allowing for tailored degradation ranging from days to months [14-16]. In all these systems, it is evident that the chemistry of the polymeric carrier plays a significant role in maintaining the function of the protein and in governing its release kinetics. This work is focused on polyanhydrides, which are biocompatible materials that have been studied extensively for use in drug and vaccine delivery [1, 3, 14, 17-28]. The polymers of interest are based on 1,6-bis(\(p\)-carboxyphenoxy)hexane (CPH) and sebacic acid (SA). In addition to their biocompatibility, the degradation of these materials is base-catalyzed [6, 29] and can be tuned allowing for drug delivery ranging from weeks (SA-rich chemistries) to months (CPH-rich chemistries) [2, 14, 15].

In this design of delivery vehicles, one can envision a large parameter space to investigate, based on the chemistry of the polymer, the type of protein drug to be encapsulated, the
desired release rate of the protein, the in vivo release environment of the protein, the required geometry of the protein encapsulation device (nanospheres for drug/vaccine delivery or films for drug-eluting implants), and the end-use application. High throughput approaches, employing combinatorial library synthesis, can be valuable to more rapidly develop and/or screen new biomaterials for controlled delivery of proteins. There are several examples in the literature of high throughput screening of biomaterials to study their interactions with proteins and cells [4, 17, 26, 30-40]. Vogel et al. developed the first high throughput approach to study combinatorial drug release kinetics from CPH:SA polyanhydride films [27]. In this study, the release profile of ethidium bromide bisacrylamide from polyanhydride films was investigated and it was shown that the more hydrophobic (CPH-rich) chemistries released the dye the slowest. The conditions used to study the release of dyes, which can withstand harsh processing (e.g., high temperature, solvent exposure, low vacuum, etc.), are not ideal for protein-based drugs. In addition, some of the colorimetric methods available to quantify dyes are not readily applicable to protein analysis.

In this work, the development of a novel and multiplexed technique to concurrently study the release kinetics of proteins from polyanhydrides with multiple varying parameters (device geometry, polymer chemistry, and pH of release medium) by employing a highly sensitive fluorescence-based assay is described. To the best of the authors’ knowledge, no combinatorial methods are currently available to evaluate the delivery of protein-based drugs from polymers. The high throughput technique described herein allowed for the rapid detection and quantification of a model fluorescent protein (Texas Red bovine serum albumin (TRBSA)) released from five different chemistries of two different CPH:SA polymer device
geometries (nanospheres and films) in media of three different pHS. This highly sensitive, fluorescence-based approach reduced the amount of protein needed (total of ~40 μg) because very small amounts of the fluorochrome-conjugated protein are necessary to get sufficient excitation, emission, and quantification as compared to alternate assays, which require significantly larger amounts of protein to obtain similar data. In addition, this novel method eliminated the need for repeated sampling, which can often introduce experimental error, and allowed for combinatorial protein release and simultaneous, rapid protein detection and quantification. High throughput methods are an invaluable resource for studying release of protein-based drugs, which are often made recombinantly and are therefore expensive and available in limited supplies. The use of multiplexed methods to study protein release kinetics will help to more readily advance the rational design and optimization of protein-based drug and vaccine delivery systems.

4.3 Materials and Methods

Materials

The chemicals utilized in monomer synthesis, 1-methyl-2-pyrrolidinone, 4-\(p\)-hydroxybenzoic acid, and 1,6-dibromohexane were purchased from Sigma Aldrich (St. Louis, MO); and sulfuric acid, was obtained from Fisher Scientific (Fairlawn, NJ). The chemicals needed for the polymerization, nanosphere fabrication, and buffer preparation include acetic anhydride, chloroform, methylene chloride, petroleum ether, pentane, monobasic potassium phosphate, dibasic potassium phosphate, sodium acetate trihydrate and glacial acetic acid, all of which were purchased from Fisher Scientific. BSA was purchased from Sigma Aldrich. The micro-bicinchoninic acid (BCA) protein assay kit was obtained from Pierce Biotechnology Inc.
Texas Red® conjugated BSA was obtained from Invitrogen (Carlsbad, CA). Deep-welled 96-well polypropylene (0.5-2.0ml) plates and sealing mats were purchased from Corning (Corning, NY).

**Polymer film library synthesis & characterization**

The CPH monomer was synthesized as described previously [28]. The SA monomer was purchased from Sigma Aldrich. CPH:SA copolymer libraries were synthesized from the corresponding monomers via a melt polycondensation reaction in multi-well substrates utilizing a robotic deposition apparatus, as reported previously [4, 25]. Copolymer chemistry and molecular weight were determined for the polymer film libraries by proton nuclear magnetic resonance (\(^1\)H NMR) spectroscopy using a Varian VXR 300 MHz spectrometer (Varian Inc., Palo Alto, CA). Each sample was dissolved in deuterated chloroform and the chemical shifts were calibrated with respect to the chloroform peak (\(\delta = 7.26\) ppm). Gel Permeation Chromatography (GPC) was also used to measure the molecular weight on the polymer film libraries. Samples were dissolved in HPLC-grade chloroform and separated on a Waters GPC chromatograph (Milford, MA) containing PL Gel columns (Polymer Laboratories, Amherst, MA). Elution times were compared to monodisperse polystyrene standards (Fluka, Milwaukee, WI). The surface chemistry of the combinatorially synthesized CPH:SA polymer film libraries (no protein) was evaluated at high throughput using Fourier transform infrared spectroscopy with a Nicolet 6700 FTIR spectrometer (Thermo Scientific) as described previously [4, 26].
Fabrication of protein encapsulated film and nanosphere libraries and nanosphere library characterization

An automated polymer synthesis/nanosphere fabrication device was designed for the initial deposition of the monomer library into a multi-vial substrate. Following synthesis, the combinatorial nanosphere library was fabricated from the polymer film library as described before [4, 26]. Additional steps were incorporated into the process for protein encapsulation. The protein (TRBSA) was initially homogenized (Tissue-Tearor™, Biospec Products, Bartlesville, OK) in chloroform for 60 s at 10,000 rpm resulting in a final concentration of 1 mg/mL. The protein/chloroform solution was robotically deposited via syringe pumps into each vial of the multi-vial film library, thus dissolving the polymer films with a resulting polymer concentration of approximately 25 mg/mL. Each solution was homogenized for 60 s at 10,000 rpm to uniformly disperse the polymer and the protein in the chloroform. They were either dried to create the protein encapsulated film library (in a deep welled, clear, polypropylene 96-well plate) or precipitated into petroleum ether and dried to create a protein-encapsulated nanosphere library. Smaller quantities of the protein-encapsulated nanospheres were weighed out and transferred to a deep-welled, clear, polypropylene 96-well plate for evaluating the release kinetics. This nano-precipitation process is a modification of a previously described method [4, 40]. Scanning electron microscopy (SEM) was performed using a JEOL JSM-840A SEM (JEOL USA Inc., Peabody, MA) to study the external morphology of the protein-loaded nanospheres by coating their surface with 200 Å of gold.

TRBSA release kinetics and protein quantification

Following fabrication of the protein-loaded film and nanosphere libraries, 1 mL of the
appropriate buffer (phosphate buffer pH 7.3, phosphate buffer pH 6.0, or acetate buffer pH 4.3) was added to each well. The well plates were sealed to prevent evaporation and incubated in a horizontal shaker at 37°C and 100 rpm for the duration of the experiment. TRBSA detection and quantification was performed incrementally throughout the study, which was terminated after one month. The protein release data is presented as a cumulative fraction of protein released, in which the amount of protein released is normalized by the total amount of protein encapsulated into the nanospheres or films. The TRBSA release was quantified using two methods: a micro-BCA assay and a high throughput fluorescence-based assay.

*Micro-bicinchoninic acid (BCA) assay*

Three hundred μL samples were withdrawn at several time points from each release well and quantified with the micro-BCA assay. Samples were run in triplicate, as described by the manufacturer (Pierce). Fresh buffer was added to the sample well to maintain constant sink conditions.

*High throughput fluorescence-based assay*

In this automated technique (i.e., no sampling), each clear, deep-welled, polypropylene 96-well plate was modified by joining each neighboring pair of wells between neighboring columns: 1 and 2, 3 and 4, 5 and 6, 7 and 8, 9 and 10, and 11 and 12 (i.e., wells A1 and A2, B1 and B2, A3 and A4, etc were joined together) (Figure 4.1). The first wells were home to the fluorescent protein-loaded film or nanospheres and the second, adjoining wells were empty. 500 μL of buffer was added to all the wells and the plate was centrifuged (100 rcf for
10 min) to localize the nanospheres to the bottom of the wells. The wells were slowly filled to the top with buffer and due to the modified well geometry, any released protein was uniformly dispersed between the two wells while keeping the films and nanospheres isolated to the first well. Each subsequent time prior to protein quantification, the polymer libraries were centrifuged (100 rcf for 10 min) to localize the nanospheres and film particulates to the bottom of the well. High throughput protein detection was performed with a Typhoon 9410 imaging system (GE Healthcare, Piscataway, NJ). The 96-well plate was placed on a flatbed scanner and a laser was directed at the bottom of the plate to excite the fluorochrome-conjugated protein. The light emission was collected through a series of filters, which was immediately output to a computer quantification program (Image Quant TL, GE Healthcare, Piscataway, NJ). A row of protein standard concentrations was included in the 96-well plate to account for the effect of subsequent scanning or light bleaching on the fluorescent protein.

4.4 Results and Discussion

In this work a novel, fluorescent, high throughput technique for studying protein release has been designed, enabling rapid data quantification in a multiplexed format, eliminating error associated with repeated sampling, and minimizing sample size requirements. This approach allowed for the simultaneous evaluation of several key parameters involved in protein release including polymer chemistry, device geometry, and pH of release medium, which were carried out using two modified 96-well release plates.

Characterization

The CPH:SA film libraries were characterized with high throughput FTIR to determine
surface chemistry and the overall accuracy of the deposition process. The results demonstrated excellent agreement between the intended molar compositions deposited and the actual molar compositions deposited into the multi-well substrate using the automated deposition apparatus (data not shown). These findings are consistent with previous work \cite{4, 26} and confirm the accuracy of the depositions apparatus. In addition, molecular weight and copolymer composition were determined with $^1$H NMR and GPC, Table 4.1, and the results were in agreement with conventionally synthesized CPH:SA copolymers \cite{2, 15, 41}. Following characterization of the polymer film libraries, the TRBSA loaded nanosphere libraries were characterized with SEM to determine shape and size. They were found to be very similar to previously published results with polyanhydride nanospheres \cite{4, 40} with an average size of $\sim$300 nm. Representative images from two selected chemistries are shown in Figure 4.2.

**Combinatorial protein release from polyanhydrides nanospheres**

Knowledge of the protein release kinetics from these carrier systems is very important for the rational design and optimization of devices for *in vivo* applications. To investigate this, the effect of polyanhydride nanosphere chemistry on TRBSA release was evaluated at high throughput using the highly sensitive fluorescence-based assay. The combinatorially measured protein release kinetics was validated with a commonly used micro-BCA assay. The multiplexed method enabled five different nanosphere chemistries ranging from 100 mole % SA to 100 mole % CPH to be simultaneously evaluated in replicates of four. The polymer chemistry played an integral role in controlling the release of TRBSA from the CPH:SA nanosphere library with the most hydrophobic chemistry (i.e., poly(CPH)) releasing the protein the slowest and the least hydrophobic chemistry (i.e., poly(SA)) releasing the
protein the most rapidly (Figure 4.3). It is well known that polymer hydrophobicity directly influences polymer erosion and protein release kinetics \cite{14, 19, 41}. This high throughput method allowed for other observations to be made simultaneously, which were consistent with previous work, including complete release of TRBSA from poly(SA) nanospheres \cite{19} and initial protein bursts of 5-20% (nanosphere chemistry dependent) \cite{15}. As discussed previously, the TRBSA release profile obtained with the fluorescence-based assay was validated with the micro-BCA assay, which yielded consistent protein release profiles for all the CPH:SA nanosphere chemistries tested (Figure 4.4). This provides evidence supporting the accuracy and reliability of the high throughput fluorescence-based assay. In fact, in most cases the micro-BCA assay demonstrated more variability in the release curve data than the fluorescence-based assay, which is likely a result of error introduced with repeated sampling and the use of a less sensitive protein detection assay. The results indicate that this method would be amenable to study protein release kinetics in other biodegradable polymer systems intended for drug or vaccine delivery. This technique can also be used to study protein release under other simulated \textit{in vitro} conditions that better mimic \textit{in vivo} applications (e.g., in the presence of serum proteins).

It is known that protein release from biodegradable polymers is affected by the pH of the polymer degradation environment \cite{6, 29, 42-45}. Polyanhydride degradation is known to be base-catalyzed \cite{6, 29}. It is important to understand the release behavior of proteins from such polymers post injection, inside the host, because the intracellular pH tends to be more acidic (pH of 4.5-6) enabling the breakdown of phagocytosed particulates in endocytic compartments \cite{46}. To this end, protein release kinetics were studied for five different
chemistries of TRBSA-encapsulated CPH:SA nanospheres with the fluorescence-based protein detection assay in release media with three different buffered pH values: 7.3 (neutral), 6.0 (mildly acidic), and 4.3 (intracellular pH) [46]. Due to the small sample size (~1 mg nanospheres) and the buffering capabilities of the release medium, it is highly unlikely that the polymer degradation will alter the pH of the release buffer. The high throughput technique allowed for concurrent, rapid protein quantification of a two-dimensional combinatorial library varying in nanosphere chemistry and pH of the release medium. The results are consistent with the base-catalyzed degradation mechanism of polyanhydrides, clearly demonstrating a reduced protein release as the pH of the release medium was lowered, as indicated in Figures 4.4-4.6. The pH level appears to more strongly affect the less hydrophobic (i.e., SA-rich) nanosphere chemistries, which is likely due to their more rapid degradation over the period of study. It is hypothesized that this effect would be evident for the more hydrophobic (CPH-rich) nanosphere chemistries if the study were carried out for a more extended period allowing for complete degradation of the polymer. While pH is not the only in vivo parameter that controls polymer degradation, this high throughput approach is amenable to simulate and study numerous other intracellular or extracellular phenomena (e.g., enzymatic degradation, serum protein adsorption).

Combinatorial protein release from polyanhydride films

The high throughput fluorescence method was also used to study the release of TRBSA from five linearly varying CPH:SA copolymer films in replicates of four, which was performed in parallel with the aforementioned nanosphere release. These studies were carried out for two reasons. First, there is interest in discerning in the effect of device geometry (films vs.
nanospheres) on protein release kinetics. As mentioned before \cite{3, 41, 47, 48}, polyanhydride devices of various geometries have been tested for \textit{in vivo} applications and the high throughput method provides a rapid way to simultaneously study the effects of polymer chemistry and device geometry. Second, it is important to demonstrate that the fluorescence technique is amenable to various device geometries. As expected, the films displayed a polymer chemistry-dependent protein release profile with the most hydrophobic (i.e., CPH-rich) chemistries releasing the protein the slowest (Figure 4.7). These results were in agreement with release profiles obtained with the micro-BCA assay (data not shown). The overall release of protein from the films was lower and resulted in a smaller initial protein burst when compared with the nanospheres, which is likely due to the reduced polymer surface area exposed to the release buffer. The variances observed with the TRBSA release profiles from the films were higher than those with the TRBSA release profiles from the nanospheres. This observation can be attributed to the tendency of films, in some cases, to delaminate and break off into non-uniform pieces, thus exposing different amounts of surface area. As stated before, this technique is amenable to investigate drug/protein release from alternate geometries such as three-dimensional scaffolds used for tissue engineering \cite{49-51} or core-shell particles used for multi-drug therapies \cite{52, 53}.

\textbf{4.5 Conclusions}

The development of a highly sensitive fluorescent technique for the simultaneous detection of protein release from biodegradable polyanhydride nanospheres and films has enabled rapid evaluation of the effects of device geometry, polymer chemistry, and pH of release medium on the protein release kinetics. The film geometry, CPH-rich chemistries, and acidic
release conditions all demonstrated the ability to significantly decrease protein release over their respective counterparts (nanosphere geometry, SA-rich chemistries, and neutral release conditions). Both the nanosphere and film systems released TRBSA protein in a sustained and polymer chemistry-dependent manner while the nanospheres also demonstrated a pH-dependent protein release. The high throughput fluorescence-based technique is amenable to other polymer and protein systems as well as alternate release environments which will allow for rational and rapid design of delivery devices. Finally, these findings contribute to the large body of evidence supporting the use of polyanhydrides as highly tunable biomaterials for the controlled delivery of drugs and proteins.

4.6 Acknowledgments

The authors would like to thank the ONR-MURI Award (NN00014-06-1-1176) for financial support. This material is based upon work supported by the National Science Foundation under Grant No. EEC 0552584.

4.7 References

5. Yoshida M, Mata J, Babensee JE. Effect of poly(lactic-co-glycolic acid) contact on
22. Kipper MJ, Narasimhan B. Molecular description of erosion phenomena in biodegradable


4.8 List of Figures

Figure 4.1: a) Schematic of modifications made to horizontal pair of wells in the 96-well polypropylene release plate, and b) actual fluorescence images of the 96-well plate while performing the TRBSA release studies from CPH:SA films (only wells with TRBSA in them are visible in the image).

Figure 4.2: SEM images of TRBSA-loaded polyanhydride nanospheres: A) poly(SA) and B) 50:50 CPH:SA.
Figure 4.3: Cumulative mass fraction of BSA released from combinatorially fabricated CPH:SA nanospheres for one month at pH 7.3 as detected by the micro-BCA assay. Error bars represent standard deviation and n=4.

Figure 4.4: Cumulative mass fraction of TRBSA released from combinatorially fabricated CPH:SA nanospheres for one month at pH 7.3 as detected by the high throughput fluorescence assay. Error bars represent standard deviation and n=4.
Figure 4.5: Cumulative mass fraction of TRBSA released from combinatorially fabricated CPH:SA nanospheres for one month at pH 6.0 as detected by the high throughput fluorescence assay. Error bars represent standard error and n=2.

Figure 4.6: Cumulative mass fraction of TRBSA released from combinatorially fabricated CPH:SA nanospheres for one month at pH 4.3 as detected by the high throughput fluorescence assay. Error bars represent standard error and n=2.
Figure 4.7: Cumulative mass fraction of TRBSA released from combinatorially fabricated CPH:SA films for one month at pH 7.3 as detected by the high throughput fluorescence assay. Error bars represent standard deviation and n=4.

4.9 List of Tables

Table 4.1: Molecular weight analysis of CPH:SA copolymer film libraries using GPC and \textsuperscript{1}H NMR.

<table>
<thead>
<tr>
<th>CPH:SA Polymer Library</th>
<th>$M_n$ (Da) from GPC</th>
<th>$M_n$ (Da) from \textsuperscript{1}H NMR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poly(SA)</td>
<td>11154</td>
<td>12555</td>
</tr>
<tr>
<td>25:75 CPH:SA</td>
<td>9692</td>
<td>10854</td>
</tr>
<tr>
<td>50:50 CPH:SA</td>
<td>13264</td>
<td>12872</td>
</tr>
<tr>
<td>75:25 CPH:SA</td>
<td>12674</td>
<td>13442</td>
</tr>
<tr>
<td>Poly(CPH)</td>
<td>16477</td>
<td>15247</td>
</tr>
</tbody>
</table>
Chapter 5: High Throughput Analysis of Protein Stability in Polyanhydride Nanoparticles

A paper published in Acta Biomateralia 2010, 6 (10), 3873-3881

L. K. Petersen, C. K. Sackett, and B. Narasimhan*

Keywords: Combinatorial, high throughput, polyanhydrides, protein stability, nanospheres

Department of Chemical and Biological Engineering, Iowa State University, 2035 Sweeney Hall, Ames, IA 50011-2230
*To whom all correspondence should be addressed
5.1 Abstract

With the complexity and fragile nature of many protein molecules used in therapeutic treatments and vaccines, devices capable of protecting and preserving the functionality of these proteins are essential. In addition, the half-lives of many vaccine antigens and therapeutic proteins are often short, especially at elevated temperatures. In this work a high throughput methodology has been developed to rapidly assess the effects of polymer chemistry and the various steps during protein delivery (i.e., encapsulation, storage, and release) from biodegradable polyanhydride nanoparticles on the stability of a model protein, bovine serum albumin. Additional factors including microenvironment pH were also investigated in this multi-parametric approach to evaluate protein stabilization. The findings indicate that the microenvironment pH caused by the acidic polymer degradation products was the most detrimental factor affecting protein stability. Nanoparticles based on 1,8-bis(p-carboxyphenoxy)-3,6-dioxaoctane and 1,6-bis(p-carboxyphenoxy)hexane maintained protein antigenicity over a range of temperatures for one month. These nanoparticles were also successful in preserving protein structure and emerged as viable candidates for use in future drug/protein stabilization and delivery applications. The combinatorial approach developed in this work allowed for a 25-fold decrease in time and a 10-fold decrease in the amount of materials needed for the investigation of protein stability when compared to conventional methods.

5.2 Introduction

The delivery of expensive and fragile protein-based drugs is challenging and poses many hurdles including limited availability and drug stabilization upon storage and administration.
Short shelf lives expedited by elevated storage temperatures limit protein functionality and use; this is especially pronounced in developing countries where resources are limited. The administration of proteins for vaccination, disease treatment, and anti-cancer therapy all require protein stabilization. It is known that small structural changes can be detrimental to protein function, leading to a decrease in the efficacy of the intended treatment \[1-3\]. In addition, repeated drug administration or surgical implantation might pose problems with patient compliance. Thus, there is a need for delivery devices that can stabilize fragile protein molecules as well as provide a sustained release to eliminate the need for repeated administration. With the large database of readily available biodegradable polymers intended for protein stabilization and delivery, new high throughput methodologies are needed for the discovery, testing, and design of these biomaterials \[4\]. These approaches are emerging in the field of biomaterials \[4-13\] to study large numbers of biomaterials in parallel for use as drug and vaccine delivery vehicles.

Polyanhydrides are a versatile class of biodegradable materials with applications in drug and vaccine delivery \[10, 14-17\]. The polyanhydrides of interest in this work are based on 1,8-bis\((p\text{-carboxyphenoxy})\)-3,6-dioxaoctane (CPTEG), 1,6-bis\((p\text{-carboxyphenoxy})\)hexane (CPH), and sebacic acid (SA); the chemical structures of the monomers are shown in Figure 5.1. These polymers are composed of anhydride bonds, which undergo hydrolysis in the presence of water as shown in Figure 5.1d). Materials based on these monomers have shown promise for applications in drug delivery with their ability to stabilize and release proteins in a controlled manner ranging from days (CPTEG-rich) to weeks (SA-rich) to months (CPH-rich) \[13, 17-21\]. Additionally, the polyanhydrides of interest can be rapidly fabricated into micro or
nanoparticles allowing for non-invasive administration via inhalation or injection. These micro and nanoparticles are promising for applications in vaccine administration, as they possess adjuvant characteristics that may be necessary for efficacious vaccination with poorly immunogenic protein antigens. However, stabilization of the antigen is a primary concern, because without the ability to deliver a fully functional antigen, protection may not be viable. Polyanhydride microparticles have been previously shown to stabilize protein antigens, specifically tetanus toxoid (TT), which confirmed the ability of TT-loaded CPH:SA microparticles to deliver functional antigen and modulate the immune response \cite{14}. Given that polyanhydrides are versatile and can be tailored for specific properties (e.g., protein stability, protein release, immune activation, adjuvant capabilities, etc.) \cite{10, 13, 14, 17, 19, 20, 22, 23}, the use of traditional, ‘one-sample-at-a-time’ approaches has and will continue to expend large amounts of time and resources for the necessary optimization of these materials for their intended applications.

There is an urgent need to develop high throughput approaches to screen and design delivery systems to keep pace with the rapid increase in the number of expensive, fragile, protein drugs that are in development for disease therapy and treatment \cite{17, 24}. The combinatorial approach for biomaterials design has emerged in the past decade as a viable method which allows for the use of reduced amounts of expensive proteins and accelerates the throughput and development of materials for numerous applications including but not limited to drug delivery, vaccine design, tissue engineering, gene therapy, etc \cite{5-11, 13, 25-33}. More recently, combinatorial studies have been carried out for the investigation of polyanhydrides in the areas of phase behavior, drug/protein release kinetics, cytotoxicity, and immune activation
However, to the best of the authors’ knowledge, this approach has not been applied to study protein stability in the polyanhydride (or any other biodegradable polymer) system.

In this work, the stability of a model protein (bovine serum albumin, BSA) upon release from polyanhydride nanoparticles of various chemistries was investigated at high throughput with an antigenicity assay. Since it is well known that protein stability could be affected by the fabrication, storage, and release conditions, high throughput methods were developed to assess BSA stability upon nanoparticle fabrication/protein encapsulation (solvent exposure, sonication, and vacuum), storage (shelf life at different temperatures), and release (polymer chemistry and pH). The multi-parametric nature of this problem and the proposed high throughput methodology is illustrated in Figure 5.2. This combinatorial method allowed for a 25-fold decrease in time and a 10-fold decrease in the materials used to fabricate libraries of polyanhydride nanoparticles and enabled the simultaneous investigation of the effect of polymer chemistry, shelf life, storage temperature, and microenvironment pH on protein stability. To better understand the mechanisms of instability caused by the chemistries that were detrimental to protein antigenicity, a high throughput multi-level structural analysis was carried out, utilizing previously described automated synthesis and fabrication techniques, to determine the source of protein degradation and improve the throughput of the experiments. The use of the combinatorial approach will lead to a more in-depth understanding of the relationship between protein stability and the multiple the steps in the delivery process, ranging from fabrication to administration. This will therefore help expedite the rational design and development of biomaterial carriers for protein stabilization and delivery.
5.3 Materials and Methods

Materials

The chemicals needed for the polymerization, nanoparticle fabrication, and buffer preparation include acetic anhydride, chloroform, methylene chloride, petroleum ether, pentane, monobasic potassium phosphate, and dibasic potassium phosphate; all were purchased from Fisher Scientific (Fairlawn, NJ). Monomer synthesis utilized the following chemicals: 1,6-dibromohexane, triethylene glycol, 4-hydroxybenzoic acid, 1-methyl-2-pyrrolidinone, 4-p- and 1,6-dibromohexane; these were purchased from Sigma Aldrich (St. Louis, MO); 4-p-fluorobenzonitrile was purchased from Apollo Scientific (Cheshire, UK); and sulfuric acid, acetonitrile, dimethyl formamide (DMF), toluene, and potassium carbonate were obtained from Fisher Scientific. Deuterated chemicals for NMR analysis (chloroform and dimethyl sulfoxide (DMSO)) were purchased from Cambridge Isotope Laboratories (Andover, MA). BSA was purchased from Sigma Aldrich. Precast 12% polyacrylamide gels (Tris-HCl), low molecular mass standards, and 2-mercaptoethanol were purchased from BioRad (Hercules, CA). Polypropylene black round bottom 96-well plates and polystyrene clear 96-well plates were obtained from Fisher Scientific. The ELISA kit for determining BSA antigenicity and the kits for making reagents and buffers were purchased from Bethyl Labs (Montgomery, TX). The primary (coating) antibody utilized in this kit was sheep antibovine albumin-affinity purified and the secondary (detection) antibody was sheep antibovine albumin-HRP conjugate.

Polymer library synthesis

The CPH and CPTEG monomers were synthesized as described previously[^34]. The SA
monomer was purchased from Sigma Aldrich. The linearly varying library in molar composition of CPH:SA prepolymer was deposited at high throughput using an automated robotic apparatus as described previously\textsuperscript{[10, 11, 13, 15]}. The prepolymer library was polymerized at 0.3 torr vacuum and the desired temperature (180 °C for CPH:SA and 140 °C for CPTEG:CPH) for 1.5 h resulting in a discrete library of CPH:SA (poly (SA), 25:75 CPH:SA, 50:50 CPH:SA, 75:25 CPH:SA, and poly (CPH)) and CPTEG:CPH (60:40 CPTEG:CPH, 50:50 CPTEG:CPH, 40:60 CPTEG:CPH, 30:70 CPTEG:CPH, 20:80 CPTEG:CPH and 10:90 CPTEG:CPH) copolymer films in replicates of four. The resulting polymer library included a total of 44 samples (11 chemistries x 4 replicates).

\textit{Protein encapsulation into nanoparticles}

The fabrication of protein-loaded nanoparticles utilized the automated robotic deposition apparatus to increase throughput. Following synthesis of the discrete polymer film libraries, lyophilized protein was dispersed in methylene chloride and added to each polymer well, thus dissolving the polymer film. The protein/polymer solution was first sonicated for 30 s at 40 Hz to uniformly disperse the protein, then precipitated into a non-solvent (pentane) to create protein-loaded nanoparticles, and finally dried in a vacuum chamber (CPH:SA) or filtered via rapid vacuum filtration (CPTEG:CPH) as described previously\textsuperscript{[10, 13, 16]}. This resulted in a 44-sample protein-loaded nanoparticle library (11 chemistries x 4 replicates).

\textit{Polymer and nanoparticle characterization}

The discrete polymer libraries were characterized with \textsuperscript{1}H nuclear magnetic resonance (NMR) spectroscopy and high throughput Fourier transform infrared (FTIR) spectroscopy.
End group analysis of the NMR spectra was used to determine molecular weight, copolymer chemistry, and chemical structure with a Varian VXR 300 MHz spectrometer (Varian Inc., Palo Alto, CA). Deuterated chloroform was used to dissolve the samples and chemical shifts were calibrated with respect to the chloroform peak (δ= 7.26 ppm). The surface chemistry of the polymer libraries was characterized using high throughput FTIR spectroscopy (Nicolet 6700 FTIR spectrometer, Thermo Fisher Scientific) as described previously [10, 13]. A polymer film library was synthesized on a 25-well silicon nitride (IR transmissive) substrate in an automated fashion with the use of a programmable mapping software package (Atlμs). This software enabled a specific sample and background map to be programmed into the FTIR sample detection, allowing for multiple FTIR spectra to be quantified without user operation. Scanning electron microscopy (SEM) was used to determine size of the polyanhydride nanoparticles.

*Enzyme-linked immunosorbent assay (ELISA): protein antigenicity*

A BSA-specific ELISA kit was used to analyze protein antigenicity, following the manufacturer’s protocol. Samples were centrifuged, and the supernatant diluted with the sample diluent (50mM Tris, 0.14 M NaCl, 0.05% Tween 20, pH 8.0) to a protein concentration in the range of 100 – 400 ng/mL. A horseradish peroxidase detection antibody was diluted to a concentration of 33.3 ng/mL. The enzyme substrate 3,3’,5,5’-tetramethylbenzidine was applied, and after the reaction was stopped with 2 M sulfuric acid, the absorbance was measured at 450 nm for high throughput analysis in a 96 well plate format. The samples were analyzed in replicates of four.
**Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE): primary structure**

SDS PAGE was used to assess changes (i.e., hydrolysis or aggregation) in the protein primary structure. Each protein sample was mixed (1:1/v:v) with a loading buffer (10% w/v SDS, 1M Tris-HCl (pH 6.8), 3 mM glycerol, 0.01% w/v bromophenol blue, and 0.05% v/v β-mercaptoethanol) and heated for 5 min at 95°C. The samples were resolved through a 12% polyacrylamide precast gel (Tris-HCl; Bio-Rad) using a constant voltage of 140 V. The gels were removed, incubated in a fixative solution (40% ethanol and 10% acetic acid) for 3 h, stained with Coomassie Blue for 30 min, destained overnight, and scanned for image analysis. All experiments were performed in replicates of four.

**FTIR spectroscopy: secondary structure**

FTIR spectroscopy was performed to determine changes in the secondary structure of the BSA protein based on a method described previously [19, 35]. To quantify these structural changes, the protein samples were deposited in high throughput using an automated robotic deposition apparatus onto a 25-well silicon nitride substrate (IR transmissive) and dried at 50 °C under vacuum. Using a Nicolet Nexus 470 Continuum infrared microscope (Thermo Fisher Scientific, Madison, WI) with a cooled MCT/A detector and an Ever-Glo source, a total of 200 scans per spectrum was collected at a resolution of 4 cm⁻¹. Dry nitrogen was used to purge the optical bench to reduce the water absorbance. For data collection and analysis, Omnic software was used in conjunction with an automated mapping program, Atlμs, to enhance speed and repeatability. Protein structural quantification and analysis was modified from a previously described procedure for determining the amount of α-helices and β-sheets of the protein [35]. The experiments were performed in replicates of four. Spectral data was
gathered from the amide I region of BSA and smoothed with an 11pt smoothing function. In all cases a linear baseline was fit to the spectral data and Gaussian curve fitting was used to determine the relative area under each peak in the amide I region. Table 5.1 provides a list of the IR peak positions and areas for the amide I region of BSA used in this structural analysis. The Atłus software was employed to automate this process.

*Fluorescence spectroscopy: tertiary structure*

Fluorescence spectroscopy was used to study the changes in protein tertiary structure with a Cary Eclipse fluorescence spectrometer (Varian, Inc., Palo Alto, CA). The samples were arranged for high throughput analysis in a 96-well plate. They were excited at a wavelength of 280 nm and a voltage of 800 V, with a resulting emission spectrum that reflects the tryptophan and tyrosine residues of the protein. The emission spectrum from 300-500 nm was analyzed for shifts in peak intensity and wavelength, which are indicative of alterations in protein tertiary structure. The experiments were performed in replicates of four.

*Statistical and data analysis*

All data (except for Figure 5.5) was statistically analyzed by using a student’s t-test with the statistical software JMP® 7 (Cary, NC). Comparisons between treatments were made and p-values of less than or equal to 0.05 were considered significant. Data in Figure 5.5 was statistically analyzed using a one-way model ANOVA with JMP® 7 to account for comparison-wise error since three treatments were studied in parallel. Comparisons between treatments were made with Tukey’s HSD to determine statistical significance and p-values of less than or equal to 0.05 were considered significant. Additionally, the data in Figures 5.4-
5.6 are presented as ‘Relative Antigenicity’. This was calculated by quantifying the epitope availability/antigenicity of the sample (BSA-specific ELISA) by dividing by the amount of protein in the sample (Micro BCA Assay). In Figure 5.8, the data is presented as “Fold Change in Absorbance”, which was calculated by quantifying the α-helix and β-sheet content per sample by dividing by the α-helix and β-sheet content of the NP (no polymer, unencapsulated protein) control.

**5.4 Results and Discussion**

It is necessary to preserve the functionality and biological activity of proteins during their encapsulation into the delivery vehicle, storage, and upon release *in vivo*. Therefore, this work focused on utilizing a combinatorial approach to rapidly assess the capabilities of polyanhydride drug delivery vehicles based on CPH:SA and CPTEG:CPH copolymer nanoparticles to preserve protein antigenicity upon release. When loss or reduction of protein antigenicity was observed, further studies were carried out to identify the cause(s) of protein instability by examining the effects of nanoparticle fabrication conditions (solvent exposure, sonication, and vacuum) and the acidic release microenvironment caused by polymer degradation.

The CPH:SA and CPTEG:CPH polymer nanoparticle libraries were synthesized in a combinatorial format, allowing for the synthesis of up to 25 different polymer chemistries in the same batch thus reducing the synthesis time by 25 fold. We add that by integrating a temperature gradient [33] or a pH gradient [13] with these libraries, further savings in time may be obtained. These polymer libraries were then rapidly precipitated with protein, forming
subsequent protein-loaded nanoparticle libraries of the desired polymer chemistries, which were also carried out at high throughput with a 25 fold reduction in time. In addition, this high throughput approach allowed for reduced sample size from the conventional 100 mg batch size $^{16, 17, 24}$ to a 10 mg batch size. This 10-fold reduction in material usage is critical when testing expensive, limited supply materials (antigens, peptides, growth factors, etc.) and also allows for analysis of the protein with different types of biomaterials. These polymer libraries were characterized by measurements of the polymer molecular weight, copolymer chemistry, and chemical structure. These properties were consistent with previous work on conventionally synthesized polymers $^{17, 19, 20, 34}$. The $^1$H NMR characterization resulted in molecular weights ranging from 10-15 kg/mol which is consistent with previously published work with combinatorially and conventionally synthesized copolymers $^{17-20, 34}$. Copolymer chemistries of both the CPTEG:CPH and CPH:SA libraries were characterized with $^1$H NMR and the CPH:SA library was further characterized with high throughput FTIR (CPTEG and CPH chemical species have indistinguishable IR spectra and could not be characterized in this manner). These methods revealed that the robotic apparatus was effective at depositing the intended molar ratios of the monomers into the discrete multi-well substrate (data not shown), which is in agreement with previously published data $^{10, 13}$. Additionally, the high throughput FTIR method employed an automated mapping software package, which significantly reduced experimental time and user error. SEM was used to assess size and morphology of the nanoparticle libraries. SEM images, representative of the chemistries studied, are shown in Figure 5.3, and are consistent with previous work employing polyanhydride nanoparticles, with particle diameters in the 300-600 nm range across all the chemistries studied $^{10, 13, 16}$. 
Initial studies investigated the stability of BSA following encapsulation and release from CPTEG:CPH and CPH:SA nanoparticle libraries. These studies examined the effect of polymer chemistry on protein stability during encapsulation and after 48 h of release. Figure 5.4 shows the effect of polymer chemistry on protein antigenicity, measured at high throughput by a protein-specific ELISA. In the CPH:SA system, it can be observed that the protein antigenicity decreased as the SA content in the copolymer increased whereas the protein released from CPTEG:CPH nanoparticles retained its antigenicity. This chemistry-dependent trend may be controlled by the pH microenvironment resulting from the degraded polymer surrounding the protein. As the libraries shift from CPTEG-rich to CPH-rich to SA-rich chemistry, there is a significant decrease in the pH of the polymer degradation products [20], exposing the protein to a much more acidic microenvironment. Acidic pH environments have previously been shown to be detrimental to protein structure, specifically to albumins like BSA [24, 35]. However, with the combined surface and bulk erosion mechanisms exhibited by polyanhydrides, water ingress into the bulk is controllable and in most cases not significant and therefore proteins would most likely only be exposed to these low pH microenvironments once they are released into surrounding solution. All nanoparticle chemistries of the CPTEG:CPH system were able to preserve the protein antigenicity presumably because of their ability to produce less acidic degradation products and to limit water ingress into the device, thus resulting in release of antigenic protein.

Next, the combinatorial libraries were utilized to study the shelf life of protein encapsulated into nanoparticles over a period of 28 days at three temperatures (4, 25, and 40 °C). The throughput of this experiment was expedited by the combinatorial approach, which allowed
for this multi-parametric (polymer chemistry, temperature, and time) investigation of protein stability. The BSA-loaded nanoparticle libraries were stored under dry conditions at the desired temperature for the desired amount of time and then incubated for 2 days in buffer to allow for protein release and analysis of the effect of storage temperature and polymer chemistry on BSA stability. The results (Figure 5.5) indicate that the antigenicity of the protein released from the nanoparticles did not significantly change over the period of 28 days for any of the chemistries tested. This suggests that the storage of the protein in polyanhydride nanoparticles is not detrimental to the protein antigenicity. When assessing the effect of temperature, there was no statistical difference in BSA antigenicity between 4 and 25 °C, however; at 40 °C all the CPTEG:CPH nanoparticle chemistries and only 75:25 CPH:SA and poly(CPH) nanoparticle chemistries were able to maintain, and in most cases exceed, preservation of the antigenic epitopes of BSA when compared to the non-encapsulated protein (NP) stored at that specific temperature. This suggests that CPTEG:CPH and the CPH-rich chemistries of the CPH:SA polymer system may be able to protect proteins at elevated temperatures when they may otherwise lose structural stability when stored alone. It is also observed in Figure 5.5 that some CPTEG:CPH polymer chemistries resulted in a statistical increase in relative antigenicity greater than one. This suggests that more epitopes of the protein may have become available and thus exposed to antibody binding upon encapsulation into CPTEG:CPH chemistries.

These findings prompted further examination of the effects of nanoparticle fabrication conditions and acidic release microenvironment on the stability of the protein. Again, parallel studies employing the combinatorial technique were carried out to rapidly investigate the
effect of the nanoparticle fabrication conditions on protein stabilization by measuring BSA antigenicity after exposure to the same conditions in the absence of polymer. This included prolonged contact with organic solvents (methylene chloride and pentane), sonication (at 40 Hz), and extended exposure to a low vacuum ($10^{-3}$ torr) chamber. These experiments revealed that the antigenicity of BSA was not affected by the processing conditions, as indicated in Figure 5.4 (bar marked “NFC”), which suggests that other factors, such as the microenvironmental pH, may play a more integral role in protein instability.

To better understand the detrimental effect on the protein released from SA-rich chemistries, BSA was exposed to the acidic degradation products of CPH:SA copolymers for an extended period of time. Under saturated conditions, aqueous solutions of the SA monomer have been reported to have a pH of 4.2 whereas aqueous solutions of the CPH monomer have a pH of 5.5\cite{20}. These studies were carried out by incubating BSA in a linearly varying gradient library of saturated CPH:SA monomers for seven days. Protein antigenicity was once again assessed with the BSA-specific ELISA and revealed a trend corresponding to a loss in antigenicity with increasing SA monomer content (decreasing pH) as shown in Figure 5.6. However, the relative decrease in antigenicity was less severe than that observed when the protein was released from the CPH:SA nanoparticles. Here, it is important to consider that the ratio of degradation products released from an eroding copolymer nanoparticle at any given time may be enriched in the more acidic/hydrophilic component, SA. This is likely due to variable backbone degradation rates of the copolymers and the increased solubility of SA \cite{20}, which may result in a more severe decrease in protein stability than observed in the corresponding monomer composition. While it is apparent that the microenvironmental pH is
playing an important role in the protein instability, other protein/polymer interactions could also be contributing to the loss in protein antigenicity. A detailed analysis of structural alterations of the protein was performed to provide a more complete explanation of how loss of protein structure is related to loss in protein antigenicity and to identify the structural levels at which the changes were occurring. The combinatorial approach provided a convenient platform for this investigation because the sample libraries were easily amendable to rapidly test many protein stability parameters (primary, secondary, and tertiary structures as well as antigenicity).

The primary structure as indicated by molecular weight of the BSA protein incubated with the monomer library as determined with SDS PAGE, shown in Figure 5.7, revealed that the primary structure of the protein was preserved when incubated with all the monomer compositions. This indicates that the changes in antigenicity as determined by the BSA-specific ELISA were not a result of structural alterations at the primary structure level upon incubation in the monomer library. While it has been reported previously [16], it is instructive to add that the hydrophobic nature of the CPH:SA chemistries likely did not induce non-covalent aggregation. This is because even the most hydrophobic chemistries (CPH-rich) studied did not reveal any high molecular weight bands in the gels (Figure 5.7).

The secondary structure of BSA was assessed by creating a combinatorial sample library of BSA from the incubation groups on a multi-well silicon nitride substrate and using transmission mode FTIR with an automated mapping program to enhance speed and repeatability. The amide I region of BSA (Table 5.1) was analyzed for α-helix and β-sheet
content, which are characteristic signatures of secondary structure \[^{[35]}\]. As shown in Figure 5.8, the $\alpha$-helix content of BSA decreased with increasing SA content. This suggests that the acidic environment resulting from SA-rich monomer compositions is detrimental to the secondary structure of the protein, which is in agreement with previously published work on the deleterious effects of acidic environments on the secondary structure of BSA \[^{[35]}\]. This is likely to be the critical factor that altered the antigenicity of BSA upon incubation with the CPH:SA monomer library (Figure 5.6). In addition, Figure 5.8 shows that the $\beta$-sheet content increased with an increase in CPH monomer content. As observed in Figures 5.4 and 5.5, this small increase in the $\beta$-sheet content with CPH-rich monomer compositions is not sufficient to cause non-covalent aggregation or alterations in the protein antigenicity.

The tertiary structure of BSA incubated with the monomer library was investigated at high throughput using fluorescence spectroscopy. This assay was used to determine if the tyrosine and tryptophan residues of the BSA were folded in their native confirmation, which is usually indicative of biological activity \[^{[17]}\]. The peak intensity and peak shift of the tyrosine and tryptophan residues were measured for each treatment and compared to that of the native protein. While there were no apparent peak shifts in wavelength over seven days of monomer incubation, there were changes in the peak intensity with increase in SA monomer content as shown in Table 5.2. This suggests that there was a decrease in tyrosine and tryptophan residue content, thus altering the overall three dimensional structure of the fully folded protein. This is in agreement with both the decrease in $\alpha$-helix content as determined by FTIR and the protein antigenicity as determined by the BSA-specific ELISA. Such changes in the tertiary structure may also constitute alterations in the primary structure of the protein;
however, such subtle differences may not be detectable by the resolution of SDS PAGE.

In this work a combinatorial approach was used for rapid examination of the effect of polyanhydride nanoparticle chemistry on protein stability and enabled an accelerated means of testing and designing delivery vehicles. Additionally, these studies helped paint an overall picture of the effect of encapsulation, storage, and release on protein stability and the causes leading to the instabilities and the structural level(s) at which these changes took place. It was demonstrated that the CPTEG:CPH nanoparticles are robust delivery vehicles capable of preserving the protein antigenicity upon encapsulation, storage (over one month at elevated temperatures), and release. This is in agreement with recently published work in which the CPTEG:CPH system showed promise in the preservation of protein function \[^{17,21,24}\]. The ability of the CPTEG:CPH chemistries to preserve protein structure is a very significant feature because it allows for the exploitation of the entire range of protein release kinetics (CPTEG-rich: days to CPH-rich: months) to fit the desired application. In the case of the CPH:SA system, CPH-rich chemistries proved to be robust delivery devices, preserving protein functionality upon encapsulation, storage, and release; on the other hand, the SA-rich chemistries proved to be detrimental to protein antigenicity. The acidic microenvironmental pH, resulting from SA-rich polymer degradation, played an integral role in protein instability. These instabilities caused by pH resulted in a loss in protein antigenicity, which was reflected by losses in both secondary and tertiary structure. In addition to the pH, other phenomena such as protein adsorption/desorption may play a role in protein degradation. While protein adsorption can occur as a function of both surface hydrophobicity and electrostatic charge, further investigation of these protein/polymer interactions is necessary to enhance our
understanding of how the SA-rich polymer chemistries are influencing protein degradation.

The combinatorial approach allowed for a 25-fold decrease in time (when compared to performing 25 “one-sample-at-a-time” experiments) and a 10-fold decrease in the amount of materials needed for the investigation of protein stability when compared to conventional methods. In addition, the use of a temperature or pH gradient as described previously \[13, 33\] could lead to further savings in time. In this work we were able to rapidly test numerous variables/aspects associated with both the nanoparticle delivery system (shelf life, temperature, polymer chemistry, processing conditions, and pH of degradation products) and the protein (antigenicity and primary, secondary, and tertiary structures) with a significant number of replicates. The trends identified in this work using the model protein, BSA, are applicable to other proteins when predicting their stability in these polymer systems. Many previous studies investigating stability of various proteins (e.g., TT, lysozyme, ovalbumin, F1-V antigen) in polyanhydrides utilized similar conventional particle fabrication techniques as those employed in this study \[14, 17-21, 23, 24\]. Therefore, the combinatorial methods described herein could easily be extended to study other proteins. Additionally, several ongoing studies with the polyanhydride system have reported trends in stability with other proteins very similar to those identified in this work \[24\]. This high throughput technique is easily amenable to investigate other materials for protein stabilization and to evaluate other factors that may contribute to protein instability. In addition, the work may also be extended to study other protein molecules (e.g., vaccines and anti-toxins) which when stored alone demonstrate structural instabilities and thus are in need of protective delivery vehicles.
5.5 Conclusions

A high throughput method was developed to study the stability of a model protein (BSA) in polyanhydride nanoparticles. It was observed that all CPTEG:CPH chemistries and CPH-rich chemistries from the CPH:SA system were capable of providing stabilizing environments for BSA. These nanoparticles were also demonstrated to preserve the antigenicity of the protein during storage, even at elevated temperatures. The acidic microenvironment of the SA degradation products from the CPH:SA library proved to be a deleterious factor that affected the antigenicity of BSA. Further structural investigations revealed that the decrease in antigenicity caused by the acidic SA degradation products was due to perturbations of the secondary and tertiary structure of the protein. The combinatorial methodologies developed in this work are amenable to rapidly design and test other biomaterial systems and to study their protein stabilization capabilities. The use of BSA as a model protein has provided a general understanding of the trends associated with protein stability and polymer chemistry and the results obtained are applicable to other proteins of interest. The versatility of this combinatorial approach can also enable the study of multiple proteins simultaneously with biodegradable polymer systems. Utilization of this rapid, multiplexed approach can expedite research with limited availability of expensive proteins and lead to rapid and rational design of biomaterials for applications in drug delivery and vaccine administration.

5.6 Acknowledgments

The authors would like to thank the ONR-MURI Award (NN00014-06-1-1176) for financial support. This material is based upon work supported by the National Science Foundation under Grant No. EEC 0552584.
5.7 References


5.8 List of Figures

Figure 5.1: Chemical structures of: a) SA; b) CPH; and c) CPTEG; d) polyanhydride hydrolysis mechanism.

Figure 5.2: Schematic describing the high throughput methodology for studying protein stability with polymer nanoparticle libraries from the initial synthesis/fabrication to storage.
over time at variable temperatures to the release and subsequent level of stability as affected by pH, temperature, and polymer chemistry. This process allows for a 25 fold savings in time and 10 fold savings in materials compared with conventional techniques.

Figure 5.3: SEM images of a) 50:50 CPTEG:CPH and b) 50:50 CPH:SA nanoparticles [13].

Figure 5.4: Antigenicity of BSA following encapsulation and release from CPH:SA and CPTEG:CPH nanoparticles and after exposure to the nanoparticle fabrication conditions (NFC). Error bars represent standard deviation (n=4). NP = no polymer, unencapsulated protein control. See adjoining table for statistical analysis. Treatments with the same letter are not statistically significant from one another. Statistical significance (stat. sig.) corresponds to $p \leq 0.05$. 

<table>
<thead>
<tr>
<th>Encapsulation Group</th>
<th>Stat. Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poly(SA)</td>
<td>E</td>
</tr>
<tr>
<td>25:75 CPH:SA</td>
<td>D</td>
</tr>
<tr>
<td>50:50 CPH:SA</td>
<td>D</td>
</tr>
<tr>
<td>75:25 CPH:SA</td>
<td>C</td>
</tr>
<tr>
<td>Poly(CPH)</td>
<td>AB</td>
</tr>
<tr>
<td>60:40 CPTEG:CPH</td>
<td>BC</td>
</tr>
<tr>
<td>50:50 CPTEG:CPH</td>
<td>A</td>
</tr>
<tr>
<td>40:60 CPTEG:CPH</td>
<td>B</td>
</tr>
<tr>
<td>30:70 CPTEG:CPH</td>
<td>AB</td>
</tr>
<tr>
<td>20:80 CPTEG:CPH</td>
<td>AB</td>
</tr>
<tr>
<td>10:90 CPTEG:CPH</td>
<td>ABC</td>
</tr>
<tr>
<td>NP</td>
<td>ABC</td>
</tr>
<tr>
<td>NFC</td>
<td>AB</td>
</tr>
</tbody>
</table>
Figure 5.5: Shelf life study of BSA encapsulated into a library of nanoparticles poly(SA), 25:75 CPH:SA, 50:50 CPH:SA, 75:25 CPH:SA, poly(CPH), 60:40 CPTEG:CPH, 50:50 CPTEG:CPH, 40:60 CPTEG:CPH, 30:70 CPTEG:CPH, 20:80 CPTEG:CPH, and 10:90 CPTEG:CPH) at three different temperatures 4, 25, and 40 °C (n=4). NP = no polymer, unencapsulated protein control. A) week 1, B) week 2, C) week 3, and D) week 4. See adjoining table for statistical analysis. Treatments with the same letter are not statistically significant from one another. Statistical significance (stat. sig.) corresponds to p < 0.05.
Figure 5.6: Antigenicity of BSA following a seven day incubation in a CPH:SA monomer library (n=4). Error bars represent standard deviation. NP = no polymer, unencapsulated protein control. See adjoining table for statistical analysis. Treatments with the same letter are not statistically significant from one another. Statistical significance (stat. sig.) corresponds to $p \leq 0.05$.

<table>
<thead>
<tr>
<th>Incubation Group</th>
<th>Stat. Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td>SA</td>
<td>D</td>
</tr>
<tr>
<td>25:75 CPH:SA</td>
<td>CD</td>
</tr>
<tr>
<td>50:50 CPH:SA</td>
<td>ABC</td>
</tr>
<tr>
<td>75:25 CPH:SA</td>
<td>A</td>
</tr>
<tr>
<td>CPH</td>
<td>A</td>
</tr>
<tr>
<td>NP</td>
<td>AB</td>
</tr>
</tbody>
</table>

Figure 5.7: Primary structure of BSA using SDS PAGE following a seven day incubation in a CPH:SA monomer library (n=4) (not all gel images are shown). NP = no polymer, unencapsulated protein control.
Figure 5.8: Secondary structure of BSA using FTIR following a seven day incubation in a CPH:SA monomer library to determine $\alpha$-helix and $\beta$-sheet content ($n=4$). Error bars represent standard deviation. NP = no polymer, unencapsulated protein control. See adjoining table for statistical analysis. Treatments with the same letter are not statistically significant from one another. Statistical significance (stat. sig.) corresponds to $p \leq 0.05$.

5.9 List of Tables

Table 5.1: Amide I region of BSA peak assignment, position, and area.

<table>
<thead>
<tr>
<th>Peak Assignment</th>
<th>Peak Position</th>
<th>Peak Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\beta$-sheet</td>
<td>1695±1</td>
<td>7±1</td>
</tr>
<tr>
<td>$\alpha$-helix</td>
<td>1657±1</td>
<td>31±1</td>
</tr>
<tr>
<td>$\beta$-sheet</td>
<td>1639±1</td>
<td>15±4</td>
</tr>
<tr>
<td>$\beta$-sheet</td>
<td>1628±2</td>
<td>8±3</td>
</tr>
</tbody>
</table>
Table 5.2: Tertiary structure of BSA with fluorescence spectroscopy, following a 7 day incubation in a CPH:SA monomer library, as determined by % change in peak intensity and shift in peak position from 340 nm.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>% Change in Peak Intensity</td>
<td>22</td>
<td>22</td>
<td>18</td>
<td>18</td>
<td>16</td>
<td>8</td>
</tr>
<tr>
<td>Shift in Peak Position (nm)</td>
<td>4</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>1</td>
</tr>
</tbody>
</table>
Chapter 6: Amphiphilic Polyanhydride

Nanoparticles Stabilize *Bacillus anthracis* Protective Antigen

A paper to be submitted for publication in *Infection and Immunity*

L. K. Petersen¹, Y. Phanse², A. Ramer-Tait², M.J. Wannemuehler², and B. Narasimhan¹

**Keywords:** PA, *Bacillus anthracis*, polyanhydride, nanoparticles, adjuvant

¹Department of Chemical and Biological Engineering, Iowa State University, Ames, IA
²Department of Veterinary Microbiology and Preventive Medicine, Iowa State University, Ames, IA 50011

*To whom all correspondence should be addressed*
6.1 Abstract

Advancements towards an improved vaccine against *Bacillus anthracis*, the causative agent of anthrax, have focused on formulations composed of aluminum hydroxide adsorbed protective antigen for anthrax (PA). However, due to the fragile nature of this antigen, stability has become a primary concern for vaccine commercialization. Thus, there is a need for a delivery system capable of preserving the functionality of PA throughout all the steps of the vaccine fabrication process through storage and until administration. In this work, we present a class of biodegradable, polyanhydride nanoparticle-based vaccine delivery adjuvants, which have previously been shown to provide controlled antigen delivery, antigen stability, immune modulation, and protection in a single dose against a live pathogen challenge. These nanoparticle vaccine delivery adjuvants demonstrated hydrophobicity dependent PA release kinetics and preserved the functionality of PA upon encapsulation, storage (over extended time and elevated temperatures) and release. Specifically, amphiphilic polyanhydride nanoparticles revealed the ability to best preserve PA functionality over extended time periods and elevated temperatures. These studies provide new insights for the use of amphiphilic nanoparticles as delivery vehicles for long-term vaccine storage as well as single-dose protective immunization against *B. anthracis* infections.

6.2 Introduction

Anthrax is a significant public health concern due to its potential as a bioterrorism and biowarfare agent. Use of the currently licensed anthrax vaccine (AVA) poses many concerns due to its high reactogenicity, multi-dose (five) immunization schedule followed by yearly boosters, and painful side effects \(^{[16]}\). The key element of the AVA vaccine involved in
immunity to the anthrax infection is the recombinant protective antigen for anthrax (PA) which has been the focus for new vaccines [14]. PA is the receptor-binding subunit for both the lethal factor (LF) and the edema factor (EF), which upon binding to the factors is responsible for the formation of the lethal toxin (LT) and the edema toxin (ET) [31].

Unfortunately, the PA protein possesses a fragile recombinant structure and is extremely unstable, especially in low pH and high temperature environments [4, 11, 25, 26]. These stability concerns have led to manufacturing roadblocks during a phase 1 clinical trial for a new anthrax vaccine based on PA [36].

It has been reported that any PA circulating in the blood stream is eliminated within six hours [17], hence the need for multiple immunizations with the AVA vaccine [31]. Methods to increase its availability to the immune system and improve the immunogenicity of PA include engineering recombinant PA, including other antigens or adjuvants in the vaccine, vaccination through alternate delivery routes, as well as the use of controlled delivery systems [31]. Currently, the AVA vaccine is administered intramuscularly and many murine in vivo studies have focused on subcutaneous delivery of PA. However, intranasal vaccination, targeting the lungs, has been reported as the ‘seemingly best route for stimulation’ [9, 31]. However, the best known mucosal adjuvants (cholera, pertussis, and edema toxin bacterial proteins and CpG) have significant limitations due to their potential toxicity in humans [8, 31].

This work focuses on a controlled delivery system for PA based on biodegradable polyanhydride nanoparticles, which provide safe intranasal delivery [10], non-specific adjuvant effect to the cells of the immune system [22, 23, 33], sustained release of encapsulated antigens [2, 5-7, 18, 21, 28], and a stabilizing environment for antigens during fabrication, storage,
and delivery\textsuperscript{[2, 5-7, 15, 18, 20, 28]}. Polyanhydrides are a class of biomaterials with excellent biocompatibility and have been studied as vaccine delivery vehicles and adjuvants\textsuperscript{[3, 13, 22, 23, 30, 32]}. Specifically, copolymers based upon sebacic anhydride (SA), 1,6-bis\((p\)-carboxyphenoxy\) hexane (CPH), and 1,8-bis\((p\)-carboxyphenoxy\)-3,6-dioxaoctane (CPTEG) have been of particular interest as adjuvants. Micro- and nanoparticles of these materials, which are suitable for inhalation or injection, have tunable antigen release kinetics\textsuperscript{[2, 5-7, 15, 18, 21, 28]}, which would provide an antigen depot \textit{in vivo}, allowing for long-term antigen exposure and eliminating the need for multiple administrations. Polyanhydride nanoparticles have demonstrated sustained release of encapsulated molecules for extended periods of time (in excess of 30 days) \textit{in vivo}\textsuperscript{[10, 19]}. Furthermore, these materials have excellent protein stabilization capabilities, and have been shown to protect and structurally preserve a wide range of proteins upon encapsulation, storage, and delivery\textsuperscript{[2, 5-7, 15, 18, 20, 28]}. These surface eroding polymers slowly release encapsulated antigen, minimizing exposure to unfavorable aqueous environments. Additional studies have shown that polyanhydride nanoparticles are capable of non-specific stimulation of immune cells\textsuperscript{[3, 22, 23, 30, 33]}. They enhance cell surface marker expression and cytokine production similar to many other adjuvants (e.g. LPS) without any toxic side effects.

The goal of this work is to design a nanoparticle-based vaccine formulation capable of encapsulating and releasing PA in a biologically functional form during fabrication, storage and release, to provide a delivery system capable of promoting a robust, high avidity, neutralizing antibody response. Nanoparticles based on 50:50 CPTEG:CPH, 20:80
CPTEG:CPH, 50:50 CPH:SA, and 20:80 CPH:SA were investigated for their ability to stabilize and release PA. The amphiphilic CPTEG:CPH nanoparticle chemistries demonstrated the ability to preserve the functionality of PA throughout all the stages of nanoparticle fabrication, storage, and controlled delivery, proving the opportunity for long-term antigen presentation to the immune system. This strategy of using amphiphilic nanoparticles as delivery vehicles presents a possible solution for long-term vaccine storage as well as single-dose protective immunization against *B. anthracis* infections.

### 6.3 Materials and Methods

#### Materials

The chemicals needed for polymerization, nanoparticle fabrication, and buffer preparation include acetic anhydride, chloroform, methylene chloride, petroleum ether, pentane, monobasic potassium phosphate, and dibasic potassium phosphate; all were purchased from Fisher Scientific (Fairlawn, NJ). Monomer synthesis utilized the following chemicals: 1,6-dibromohexane, tri-ethylene glycol, 4-hydroxybenzoic acid, 1-methyl-2-pyrrolidinone, 4-\(p\)-fluorobenzonitrile was purchased from Apollo Scientific (Cheshire, UK); and sulfuric acid, acetonitrile, dimethyl formamide (DMF), toluene, and potassium carbonate were obtained from Fisher Scientific. Deuterated chemicals for NMR analysis (chloroform and dimethyl sulfoxide (DMSO)) were purchased from Cambridge Isotope Laboratories (Andover, MA). Precast 12% polyacrylamide gels (Tris-HCl), low molecular mass standards, and 2-mercaptoethanol were purchased from BioRad (Hercules, CA). PA and LF were obtained from BEI Resources (Manassas, VA).
**Polymer synthesis, nanoparticle fabrication, protein encapsulation, and characterization**

The CPH and CPTEG monomers were synthesized as described previously\[^{29,35}\]. The SA monomer was purchased from Sigma Aldrich (St. Louis, MO). Pre-polymer and polymers were synthesized as described before\[^{12,28}\]. The CPTEG:CPH and CPH:SA copolymers were formed into nanoparticles as described previously\[^{20,21,23}\]. A 2% (w/w) PA loading into the nanoparticles was used in the experiments of Figures 6.1-6.4 and a 0.8% PA loading was used in the experiments of Figures 6.5-6.6. These loading percentages were chosen due to protein detection limitations of the assays and techniques required by each experiment.

Polymer molecular weight, chemical structure, and chemical composition were determined with $^1$H NMR spectroscopy using a Varian VXR 300 MHz spectrometer (Varian Inc., Palo Alto, CA). Nanoparticle size and morphology were characterized with scanning electron microscopy (SEM) (JEOL 840 A, JEOL Peabody, MA).

**In vitro protein release kinetics**

PA-loaded nanoparticles were placed in microcentrifuge tubes with 0.1 mM PBS buffer (pH 7.6). Samples were sonicated for uniform nanoparticle distribution and placed in a shaker/incubator at 37 °C. Sample supernatants were removed incrementally over time to measure the amount of released PA with the micro bicinchoninic acid (BCA) assay. Equivalent volumes of PBS buffer were added following supernatant removal to maintain perfect sink conditions. Data is presented as percent fraction released, which was determined by dividing the amount released at each time point by the total amount encapsulated into the nanoparticles\[^{2,5-7,15,18,21,28}\].
In vitro pH study

Blank nanoparticles were placed in centrifuge tubes with 0.1 mM PBS buffer (pH 7.6). Samples were sonicated for uniform nanoparticle distribution and placed in a shaker/incubator at 37 °C. The pH measurements were carried out incrementally to monitor the buildup of acidic degradation products.

Protein release and in vivo administration of released protein

PA-loaded nanoparticle samples were incubated in PBS buffer for 14 days at 4 °C in dialysis cassettes to accumulate enough PA for immunization and to prevent temperature induced instability. Protein concentration was determined with the micro-BCA assay and adjusted for all samples. Equal amounts of PA (10 μg) released from the different nanoparticles studied (20:80 CPTEG:CPH, 50:50 CPTEG:CPH, and 20:80 CPH:SA) and PA alone were adsorbed to imject alum (Fisher Scientific) for 30 min. A/J mice (Jackson Laboratories, Bar Harbor, Maine) were immunized subcutaneously in the nape of the neck with 10 μg of PA absorbed to alum from each of the above formulations (3 mice per group), boosted on day 15 with 10 μg of PA, euthanized on day 21, and serum collected from a cardiac puncture. All mice were housed under specific pathogen-free conditions where all bedding, caging, and feed were sterilized prior to use. All animal procedures were conducted with the approval of the Iowa State University Institutional Animal Care and Use Committee.

Enzyme-linked immunosorbent assay (ELISA): PA-specific serum antibody response

PA-specific serum antibody response (total IgG [H+L]) was analyzed by ELISA, modified from a method described previously \[^{32}\]. High binding 96 well plates were coated with 0.5
μg/mL PA overnight at 4 °C. Following antigen incubation, the plates were blocked for 2 h (PBS containing 0.5% Tween 20 (PBS-T) and 2% gelatin) at room temperature, washed three times in PBS-T, and then incubated with mouse serum for 24 h at dilutions from 1:1,000 to 1:1,000,000. The following day the plates were washed three times in PBS-T, incubated with the alkaline phosphatase-conjugated secondary IgG [H+L] antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) for 2 h at room temperature, washed again, and incubated with alkaline phosphatase substrate buffer (Sigma Aldrich) for plate development for 2 h at room temperature. The absorbance was measured as an optical density (OD) at 405 nm using a Spectramax 190 Plate Reader (Molecular Devices, Sunnyvale, CA). The samples were analyzed in triplicate.

**Enzyme-linked immunosorbent assay (ELISA): serum antibody avidity**

Serum antibody avidity was determined by modifying a method described previously [32]. The avidity assay was carried out similar to the ELISA serum antibody response protocol described above. Following serum incubation for 24 h at a dilution of 1:1,000,000, sodium thiocyanate was incubated with increasing dilutions ranging from 0 to 5 M for 20 min at room temperature. Plates were washed four times in PBS-T and detection of bound serum antibody followed as described above. Relative avidity index is defined as the concentration of sodium thiocyanate required to decrease the optical density of each sample with 0 M sodium thiocyanate by 50%.
**Lethal toxin biological assay**

The lethal toxin assay was modified from a previously described method \[11\]. The murine macrophage-like cell line RAW 264.7 obtained from ATCC (Manassas, VA) were maintained in culture at 37°C in a 5% CO₂ humidity using high glucose DMEM medium (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated FBS (Valley Biomedical, Winchester, VA), 100 IU/ml penicillin and 10 μg/ml streptomycin (Mediatech, Herndon, VA). For the lethal toxin assay, RAW cells were transferred to a 96 well plate (100,000 cells per well) grown to approximately 90% confluency in 100 μL medium over two days. Treatments (released PA, PA-loaded nanoparticles, medium only control, PA control, LF control, cell lysis buffer control) were added to the cells with 0.3 μg/mL LF for 6 h unless otherwise stated. The amount of released protein was calculated from the release curve in Figure 6.1. This assay was also utilized to investigate antibody neutralization as well as shelf life of the nanoparticle vaccines. For antibody neutralization assessment, sera from the different immunized mouse groups was added to the RAWs along with PA (0.8 μg/mL) and LF (0.3 μg/mL). The samples were then allowed to incubate for 6 h after which the MTT assay was used to determine cell viability. Optical density (OD) was measured at 570 and 690 nm. Antibody neutralization was considered significant when OD values measured 3X higher than the saline only control. Data is presented as % residual activity, as determined by Equation 1:

\[
\text{% residual activity} = \frac{\text{OD}_{\text{sample}} - \text{OD}_{\text{neg control}}}{\text{OD}_{\text{pos control}} - \text{OD}_{\text{neg control}}} \quad \text{Eq. 1}
\]
In vitro release of PA for analysis of activity, antigenicity and structure

Groups tested for their effect on PA stability were nanoparticles of 20:80 CPTEG:CPH, 50:50 CPTEG:CPH, 20:80 CPH:SA, and 50:50 CPH:SA, PA (positive control stored at -20 °C), and nanoparticle fabrication conditions (NFC) which included sonication, solvent exposure (methylene chloride and pentane), and vacuum drying. All groups were sonicated and placed in microcentrifuge tubes with 0.1 mM PBS buffer (pH 7.6) in a shaker/incubator at 37 °C. PA was released from the nanoparticles for two days to accumulate enough protein for characterization. Following the release, sample supernatants were removed, PA concentration quantified with the micro-BCA assay, concentrations adjusted uniformly to 60 μg/mL and analyzed for PA antigenicity, activity, and structure alterations.

Enzyme-linked immunosorbent assay (ELISA): PA antigenicity

High binding 96 well plates were coated with 0.5 μg/mL of released PA overnight at 4 °C and incubated with serum collected from mice immunized with released PA from 50:50 CPTEG:CPH nanoparticles at a dilution of 1:1,000. The detection of antigenic PA antibody was carried out using an ELISA as described above.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE): primary structure

SDS PAGE was used to assess changes in the protein primary structure. Each protein sample was mixed 1:1 (v/v) with a loading buffer (10% w/v SDS, 1M Tris-HCl (pH 6.8), 3 mM glycerol, 0.01% w/v bromophenol blue, and 0.05% v/v β-mercaptoethanol) and heated for 5 min at 95 °C. The samples were resolved through a 12% polyacrylamide precast gel (Tris-HCl; Bio-Rad, Richmond CA) using a constant voltage of 140 V. The gels were removed,
incubated in a fixative solution (40% ethanol and 10% acetic acid) for 3 h, stained with flamingo fluorescent gel stain overnight, and then scanned for image analysis (Typhoon™ Variable Mode Imager 9400, GE Healthcare, Piscataway, NJ). All experiments were performed in replicates of four.

Circular dichroism: secondary structure

Far UV circular dichroism (CD) (190–250 nm) was used to study the changes in protein secondary structure. All CD spectra were collected for samples on a Jasco J-170 Spectropolarimeter (Easton, MD). The emission spectrum from 200-260 nm was analyzed for shifts in molar ellipticity and wavelength, which are indicative of alterations in protein secondary structure. The experiments were performed in replicates of four.

Fluorescence spectroscopy: tertiary structure

Fluorescence spectroscopy was used to study the changes in protein tertiary structure with a Cary Eclipse fluorescence spectrometer (Varian, Inc., Palo Alto, CA). The samples were excited at a wavelength of 280 nm and a voltage of 800 V, with a resulting emission spectrum that corresponds to the tryptophan and tyrosine residues of the protein. The emission spectrum from 300-500 nm was analyzed for shifts in peak intensity and wavelength, which are indicative of alterations in protein tertiary structure. The experiments were performed in replicates of four.

Shelf life stability

The storage of PA was studied while loaded into the nanoparticles (dry) (labeled by polymer
chemistry), dissolved in PBS buffer (labeled “PBS”), and adsorbed to alum (labeled “Alum”). All groups were stored under desiccant at four different temperatures, 40, 25, 4, and -20 ºC, for two months and tested for their activity at incremental time points. Following the storage time the groups were tested for their ability to preserve PA activity using the LT assay as described before [11]. In these studies, 125 μg/mL of PA-loaded nanoparticles was utilized because a 6 h release (assay incubation time) resulted in ~0.1 μg/mL PA (equivalent to the PA control concentration).

Statistical analysis

Statistical analysis was carried out using a one-way model ANOVA with JMP® 7 (Cary, NC) to account for comparison-wise error since three treatments were studied in parallel. Comparisons between treatments were made with Tukey’s HSD to determine statistical significance (data from Figures 6.3 and 6.5 was log transformed for statistical analysis).

6.4 Results

Polymer and nanoparticle characterization

Polymer characterization by 1H NMR and GPC indicates that the polymer molecular weights were within the desired ranges of 9,000-15,000 Da, which agrees with previous work [1, 12, 29]. The structure and composition of the polymers was also confirmed using NMR and the results were as expected. SEM images (data not shown) indicated that protein-loaded and blank nanoparticles resulted in similar size (~212 ± 43 nm) and morphology, consistent with other work [20-22, 32, 34]. Particle yield was 80%. 
In vitro PA release and pH of nanoparticle degradation are dictated by chemistry

Release of PA and change in pH due to nanoparticle degradation was studied for two months and one month, respectively. We observed chemistry-dependent degradation kinetics, which controlled the release of the encapsulated protein as well as the buffer acidification. Increasing the content of SA or CPTEG resulted in a more rapid release of encapsulated PA. In contrast, the CPH-rich chemistries provided a more long-term PA release profile as shown in Figure 6.1A. This is in agreement with release kinetics of several other proteins from polyanhydride nanoparticles, which demonstrate chemistry-dependent release profiles \[2, 5-7, 15, 21, 28, 38\]. The effect of the polymer chemistry is also evident in the pH profile of the degrading nanoparticles, as shown in 6.1B. The acidic monomers produced from the degradation of CPH:SA nanoparticles have been shown to be water soluble \[7\] and result in a greater decrease in pH (\(\Delta p\text{H} = 2.5\)) as compared with that of the CPTEG:CPH monomers (\(\Delta p\text{H} = 0.6\)). Our results are consistent with previous findings \[7, 28\].

In vivo immunization with PA released from amphiphilic CPTEG:CPH nanoparticles results in high titer, high avidity, neutralizing antibody

In order to determine the immunogenicity of the released protein, mice were immunized with PA released from the nanoparticles, adsorbed to alum. Antibody response to the released protein was characterized in terms of titer, avidity, and neutralizing ability. The antibody titers (Figure 6.2A), relative avidity (Figure 6.2B), and neutralizing antibody titers (Figure 6.2C) indicate that PA released from CPH:SA nanoparticles produced a weak humoral response, while that released from the CPTEG:CPH nanoparticles resulted in the induction of high titer antibody. This indicates that the amphiphilic CPTEG:CPH nanoparticles preserved
the immunogenicity of the released PA. While the overall and neutralizing antibody titers for PA released from CPTEG:CPH nanoparticles remained indistinguishable from the positive control (i.e., PA), the antibody avidity was notably less.

Protein activity and structural stability was preserved by amphiphilic CPTEG:CPH nanoparticles

The stability of PA released from the nanoparticles was evaluated and is summarized in Figure 6.3. Specifically, Figure 6.3A and Figure 6.3B depict PA antigenicity and activity, respectively. Our analysis revealed that PA released from amphiphilic 50:50 CPTEG:CPH nanoparticles retained almost 80% of its functional activity and antigenicity. PA released from 20:80 CPTEG:CPH nanoparticles retained only 52% of its activity and antigenicity while CPH:SA-containing nanoparticles preserved very little PA activity and antigenicity. Nanoparticle fabrication conditions had little to no effect on the antigenicity or activity of PA (Figure 6.3).

To better understand the loss in antigenicity and activity, a detailed structural evaluation of PA was carried out as shown in Figure 6.4. The primary structure of PA from SDS-PAGE studies (Figure 6.4A) appears to be altered for all conditions except for the positive control (PA) and NFC. The primary band for PA appears at 83 kDa, which can be clearly identified in the PA control lane (Figure 6.4A). While this main band at 83 kDa was observed for PA released from both CPTEG:CPH chemistries, the band was significantly weaker in intensity. In contrast, no bands were observed for PA released from the CPH:SA nanoparticles. Since no high molecular weight bands were observed in the gel image, it is hypothesized that the
PA underwent degradation rather than aggregation.

The CD spectra for PA showed a minimum at approximately 208-210 nm and a shoulder at 215-217 nm (Figure 6.4B). This is consistent with PA secondary structure from CD spectra reported previously and indicates that PA consists mainly of a mixture of α-helices and β-sheets \[^{[11, 24]}\]. Figure 6.4B reveals that only the NFC and 50:50 CPTEG:CPH nanoparticles were able to preserve the secondary structure. All other conditions (20:80 CPTEG:CPH, 20:80 CPH:SA, and 50:50 CPH:SA) resulted in a significant increase in molar ellipticity (Δε), signifying a loss in secondary structure.

Tertiary structure analysis, performed with intrinsic fluorescence spectroscopy, displayed a red shift in peak position for the PA released from all nanoparticle formulations (Figure 6.4C). This peak shift indicates exposure to a more polar environment indicative of protein unfolding \[^{[11]}\]. However, PA peak intensity was best preserved by the amphiphilic 50:50 CPTEG:CPH nanoparticles and to a lesser extent by 20:80 CPTEG:CPH nanoparticles. The nanoparticle fabrication conditions appeared to have no detrimental effects on the tertiary structure of PA.

*PA functionality upon release from nanoparticles is maintained in all chemistries in a dose and time dependent manner*

An *in vitro* cytotoxicity assay was employed to assess the ability of PA-loaded nanoparticles to preserve the biological function of PA directly after its release. Moreover, this functional assay eliminated the effects of any structural changes that may have occurred to the PA
during the two-day release experiment. Six h of co-culture with the RAW264.7 cells and 125 μg/mL of PA-loaded nanoparticles was determined to provide a concentration of released PA equivalent to that of the positive control (i.e., only PA). Because there was no statistical difference in the amount of PA released between the different nanoparticle chemistries in the first 6 h (as shown in Figure 6.1), direct comparisons between the various groups can be made. Nanoparticles released PA in a time- and nanoparticle concentration-dependent manner (Figure 6.5). More importantly, nanoparticle chemistry clearly influenced the biological activity of PA. Specifically, amphiphilic 50:50 CPTEG:CPH nanoparticles released fully functional PA after only 4 h of incubation at 62.5 μg/mL. Although 50:50 CPTEG:CPH nanoparticles appeared to have a superior ability to preserve PA activity (Figure 6.5), all other nanoparticle chemistries released PA that possessed a similar level of activity as the positive control when incubated for 6 h at a concentration of 125 μg/mL (Figure 6.5C).

*Shelf life stability of PA encapsulated into 50:50 CPTEG:CPH, 20:80 CPTEG:CPH and 20:80 CPH:SA is superior to that of PA adsorbed to alum*

The ability of a PA-containing vaccine to retain full activity upon storage remains a primary concern. Therefore, we investigated the shelf life storage capabilities of PA loaded into polyanhydride nanoparticles (dry), PA dissolved in PBS buffer (pH 7.6), and PA adsorbed to alum and then stored at 40, 25, 4, and -20 °C. It is known that adjuvant solutions containing alum are acidic (pH = 5.6) \(^{[37]}\), so the PBS buffer group was included to investigate the effect of storage temperature at a neutral pH on the stability of PA. After one week of storage at 40 °C, the biological activity of PA adsorbed to alum and PA stored in PBS buffer was
completely lost (Supplementary Figure 6.1). Following two weeks of storage at 25 °C, PA adsorbed to alum lost half of its activity, and after one month, it lost all of its activity (Supplementary Figure 6.1). After two months of storage at 25 and 40 °C, only PA released from 50:50 CPTEG:CPH and 20:80 CPH:SA nanoparticles was capable of maintaining almost 100% of its biological activity (Figure 6.6). PA adsorbed to alum lost all its functionality when tested after two months of storage (Supplementary Figure 6.1). Nanoparticles made of 20:80 CPTEG:CPH nanoparticles were capable of preserving 100% of PA activity under cold storage and partial activity at the two elevated temperatures. In contrast, nanoparticles made of 50:50 CPH:SA were ineffective at stabilizing PA under any storage temperatures above freezing.

6.5 Discussion

Next-generation anthrax vaccines need to possess several attributes, including storage stability and reduced immunization frequency. In this work, we have demonstrated that polyanhydride nanoparticle-based controlled delivery systems can provide these attributes. In particular, our studies have shown that amphiphilic polyanhydride nanoparticles provide controlled antigen release over several weeks, high titer total and neutralizing antibody production, and extended shelf lives at tropical temperatures.

Long term, repeated antigen exposure (depot effect) is essential for the development of immunological memory, which is why the AVA vaccine regimen consists of five immunizations with yearly boosters [31]. In the event of a bioterrorism attack, this long-term vaccination strategy is not a viable option; thus there is a need for a vaccine capable of
mimicking the current immunization regimen in a single dose. Such a single dose vaccine will also improve patient compliance. The nanoparticle-based delivery system demonstrated long term release kinetics for over two months, dictated by polymer chemistry, as shown in Figure 6.1A. These long-term release kinetics have also been demonstrated in vivo\textsuperscript{19}.

While controlled release kinetics enable long-term antigen exposure, it is essential that the antigen remain intact during fabrication, long-term storage, and release, in order to generate a protective immune response. The current vaccine adjuvant approved for human use in the U.S., alum, is known to produce an unstable acidic micro-environment for pH sensitive proteins\textsuperscript{37}. While the adsorbed antigen can be better protected by the less acidic microenvironment pH on the surface of alum\textsuperscript{37}, any antigen in the bulk could be rendered inactive (i.e., pH sensitive antigens such as PA) due to the increased acidic environment (pH $\sim$5.6). In addition to pH sensitivity, PA has also demonstrated sensitivity to increased temperature; an effect that is exacerbated as the conditions become more acidic\textsuperscript{11}. In this work, we demonstrated the superior PA stabilization capabilities of a nanoparticle-based delivery system that preserved the biological activity of PA during fabrication, delivery, and storage, overcoming several of the problems associated with current vaccine strategies.

Nanoparticle fabrication conditions (solvent exposure, sonication, and vacuum drying) had no effect on the structure, antigenicity, or activity of PA. This renders the nanoparticle fabrication process safe for PA and is in agreement with similar results for other proteins exposed to these conditions\textsuperscript{20}. The pH and temperature, however, has been reported to be a more influential factor on the stability of PA\textsuperscript{4,11,24,26,27}. As shown in Figure 6.1B, the pH
of CPTEG:CPH nanoparticles only drops to approximately 7.0 and is maintained at that value for over 30 days; on the other hand, the pH of CPH:SA nanoparticles decreases to 5.25, which is similar to alum, within the first few days of PA release. This acidic microenvironment caused by the CPH:SA nanoparticle degradation products can be correlated with the losses in antigenicity and activity (Figure 6.3) and structure (Figure 6.4) of PA after release from these nanoparticles. This is also reflected in the low levels of total and neutralizing antibody titers and low antibody avidity observed in Figure 6.2. Detailed structural analysis studies demonstrated that significant alterations in the primary, secondary and tertiary structure of PA are important determinants of the loss in antigenicity, activity and antibody production (Figure 6.5). Consistent with these results, it has been reported that thermally-induced ellipticity changes in PA occurred in acidic environments \[4, 11, 26, 27\] and PA under low pH and high temperature conditions is structurally modified to a molten globular state \[11\]. As observed in this work, this modified state is rendered inactive and non-immunogenic.

Amphiphilic CPTEG:CPH nanoparticles, specifically 50:50 CPTEG:CPH, provided an effective stabilizing environment for PA. Nearly 80% of PA antigenicity and activity was preserved upon release from 50:50 CPTEG:CPH (Figure 6.3). The total and neutralizing antibody titers for PA released from 50:50 CPTEG:CPH nanoparticles were indistinguishable from the positive control (Figure 6.2). From the detailed structural analysis, it is likely that the alterations in the tertiary structure of PA released from 50:50 CPTEG:CPH nanoparticles (Figure 6.4C) may be responsible for the slight loss in antibody avidity (Figure 6.2) and antigenicity and activity (Figure 6.3). These alterations are likely a result of the slight
decrease in pH at 37 °C as reported by Jiang et al.\textsuperscript{[11]} When administered \textit{in vivo} (i.e. intranasally), this acidic microenvironment would be readily eliminated, thereby reducing pH-dependent alterations of PA. Based upon these results and previous \textit{in vivo} protection reports \textsuperscript{[32]}, we hypothesize that when PA-loaded nanoparticles are injected \textit{in vivo} they will adjuvant the immune response by providing an antigen depot to enable the development of high avidity antibody and long term immune memory.

Successful vaccine formulations provide a sustained release of functional protein over time, while limiting the number of doses needed. All nanoparticle chemistries revealed dose- and time-dependent release of functional PA (Figure 6.5), suggesting that they are viable candidates for releasing functional PA in a vaccine formulation. However, the amphiphilic 50:50 CPTEG:CPH once again outperformed the other formulations, demonstrating the ability to best preserve the function of PA.

One of the most difficult challenges to overcome in vaccine design and development is long-term storage capabilities. This is an immediate concern in developing countries with limited cold storage. In this work, we tested storage of dry PA-loaded nanoparticles over two months at elevated temperatures. All nanoparticle chemistries, except for 50:50 CPH:SA, outperformed alum in preserving the stability of PA at 40, 25, and 4 °C (Figure 6.6). Since the build-up of acidic degradation products leading to a decrease in pH was largely eliminated from these studies, it is hypothesized that the hydrophobic 50:50 CPH:SA nanoparticle chemistry induced hydrophobic interactions with PA, leading to non-covalent aggregation. These same formulations also proved to be superior to PA stored in PBS buffer.
at 40 °C (Figure 6.6). The changes observed with PA stored with alum are hypothesized to be a result of the low pH environment combined with the storage temperature. In contrast, PA stored in PBS buffer at a neutral pH is only likely to be affected by the elevated temperature. The 50:50 CPTEG:CPH and 20:80 CPH:SA nanoparticles revealed surprising similar capabilities to preserve PA at 40 and 25 °C for the entirety of our study, demonstrating enhanced protein stabilization as compared to lyophilized PA stored with stabilizers after only one month \[^{11}\]. The ability of 20:80 CPH:SA nanoparticles to stabilize PA was surprising based upon the lack of a robust antibody response following immunization with PA released from this formulation. However, it is to be noted that the time course of this study was short, thereby reducing the buildup of acidic degradation products that could negatively affect stability.

6.6 Conclusions

Together, the studies reported herein demonstrate the potential use of the polyanhydride nanoparticle platform in next-generation anthrax vaccines. These nanoparticles provide the ability to control protein release kinetics, which would provide an \textit{in vivo} antigen depot capable of long-term antigen presentation. Additionally, the particles are capable of preserving PA activity upon fabrication, storage at elevated temperatures, and after release. Of all the formulations tested, amphiphilic 50:50 CPTEG:CPH nanoparticles demonstrated the best combination of characteristics compatible for PA preservation and release, making it an ideal candidate for a future, single-dose anthrax vaccine. We are currently investigating formulations based on these particles for protective capability upon a lethal challenge.
6.7 Acknowledgments

This material is based upon work supported by the National Institutes of Health, grant R03 AI076855-01A1.

6.8 References


6.9 List of Figures

![Figure 6.1: PA is released from polyanhydride nanoparticles in a chemistry dependent manner with 20:80 CPH:SA and 50:50 CPTEG:CPH releasing the fastest. A) Release of PA from polyanhydride nanoparticles over 60 days and B) the change in pH of PBS buffer (pH 7.6, 0.1 mM) from nanoparticle degradation over 30 days. Error bars represent standard deviation of 3 independent experiments.](image-url)
Figure 6.2: Mice immunized with PA released from 50:50 and 20:80 CPTEG:CPH, adjuvanted with alum, developed the highest antibody titers, the most avid antibody response, and highest neutralizing antibody titers. A) Antibody titers, B) antibody avidity, and C) antibody neutralization titers from immunized mice. Data is representative of pooled samples of 3 replicates for each treatment.
Figure 6.3: CPTEG:CPH nanoparticle chemistries release actively and antigenically intact PA. A) Residual antigenicity and B) residual activity of PA after a 2 day release from polyanhydride nanoparticles. The direct ELISA was performed with 0.5 μg/mL PA released from the nanoparticles. The biological assay was performed by incubating 6.4 μg/mL PA released from polyanhydride nanoparticles with 0.3 μg/mL LF with RAW cells for 6 h. Error bars represent standard deviation of 3 replicates performed in A) 3 independent and B) 1 independent experiment(s). Treatments with different letters are significantly different from one another at p < 0.0003.
Figure 6.4: 50:50 CPTEG:CPH best preserves the structural integrity of PA while CPH:SA nanoparticle chemistries cause a loss in primary, secondary and tertiary structural integrity. A) SDS-PAGE analysis of released PA, B) secondary structure as measured by circular dichroism (CD), and C) tertiary structure as measured by fluorescence spectroscopy after a 2 day release from polyanhydride nanoparticles. Data is representative of 4 independent experiments.
Figure 6.5: PA-loaded polyanhydride nanoparticles are capable of releasing fully functional PA in a dose and time dependent manner dictated by polymer release kinetics and stability of PA. A) 31.25, B) 62.5, C) 125, and D) 250 μg/mL polyanhydride nanoparticles incubated for 2, 4, or 6 h with LF (0.3 μg/mL) for the biological toxicity assay. Error bars represent standard deviation and of 4 replicates performed in 2 independent experiments. Treatments within each time point with different letters are significantly different from one another at p < 0.048.
Figure 6.6: PA-loaded polyanhydride nanoparticles are capable of preserving the activity of PA for 2 months when stored at 40, 25, 4, and -20 °C. Nanoparticle made of 50:50 CPTEG:CPH and 20:80 CPH:SA preserved full functionality of PA under all conditions tested. The biological assay was performed by incubating 125 μg/mL nanoparticles with 0.3 μg/mL LF with RAW cells for 6 h. Error bars represent standard deviation of 3 replicates performed in 1 independent experiment. Treatments with different letters are significantly different from one another at p < 0.039.
Supplementary Figure 6.1: PA activity is sensitive to high temperature when stored adsorbed to alum A) or in PBS buffer B) over incremental time points (7, 14, 28, and 58 days). The biological assay was performed by incubating the storage groups (at a concentration of 0.1 μg/mL PA) with 0.3 μg/mL LF and RAW cells for 6 h. Error bars represent standard deviation of 3 replicates performed in 1 independent experiment. Treatments with different letters are significantly different from one another at p < 0.048.
Chapter 7: High Throughput Cell-based Screening of Biodegradable Polyanhydride Libraries

A paper published in Combinatorial Chemistry and High Throughput Screening 2009, 12 (7), 634-645

Andrew F. Adler\textsuperscript{a,b,+}, Latrisha K. Petersen\textsuperscript{a,+}, Jennifer H. Wilson\textsuperscript{c}, Maria P. Torres\textsuperscript{a}, Jon B. Thorstenson\textsuperscript{a}, Stuart W. Gardner\textsuperscript{d}, Surya K. Mallapragada\textsuperscript{a}, Michael J. Wannemuehler\textsuperscript{c}, and Balaji Narasimhan\textsuperscript{a,*}

\textbf{Keywords:} Combinatorial, high throughput, polyanhydrides, cytotoxicity, polymer films

\textsuperscript{a}Department of Chemical and Biological Engineering, Iowa State University, 2035 Sweeney Hall, Ames, IA 50011-2230
\textsuperscript{b}Department of Biomedical Engineering, Northwestern University, 2145 Sheridan Road, Evanston, IL 60208-3107
\textsuperscript{c}Department of Veterinary Microbiology and Preventive Medicine, Iowa State University, Ames, IA 50011
\textsuperscript{d}Department of Statistics, Iowa State University, Ames, IA 50011
\textsuperscript{+}Both these authors contributed equally to this work
\textsuperscript{*}To whom all correspondence should be addressed
7.1 Abstract

A parallel screening method has been developed to rapidly evaluate discrete library substrates of biomaterials using cell-based assays. The biomaterials used in these studies were surface-erodible polyanhydrides based on sebacic acid (SA), 1,6-bis(p-carboxyphenoxy)hexane (CPH), and 1,8-bis(p-carboxyphenoxy)-3,6-dioxaoctane (CPTEG) that have been previously studied as carriers for drugs, proteins, and vaccines. Linearly varying compositional libraries of 25 different polyanhydride random copolymers (based on CPH:SA and CPTEG:CPH) were designed, fabricated, and synthesized using discrete (organic solvent-resistant) multi-sample substrates created using a novel rapid prototyping method. The combinatorial libraries were characterized at high throughput using infrared microscopy and validated using $^1$H NMR and size exclusion chromatography. The discrete libraries were rapidly screened for biocompatibility using standard SP2/0 myeloma, CHO and L929 fibroblasts, and J774 macrophage cell lines. At a concentration of 2.8 mg/mL, there was no appreciable cytotoxic effect on any of the four cell lines evaluated by any of the CPH:SA or CPTEG:CPH compositions. Further, the activation of J774 macrophages was evaluated by incubating the cells with the polyanhydride libraries and quantifying the secreted cytokines (IL-6, IL-10, IL-12, and TNF-α). The results indicated that copolymer compositions containing at least 50% CPH induced elevated amounts of TNF-α. In summary, the results indicated that the methodologies described herein are amenable to the high throughput analysis of synthesized biomaterials and will facilitate the rapid and rational design of materials for use in biomedical applications.
7.2 Introduction

Biodegradable polymers have been used in biomedical applications ranging from drug delivery \[1\], to sutures, to bio-absorbable prostheses \[2\], to tissue culture scaffolding \[3\], and stents. Their use in a broad range of applications stems largely from their uniquely versatile and tunable degradation behavior. In particular polyanhydrides have been widely used as carriers for drugs, proteins, and antigens. The Gliadel® wafer, a U.S. FDA approved polyanhydride based drug delivery system, has been used in the treatment of brain tumors. Their history of positive impact for human use \[4\] along with their chemical diversity makes them ideal candidates for protein and vaccine delivery. Today, many candidate drugs are proteins, which are more susceptible to various forms of deactivation due to their chemical and physical environment \[5-7\]. To ensure protein stability and functionality, it is highly desirable to provide stable microenvironments for these protein-based drugs during storage and \textit{in vivo} delivery. Due to their versatile chemistries and degradation rates, polyanhydrides are excellent candidates for protein stabilization and delivery.

Previous reports from this laboratory \[5, 8, 9\] have indicated that multiple proteins and antigens can be stably encapsulated into and released from micro/nanospheres composed of random copolymers of sebacic acid (SA) (Figure 7.1 a), 1,6-bis-(\(p\)-carboxyphenoxy)hexane (CPH) (Figure 7.1 b), and 1,8-bis-(\(p\)-carboxyphenoxy)-3,6-dioxaoctane (CPTEG) (Figure 7.1 c). Specifically, encapsulation of model proteins into these polyanhydrides preserves the primary and secondary structure of proteins \[5\] and maintains immunogenicity of biologically active antigens \[8,10\]. CPH:SA devices degrade by surface erosion, rather than the bulk erosion seen in polyesters. Surface-eroding devices are able to exclude water from the
interior during degradation, offering more protection for sensitive payloads \cite{11,12}. This unique feature has also been observed \textit{in vivo}, favoring the use of polyanhydrides in human medicine \cite{5,13,14}. Additionally, CPTEG:CPH copolymers can be tailored to degrade from bulk-eroding to surface-eroding mechanisms, thereby enabling highly tunable release kinetics for controlled drug delivery \cite{15}. Compared to polyesters, the degradation products of polyanhydrides are less soluble di-carboxylic acids, resulting in a less acidic micro-environment, which enhances the long-term stability of encapsulated proteins \cite{11}.

Polyanhydrides have also been used as adjuvants in vaccine delivery devices \cite{8,16}. A persistent, near zero-order drug release can be obtained, and the polyanhydride chemistry can be used to control the immune response to an antigen. Prolonged presentation of an active antigen, as afforded by the polyanhydrides' protection and controllable degradation, results in greater balance between the humoral and cell-mediated responses compared to bolus delivery of the antigen \cite{8,17}. Humoral or Th2 response is accompanied by antibody production, while cell-mediated, or Th1 response, is characterized by macrophage and cytotoxic T cell maturation and activity \cite{18}. Such a balanced response is known to play an important role in the achievement of protective immunity against many intra-cellular pathogens.

Modulation of the release of encapsulated substances is a critically important function of these polymeric devices, and is credited to the unique chemistry and kinetics of polyanhydride degradation in aqueous environment. These polymers have been shown to degrade via hydrolysis to their acidic monomers \cite{13,19} over a period of years for poly(CPH),
weeks for poly(SA) \[^{20,21}\] and days for poly(CPTEG) \[^{15}\]. These degradation times can be modulated by synthesizing copolymers with various combinations of monomers as dictated by application. Less hydrophobic systems (CPTEG and SA rich) tend to degrade more quickly than more hydrophobic systems (CPH rich) \[^{1,15,22-24}\]. Initial burst release from microspheres can also be achieved for certain CPH:SA compositions, and release kinetics can be additionally modified with altered microsphere size \[^{10}\]. Simple changes such as the number of glycolic oxygens in the polymer’s backbone result in similar differences \[^{15,24}\].

Thus, choosing the right polymer chemistry for an application requires navigation of a large parameter space (composition, concentration, synthesis conditions, etc.), which if approached with classical macroscale, single-synthesis methods would require unacceptable amounts of time and cost. Thus, there is a significant need to develop high throughput combinatorial methods aimed to satisfy the demand for rapid discovery without needlessly sacrificing accuracy, time, or cost.

Combinatorial discovery was thrust into the limelight with application in the pharmaceutical industry \[^{2,25}\]. Since then, there has been much interest in the development of combinatorial methods for materials science \[^{2}\]. Recently, the hurdles of expense \[^{26,27}\], characterization \[^{25}\], and availability \[^{28}\] are being overcome; wide accessibility greatly increases combinatorial methods’ efficacy in allowing more work to be done in parallel. Past combinatorial work in biodegradable polymers has focused largely on characterization of physical properties, such as glass transition temperature \[^{29}\] and elastic modulus \[^{30}\], and drug delivery \[^{28}\]. Cell-based combinatorial methods have also been used to study phenomena such as cell adhesion \[^{31}\] and protein adsorption \[^{32}\]. However to our knowledge, none of the past studies present a rapid
prototyping, parallel method for combinatorial synthesis and in vitro investigation of cellular interactions. When cells come into contact with biodegradable polymers in vivo, initial interactions and activation can be triggered by surface chemistry and there is a need for the design of in vitro tests that will allow for both combinatorial synthesis of biomaterials and robust cell based assays to assess the initial cell-biomaterial interaction.

The present work describes the development of a new photolithographic method to design biomaterial libraries for rapid screening by cell-based assays. The speed, low cost, ease of use, and reproducibility of this method allowed for efficient cytotoxic screening and assessment of immune cell activation by discrete libraries of compositionally and concentrationally variant CPH:SA or CPTEG:CPH copolymers. Utilization of this method will allow for flexible and rapid characterization of cell-biomaterial interactions prior to the in vivo studies required for human or animal use [32].

7.3 Materials and Methods

Materials

HPLC Grade Chloroform, 100 mm plastic Petri dishes, and 500 x 750 x 1mm pre-cleaned glass microscope slides were acquired from Fisher Scientific (Fairlawn, NJ). 200 proof ethanol was purchased from Chemistry Stores (Ames, IA). Norland Optical Adhesive 81 (NOA 81) was purchased from Norland Products (Cranbury, NJ). Sebacic acid and 1,6-bis-(p-carboxyphenoxy)hexane prepolymers were prepared with slight modification to well known syntheses [1]. CPTEG diacid was prepared as described previously [15]. Deuterated NMR solvents (DMSO and chloroform) were purchased from Cambridge Isotope
Laboratories (Andover, MA). MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assays for cytotoxicity screening were obtained from Sigma Aldrich (St. Louis, MO). Sp2/0 mouse myeloma cells were obtained from stock cultures maintained by Iowa State University’s Hybridoma Facility, J774A (J774) mouse macrophage cell line was a gift from Dr. Jesse Hostetter of the Department of Veterinary Pathology at ISU and Chinese Hamster Ovary (CHO) cells were a gift from Michael Kimbler (Department of Biomedical Sciences at Iowa State University). L929 cells were purchased from American Type Culture Collection (Manassas, VA).

Multi-well library fabrication

Rapid prototyping was used to fabricate discrete solvent-resistant multi-well substrates capable of containing large enough fluidic volumes to support cell proliferation (Figure 7.2). This procedure is an adaptation of existing photolithographic techniques utilizing frontal-polymerizing optical resists described elsewhere. Major modifications were made to enable ease of use, speed, durability, reproducibility and cost. The method uses a thiolene-based, UV curing optical adhesive (NOA 81). Mechanistically, this involves the addition of a thyl radical to a vinyl functional group, followed by radical transfer from the ensuing carbon radical to a thiol functional group. The extremely fast curing in the presence of oxygen using UV light, without the need for traditionally required photoinitiators means that fairly complex structures can be prototyped very easily and rapidly using a simple mask. In this work, the desired 5x5 array of discrete wells was created using a photomask designed with a readily available drawing program and printed on a commercially available laser printer. The mask was then copied as many times as desired onto transparencies with a
photocopier. Four of the transparencies were overlaid (to ensure complete opacity) and affixed to a standard glass microscope slide with tape. Wells were given lateral dimensions of 5 x 5 mm, which when combined with a wall height of 2 mm produced space for liquid volumes of 200 μL. To our knowledge, this represents a much greater well capacity than in any previous work.

Spacers were taped to regions of a second glass slide which fall outside the desired area of structure formation and served as an upper limit to feature height. The corners of the glass slides were removed with a glass cutter to allow them to fit within standard Petri dishes. Aluminum foil was cut into circles, flattened and laid into standard disposable Petri dish lids. The layer of aluminum served as a surface which could be easily removed from the polymerized NOA 81. Previous work utilized a polydimethylsiloxane release layer (PDMS Sylgard 184 Base and PDMS Sylgard 184 Cure (Dow Corning, Midland, MI)) \[28, 33, 34, 36\] that requires 12 h to cure, is more expensive, and produced irregular and rounded features due to its uneven surface. NOA 81 was then poured slowly onto the flat aluminum foil to a depth slightly less than the height of the spacers. The slide with spacers was then slowly laid down onto the NOA 81 layer, allowing capillary action to wet only the lower glass surface smoothly and with minimal bubble formation. The stack was then exposed to a collimated long-wave UV source at an intensity of 10 mW/cm² for 7 min. In order to provide increased resistance to the chlorinated solvents \[39\], high temperature, and high vacuum the substrate must endure during polyanhydride synthesis, it was necessary to polymerize a thin layer of NOA 81 in the bottom of each well. This was achieved by removing the photomask and exposing the naked substrate to 10 mW/cm² intensity UV light for 5 seconds. After precure,
the aluminum foil layer was carefully peeled away from the structure and any unreacted NOA 81 remaining in the wells was removed with a blast of compressed air. Liberal amounts of ethanol from a spray bottle helped to more sharply define the features. After the ethanol evaporated, the substrates were postcured under UV light for 17 min at 10 mW/cm$^2$. Finally, the substrates were thermally cured at 80°C for 12 h.

*Prepolymer solution deposition*

Libraries of varying concentration or mole fraction of CPH:SA and CPTEG:CPH copolymers were rapidly deposited using robotics. Two programmable syringe pumps (New Era Pump Systems, Farmingdale, NY) in conjunction with three programmable motorized stages arranged orthogonally (Zaber Technologies, Richmond, British Columbia, Canada) served to fully automate depositions. The pumps and syringes were controlled by third-party macro software operating on the actuators’ respective consoles. Complete 5 x 5 depositions were routinely completed in approximately 5 minutes.

*Combinatorial synthesis of polyanhydrides*

CPH:SA and CPTEG:CPH copolymer films were synthesized in the discrete well substrate from their corresponding prepolymers. CPH and SA prepolymers were dissolved in chloroform and deposited into the wells in various molar ratios. CPTEG:CPH prepolymers were dissolved in acetic anhydride and deposited in the same manner. With both systems the libraries were placed in a vacuum oven preheated to the necessary temperature (180°C for CPH:SA and 140°C for CPTEG:CPH) and 0.3 torr vacuum for the polycondensation reaction to occur. After synthesis, the polymer libraries were stored in desiccators.
Characterization

Copolymer structures were characterized by $^1$H NMR in deuterated chloroform on a Varian VXR 300 MHz spectrometer (Palo Alto, CA). Molecular weights were measured by gel permeation chromatography (GPC). GPC samples were dissolved in HPLC-grade chloroform and separated using PL Gel columns from Polymer Laboratories (Amherst, MA) on a Waters GPC system (Milford, MA). 50 μL samples were eluted at 1 mL/min. Elution times were compared to monodisperse polystyrene standards (Fluka, Milwaukee, WI) and used to determine number averaged molecular weights ($M_n$), and polydispersity indices. Fourier Transform Infrared (FTIR) spectroscopy was conducted on a Nicolet Continuum infrared microscope (Thermo Scientific, Madison, WI) in order to verify the ability of the robotics to accurately deposit linearly varying composition gradients. Two hundred scans were collected for each data point at a resolution of 4 cm$^{-1}$ and nitrogen purge flowrate of 30 SCFH.

High throughput cytotoxicity screening

Cell viability in the presence of CPH:SA and CPTEG:CPH films was assessed using MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Cells were grown to near confluency in either cRPMI (RPMI 1640 containing L-glutamine (Mediatech, Herndon, VA) supplemented with 1% non-essential amino acids (Mediatech), 1% sodium pyruvate (Mediatech), 2% essential amino acids (Mediatech), 25 mM HEPES buffer (Mediatech), 100 units/mL penicillin, 0.1 mg/mL streptomycin (Mediatech), 0.05 mg/mL gentamycin (Gibco), 1% L-glutamine (Mediatech), 5 x 10$^{-5}$ M 2-mercaptoethanol (Sigma), and 10% heat-inactivated fetal bovine serum for Sp2/0 cells or in Dulbecco’s Modified Eagle Medium or Ham’s F12 with 4.5 g/L glucose (Gibco, Invitrogen, Carlsbad, CA) supplemented with 1%
non-essential amino acids (Mediatech), 1% sodium pyruvate (Mediatech), 25 mM HEPES buffer (Mediatech), 100 units/mL penicillin, 0.1 mg/mL streptomycin (Mediatech), 1% L-glutamine (Mediatech), and 10% heat-inactivated fetal bovine serum for J774 and CHO cells. L929 cells were grown to confluency in Dulbecco’s Modified Eagle Medium with 4.5 g/L glucose (Gibco, Invitrogen, Carlsbad, CA) supplemented with 1% non-essential amino acids (Mediatech), 1% sodium pyruvate (Mediatech), 2% Sodium Bicarbonate (10% solution) (Mediatech), 100 units/mL penicillin, 0.1 mg/mL streptomycin (Mediatech), 1% L-glutamine (Mediatech), and 10% heat-inactivated horse serum. Cells were maintained in a humidified incubator set at 37°C, 5% CO2. J774 cells were removed from culture flasks with gentle scraping using sterile cell scrapers (Fisher). CHO and L929 cells were removed from culture flasks by treatment with Trypsin-EDTA solution (Gibco). To generate control wells displaying 100% cytotoxicity, cells were lysed with a cell lysis buffer (Qiagen) containing HCl (1 N), 2% sodium di-lauryl sulfate and 5% Triton 100x (LKB Instruments, Gaithersburg, MD).

The multi-well substrates were incubated in 100 mm Petri dishes atop sterilized gauze pads cut to fit the Petri dish and saturated with sterile phosphate buffered saline (PBS, pH 7.4) in order to create a humid microenvironment and minimize evaporation. 80μL aliquots of 5.0 x 10^5 cells/mL were incubated for 18 to 24 h with the desired polyanhydride films in each well of the multi-well substrates. 10 μL of MTT salt solution was added to each well. Plates were returned to the incubator for 2-3 h. The solution was then transferred to a 96 well polystyrene assay plate, and each NOA 81 well was washed with 80 μL acidic alcohol solution, which was then added to the corresponding well within the assay plates. Optical density (OD) at
570 nm minus the background OD at 690 nm was measured using a Spectramax 190 Plate Reader (Molecular Devices, Sunnyvale, CA). Assays were performed on duplicate plates on three separate occasions for each cell type and polymer composition (CPH:SA or CPTEG:CPH), so in total there were twenty-four assays performed with each composition, six for each cell line. The polyanhydride films were also incubated with the MTT solution alone in the absence of cells. The OD measurements were similar to that of the background OD suggesting that the presence of the polyanhydride copolymer films in the library wells did not interfere with the OD measurements.

**Cytokine secretion from J774 cells**

In order to assess immune activation, cytokine production from J774 macrophages was measured following incubation of the cells in the presence of the discrete polyanhydride libraries in the multi-well substrates. Dissolved polymer was deposited to create 4 replicate wells within the 25-well grid. Three wells were left blank (i.e., no polymer deposited) and two wells were treated with 2 μg/mL of lipopolysaccharide (Phenol water extract from *E. coli*, Sigma) (LPS), which served as a positive control. J774 cells were grown in media as described above to near confluency and removed from the tissue culture flasks using sterile cell scrapers. Cells were washed once by centrifugation and diluted to 1.25 x 10⁶ cells/mL. 100 μL of diluted cells added to each well, as described above. At 48 h, cell free supernatants were collected and stored at -20°C until assayed. Secretion of IL-6, IL-10, IL-12 and TNF-α was analyzed using a multiplexed fluorescence-based assay (FlowMetric System, Luminex, Austin, TX). Assays were performed on duplicate plates with the J774 cell line on at least three separate occasions for each polymer composition. The results for the background (no
polymer) and LPS wells were compared to cells grown in conventional 48 well tissue culture plates.

Statistical analysis

Analysis was performed with the aid of SAS statistical software (SAS Institute, Cary, NC). In the linear mixed models, the Kenwood-Rodgers method was used for calculating degrees of freedom. The cytotoxicity study consisting of varying concentrations (mg/mL) of 50:50 copolymer was fit with a linear model using concentration (mg/mL) as the fixed term. Alternatively, assessment of cytotoxicity based upon changes in polymer composition (%) CPH) was performed using a linear mixed model. The fixed term in the composition analyses was mol % CPH and the plate was assigned as the random term in the mixed model. In the analysis of cytokine secretion, a linear mixed model was used in which the mol % CPH was set as the fixed term and plate and day as random terms in the mixed model.

7.4 Results

Nuclear magnetic resonance (1H NMR)

NMR analysis was performed to ensure that the substrate did not interfere in the parallel synthesis of the copolymer libraries. Polyanhydride libraries with concentrations three times higher than that used in the cytotoxicity assays were used in characterization to produce signals large enough to interpret. Representative spectra and lettered chemical shifts denoted in Figure 7.3 for CPH:SA and Figure 7.4 for CPTEG:CPH are marked as such by comparison to previous work [1, 22]. NMR spectra showed no difference between conventional syntheses methods of similar volumes performed in glass vials and the new combinatorial method
performed in the multi-well substrates. Mole fraction gradients in the CPH:SA and CPTEG:CPH systems produced the expected changes in $^1$H NMR peak area. That is, peaks corresponding to the CPH portion of the copolymer (Peaks a-e in Figure 7.3) became less intense when compared to SA peaks (Peaks f-I in Figure 7.3) as the CPH content was reduced. Similar results were obtained with the CPTEG:CPH system (Figure 7.4). This suggests that the multi-well substrate did in fact isolate discrete copolymer compounds with minimal cross-contamination, and that the deposition robot created a smooth compositional gradient. It is also important to note that the end group peaks in these spectra are relatively small, suggesting that the syntheses did in fact drive off much of the prepolymeric acid groups, producing long chain polymers.

*Molecular weights by gel permeation chromatography (GPC) and $^1$H NMR*

GPC was performed on various CPH:SA copolymers following synthesis in the wells and their molecular weights were compared to that of conventionally synthesized copolymers. As shown in Table 7.1, the copolymers had number-average molecular weights suitable for their processing into delivery devices such as tablets and microspheres, and the average polydispersity index was 2.2. Similar results were obtained with the CPTEG:CPH system. In this case, the molecular weights were determined by end-group analysis using $^1$H NMR spectra (Table 7.2, for example). These values are consistent with previous work on polyanhydrides synthesized by conventional polycondensation, and add further support to the success of the high throughput synthesis of polymers in the discrete wells [10, 28, 40, 41].
Fourier transform infrared (FTIR) spectroscopy

A typical IR spectrum of a CPH:SA copolymer is shown in Figure 7.5. The characteristic peak chosen for CPH is sharply defined at 1605 cm\(^{-1}\) and represents the aromatic ring stretching of the CPH moiety. The feature chosen for poly(SA) is at 1810 cm\(^{-1}\) and represents the carboxyl anti-symmetric stretching of the aliphatic-aliphatic anhydride bond \[^{42}\].

Each of the characteristic peaks was mathematically fitted with a standard normal distribution in order to calculate the area occupied by each band. The calculated peak areas for CPH (1605 cm\(^{-1}\)) and SA (1810 cm\(^{-1}\)) were divided by one another and further divided by their respective monomer molecular weights in order to account for the effect that differing values of \(M_r\) have on the area of the characteristic peak. The peak areas were also divided by their respective number of occurrences of characteristic peak functionality per monomer. Since the characteristic functionality for CPH (aromatic ring) and SA (aliphatic-aliphatic anhydride bond) both occur twice per monomer unit, the effect can be factored out. The measured area ratios were compared to a previously prepared calibration to elucidate the actual CPH mole fraction. Figure 7.6 shows the resulting CPH:SA composition gradient as a function of position in the 5x4 array. The CPTEG:CPH libraries were characterized using NMR. The linearity of the profile spanning the library demonstrates not only the accuracy of the deposition apparatus but also the viability of the thiolene/silicon substrate as a platform for high-throughput transmission FTIR characterization and parallel synthesis of polyanhydrides.
**MTT cytotoxicity screening**

The MTT assay was chosen for screening of the polyanhydrides’ cytotoxicity due to its prevalent use in biocompatibility studies, with a decrease in cell viability being correlated to a decrease in optical density (OD) \[^{[43-46]}\]. As the number of metabolically active cells decrease, the amount of tetrazolium rings cleaved and converted to a precipitating product (purple formazan crystals) is reduced, resulting in a relatively lower OD. Conversely, under favorable conditions, cells divide rapidly, thus more cells actively cleave tetrazolium rings resulting in a relatively higher OD. Preliminary tests were run to ensure that there was no interference of the MTT assay with the multi-well substrates. In order to estimate the polyanhydride concentrations at which cytotoxicity is observed, a concentration gradient from 0 to 28 mg/mL 50:50 CPH:SA and 50:50 CPTEG:CPH was combinatorially synthesized and assayed at high throughput by incubating Sp2/0 mouse myeloma cells in the wells for 18 h. The data points in Figure 7.7 represent the mean relative OD across four plates for a given concentration whereas the solid line depicts the estimate from the fit of the linear model to the data. The results presented in Figure 7.7 (top) display the linear fit of a quadratic model to the CPH:SA concentrations. The linear term has a slope of 0.029 with a standard error of 0.007 (\(p\)-value = 0.0002) and the quadratic term is -0.002 with a standard error of 0.0003 (\(p\)-value < 0.0001). Ninety one observations were used in the model resulting in 88 degrees of freedom for the error term. The results of fitting a linear model to the CPTEG:CPH compositions (Figure 7.7 (bottom)) has a slope of -0.019 with a standard error of 0.003 (\(p\)-value < 0.0001). Eighty eight observations were used for the CPTEG:CPH model resulting in 86 degrees of freedom in the error term.
Low concentrations of CPH:SA appear to be stimulatory to the cells as indicated by a mean OD greater than that obtained for cells cultured in wells containing no polymer (Figure 7.7 (top)). However, at higher concentrations (i.e., above 20 mg/mL, which is well above that which would be used physiologically [8]), CPH:SA copolymers were shown to be cytotoxic to Sp2/0 cells. By altering the hydrophobic/hydrophilic properties of the copolymer (i.e., inclusion of CPTEG instead of SA) the overall cytotoxicity was reduced. Taken together, these results indicated that the biocompatibility of polyanhydride copolymers was relatively high. The studies of CPH:SA cytotoxicity determined that a concentration of 2.8 mg/mL was the maximal concentration at which no cytotoxicity was observed. The concentration at which cytotoxicity does not occur was estimated as two standard errors below the mean OD for Sp2/0 cells incubated in the absence of any polymer. The results depicted in Figure 7.7 suggest that the concentration at which little or no cytotoxicity is observed may be much higher. To this end, all subsequent studies to evaluate the biological effect of a discrete copolymer library were performed using wells containing 2.8 mg/mL.

In order to evaluate the effect of varying the fraction of CPH:SA or CPTEG:CPH, multi-well substrates were made in a 5 x 5 format and 2.8 mg/mL copolymer was combinatorially synthesized and deposited in a linear gradient ranging from 100 mol % CPH to 100 mol % SA or CPTEG. A few wells in each plate were left blank to control for the effect of incubating the cells in the NOA 81 substrate wells. Four separate cell lines were used to evaluate the cytotoxicity of the discrete polyanhydride libraries. After incubating the cells in the wells for 18 to 24 h, cytotoxicity was evaluated with MTT. The results in Figures 7.8 and 7.9 show that there was no discernable effect of copolymer composed of either CPH:SA or
CPTEG:CPH on Sp2/0 myeloma cells. Similarly, these copolymer formulations did not induce cytotoxicity for CHO and L929 fibroblast cell lines or J774 macrophages (data not shown). Table 7.3 summarizes the findings of the linear mixed model fit for both CPH:SA and CPTEG:CPH copolymers for all the cell lines tested. With the CPH:SA compositional gradients, for Sp2/0 and CHO cells, though the estimated slope obtained using a linear model was significant for some formulations (p < 0.05), the magnitude of the slope was essentially zero (0.0006 and 0.0016, respectively), indicating that there was no biologically significant effect.

High throughput cytokine screening

In order to evaluate the effect of differing copolymer compositions on the activation of antigen presenting cells, J774 macrophage cells were incubated in the multi-well substrates with discrete compositions of CPH:SA copolymers. After 48 h, cell culture supernatants were collected and analyzed for the secretion of the following cytokines: IL-6, IL-10, IL-12, and TNF-α by capture immunoassay. For data analysis, a linear mixed model was fit in order to predict the effect of increasing % of CPH in the CPH:SA copolymer on the cytokine secretion.

Following stimulation with 2 μg/mL LPS, results indicated that the incubation of J774 cells in the multi-well substrate did not affect the secretion of the measured cytokines when compared to that secreted by LPS-stimulated J774 cells incubated in 48 well tissue culture plates (data not shown). While the composition of the copolymer in the wells did not affect the secretion of IL-10 (Figure 7.10, panel B) or IL-12 (data not shown), copolymer
compositions containing greater than 50% CPH reduced the amount of IL-6 secreted from background levels (Figure 7.10, panel A). Correspondingly, the linear mixed model (Figure 7.10, panel D) does predict a modest linearly decreasing rate (-0.8269) of IL-6 production with increasing percentages of CPH but essentially no change in IL-10 or IL-12 production (predicted slopes of -0.2382 and -0.0093, respectively). In contrast, the copolymer compositions rich in CPH enhanced the secretion of TNF-α (Figure 7.10, panel C), which was corroborated with the prediction of the fit linear mixed model with a slope of 11.7084 (Figure 7.10, panel D). Collectively, these data suggest that the varying chemical compositions differentially regulate/affect the secretion of IL-6, IL-10, and TNF-α.

7.5 Discussion

Polyanhydrides, as stated earlier, are generally accepted as being highly biocompatible materials. The goal of these experiments was not to simply confirm the biocompatible nature of CPH:SA and CPTEG:CPH copolymers, but to illustrate a high throughput method for screening of degradable biomaterials using a novel multi-well substrate and combinatorial polymer synthesis. Taken together, these results are consistent with numerous conventional (one-sample-at-a-time) in vitro and in vivo studies that attest to the biocompatibility of polyanhydride systems [14, 47-49]. The highly generalized method developed here will be invaluable in the rapid testing and discovery of optimally tuned biomaterial compositions for drug delivery. Discrete libraries of virtually any geometry can be rapidly produced to screen polymers at any desired resolution. Similarly, the thermally cured multi-well substrate has been newly demonstrated to be highly robust during synthesis at high temperature in the presence of chloroform and acetic anhydride. Thus, many different types of polymer libraries
can be created via melt polycondensation in many solvents.

Such a robust, rapid, and generalized method has great potential in various applications. A simple modification of the study could allow for time-dependent parallel monitoring of polyanhydride degradation and cytotoxicity. The study of drug and protein release kinetics would particularly benefit from a combinatorial methodology, as unique release mechanisms have been demonstrated at various copolymer compositions \[^{[1, 5, 11]}\]. The discrete multi-well substrate may also find use in high throughput screening of biodegradable tissue scaffolding materials \[^{[50]}\] and integration with microfluidics \[^{[35]}\], thus providing a unique combination of generalized high throughput testing, rational design, and discovery of new biomaterials.

### 7.6 Conclusions

A rapid method has been outlined for prototyping discrete library substrates for high throughput cytotoxicity and cell activation screening of polyanhydride libraries. This methodology has been validated with NMR and GPC characterization of rapidly synthesized and deposited libraries of polyanhydride chemistries based on CPH:SA and CPTEG:CPH copolymers. The libraries were characterized by high throughput techniques using FTIR spectroscopy and biological responses of various cell lines. There was no observed cytotoxic effect for any CPH:SA or CPTEG:CPH composition at a concentration higher than that expected to be used for a variety of \textit{in vivo} applications \[^{[8]}\]. While the biocompatibility of polyanhydrides has long been established, the methods described herein show the compatibility of the polymers synthesized combinatorially and of the cell based assays. The results from the cytokine secretion studies confirmed the importance of polymer chemistry
on the activation of antigen presenting cells. Many of the promiscuous innate immune receptor ligands share the common feature of hydrophobic domains \[51\]. While it is known that microspheres of various chemistries (i.e. PLGA, PLA and poly-amino acid derivatives) are efficient at delivering protein antigens to antigen presenting cells \[52\] and that PLGA microspheres can increase activation markers on the surface of antigen presenting cells \[53\], these previous studies have not systematically evaluated the effect of varying polymer chemistry on immune cell activation. In contrast, the present studies demonstrated that copolymers containing higher hydrophobic (CPH) content induced greater TNFα or pro-inflammatory responses than that observed with less hydrophobic polymers. The level of activation and the behavior of antigen presenting cells such as macrophages and dendritic cells are key factors in the induction and maintenance of a Th1 (cellular) or Th2 (humoral) immune responses \[18\] and thus underscores the potential of polyanhydrides as effective adjuvants to modulate immune responses \[8\]. The methods described herein lay a foundation for establishing rapid screening protocols of biomaterials and for quantifying the role of biomaterial chemistry not only in cellular toxicity but also immune activation. These results also add to the large body of evidence supporting the use of polyanhydrides as biocompatible materials for use in drug delivery devices.

7.7 Acknowledgments

B.N. and M.J.W. gratefully acknowledge financial support from the U.S. Department of Defense – Office of Naval Research (ONR Award # N00014-06-1-1176). This material is based upon work supported by the National Science Foundation under Grant No. EEC 0552584 to B.N. M.P.T. acknowledges financial support from NIH-NCI via the Ruth L.
Kirschstein Fellowship. Special thanks to Andrea Dorn for her performance of the Luminex cytokine assays.

7.8 References

51. Seong SY, Matzinger P. Hydrophobicity: an ancient damage-associated molecular pattern

7.9 List of Figures

Figure 7.1: Chemical structures of a) poly(SA); b) poly(CPH); and c) poly(CPTEG).
Figure 7.2: Schematic of photolithographic design of discrete thiolene-based multi-well substrates.

Figure 7.3: Proton NMR spectrum for combinatorially synthesized 50:50 CPH:SA copolymer showing chemical shifts.
Figure 7.4: Proton NMR spectrum for combinatorially synthesized 50:50 CPTEG:CPH copolymer showing chemical shifts.

Figure 7.5: Representative FTIR spectrum of a 60:40 CPH:SA copolymer with characteristic absorbances of 1605 cm\(^{-1}\) for the CPH aromatic peak and 1810 cm\(^{-1}\) for the SA aliphatic peak.
Figure 7.6: Comparison of predicted and experimentally measured composition in a 5x4 discrete library (95% confidence intervals).

Figure 7.7: Cell (Sp2/0 mouse myeloma) viability as a function of 50:50 CPH:SA (left) and CPTEG:CPH (right) concentration. The solid line depicts the fitted quadratic (for CPH:SA, n = 91) or linear (for CPTEG:CPH, n = 88) model respectively. The data points are the means for each concentration tested.
Figure 7.8: Effect of CPH:SA copolymer composition on cytotoxicity of Sp2/0 myeloma cells. $5 \times 10^5$ cells were incubated for 18 h on 25 multi-well substrate plates with a compositional library of CPH:SA and cytotoxicity was evaluated with MTT assay. Data is represented as mean OD ± SEM for replicate plates. The filled bar indicates cells incubated on multi-well substrate plates without any copolymer. SEM = Standard Error of the Mean.
Figure 7.9: Effect of CPTEG:CPH copolymer composition on cytotoxicity of Sp2/0 myeloma cells. 5 x 10^5 cells were incubated for 18 h on 25 multi-well substrate plates with a compositional library of CPTEG:CPH and cytotoxicity was evaluated with MTT assay. Data is represented as mean OD ± SEM for replicate plates. The filled bar indicates cells incubated on multi-well substrate plate without any copolymer.
Figure 7.10: Cytokine secretion from J774 macrophages incubated in multi-well substrates containing 2.8 mg/mL of discrete compositions of CPH:SA was measured by capture immunoassay. Data depicted is the mean ± SEM. A) IL-6, B) IL-10, C) TNF-α, and D) statistical summary of linear mixed model fit to the experimental data. Data is representative of two replicate plates on two separate experiments. D.F. = degrees of freedom.
7.10 List of Tables

Table 7.1 Number average molecular weight and polydispersity index of combinatorially synthesized CPH:SA copolymers obtained by GPC.

<table>
<thead>
<tr>
<th>CPH:SA Copolymer</th>
<th>$M_n$ (Da)</th>
<th>Polydispersity index</th>
</tr>
</thead>
<tbody>
<tr>
<td>0:100</td>
<td>11200</td>
<td>2.2</td>
</tr>
<tr>
<td>10:90</td>
<td>12700</td>
<td>2.5</td>
</tr>
<tr>
<td>20:80</td>
<td>9700</td>
<td>2.1</td>
</tr>
<tr>
<td>50:50</td>
<td>10800</td>
<td>2.4</td>
</tr>
<tr>
<td>80:20</td>
<td>13300</td>
<td>2.0</td>
</tr>
<tr>
<td>90:10</td>
<td>16500</td>
<td>2.0</td>
</tr>
</tbody>
</table>

Table 7.2 Number average molecular weight of combinatorially synthesized 50:50 CPTEG:CPH copolymers obtained by $^1$H NMR.

<table>
<thead>
<tr>
<th>50:50 CPTEG:CPH Trial</th>
<th>$M_n$ (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10900</td>
</tr>
<tr>
<td>2</td>
<td>13500</td>
</tr>
<tr>
<td>3</td>
<td>10500</td>
</tr>
<tr>
<td>4</td>
<td>13500</td>
</tr>
<tr>
<td>5</td>
<td>14200</td>
</tr>
<tr>
<td>6</td>
<td>13800</td>
</tr>
</tbody>
</table>
Table 7.3 Summary of linear fit model to data from compositional cytotoxicity experiments.

Estimated slope is the slope of the linear mixed model fit to each cell type for each copolymer composition tested. D.F. = degrees of freedom.

<table>
<thead>
<tr>
<th>Copolymer Type</th>
<th>Cell Type</th>
<th>Estimated Slope of Linear Model</th>
<th>Standard Error of Linear Model</th>
<th>D.F.</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPH:SA</td>
<td>Sp2/0</td>
<td>0.0006</td>
<td>0.0003</td>
<td>62</td>
<td>0.0326</td>
</tr>
<tr>
<td>CPH:SA</td>
<td>J774</td>
<td>0.0002</td>
<td>0.0002</td>
<td>186</td>
<td>0.2494</td>
</tr>
<tr>
<td>CPH:SA</td>
<td>L929</td>
<td>0.0005</td>
<td>0.0003</td>
<td>136</td>
<td>0.1503</td>
</tr>
<tr>
<td>CPH:SA</td>
<td>CHO</td>
<td>0.0016</td>
<td>0.0006</td>
<td>125</td>
<td>0.0051</td>
</tr>
<tr>
<td>CPTEG:CPH</td>
<td>Sp2/0</td>
<td>0.0009</td>
<td>0.0006</td>
<td>62</td>
<td>0.1258</td>
</tr>
<tr>
<td>CPTEG:CPH</td>
<td>J774</td>
<td>0.0005</td>
<td>0.0003</td>
<td>62</td>
<td>0.0967</td>
</tr>
<tr>
<td>CPTEG:CPH</td>
<td>L929</td>
<td>0.0010</td>
<td>0.0012</td>
<td>62</td>
<td>0.3961</td>
</tr>
<tr>
<td>CPTEG:CPH</td>
<td>CHO</td>
<td>0.0006</td>
<td>0.0005</td>
<td>83</td>
<td>0.2471</td>
</tr>
</tbody>
</table>
Chapter 8: The Simultaneous Effect of Polymer Chemistry and Device Geometry on the \textit{In vitro} Activation of Murine Dendritic Cells

A paper published in Biomaterials 2009, 30 (28), 5131–5142

L. K. Petersen$^1$, L. Xue$^2$, M.J. Wannemuehler$^3$, K. Rajan$^2$, and B. Narasimhan$^{1,*}$

\textbf{Keywords:} Polyanhydrides, murine dendritic cells, adjuvants, vaccine delivery, nanospheres

$^1$Department of Chemical and Biological Engineering, Iowa State University, Ames, IA 50011
$^2$Department of Materials Science and Engineering, Iowa State University, Ames, IA 50011
$^3$Department of Veterinary Microbiology and Preventive Medicine, Iowa State University, Ames, IA 50011
$^{*}$Corresponding author
8.1 Abstract

Poylanhydrides are a promising class of biomaterials for use as vaccine adjuvants and as multi-component implants. Their properties can be tailored for such applications as controlled drug release, drug stability, and/or immune regulation (adjuvant effect). Understanding the induction of immunomodulatory mechanisms of this polymer system is important for the design and development of efficacious vaccines and tissue compatible multi-component implantable devices using this polymer system. This study describes the development of a rapid multiplexed method for the investigation of the adjuvanticity of polyanhydride nanospheres and films using murine dendritic cells (DCs). To assess the immune response, cell surface markers including MHC II, CD86, CD40, and CD209 and cytokines including IL-6, IL-12p40, and IL-10 were measured. The DCs incubated with nanospheres displayed enhanced expression of all the surface markers and the production of IL-12p40 compared to DCs incubated with polymer films in a chemistry-dependent manner. This suggests that polyanhydrides of various chemistries and device geometries can be tailored to achieve desired levels of immune cell activation for specific applications. The observed biocompatibility and activation of DCs by polyanhydride devices supports their inclusion in vaccine delivery devices as well as in multi-component medical implants.

8.2 Introduction

Current advancements in the design and development of implantable/injectable biomaterial devices have focused on the need for a better understanding of their adjuvant effect on the host’s innate and adaptive immune responses. An adjuvant is an agent that, while having no intrinsic antigenicity, is able to simulate the immune system. Activation of the immune
system may or may not be desirable based on the intended application; injectable vaccine delivery devices will benefit by an enhanced immune response whereas implantable tissue engineering constructs will ideally avoid immune activation [1-3].

The cells of the immune system principally responsible for inducing a primary immune response and enhancing a secondary immune response are dendritic cells (DCs) [2, 3]. The DCs play a major role in antigen presentation, which is a necessary step for induction of long term memory and protective immunity. Antigen processing and presentation is activated by cellular engagement of pathogen-associated molecular patterns (PAMPs) with Toll-like and other pattern recognition receptors on the host cell [1, 2, 4]. Many cell surface molecules such as MHC II, MHC I, and co-stimulatory molecules such as CD80, CD86, and CD40 are essential for antigen presentation by the DCs in order to activate T cells and to induce clonal expansion. It is also thought that adhesion molecule such as CD209 may play an important role in T cell activation and DC adhesion [5]. Activated DCs may also produce cytokines such as IL-4, IL-5, IL-10, IL-12, IL-6, TNF-α, TNF-β, and/or IFN-γ, which are chemical messengers capable of mediating several facets of the immune response [2]. Since very few DCs are necessary for the induction of a robust immune response [6], understanding these mechanisms of immune activation is critical for the rational design of vaccines and development of multi-component implants.

Previous studies by Babensee and co-workers have investigated the adjuvant effect of the PLGA polymer system [7-10]. Specifically, work by Yoshida et al. focused on the adjuvant activity of PLGA films and microparticles [11]. The investigators found that the PLGA
microparticles did not enhance the expression of either CD80 or CD86; however, films were capable of significantly enhancing the expression of CD80. They also demonstrated that PLGA microparticles and films significantly enhanced the production of IL-6 and TNF-α over the non-stimulated DCs. More recently, Torres et al. assessed the adjuvant properties of polyanhydride microparticles and reported that the activated DCs were characterized by the enhanced expression of MHC II, CD86, CD40 and CD209 and by increased production of IL-12p40 and IL-6 [12].

Biodegradable polymeric particles are a promising class of adjuvants because of their ability to preserve the activity of the protein antigen, release the antigen in a controlled manner, and modulate the immune response (adjuvant effect). This makes them promising candidates for vaccine delivery with the potential to reduce the number of doses necessary to induce protective immunity [13, 14]. In particular, polyanhydrides are a class of surface-eroding, biocompatible materials which have shown much promise for these applications with their ability to provide a controlled drug release that is mediated by the chemistry of the polymer, and, unlike polyesters, correlates closely with the polymer degradation profile [15-23]. The FDA has approved the use of a specific polyanhydride (called the Gliadel® wafer) to locally deliver anti-cancer drugs to the brain [17]. The polyanhydride chemistries of interest in this work, which are closely related to the FDA-approved polymer, are based on sebacic acid (SA) and 1,6-bis(p-carboxyphenoxy)hexane (CPH). Biocompatibility tests of these polymers have shown that they produce degradation products that are both non-mutagenic and non-cytotoxic, which makes a strong case for their use in vivo [24, 25]. When copolymerized, the specific chemistry of the CPH:SA copolymer plays an integral role in modulating many
aspects related to drug and protein release and immune response. The copolymer composition controls the hydrophobicity of the polymer, allowing for tunable release kinetics (CPH degradation is much slower than SA degradation), protein stabilization, and immune modulation \[^{[12, 18, 19, 26]}\]. Microspheres based on CPH:SA polyanhydrides have been previously shown to be promising vaccine carriers because of their adjuvant capabilities and immunomodulatory behavior both \textit{in vitro} and \textit{in vivo}, which are dependent on the chemistry of the polymer \[^{[12, 26]}\]. Work by Kipper et al. demonstrated that a single dose of tetanus toxoid (TT)-loaded 20:80 CPH:SA microspheres induced a dominant IgG1 immune response while TT-loaded 50:50 CPH:SA microspheres induced a more balanced IgG2a:IgG1 anti-TT immune response \[^{[26]}\].

Many previous studies in the design and development of polyanhydrides and other biomaterials have been carried out in a conventional, one sample at a time format. This inefficient and laborious process consumes a large amount of time and resources, which is often not a viable option in the rapidly advancing world of nanomedicine. Viruses evolve with speeds far superior to the rate of medical technological advancements and new forms of cancer and disease are discovered every day. These rapidly advancing problems demand combinatorial approaches and advanced data mining techniques to accelerate the design and discovery of new materials for biomedical applications \[^{[27-34]}\]. Initial studies employing this combinatorial methodology to study polyanhydrides have focused on the rapid synthesis, assessment of phase behavior, drug dissolution, and cytotoxicity of polymer films with cell lines \[^{[35-38]}\]. The cytotoxicity study demonstrated that the polymer films were non-cytotoxic up to a total mass of 0.28 mg covering a 0.32 cm\(^2\) surface area in a volume of 100 μL,
resulting in a polymer concentration (~2.8 mg/mL) that exceeds the typical dosage for many in vivo applications [26]. The primary focus of this work was to assess and compare the adjuvant effect of CPH:SA nanosphere and film systems, using a rapid, multiplexed approach with the use of advance informatics techniques for the rational design and optimization of vaccine adjuvants and tissue engineering platforms.

8.3 Materials and Methods

Materials

The chemicals utilized in monomer synthesis include potassium carbonate, dimethyl formamide, toluene, sulfuric acid, acetic acid, and acetonitrile, which were purchased from Fisher Scientific (Fairlawn, NJ); 1-methyl-2-pyrrolidinone, 4-p-hydroxybenzoic acid, 1,6-dibromohexane, and tri-ethylene glycol were purchased from Sigma Aldrich (St. Louis, MO); and 4-p-fluorobenzonitrile was obtained from Apollo Scientific (Cheshire, UK). The chemicals needed for the polymerization, nanosphere fabrication, and buffer preparation include acetic anhydride, chloroform, methylene chloride, petroleum ether, monobasic potassium phosphate, dibasic potassium phosphate, sodium acetate trihydrate and glacial acetic acid, which were purchased from Fisher Scientific. Sebacic acid (99%), E. coli lipopolysaccharide (LPS), β-mercaptoethanol, and rat immunoglobulin (rat IgG) were purchased from Sigma Aldrich. The materials required for the DC culture medium include: RPMI 1640, penicillin-streptomycin, HEPES buffer, and L-glutamine, purchased from Mediatech (Herndon, VA); granulocyte macrophage colony stimulating factor (GMCSF), purchased from PeproTech (Rocky Hill, NJ); and heat inactivated fetal calf serum, purchased from Valley Biomedical (Winchester, VA). The materials needed for flow cytometry were:
unlabeled mouse IgG, purchased from Pharmingen, Becton Dickinson (Franklin Lakes, NJ); mouse serum and unlabeled CD36/16 FcγR, purchased from Southern Biotech (Birmingham, AL); unlabeled hamster IgG, phycoerythrin-Cy5 (PE/Cy5) conjugated anti-mouse CD11c (clone N418), Alexa Fluor® 700 anti-mouse CD11c (clone N418), fluorescein isothiocyanate (FITC) conjugated anti mouse/rat MHC Class II (I-Ek) (clone 14-4-4S), allophycocyanin (APC) anti-mouse CD40 (clone 1C10), biotin conjugated anti-mouse CIRE (DC-SIGN or CD209) (clone 5H10), and phycoerythrin-Cy7 (PE/Cy7) anti-mouse CD86 (clone GL-1); and corresponding isotypes: PE/Cy5 rat IgG2a κ (clone eBR2a), Alexa Fluor® 700 conjugated Armenian hamster IgG (clone eBio299Arm), FITC IgG2a κ (clone eBM2a), APC rat IgG2a κ (clone eBR2a), biotin conjugated rat IgG2a (clone eBR2a), and PE/Cy7 conjugated rat IgG2b (clone KLH/G2b-1-2); and APC-Cy7-conjugated streptavidin. All of these reagents were purchased from e-Bioscience (San Diego, CA).

**Polymer film synthesis**

1,6-bis(\(p\)-carboxyphenoxy)hexane (CPH) monomer was synthesized as described previously \[^{[39]}\]. Sebacic acid (SA) monomer was purchased from Sigma Aldrich. The linearly varying, discrete library of CPH:SA polyanhydride films were synthesized in replicates of five from the corresponding monomers via a melt polycondensation reaction in thiolene based multi-well substrates utilizing a multiplexed robotic deposition apparatus, reported previously by Adler et al \[^{[35]}\]. A modification was made to the original size of the multi-well substrate previously reported. The size was increased to that of a 24 well-plate and included 24 wells which allowed for larger films and more cell growth area.
Film characterization

All film characterization studies evaluated polymer synthesized in the full range of possible copolymers chemistries varying from 100 % SA to 100 % CPH. Five chemistries, poly(SA), 25:75 CPH:SA, 50:50 CPH:SA, 75:25 CPH:SA, and poly(CPH), were investigated in these studies. Polymer functionality and molecular weight was determined by proton nuclear magnetic resonance spectroscopy ($^1$H NMR) using a Varian VXR 300 MHz spectrometer (Varian Inc., Palo Alto, CA). Number average molecular weights, weight average molecular weights, and polydispersity indices were determined using gel permeation chromatography (GPC). Samples were first dissolved in HPLC-grade chloroform and then separated through a Waters GPC chromatograph (Milford, MA) containing PL Gel columns (Polymer Laboratories, Amherst, MA). Retention times were compared to polystyrene standards (Fluka, Milwaukee, WI). The surface chemistry of the synthesized polymer film libraries was rapidly evaluated at using Fourier transform infrared spectroscopy (FTIR) with a Nicolet 6700 FTIR spectrometer (Thermo Fisher Scientific). The profile of the composition in each well was obtained using transmission mode FITR on a silicon nitride wafer substrate (University Wafer, South Boston, MA). By identifying the characteristic peaks from each sample (CPH aromatic peak at 1605 cm$^{-1}$ and SA aliphatic peak at 1850 cm$^{-1}$) the relative ratios between the monomer species were used to identify the corresponding compositions [37]. This was calculated by using Gaussian curve fitting and integration with the OMNIC software. The areas under each curve were used to determine the molar ratio between the two monomers.
Nanosphere fabrication

An automated nanosphere fabrication apparatus was designed to create a multiplexed library of CPH:SA nanospheres from the synthesized polymer film library. This process requires a modification to the film library deposition described previously \cite{35}. In the modified process, the thiolene based multi-well substrate was replaced by a multi-vial substrate to allow for larger holding volumes. The apparatus controlling the automated nanosphere fabrication process consisted of a series of 4 linear actuators (Zaber Technologies, Richmond, British Columbia, Canada) and 3 programmable syringe pumps (New Era Pump Systems, Farmingdale, NY) controlled by a third-party macro software operating on the actuators’ respective consoles. This fully automated process was initiated by chloroform solution deposition into each vial of the multi-vial substrate, thus dissolving the polymer films with a resulting polymer concentration of approximately 25mg/mL. Each solution was then homogenized for 60 s at 10,000 rpm to completely dissolve the polymer in the chloroform. After that petroleum ether was dispensed into 15 mL tubes, each tube corresponding to a separate copolymer vial. Next, the polymer solution was withdrawn from a vial and dispensed into its corresponding tube of petroleum ether. During this time a nanoprecipitation process occurs in which the dissolved polymer is rapidly precipitated out in the presence of the non-solvent (petroleum ether) and the nanospheres are formed. The optimal ratio of solvent to non-solvent was identified to be 1:40 for the fabrication of an average size of 300 nm polyanhydride particles. The nanospheres were characterized with scanning electron microscopy (SEM) to analyze size and surface morphology using a JEOL 480A SEM (JEOL USA Inc., Peabody, MA).
Culture and stimulation of DCs

Bone marrow derived dendritic cells (BMDCs) were prepared from bone marrow cells isolated from the femurs, tibias, humerus’ and iliums of C57BL/6 mice (obtained from Harlan Sprague Dawley and housed within the ISU Laboratory Animal Resource Facility, Ames, IA) as previously described [40]. Following mouse euthanization and bone excision, attached tissue and muscle were dissected away. The ends of the bone were cut and the marrow was flushed out using a syringe fitted with a 30 gauge needle. Each bone as flushed with 5 mL of RPMI medium containing 1% pen/strep. Large particulates were allowed to settle and then removed. After centrifugation, the cells were resuspended in DC medium (RPMI containing 1% L-glutamine, 1% penicillin-streptomycin solution, 2% HEPES, 0.5% gentamicin, 0.1% β-mercaptoethanol, and 10% heat inactivated fetal bovine serum (FBS) supplemented with GM-CSF (10 ng/mL). The cells were then placed in T75 cell culture flasks in 10 mL of DC medium containing 10 ng/mL GMCSF and incubated at 37 ºC under 5% CO₂ atmosphere. On day 3, 10 mL of fresh DC medium with 10 ng/mL GMCSF was added. On day 6, 10 mL of the culture medium was harvested and placed in a 15 mL centrifuge tube. After centrifugation, the supernatant was decanted and the cells were resuspended into 10 mL of fresh DC medium containing 10 ng/mL GMCSF and re-inoculated into the original flask. On day 8, DCs were removed from the flasks, counted, resuspended in fresh DC medium, and transferred to 24-well plates (1 x 10⁶ cells/well). On day 9, a portion of the cells were stained for CD11c to determine the percentage of DCs. On the same day the remaining DCs were incubated with the different stimulation treatments (films or nanospheres). The DCs were treated with CPH:SA copolymer films and nanospheres of the following chemistries: poly(SA), 13:87 CPH:SA, 25:75 CPH:SA, 37:63
CPH:SA, 50:50 CPH:SA, and 63:37 CPH:SA. Polyanhydride nanospheres were incubated at a concentration of 0.125 mg/mL (2 cm² cell growth area). Non-stimulated (NS) DCs and LPS (200 ng/mL) treated DCs were used as negative and positive controls, respectively. After the addition of the stimulants, the DC cultures were incubated for an additional 48 h (37°C, 5% CO₂) at which time the supernatants and DCs were harvested for cytokine production and cell surface marker expression, respectively. Cell viability was assessed by measuring trypan blue exclusion using a hemocytometer and light microscope.

**Cell surface markers**

The expression of cell surface markers including CD11c, CD86, CD40, MHC II, and CD209 were assessed after the 48 h incubation period of the DCs with the stimulation treatments. The adherent DCs were harvested from the culture dishes with vigorous pipetting, placed in polystyrene tubes (BD FALCON™, Franklin Lakes, NJ), centrifuged (250 rcf, 10 min, 4°C), and resuspended in Fc blocking solution consisting of PBS buffer with 0.1% anti-CD16/CD32, 0.1% unlabeled hamster IgG, 1% rat IgG, 1% mouse serum, 0.1% sodium azide, and 1% FBS. After blocking, the DCs were stained and fixed for evaluation of cell surface markers using monoclonal antibodies against CD11c, MHCII, CD86, CD40, and CD209. The samples were analyzed using a Becton-Dickinson FACSCanto flow cytometer (San Jose, CA) and FlowJo (TreeStar Inc, Ashland, OR).

**Cytokine release**

Following incubation of the DCs with the stimulation treatments for 48 h, 200 μL of the supernatants were collected and assayed for the presence of IL-6, IL-10, and IL-12p40 using
the Luminex® Multiplex assay system (Austin, TX).

*Statistical analysis*

All data was statistically analyzed by using a one-way model ANOVA with the statistical software JMP® 7 (Cary, NC). Comparisons between treatments were made with Tukey’s HSD to determine statistical significance, and p-values of less than or equal to 0.05 were considered significant. All data was log transformed for use of the one-way model ANOVA.

*Principal component analysis (PCA)*

Principal Component Analysis (PCA), which is a dimension reduction technique, is typically used to represent directions with maximum variability and provide a simpler and more parsimonious description of the covariance structure[^41-43]. In this work PCA was used in a blinded fashion to uncover the latent features of the stimulation data and explain the relationships between polymer chemistry and cell marker expression and cytokine production. PCA accomplished data reduction and facilitated interpretation by reducing the six original variables (CD209, CD40, CD86, MHC II, IL-6, and IL-12p40) to two principal components. The principal components are linear combinations of the original six variables, and make up new axes that represent the directions with maximum variability. The projection of the original six-dimensional data to the two-dimensional space constructed by the first principal component (PC1) and the second principal component (PC2) helps to visualize and interpret data and often reveals relationships that are not previously suspected, thereby allowing for interpretations that would not ordinarily result[^41,42]. Before PCA is applied on the data, the data was preprocessed by normalization, detection and elimination of outliers,
and missing values.

The replicate experiments were conducted on several different days and to eliminate the
effect of the day of experiment, the flow cytometry data for surface maker expression and the
Luminex data for cytokine production were normalized according to Equation 1:

$$X_{\text{norm}} = \frac{X - NS}{X_{\text{max}} - NS}$$   \text{Eq. 1}

Here, $X_{\text{norm}}$ is the normalized value of the sample being analyzed (either normalized mean
fluorescence intensity (MFI) of surface marker or normalized concentration of cytokine), $NS$
is the MFI or concentration value of the non-stimulated group, $X$ is the MFI or concentration
value of the sample being analyzed, and $X_{\text{max}}$ was derived from either the MFI value induced
by poly(SA) nanospheres for cell surface marker expression or from the cytokine
concentration produced by 63:37 CPH:SA nanospheres. This normalization method has been
reported in the literature [44].

The elimination of outliers is very important since outliers can dramatically change the data
variance. In this study, the outliers were detected using boxplots. A boxplot is a convenient
way of graphically depicting groups of numerical data through their smallest observation,
lower quartile, median, upper quartile and largest observation. The IQR (interquartile range)
is the range between the lower quartile and the upper quartile. Conventionally, any
observation that lies out of 1.5 IQR from the lower quartile or the upper quartile is regarded
as an outlier. Outliers were replaced using mean values of the stimulation data with the same
copolymer chemistry.
Each observation is a six-dimensional data point with two dimensions of cytokines and four dimensions of cell markers. Conventionally, the observations with more than 30% missing values are deleted. For example, if an observation for a specific CPH:SA nanosphere treatment has only values for IL-6 and IL-12p40 and no measured values for CD209, CD40, CD86 and MHC II, this observation was deleted.

8.4 Results

Discrete, combinatorial film libraries, linearly varying in copolymer composition of the CPH:SA system, were characterized with FITR using an automated method to verify that the deposition apparatus was depositing the intended molar volumes of monomer into each well. The observed results, shown in Figure 8.1, are in good agreement with the intended deposition of the molar composition of the copolymer chemistries. The copolymers were also characterized with GPC to determine molecular weight and $^1$H NMR to verify copolymer structure, chemistry, and molecular weight. These findings, shown in Table 8.1, are in good agreement with the properties of conventionally synthesized polymers $^{[17]}$. Following synthesis of the polymer films, nanospheres of six copolymer compositions were rapidly fabricated using a nanoprecipitation technique. The multiplexed method enabled the simultaneous fabrication of nanospheres ~100 times faster than conventional “one sample at a time” nanoprecipitation methods $^{[45]}$. Scanning electron photomicrographs of nanospheres of six copolymer compositions are shown in Figure 8.2. It can be observed that the size, between 200 nm and 500 nm, and relative roughness are similar across polymer chemistries. These sizes and morphologies are in good agreement with those of conventionally synthesized nanospheres $^{[45]}$. 
To assess activation of CD11c+ DCs by the CPH:SA film and nanosphere libraries, flow cytometry was used to measure levels of cell surface expression of co-stimulatory molecules CD86 and CD40, integrin CD209, and major histocompatibility complex molecule MHC II. Supernatants were also collected and analyzed for cytokine production, including IL-10, IL-12p40p40, and IL-6. LPS was used as a positive control and a non-stimulated group with medium only acted as a negative control. Prior to use in cell stimulation assays, the BMDC cultures were shown to be >90% positive for CD11c (data not shown).

The results from the analysis of cell surface marker expression following the 48 h stimulation period suggest differential regulation of the selected markers depending on the polymer chemistry and the relative hydrophobicity of each formulation. This effect was observed with both films and nanospheres, as shown in Figures 8.3-8.6 (statistical analysis of the data in these figures is shown in Table 8.2). In these figures, histograms depicting a representative profile of expression for the given cell surface marker are shown together with a normalized graphical compilation of all the replicates tested for each treatment. The SA-rich (least hydrophobic) chemistries demonstrated the ability to best promote the expression of MHC II, CD86, CD40, and CD209. The chemistry-dependent up-regulation of the cell surface markers in comparison to the non-stimulated control is clearly observed with SA-rich nanosphere and film compositions. Enhanced expression of these markers clearly correlates with the amount of SA in the films or nanospheres, exhibiting a maximum for the poly(SA) films and nanospheres. In addition to the MFI measurements, measurements of cells as percent positive for each of the specific markers was performed, and the results were consistent with the observed MFI for each specific cell surface marker (data not shown).
Although the trends observed between the film and nanosphere systems with respect to the copolymer chemistry were consistent, it is apparent that there are differential levels of cell surface marker up-regulation between the two geometries. More specifically, nanospheres more effectively up-regulated expression of all cell surface markers in comparison to films. The level of up-regulation of the cell surface markers induced by the nanospheres is markedly higher for MHC II (Figure 8.3) and CD86 (Figure 8.5) and even more pronounced for CD40 (Figure 8.4) and CD209 (Figure 8.6) relative to the changes in cell surface marker expression induced by the films. This is also noted in the histograms by the increased shift from the isotype control between nanosphere and film geometries. In comparison with LPS, poly(SA) nanospheres induced greater up-regulation of CD209 and lower up-regulation of CD40. LPS is not known to up-regulate CD209, so the low level of expression was not unexpected [4, 6, 46].

Supernatants were collected and assessed for cytokine concentrations. The results suggest differential cytokine production (enhancement of IL-6 and regulation of IL-12p40) in response to the composition of CPH:SA nanospheres and films. However, the trend for cytokine production is counter to the trend observed with the cell surface marker expression. The more hydrophobic or CPH-rich nanosphere chemistries appear to better promote production of IL-6 and IL-12p40 as shown in Figure 8.7. Consistent with the nanosphere data, a chemistry-dependent trend is observed in cytokine production with the film chemistries with the highest concentrations of IL-6 corresponding to cells stimulated with CPH-rich chemistries as shown in Figure 8.7 (statistical analysis is shown in Table 8.2). In the case of IL-12p40, the CPH-rich chemistries inhibited the background production to a
lesser extent than the SA-rich chemistries (Figure 8.7). The CPH:SA films considerably promoted IL-6 production over that of the corresponding CPH:SA nanospheres. Interestingly, IL-6 production was increased by DCs stimulated by the film library, while IL-12p40 secretion was down-regulated by the film library in comparison to the non-stimulated group. IL-10 was not produced by any of the treatment groups, so the data is not included in the figures or histograms.

PCA was used to analyze the cytokine production and the cell surface marker expression data and to draw inferences about the complexities of the immune activation. The use of refined data mining techniques is necessary to analyze the large data sets from flow cytometry and Luminex assays in parallel, and will be the subject of future studies. PCA enables the simultaneous investigation of the relationship between the multiple variables of this complex system, including polymer chemistry, device geometry (nanosphere and film), cytokine production, and cell surface marker expression. Figure 8.8 depicts the nanosphere system analyzed with PCA and shows that the nanospheres enhance the expression of all cell surface markers and production of all cytokines (except IL-10), which is seen by the strong chemistry-dependent (polymer chemistry is depicted by % CPH in the PCA biplots) correlation with the plot vectors. This analysis revealed that the polyanhydride nanospheres enhanced the expression of the co-stimulatory molecules and the IL-12p40 cytokine, which is important for induction of an immune response, more effectively than the films did as shown in Figure 8.9. This can be observed by the indicated nanosphere chemistries clustered in parallel to the cell surface marker and IL-12p40 vectors. This suggests that the cell surface interaction with a polymer film is not sufficient to promote DC activation. The film library
was also assessed with PCA and the biplot in Figure 8.10 illustrates the low level of cell
surface marker expression which is achieved by DC interactions with films. This is indicated
by the clustering of the cell surface maker vectors around the central axis while the cytokine
production vectors (IL-6 and IL-12p40) are more outstretched demonstrating a better
correlation to the ability of the film chemistry (i.e., % CPH) to affect cytokine production.
This suggests that the film device geometry is poor at enhancing cell surface marker
expression but is more effective at regulating cytokine production (i.e., activating IL-6
production but inhibiting IL-12p40 production).

8.5 Discussion

The ability of a polymer system to both modulate its effect on the immune response and
deliver a drug or antigen in a controlled fashion provides an ideal platform for both vaccine
adjuvants and multi-component implants. Many factors influence the innate and adaptive
immune responses, including the activation of DCs. This study was designed to utilize the
known measures of DC activation as a means to more rationally design biodegradable
adjuvants for vaccine development. Adjuvant properties such as enhancing immunogenicity
have been investigated for many individual polyanhydride polymer formulations \cite{11, 12};
however, to our knowledge this study represents the first time these analyses have been
performed to assess a multiplexed polyanhydride library. Likewise, we are not aware of any
reported methods that have multiplexed the fabrication of polymer nanospheres. The rapid
technique for nanosphere fabrication described in this work may be applicable to fabricate
nanospheres of other degradable or non-degradable polymers. It can also be easily extended
to synthesize hundreds of nanospheres of various chemistries by either increasing the number
of wells in the substrate or by fabricating a large number of multi-well substrates. The concomitant use of this multiplexed technique and the informatics analysis will allow us to rapidly optimize the CPH:SA polyanhydride system for use in tissue engineering (where biocompatibility is desirable) or vaccine delivery (where enhanced innate immune responses are desirable).

The multiplexed method described herein was able to clearly demonstrate that the chemistry of the polymer played a major role in up-regulating cell surface marker expression and cytokine production when films or nanospheres were used to stimulate DCs. The least hydrophobic (i.e., SA-rich) nanosphere chemistries were most effective at enhancing the expression of cell surface markers, while the most hydrophobic (i.e., CPH-rich) chemistries were most successful at enhancing cytokine production. With MHC II, CD86, and CD40 all playing critical roles in the induction of adaptive immunity through activation of CD4+ T cells, it would be preferable for an effective vaccine to enhance their expression [2]. CD209 is a marker with possible implications associated with activation of the immune system because it functions as a cell adhesion receptor that mediates DC migration and T cell activation [5]. In addition, IL-12 and IL-10 play important roles in adaptive immunity through enhancing or inhibiting events leading to the differential effector functions of CD4+ T cells [2]. The cytokine IL-6 is important to the innate immune response, contributing to systemic inflammation [5]. The multi-faceted immune responses associated with these various cell surface markers and cytokines are all directly correlated with the adjuvanticity of the CPH:SA polymer system. The PCA biplot (Figure 8.8) suggests that DC activation would be most optimal at intermediate CPH:SA nanosphere compositions (such as 37:63 CPH:SA)
which would simultaneously enable adequate production of the cytokines IL-12p40 and IL-6 and cell surface expression of MHC II, CD86, CD209 and CD40. These findings are consistent with previous observations in as much as IL-10 is primarily responsible for inhibiting activated DCs through the inhibition of IL-12 production and MHC II expression [5]. The ability of the relatively hydrophobic polyanhydrides used in this study to activate DCs is in agreement with Matzinger’s “danger signal” hypothesis in which hydrophobic molecules are internalized and induce activation of innate immune cells [47].

While significantly different cellular mechanisms are engaged by cells encountering nanospheres (internalization and intracellular interaction) versus those interacting with films (extracellular interaction), it is still important to understand the immune activation in the two geometries because their end-use applications may activate vastly different immune mechanisms. It appears that both the hydrophobic nature and the device geometry (film or particle) of this polymer system play important roles in DC activation processes. The hydrophobicity of the polymer alone was not enough to activate the DCs as demonstrated by the low levels of cell surface marker expression induced by the polyanhydride films. Particle internalization or phagocytosis, possibly mediated by hydrophobicity, appears to be necessary to achieve an enhanced DC activation as seen in Figure 8.9. Using confocal microscopy, it has been demonstrated that CPH:SA nanospheres are internalized by phagocytic cells [45]. However, internalization may not be necessary for production of IL-6, as an increased production of this cytokine is observed when the DCs are incubated with polyanhydride films. This is in agreement with previous studies with PLGA microparticles and films in which the inhibition of DC phagocytosis did not affect IL-6 production [11].
Surface interactions between the DCs and the CPH-rich polymer film treatments may play an important role in the production of this cytokine. Overall, the results suggest that the specific chemistry of the polyanhydride nanospheres and films dictate the differential level of DC activation. These conclusions are broadly supported by previous work [45], which indicates that polyanhydride nanospheres are differentially engulfed by phagocytic cells based on polymer chemistry.

In some cases, the cellular activation may not be a desirable feature for a biomaterial. For the body to accept an implant and resume tissue regeneration it is integral for such a device to avoid activating the immune system. The CPH:SA film system is a promising candidate for tissue engineering applications as the PCA biplot depicted its low level of immune activation both with cytokine production (except IL-6) and cell surface maker expression. In addition, as mentioned previously these films have shown outstanding cellular biocompatibility at concentrations of 2.8 mg/mL [35]. Despite the ability of CPH-rich film chemistries to enhance production of IL-6, the PCA analysis has shown that an intermediate or slightly SA-rich film chemistry (i.e., 25:75 CPH:SA) would have the least likelihood of producing an adjuvant effect or inducing a potent immune response (Figure 8.10). It should be emphasized that the interpretation of the PCA plots were made following blind data analyses. The fact that the clustering of data is consistent with expected observations simply confirms the robustness of the data analysis techniques. In this work the data dimensionality reduction techniques were used for classification purposes. With the availability of more experimental data, statistical learning tools can be introduced that will provide predictive information.
In the future, it will be necessary to study antigen presentation of DCs \textit{in vivo} following stimulation by antigen-loaded polyanhydride nanospheres. Further, it will be critical to elucidate the mechanisms of DC activation as related to other immune effector cells and induction of protective immunity.

\section*{8.6 Conclusions}

The CPH:SA polyanhydride system is a promising candidate for applications in drug/vaccine delivery and tissue engineering. It has been shown that the CPH:SA polymer film system provides a gentle, biocompatible environment necessary for multi-component implants; in contrast, the CPH:SA nanosphere system provides an adjuvant effect by enhancing DC activation. The informatics analysis employed in this study defined the clear differences between these two effects (chemistry and device geometry) and showed that the CPH:SA system has immunomodulatory capabilities to regulate both cellular expression of surface markers and production of cytokines. This rapid and multiplexed investigation of the adjuvant effect (five different polymer film chemistries and six different nanosphere chemistries with multiple replicates) was made possible by the use of a novel multiplexed approach to fabricate nanospheres and films and screen their interactions with immune cells. This generality of the technique paves the path for rational design and development of biomaterials for specific applications in drug/vaccine delivery and tissue engineering.

\section*{8.7 Acknowledgments}

The authors would like to thank the ONR-MURI Award (NN00014-06-1-1176) and the ISU Institute for Combinatorial Discovery for financial support. We would also like to thank
Andrea Dorn for her technical assistance with the Luminex assay and Shawn Rigby for his technical assistance with flow cytometry.

8.8 References

36. Vogel BM, Mallapragada SK, Narasimhan B. Rapid synthesis of polyanhydrides by
Figure 8.1: High throughput FTIR analysis of the polymer chemistry (% CPH) varying across the CPH:SA film library. The line shows the intended copolymer compositions, which are 0, 25, 50, 75, and 100% CPH for wells 1-5 respectively.
Figure 8.3: Analysis of MHC II expression by C57BL/6 DCs with histograms comparing between treatments in light grey and the isotype non-specific control in dark grey. Treatments represented in the histograms include a) LPS, b) NS, c) poly(SA) nanospheres, d) 25:75 CPH:SA nanospheres, e) 63:37 CPH:SA nanospheres, f) poly(SA film), g) 50:50 CPH:SA(film), and h) poly(CPH) film. Below the histograms is the complete set of results for MFI expression of C57BL/6 DC cell surface marker MHC II by multiplexed CPH:SA libraries. Data is representative of a minimum of 3 replicates per stimulation group. Errors bars indicate standard error. See Table 8.2 for statistical analysis.
Figure 8.4: Analysis of CD40 expression by C57BL/6 DCs with histograms comparing treatments in light grey and the isotype non-specific control in dark grey. Treatments represented in the histograms include a) LPS, b) NS, c) poly(SA) nanospheres, d) 25:75 CPH:SA nanospheres, e) 63:37 CPH:SA nanospheres, f) poly(SA) film, g) 50:50 CPH:SA film, and h) poly(CPH) film. Below the histograms is the complete set of results for MFI expression of C57BL/6 DC cell surface marker CD40 by multiplexed CPH:SA libraries. Data is representative of a minimum of 3 replicates per stimulation group.
Figure 8.5: Analysis of CD86 expression by C57BL/6 DCs with histograms comparing between treatments in light grey and the isotype non-specific control in dark grey. Treatments represented in the histograms include a) LPS, b) NS, c) poly(SA) nanospheres, d) 25:75 CPH:SA nanospheres, e) 63:37 CPH:SA nanospheres, f) poly(SA) film, g) 50:50 CPH:SA film, and h) poly(CPH) film. Below the histograms is the complete set of results for MFI expression of C57BL/6 DC cell surface marker CD86 by multiplexed CPH:SA libraries. Data is representative of a minimum of 3 replicates per stimulation group. Errors bars indicate standard error. LPS (positive control) normalized value is 3.6. See Table 8.2 for statistical analysis.
Figure 8.6: Analysis of CD209 expression by C57BL/6 DCs with histograms comparing between treatments in light grey and the isotype non-specific control in dark grey. Treatments represented in the histograms include a) LPS, b) NS, c) poly(SA) nanospheres, d) 25:75 CPH:SA nanospheres, e) 63:37 CPH:SA nanospheres, f) poly(SA) film, g) 50:50 CPH:SA film, and h) poly(CPH) film. Below the histograms is the complete set of results for MFI expression of C57BL/6 DC cell surface marker CD209 by multiplexed CPH:SA libraries. Data is representative of a minimum of 3 replicates per stimulation group. Errors bars indicate standard error. See Table 8.2 for statistical analysis.
Figure 8.7: Production of IL-6 and IL-12p40 by C57BL/6 DCs upon stimulation with multiplexed CPH:SA libraries. Data is representative of a minimum of 3 replicates per stimulation group. LPS (positive control) normalized value is 108 for IL-6 and 75 for IL-12p40. The 63:37 CPH:SA nanosphere and non stimulated average concentrations before normalization for IL-6 were 406 pg/mL and 30 pg/mL respectively and for IL-12p40 were 1130 pg/mL and 335 pg/mL respectively. Errors bars indicate standard error. See Table 8.2 for statistical analysis.
Figure 8.8: PCA biplot of CPH:SA nanosphere stimulation of all the cell surface markers, CD209, CD40, CD86 and MHC II and both cytokines IL-6 and IL-12p40. The plot maps out high dimensional correlations permitting one to track the relative influences of varying the polymer chemistry. For example, the cluster marked “cell surface markers” indicates its inverse correlation with increasing CPH concentration. On the other hand cytokine production is moderately correlated with CPH concentration. The choice of the principal components is based on standard statistical procedures. The first principal component (PC1) explains 19.54% data variance, and the second principal component (PC1) explains 55.99% data variance which together account for 75.39% of the data variance, meaning that PC1 and PC2 could replace the original six variables (CD209, CD40, CD86, MHC II, IL-6, and IL-12p40) with little loss of information.
Figure 8.9: PCA biplot comparing the polyanhydride nanosphere and film systems showing inverse correlations with clusters in opposite quadrants. The IL-12p40 production is strongly associated with the nanospheres while the IL-6 production is more associated with the film geometry. The first principal component (PC1) explains 55.14% data variance, and the second principal component (PC2) explains 30.82% data variance which together accounts for 85.89% of the data variance, meaning that PC1 and PC2 could replace the original six variables (CD209, CD40, CD86, MHC II, IL-6, and IL-12p40) with little loss of information.
Figure 8.10: PCA biplot of CPH:SA film stimulation of all cell surface markers, CD209, CD40, CD86 and MHC II and both cytokines IL-6 and IL-12p40. This PCA projection shows the strong influence of cytokine production with polymer chemistry (as noted by their large PCA vectors) compared to the cell surface vectors of nearly zero or very small magnitudes. The first principal component (PC1) explains 88.41% data variance, and the second principal component (PC1) explains 9.52% data variance which together account for 97.93% of the data variance, meaning that PC1 and PC2 could replace the original six variables (CD209, CD40, CD86, MHC II, IL-6, and IL-12p40) with little loss of information. As depicted in the figure, most of the data variance is attributed to the two cytokines, IL-6 and IL-12p40.
8.10 List of Tables

Table 8.1: Molecular weight analysis of the CPH:SA polymer film library using GPC and $^1$H NMR.

<table>
<thead>
<tr>
<th>CPH:SA Polymer Film Library</th>
<th>$M_n$ (Da) from GPC</th>
<th>$M_n$ (Da) from $^1$H NMR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poly(SA)</td>
<td>11154</td>
<td>12555</td>
</tr>
<tr>
<td>25:75 CPH:SA</td>
<td>9692</td>
<td>10854</td>
</tr>
<tr>
<td>50:50 CPH:SA</td>
<td>13264</td>
<td>12872</td>
</tr>
<tr>
<td>75:25 CPH:SA</td>
<td>12674</td>
<td>13442</td>
</tr>
<tr>
<td>Poly(CPH)</td>
<td>16477</td>
<td>15247</td>
</tr>
</tbody>
</table>
Table 8.2: Statistical analysis of cell surface marker expression and cytokine production corresponding to Figures 8.3-8.7. Treatments with the same letter are not statistically significant from one another. Statistical significance corresponds to $p \leq 0.05$.

<table>
<thead>
<tr>
<th></th>
<th>MHC II Figure 8.3</th>
<th>CD40 Figure 8.4</th>
<th>CD86 Figure 8.5</th>
<th>CD209 Figure 8.6</th>
<th>IL-6 Figure 8.7</th>
<th>IL-12p40 Figure 8.7</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPS</td>
<td>BCD</td>
<td>ABC</td>
<td>A</td>
<td>CDE</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>NS</td>
<td>E</td>
<td>F</td>
<td>E</td>
<td>E</td>
<td>F</td>
<td>C</td>
</tr>
<tr>
<td>Poly(SA) Nano</td>
<td>A</td>
<td>A</td>
<td>AB</td>
<td>A</td>
<td>E</td>
<td>C</td>
</tr>
<tr>
<td>13:87 CPH:SA Nano</td>
<td>AB</td>
<td>AB</td>
<td>AB</td>
<td>AB</td>
<td>CDE</td>
<td>BC</td>
</tr>
<tr>
<td>25:75 CPH:SA Nano</td>
<td>BC</td>
<td>ABCD</td>
<td>AB</td>
<td>ABC</td>
<td>E</td>
<td>BC</td>
</tr>
<tr>
<td>37:63 CPH:SA Nano</td>
<td>BCD</td>
<td>ABCD</td>
<td>ABC</td>
<td>ABCD</td>
<td>CDE</td>
<td>BC</td>
</tr>
<tr>
<td>50:50 CPH:SA Nano</td>
<td>CDE</td>
<td>CDEF</td>
<td>BCD</td>
<td>BCDE</td>
<td>CDE</td>
<td>BC</td>
</tr>
<tr>
<td>63:37 CPH:SA Nano</td>
<td>DE</td>
<td>CDEF</td>
<td>CDE</td>
<td>DE</td>
<td>CD</td>
<td>B</td>
</tr>
<tr>
<td>Poly(SA) Film</td>
<td>BC</td>
<td>ABCDE</td>
<td>ABC</td>
<td>BCDE</td>
<td>DE</td>
<td>D</td>
</tr>
<tr>
<td>25:75 CPH:SA Film</td>
<td>BCDE</td>
<td>BCDEF</td>
<td>BCDE</td>
<td>DE</td>
<td>BCDE</td>
<td>D</td>
</tr>
<tr>
<td>50:50 CPH:SA Film</td>
<td>CDE</td>
<td>DEF</td>
<td>BCDE</td>
<td>DE</td>
<td>BCD</td>
<td>CD</td>
</tr>
<tr>
<td>75:25 CPH:SA Film</td>
<td>CDE</td>
<td>EF</td>
<td>DE</td>
<td>DE</td>
<td>BC</td>
<td>CD</td>
</tr>
<tr>
<td>Poly(CPH) Film</td>
<td>DE</td>
<td>EF</td>
<td>DE</td>
<td>E</td>
<td>B</td>
<td>C</td>
</tr>
</tbody>
</table>
Chapter 9: Amphiphilic Polyanhydride Films

Promote Neural Stem Cell Adhesion and Differentiation

A paper submitted for publication in Tissue Engineering

Latrish K. Petersen1, Jisun Oh2, Donald S. Sakaguchi2, Surya K. Mallapragada1, and Balaji Narasimhan1*

Keywords: Neural stem cells, polyanhydride films, dendritic cells, implant coatings

1 Department of Chemical and Biological Engineering, Iowa State University, Ames, Iowa 50011, USA
2 Department of Genetics, Development & Cell Biology, Iowa State University, Ames, Iowa 50011, USA
*To whom all correspondence should be addressed
9.1 Abstract

Several challenges currently exist for rational design of functional tissue engineering constructs within the host, which include appropriate cellular integration, avoidance of bacterial infections, and low inflammatory stimulation. This work describes a novel class of biodegradable, amphiphilic polyanhydrides with many desirable protein-material and cell-material attributes capable of confronting these challenges. The biocompatible amphiphilic polymer films were shown to release laminin in a stable and controlled manner, promote neural cell adhesion and differentiation, and evade inflammatory responses of the immune system. Using high throughput approaches, it was shown that polymer chemistry plays an integral role in controlling cell-film interactions, which suggests that these polyanhydrides can be tailored to achieve the desired cell adhesion and differentiation while minimizing immune recognition. These findings have important implications for development of engineered constructs to regulate differentiation and target the growth of transplanted cells in stem cell-based therapies to treat nervous system disorders.

9.2 Introduction

Biodegradable polymeric scaffolds and implant coatings have been used widely to facilitate implant tissue integration while defending against bacterial burdens \cite{1,2}. Polymers provide the ability to mechanically and chemically control cellular integration through structure and surface functionalization with or without controlled release of biological cues (growth factors, adhesion molecules, etc.) \cite{1,2}. Functionalization or controlled release properties can also be used to inhibit bacterial growth and adhesion \cite{1,3}. Proper cellular integration, limiting fibrosis (adhesion of fibroblasts which results in poor implant binding), and low
inflammatory stimulation are essential for polymer implant or scaffold viability within the host \[1, 2\].

Many current polymer systems lack the ability to provide controlled release of biologically active molecules and some fragile proteins may lose their function when encapsulated in biodegradable polymers such as poly(lactide-co-glycolide) (PLGA) \[4-6\]. In addition, with bulk eroding polymers such as PLGA, the structural integrity of the device may be compromised because once the molar mass is low enough, there is a rapid loss of mechanical properties \[2, 7, 8\]. In contrast, surface eroding polymers are characterized by a slow loss of mechanical stability over time, which would reduce the possibility of scaffold collapse before new tissue formation and integration with the host \[2\]. In addition to mechanical integrity, it is important for the polymer construct to evade clearance by the immune system and avoid an inflammatory response. Inflammation and immune activation can lead to the buildup of fibroblasts which inhibit tissue integration and implant acceptance by the host \[1, 2\]. Thus, it would be desirable to design surface eroding polymeric constructs for tissue engineering that are capable of modulating the inflammatory response of the immune system to provide a controlled environment conducive to cellular growth and regeneration.

Polyanhydrides have been studied extensively as vehicles for protein and vaccine delivery over the past decade \[9-27\]; however, little work to date has investigated their use in tissue engineering applications. These surface eroding polymers are biologically inert, non-toxic, and non-mutagenic and are capable of providing sustained release kinetics of encapsulated proteins \[11-13, 16, 18, 19, 23\]. Additionally, polyanhydrides have been shown to stabilize a wide
range of biologically active molecules, such as F1-V (vaccine antigen for pneumonic plague), PA (vaccine antigen for anthrax), lipocalin-2, ovalbumin, lysozyme, and tetanus toxoid (vaccine antigen for tetanus) [11-14, 16, 18, 20, 23, 28]. Specifically, polyanhydrides based upon 1,6-bis(p-carboxyphenoxy)hexane (CPH) and 1,8-bis(p-carboxyphenoxy)-3,6-dioxaoctane (CPTEG) have been studied extensively because of their amphiphilic properties, controlled protein release and protein stabilization capabilities, immune modulation, and cellular compatibility [9, 21, 22, 24, 25, 27]. These polymers can also be functionalized to target specific cellular receptors [26]. Upon degradation, CPTEG:CPH copolymers result in small changes in pH (7.6 to 7.1) unlike other systems (i.e., PLGA), which result in much greater decreases in pH to as low as 2.6 [18, 29-32]. These properties make this polymer system a promising candidate for tissue engineering applications combined with targeted delivery and/or surface functionalization.

The focus of this work was to investigate amphiphilic CPTEG:CPH polyanhydride copolymer films for their ability to control protein release and stability while providing a non-toxic, immune-stealthy, and cell conducible environment for neural stem cell growth and adhesion. The findings indicate that CPTEG:CPH copolymer films provide an ideal non-fouling substrate for cellular exclusion, but with the incorporation of extracellular matrix proteins, cellular adhesion is restored. Additionally, by altering the chemistry of the polymer, this polymer system was capable of modulating immune stimulation, protein release kinetics, and cellular differentiation. These studies were performed using a high throughput, combinatorial approach, which enabled the rapid assessment of protein-polymer and cell-polymer interactions.
9.3 Materials and Methods

Polymer library synthesis and characterization

CPH and CPTEG monomers were synthesized as described previously\textsuperscript{[33, 34]}. CPTEG:CPH copolymer film libraries were synthesized via a melt polycondensation reaction in multi-well substrates utilizing a robotic deposition apparatus, as reported previously\textsuperscript{[19, 20, 22, 34]}. Copolymer chemistry and molecular weight were determined by proton nuclear magnetic resonance (\textsuperscript{1}H NMR) spectroscopy using a Varian VXR 300 MHz spectrometer (Varian Inc., Palo Alto, CA). Gel permeation chromatography (GPC) was also used to measure the polymer molecular weight with a Waters GPC chromatograph (Milford, MA).

Fabrication of protein encapsulated and blank (no protein) film libraries

Blank and laminin-loaded combinatorial film libraries were fabricated from the polymer libraries as described previously\textsuperscript{[19, 22]}. Briefly, laminin and methylene chloride were added to the polymer film library, sonicated, and the protein/polymer solution in each well was separately dispensed onto glass coverslips and allowed to dry resulting in a protein-encapsulated film library.

High throughput protein release

Following encapsulation of laminin into the polymer films, the films were placed in a 24-well polystyrene plate, and 2 mL PBS buffer (0.1 mM, pH 7.6) added to each well. The plate was sealed to prevent evaporation and incubated in a horizontal shaker at 37°C and 100 rpm for the duration of the experiment. At incremental time points, samples of the supernatant were removed and replaced with fresh PBS buffer to maintain constant sink conditions.
Laminin concentration in each sample was determined with the micro bicinchoninic acid (BCA) assay (Thermo Scientific, Rockford, IL), which was carried out in triplicate. Protein release at each time point was normalized by the total amount of protein encapsulated into the film which is presented as mass fraction of released laminin [19].

Culture and stimulation of dendritic cells (DCs)

All procedures involving animals were conducted in accordance with procedures that were approved by the Iowa State University Institutional Animal Care and Use Committee. Bone marrow derived dendritic cells (BMDCs) were isolated from C57BL/6 mice (ISU Laboratory Animal Resource Facility, Ames, IA) and cultured using a previously developed method [21, 22, 24, 26, 27]. BMDCs were grown in DC culture medium (RPMI containing 1% L-glutamine, 1% penicillin-streptomycin solution, 2% HEPES, 0.5% gentamycin, 0.1% β-mercaptoethanol, and 10% heat inactivated fetal bovine serum (FBS)) supplemented with granulocyte macrophage colony stimulating factor (GM-CSF) (10 ng/mL). After nine days of culture, DCs were incubated for 48 h with the different treatments: polymer films (poly(CPTEG), 75:25 CPTEG:CPH, 50:50 CPTEG:CPH, 25:75 CPTEG:CPH, and poly(CPH), at a concentration of 1.5 mg/coverlip), no stimulation (NS, negative control), and lipopolysaccharide (LPS, 200 ng/mL, positive control).

Cell surface marker expression

The expression of cell surface markers including CD11c, CD86, CD40, MHC I, and MHC II was assessed with flow cytometry as described elsewhere [22, 27]. The samples were run on a Becton-Dickinson FACSCanto flow cytometer (San Jose, CA) and analyzed with FlowJo.
Cytokine production

Following incubation with the treatments, supernatants from DC cultures were collected and assayed for cytokines, TNF-α, IL-6, and IL-12p40, using the Luminex® Multiplex assay (Austin, TX) [22, 27].

MTT cellular toxicity assay

The CellTiter 96® Non-Radioactive Cell Proliferation Assay MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Promega, Madison, WI) was used to determine the cellular viability of DCs incubated with the polymer films for 48 h. The DCs were incubated with the MTT assay for 3 h and the cell viability was quantified by measuring the optical density.

Culture and stimulation of human neural progenitor cells

Human neural stem/progenitor cells (hNPCs), isolated from 16-18-week fetal cortex, were purchased from Lonza (Allendale, NJ) [35]. The hNPCs were cultured as neurospheres in maintenance medium (a proprietary medium, Bulletkit, Lonza) further supplemented with 10 ng/mL human recombinant basic fibroblast growth factor (bFGF) (Promega, Madison, WI) and 20 ng/mL epidermal growth factor (EGF) (Gibco, Grand Island, NY) [35]. This maintenance medium is referred to as MM. At day 0 the cells were plated at ~10 neurospheres per well (each well containing a 12-mm glass coverslip coated with the different polymer film treatment groups) in a 24 well plate in 500 μL of differentiation
medium (a proprietary medium, Bulletkit, Lonza) further supplemented with 20 ng/mL recombinant human brain-derived neurotrophic factor (BDNF; Sigma-Aldrich, St. Louis, MO). The differentiation medium is referred to as DM. The polymer film treatment groups included poly(CPTEG), 75:25 CPTEG:CPH, 50:50 CPTEG:CPH, and 25:75 CPTEG:CPH (blank or 1% laminin loaded in the polymer film) at a polymer coating of 1.5 mg/cover slip. The control treatment groups included laminin-coated coverslips incubated either in DM with BDNF as a positive control (laminin DM) or in MM as a negative control (laminin MM). Cultures with the different treatment groups were maintained for 7 days. Culture media were replenished with 350 μL of fresh media at days 3 and 5.

**Immunocytochemistry on hNPCs**

Following the seven day incubation period, the hNPCs were immunolabeled. The coverslips containing the cells were rinsed in 0.1 M phosphate (PO₄) buffer and then fixed with 4% paraformaldehyde in 0.1 M PO₄ buffer for 30 min. Following fixation, the cells were washed in PBS and then incubated with the primary TUJ1 antibody (mouse monoclonal IgG, R&D Systems, Minneapolis, MN) diluted (1:200) in blocking solution {0.4% BSA (Sigma Aldrich), 0.2% Triton X-100 (Fisher Scientific), and 2.5% normal goat serum (Jackson ImmunoResearch Inc., West Grove, PA)} overnight at 4°C. The next day the cells were rinsed in PBS and incubated with a goat anti-mouse secondary antibody (Cy5-conjugated, Jackson ImmunoResearch Inc.) diluted (1:500) in the same blocking solution for 2 h. Cells on the coverslips were rinsed in PBS and then incubated with a Alexa Fluor 488 (AF488)-conjugated primary MAP2B antibody (mouse monoclonal IgG, BD Pharmingen, San Diego, CA) and a Cy3-conjugated primary GFAP antibody (mouse monoclonal IgG, Sigma-Aldrich,
St. Louis, MO) diluted in the blocking solution (1:18 and 1:400, respectively) overnight at 4°C. The following day cells on the coverslips were rinsed in PBS. After nuclei were counterstained with 1 μM 4′,6-diamidino-2-phenylindole dilactate (DAPI) and the preparations were mounted on microscope slides with Gel Mount (Fisher Scientific) for fluorescence microscopy analysis.

**Microscopy, image acquisition, and data analysis**

Epifluorescence microscopy was carried out with an upright fluorescence microscope (Nikon Microphot FXA, Nikon Inc., Garden City, NY) equipped with a Retiga 2000R digital camera controlled by QCapture software (QImaging, Surrey, BC, Canada). A minimum of eight images with an average of 222 cells per image was taken per fluorochrome per treatment group using a 20X objective. Image J software (NIH, Bethesda, MD) was used to analyze the microscopy images. To calculate the percentage of immunoreactive cells, the number of cells immunoreactive for each antibody was divided by the total number of cells (DAPI-stained nuclei).

**Statistical analysis**

JMP software (SAS Institute, Cary, NC) was used to make comparisons between different treatments were determined using a model ANOVA with Tukey’s HSD as indicated by letters above each bar. Differing letters represent difference between treatments. Comparisons between treatments and controls were made using the student’s T-test and indicated by an asterisk above the bars.
9.4 Results

Polymer characterization and protein release

The NMR and GPC characterization indicated that polymer molecular weight, structure, and composition were as intended and in agreement with previous studies [23, 34, 36]. High throughput laminin release studies from CPTEG:CPH copolymer films (Figure 9.1) were carried out because laminin is an adhesion protein for cellular binding to substrates [37-39]. Figure 9.1 shows that laminin was released in a controlled manner over time as a function of polymer chemistry. By increasing the content of the less hydrophobic CPTEG in the copolymer, protein release rates were higher.

Polyanhydride libraries are biocompatible and induce low levels of immune response

To test the toxicity of polyanhydride film libraries to primary cells, dendritic cells (DCs), which are professional antigen presenting cells of the immune system, were incubated with varying concentrations of 50:50 CPTEG:CPH copolymer films and cell viability was examined by the MTT assay (Figure 9.2A). All polymer concentrations below 16.9 mg/mL resulted in no statistical difference for the cell viability compared to the “no polymer” (NP) positive control. This result signifies that a wide range of polymer coating or scaffold thicknesses could be utilized with this system.

To test the effects of the film libraries on induction of an immune response, DCs were incubated on the polymer films for two days. Supernatants were analyzed for secreted cytokines, TNF-α, IL-6, and IL-12p40, and cells were stained for expression of cell surface markers, MHC I, MHC II, CD40 and CD86, and analyzed by flow cytometry. Secretion of
these cytokines and expression of these cell surface markers are indicators of immune activation and DC maturation \[^{[40]}\]. The results showed that the CPTEG:CPH polymer films cause DCs to down-regulate cytokine secretion (IL-12p40 and IL-6) compared to the NP control (Figure 9.2B). TNF-\( \alpha \) was below detection limits for all treatments except LPS. Additionally, low levels of cell surface marker expression were observed (Figure 9.2C). Both cell marker expression and cytokine production appeared to be modulated in a chemistry-dependent manner. These findings suggest that there may be an optimal copolymer composition, such as 50:50 CPTEG:CPH, which is capable of achieving the lowest level of immune stimulation.

\textit{Adhesion of hNPCs to the polymer library is controlled by polymer chemistry}

Human neural stem/progenitor cells (hNPCs) were incubated for seven days with the polymer film library and investigated for their ability to adhere and differentiate. Initial studies evaluated the adhesion of hNPCs on blank or laminin-encapsulated polymer film libraries including poly(CPTEG), 75:25 CPTEG:CPH, 50:50 CPTEG:CPH, and 25:75 CPTEG:CPH. The results indicated that blank polymer films provided a non-fouling background preventing cellular adhesion with all polymer chemistries (data not shown). However, when laminin was encapsulated into the polymer films, cellular adhesion was restored in for CPH-rich polymer film chemistries (data not shown). These results indicate that the biological functionality of laminin was preserved upon encapsulation into the polymer films. In addition, CPTEG-rich polymer chemistries resulted in minimal cellular adhesion (even with laminin encapsulated into the polymer) (data not shown), which is likely a result of the ethylene glycol \[^{[1,41]}\] incorporated into the polymer backbone.
Differentiation of hNPCs is controlled by polymer chemistry

The three polymer film chemistries with adequate cellular adhesion (75:25 CPTEG:CPH, 50:50 CPTEG:CPH, and 25:75 CPTEG:CPH) were studied for their influence on hNPC differentiation after a 7 day co-incubation. Figure 9.3 suggests that film chemistry controlled the differentiation of hNPCs with 50:50 CPETG:CPH resulting in the lowest percentage of hNPCs immunoreactive for microtubule associated protein 2B (MAP2B), a marker of immature neurons. The other two chemistries resulted in significantly higher percentages of MAP2B-immunoreactive cells. The percentages of cells immunoreactive for beta III tubulin (TUJ1, marker of developing neurons) or glial fibrillary acidic protein (GFAP, glial cell marker) revealed increasing trends when in the presence of CPTEG-rich chemistries. The data in Figure 9.3 suggest that 75:25 CPTEG:CPH polymer films enhance the most hNPC differentiation into both astrocytes and neurons compared with other film treatments and control treatments {positive control: laminin-coated glass coverslip with NP film in differentiation media (laminin DM); negative control: laminin-coated glass coverslip with NP film in maintenance media (laminin MM)}.

Supplementary Figure 9.1 presents the percentages of hNPCs that were subcellular immunolabeled for MAP2B (Supplementary Figure 9.1A) and TUJ1 (Supplementary Figure 9.1B), respectively. The data in Supplementary Figure 9.1A suggest that higher fraction of hNPCs were undergoing neuronal differentiation when they were incubated on CPTEG-rich polymer films (75:25 and 50:50 CPTEG:CPH) than on 25:75 CPTEG:CPH films. In addition, the percentage of cells immunoreactive for MAP2B in both cell body and neurites was higher when the cells were cultured on 75:25 CPTEG:CPH films than CPH-rich polymer films.
Supplementary Figure 9.1B also demonstrated increasing trends for the percentages of TUJ1-positive hNPCs (immunolabeled in both cell body and neurites) when incubated with increasing CPTEG-content in the polymer film.

Images of each treatment group and marker can be observed in Figure 9.4 and corroborate the data presented in Figure 9.3. By quadruple labeling the hNPCs (DAPI-nucleus, AF488-MAP2B, Cy5-TUJ1, and Cy3-GFAP), double positive cell populations for the differentiation markers were assessed as shown in Figure 9.5. Figure 9.5A indicates that cells immunoreactive for MAP2B are also largely positive for TUJ1 or GFAP, especially when incubated on CPH-rich polymer films. However, cells immunoreactive for TUJ1 or GFAP are less positive for each other, but follow the same chemistry dependent trend (Figures 9.5B and 9.5C). The hNPC populations positive for MAP2B appeared to be more influenced by polymer film chemistry in their immunoreactivities for TUJ1 or GFAP than populations positive for TUJ1 or GFAP, which only resulted in chemistry dependent trends for co-labeling with GFAP or TUJ1, respectively. When examining the entire hNPC population (based upon DAPI count) (Supplementary Figure 9.2), the data indicate an opposite chemistry trend with CPTEG-rich polymer films influencing the most double positive expression of cells for TUJ1 and GFAP, and GFAP and MAP2B. In contrast, double positive cells for both neuronal makers, TUJ1 and MAP2B, trended toward CPH-rich chemistries.

9.5 Discussion

Many current approaches to tissue engineering scaffolds or implant coatings have focused on the use of surface functionalization or controlled release of biological signals to promote
cellular adhesion, growth, vascularization, differentiation, etc. and/or to inhibit the growth and viability of infectious microorganisms \cite{1,2}. In this regard, CPTEG:CPH copolymers represent a biodegradable delivery system capable of both controlled release and surface functionalization. In this work these copolymers were demonstrated to release laminin in a chemistry-dependent manner, which was a strong function of the hydrophobicity of the polymer film (Figure 9.1). All the polyanhydride chemistries tested in this work demonstrated the ability to preserve the functionality of laminin. This data is consistent with other work from our laboratory on the protein stabilization capabilities of this amphiphilic polymer system \cite{11,16,18,20,23}. In those studies, the stabilization of fragile recombinant proteins as well as more robust globular proteins was shown to be strongly dependent upon polymer chemistry, with the amphiphilic chemistries providing the best environment for preservation of protein activity (and structure). Recently, Carrillo-Conde et al. demonstrated that CPTEG:CPH nanoparticles can be functionalized with carbohydrates with an eye towards targeting DCs for immune activation \cite{26}. That approach could be applied to functionalize the polymer films in this work for promoting cell-specific adhesion and growth and/or for reducing bacterial adhesion and growth. Bactericidal activity of a tissue engineering scaffold or implant coating would be a key advancement for reducing implant infections, especially intracellular bacteria, which cannot be cleared by antibiotics and pose a threat to implant viability \cite{1}.

Biocompatibility and immune acceptance, specifically low inflammatory responses, of the implant by the host often dictate acceptance of the implant and in vivo viability. Materials capable of inducing low immune recognition would be ideal candidates for tissue engineering
applications as coatings to non-inflammatory implants or implants themselves. DCs were used to study the effect of CPTEG:CPH polymer films on cellular toxicity, expression of surface markers responsible for DC maturation and antigen presentation (MHC II, MHC I, CD86 and CD40), and secretion of both inflammatory and immune activating cytokines (TNF-α, IL-6, and IL-12p40). This work has demonstrated that the CPTEG:CPH polymer film system modulated the immune response of DCs in a chemistry dependent manner (Figure 9.2). Moderate to low CPTEG-containing copolymer compositions demonstrated simulation levels no higher than the negative (NP) control group. This indicates that these films could be stealth-like to the immune system. Furthermore, all the chemistries studied maintained background levels or decreased production of the pro-inflammatory cytokines TNF-α and IL-6 (Figure 9.2B). These data suggest that these films may elicit weak inflammatory responses in vivo. In other related work from our laboratories, when nanoparticles made of these polymers were administered intranasally or subcutaneously, very low levels of inflammation were observed [42]. Since nanoparticles can be phagocytosed, it is reasonable to expect that the same chemistry in non-phagocytic geometries (i.e., film, coating, scaffold) would elicit even lower levels of inflammation similar to what was observed in vitro [22].

Stealth-like behavior enabling protection from immune activation and clearance by the immune system are important characteristics for implant or scaffold acceptance and tissue integration. However, it is also important that cell survival, adhesion, and differentiation within the polymer are conducive for cellular integration and tissue regeneration. As shown in Figure 9.3A, the CPTEG:CPH films were biocompatible at concentrations thirty-fold
higher than those administered \textit{in vivo} \cite{15, 43}. Adhesion and differentiation of hNPCs was also enabled by this system in a chemistry-dependent manner. Adhesion was not favored for the CPTEG-rich chemistries due to the ethylene glycol content in the backbone of poly(CPTEG). This is consistent with other studies in which low protein adsorption was observed on the surface of microparticles composed of CPTEG-rich chemistries \cite{10}.

Cellular differentiation also appears to be influenced by chemistry with CPTEG-rich compositions influencing the most overall expression of cells positive for neuronal markers (Figures 9.3 and 9.4). This suggests that 75:25 CPTEG:CPH has the ability to enhance differentiation of hNPCs into neurons and glial cells. In addition, 50:50 CPTEG:CPH may also be able to promote neuronal differentiation as indicated by the higher fraction of hNPC cell bodies immunoreactive for MAP2B (Supplementary Figure 9.1A). This work demonstrates the capabilities of CPTEG:CPH polymer films to control cellular adhesion and differentiation in a chemistry-dependent manner while providing a system capable of releasing biologically relevant proteins for further controlling cellular behavior. By employing a combinatorial platform approach, these protein-material and cell-material interactions were rapidly screened, which will facilitate the rational design of tissue engineering systems composed of biodegradable polyanhydrides.

\textbf{9.6 Conclusions}

Amphiphilic polyanhydride films provide a biologically tunable environment capable of overcoming many of the challenges associated with tissue engineering constructs. They were capable of preserving protein structure while controlling protein release, which is integral for
dictating cellular behavior (adhesion, differentiation, etc.). The polymer films also demonstrated immune stealth characteristics indicated by background level stimulation of DCs and low inflammatory signaling. These attributes are important for implant host acceptance, whereas other properties promoting tissue specific cell interactions are vital for implant integration into the host. This amphiphilic polymer film system also demonstrated the capability to promote and control hNPC adhesion and differentiation. A copolymer containing 50% CPTEG and 50% CPH may present optimal properties for neural tissue engineering with hNPCs by limiting immune recognition while providing a conducible environment for cellular adhesion and differentiation. These amphiphilic polymers may provide an excellent platform technology for tissue engineering constructs.

9.7 Acknowledgments

The authors would like to acknowledge financial support from the ONR-MURI Award (NN00014-06-1-1176). The authors would also like to thank Shawn Rigby for his expertise in flow cytometry and Christian Tormos for his help with data analysis.

9.8 References

5. Fu K, Pack DW, Klibanov AM, Langer R. Visual evidence of acidic environment within
38. Tong YW, Shoichet MS. Enhancing the neuronal interaction on fluropolymer surfaces with mixed peptides or spacer group linkers. Biomaterials 2001;22(10):1029-1034.

9.9 List of Figures

![Graph](image)

Figure 9.1: Fractional release of laminin from CPTEG:CPH copolymer film libraries. The CPTEG-rich films released laminin most rapidly, whereas CPH-rich films released laminin the slowest. Error bars represent standard deviation and six independent experiments were carried out.
Figure 9.2: Biocompatibility and immune stimulation capabilities of CPTEG:CPH polymer films. A) Viability of DCs incubated with 50:50 CPTEG:CPH films at varying concentrations; B) cytokine production (TNF-α was below detection limits for all treatments except LPS) and C) cell marker expression of DCs incubated with polymer film libraries. Average IL-12p40, IL-6, and TNF-α concentrations for cells stimulated with LPS were 55900, 101500, and 4600 pg/mL respectively. In A), asterisks indicate statistical significance from the NP control group and in B) and C), letters indicate statistical significance between treatments. Three independent experiments were carried out with replicates of two in each experiment, and error bars represent standard error of the mean.
Figure 9.3: Percentage of hNPCs displaying neuronal (MAP2B and TUJ1) or glial (GFAP) differentiation. The CPTEG-rich films best enhanced hNPC differentiation. Letters indicate statistical significance between treatments, eight different images were analyzed with an average of 222 cells per image, and error bars represent standard deviation.
Figure 9.4: Representative epifluorescent images of hNPCs stained for DAPI (blue, column 1), MAP2B (neuronal marker, AF488 green, column 2), TUJ1 (neuronal marker, Cy5 red, column 3), and GFAP (glial cell marker, Cy3 yellow, column 4), after incubation with 25:75 CPTEG:CPH, 50:50 CPTEG:CPH, 75:25 CPTEG:CPH, laminin-coated coverslip in DM (positive control, Laminin DM), and laminin-coated coverslip in MM (negative control, Laminin MM). The hNPCs readily adhered to CPH-rich polymer films and strongly expressed differentiation markers.
Figure 9.5: CPH-rich chemistries resulted in the most double positive hNPC populations for MAP2B + TUJ1, MAP2B + GFAP, TUJ1 + GFAP, and GFAP + TUJ1 (MAP2B and TUJ1 = neuronal markers and GFAP = glial cell marker). A) The percentage of MAP2B⁺ hNPCs also positive for TUJ1 or GFAP; B) the percentage of TUJ1⁺ hNPCs also positive for MAP2B or GFAP; and C) the percentage of GFAP⁺ hNPCs also positive for MAP2B or TUJ1. Letters indicate statistical significance between treatments, eight different images were analyzed with an average of 200 cells per image, and error bars represent standard deviation.
Supplementary Figure 9.1: CPH-rich films best enhanced hNPC differentiation. Subcellular immunolabeling (cell body and neurite = complete differentiation, cell body only = early stage differentiation, total = cell body and neurite + cell body) indicating variable levels of neuronal differentiation for A) MAP2B and B) TUJ1. Letters indicate statistical significance between treatments, eight different images were analyzed with an average of 200 cells per image, and error bars represent standard deviation.
Supplementary Figure 9.2: CPTEG-rich films best promoted the expression of total double positive hNPCs for TUJ1 + GFAP and GFAP + MAP2B (MAP2B and TUJ1 = neuronal markers and GFAP = glial cell marker). The percentages of total hNPCs double positive for MAP2B, TUJ1, and GFAP were compared among the polymer chemistries. Letters indicate statistical significance between treatments, eight different images were analyzed with an average of 200 cells per image, and error bars represent standard deviation.
Chapter 10: Amphiphilic Polyanhydride

Nanoparticle Adjuvants Activate Innate Immune Responses in a Pathogen-Mimicking Manner

A paper submitted for publication in *Biomaterials*

L. K. Petersen¹, A. E. Ramer-Tait², S. R. Broderick³,⁴, C. Sun Kong³,⁴, B. D. Ulery¹, K. Rajan³,⁴, M. J. Wannemuehler², and B. Narasimhan¹

**Keywords:** Combinatorial, high throughput, polyanhydrides, vaccine adjuvants, nanoparticles

¹Department of Chemical and Biological Engineering, Iowa State University, Ames, IA 50011
²Department of Veterinary Microbiology and Preventive Medicine, Iowa State University, Ames, IA 50011
³Department of Materials Science and Engineering, Iowa State University, Ames, IA 50011
⁴Institute for Combinatorial Discovery, Iowa State University, Ames, Iowa 50011
*To whom all correspondence should be addressed*
10.1 Abstract

Techniques in materials design, immunophenotyping, and informatics can be valuable tools for using a molecular based approach to design vaccine adjuvants capable of inducing protective immunity that mimics a natural infection but without the toxic side effects. This work describes the molecular design of amphiphilic polyanhydride nanoparticles that activate antigen presenting cells in a pathogen-mimicking manner. Biodegradable polyanhydrides are well suited as vaccine delivery vehicles due to their adjuvant-like ability to: 1) enhance the immune response, 2) preserve protein structure, and 3) control protein release. The results of these studies indicate that amphiphilic nanoparticles possess pathogen-mimicking properties as evidenced by their ability to activate dendritic cells similarly to LPS. Specific molecular descriptors responsible for this behavior were identified using informatics analyses, including the number of backbone oxygen moieties, percent of hydroxyl end groups, polymer hydrophobicity, and number of alkyl ethers. Additional findings from this work suggest that the molecular characteristics mediating APC activation are not limited to hydrophobicity but vary in complexity (e.g., presentation of oxygen-rich molecular patterns to cells) and elicit unique patterns of cellular activation. The approach outlined herein demonstrates the ability to rationally design pathogen-mimicking nanoparticle adjuvants for use in next-generation vaccines against emerging and re-emerging diseases.

10.2 Introduction

Successful vaccines induce protective immune responses that mimic those induced by a natural infection but do not elicit the negative effects associated with disease [1]. To do so, they must signal to the innate immune system using mechanisms similar to those employed
by pathogens. Upon infection, the innate immune system is activated by pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharide (LPS) found on the surface of gram negative bacteria, which interact with Toll-like receptors (TLRs) and other pattern recognition receptors (PRRs) on the surface of antigen presenting cells (APCs), including dendritic cells (DCs) \[^2, 3\]. PAMPs contain repetitive molecular patterns that are recognized as “danger signals” by the host \[^3\]. Moreover, PAMPs are often comprised of insoluble, hydrophobic moieties and are presumed to interact with PRRs, thereby providing signals that activate the innate immune system \[^4, 5\]. In contrast, the soluble antigens and adjuvants found in current vaccine formulations cannot provide the same degree of continued stimulation. An opportunity exists to exploit the material properties of biodegradable polymers in order to rationally design vaccine adjuvants that mimic the behavior of pathogens, including prolonged in vivo residence times capable of providing extended immune activation and continued stimulation of APCs, without inducing the deleterious effects of disease.

Polyanhydrides are biodegradable materials that have been well documented to provide sustained delivery of proteins and stabilization of vaccine antigens \[^6-12\]. Copolymers based upon sebacic acid (SA), 1,6-bis-(p-carboxyphenoxy)hexane (CPH), and 1,8-bis-(p-carboxyphenoxy)-3,6-dioxaoctane (CPTEG) have been studied as antigen carriers and adjuvants. These copolymers degrade into non-toxic, non-mutagenic degradation products and have demonstrated biocompatibility both in vivo and in vitro at concentrations expected for human use \[^13-16\]. Some amphiphilic polyanhydrides have also been reported to exhibit adjuvant characteristics capable of enhancing the adaptive immune response \[^13, 16\].
When designing vaccine adjuvants, it is integral to understand the molecular properties of the adjuvant responsible for immune cell activation that would facilitate the induction of long-lived, protective immunity. In this work, we investigated the molecular properties of polyanhydride nanoparticles responsible for their robust stimulation of DCs. A simultaneous investigation of nanoparticle internalization and activation of DCs was complemented by informatics analysis to identify important polymer descriptors responsible for mimicking the adjuvant capabilities of the PAMP, LPS. While many previous studies have investigated the expression of cell surface markers and production of cytokines caused by stimulation with polymeric adjuvants [12, 17, 18], this is the first study to report the complex relationship between nanoparticle internalization, DC activation, and polymer chemistry (i.e., through polymer descriptors). This is also the first study to present a direct comparison of cellular activation between DCs that have engulfed the nanoparticles and those that have not in the same culture. These studies identified molecular descriptors of polyanhydrides that are responsible for DC activation in a manner that closely resembles a pathogen-mimicking profile. The results indicate that polyanhydride nanoparticles may provide ‘danger signals’ to APCs through specific molecular descriptors resulting in innate immune activation.

10.3 Materials and Methods

Materials

The chemicals utilized in the monomer synthesis include: 1,6-dibromohexane, tri-ethylene glycol, 4-hydroxybenzoic acid, 1-methyl-2-pyrrolidinone, 4-p- and 1,6-dibromohexane; these were purchased from Sigma Aldrich (St. Louis, MO); 4-p-fluorobenzonitrile was purchased from Apollo Scientific (Cheshire, UK); and sulfuric acid, acetonitrile, dimethyl formamide
(DMF), toluene, and potassium carbonate were obtained from Fisher Scientific (Fairlawn, NJ). Chemicals for the polymerization and nanoparticle fabrication include petroleum ether, pentane, acetic anhydride, chloroform, and methylene chloride; all were purchased from Fisher Scientific. Deuterated chemicals for NMR analysis include chloroform and dimethyl sulfoxide (DMSO; Cambridge Isotope Laboratories, Andover, MA). β-mercaptoethanol, *E. Coli* lipopolysaccharide (LPS), and rat immunoglobulin (rat IgG) were purchased from Sigma Aldrich and brefeldin A from eBioscience (San Diego, CA). The materials required for the DC culture medium include: granulocyte macrophage colony stimulating factor (GM-CSF), purchased from PeproTech (Rocky Hill, NJ); HEPES buffer, RPMI 1640, penicillin-streptomycin, and L-glutamine, purchased from Mediatech (Herndon, VA); and heat inactivated fetal calf serum, purchased from Atlanta Biologicals (Atlanta, GA). Materials used for flow cytometry included: permeabilization buffer (10x) and intracellular (IC) fixation buffer, purchased from eBioscience (San Diego, CA); unlabeled anti-CD16/32 FcγR, purchased from Southern Biotech (Birmingham, AL); unlabeled hamster IgG, peridinin-chlorophyll proteins-Cy5.5 (PerCP/Cy5.5) conjugated anti-mouse IL-12/23 p40 (clone C17.8), phycoerythrin (PE) conjugated anti-mouse IL-6 (clone MP5-20F3), biotin conjugated anti-mouse MHC II (I-Ex) (clone 14-4-4S), phycoerythrin-Cy5 (PE/Cy5) conjugated anti-mouse CD11c (clone N418), fluorescein isothiocyanate (FITC) conjugated anti mouse/rat MHC Class II (I-Ex) (clone 14-4-4S), PE conjugated anti-mouse MHC Class I (H-2Kd/H-2Dd)(clone 34-1-2S), allophycocyanin (APC) anti-mouse CD40 (clone 1C10), phycoerythrin-Cy7 (PE/Cy7) anti-mouse CD86 (clone GL-1), Alexa Fluor® 700 anti-mouse CD11c (clone N418), and biotin conjugated anti-mouse CIRE (DC-SIGN or CIRE) (clone 5H10; and corresponding isotypes: PerCP-Cy5.5 conjugated rat IgG2a κ, PE conjugated rat IgG2 κ,
biotin conjugated mouse IgG2a κ, PE/Cy5 rat IgG2a κ (clone eBR2a), PE-conjugated rat 
IgG2a (clone eBR2a), APC rat IgG2a κ (clone eBR2a), PE/Cy7 conjugated rat IgG2b (clone 
KLH/G2b-1-2), Alexa Fluor® 700 conjugated Armenian hamster IgG (clone eBio299Arm),
and biotin conjugated rat IgG2a (clone eBR2a); and APC-Cy7-conjugated streptavidin. All of 
these reagents were purchased from eBioscience. Cadmium selenide quantum dots (QDs) 
(emission at 630nm) were a gift from Dr. Aaron Clapp at Iowa State University.

*Polymer synthesis, nanoparticle fabrication, and characterization*

SA monomer was purchased from Sigma Aldrich. 1,6-bis(p-carboxyphenoxy)hexane (CPH) 
monomer, 1,8-bis(p-carboxyphenoxy)-3,6-dioxaocatane (CPTEG), and conventional 
CPTEG:CPH and CPH:SA polymers were synthesized as described previously [19-22].
Combinatorial discrete libraries of CPTEG:CPH and CPH:SA copolymers were synthesized at high throughput as described previously [9, 10, 17, 18]. Following synthesis, the polymers were 
dissolved in a solvent (methylene chloride), QDs added to the dissolved polymer (nothing 
was added at this step for blank particles), dispersed by sonication at 40 Hz for 30 s, and then 
rapidly precipitated into a non-solvent (pentane) for the formation of nanoparticle libraries [9, 
10, 18]. These libraries were dried under vacuum drying or via rapid filtration and stored under 
dry conditions until use in the cellular assays. Conventional nanoparticles were fabricated 
with previously described methods [16, 23]. The polymer libraries were characterized by 1H 
nuclear magnetic resonance (NMR) spectroscopy. The nanoparticle libraries were imaged 
using scanning electron microscopy (SEM) to assess size and morphology.
Culture and stimulation of murine DCs

All experiments involving animals were carried out in accordance with procedures approved by the Iowa State University Institutional Animal Care and Use Committee. DCs were grown as described previously \cite{12,18} and stimulated with either 200 ng/mL lipopolysaccharide (LPS; a positive control), 125 μg/mL of QD-loaded CPTEG:CPH copolymer nanoparticles of various molar ratios (60:40, 50:50, 40:60, 30:70, 25:75, 20:80, and 10:90), QD-loaded CPH:SA copolymer nanoparticles of various molar ratios (0:100, 13:87, 25:75, 37:63, 50:50, and 63:37) or left untreated (NS; non-simulated, negative control). Treatments were applied to the DCs on day nine post-harvest and incubated for 48 h. Flow cytometry was then performed to assess the phenotype of the DCs. In all cases, cells were > 90% positive for the DC specific marker, CD11c (data not shown). To account for QD release due to particle degradation which would result in cells “false positive” for particles, a released QD control (background) was subtracted from each treatment group. In this control experiment, QD-loaded nanoparticles of all chemistries were allowed to incubate in DC culture medium for 48 h. After the incubation, the particles were centrifuged and the supernatants containing the released QDs were added to DCs for 48 h to account for any fluorescence caused by the uptake of released QDs as opposed to internalization of QD-loaded nanoparticles. For experiments investigating cytokine production via flow cytometry, brefeldin A was administered at the same time as the treatments to prevent secretion of the protein from the cells.

For the experiments investigating cytokine production, brefeldin A was administered at the same time point as the treatments to prevent protein transport out of the cells. On day 11,
supernatants were collected for cytokine analysis and cells were stained for flow cytometry.

Flow cytometry

After stimulation, DCs were assessed for the expression of MHC I, MHC II, CD40, CD86, and CIRE as described previously \(^{12,18}\). To detect the intracellular cytokines IL-12p40 and IL-6, cells were labeled according to the manufacturer’s recommended protocol (eBioscience). No differences were observed in expression of MHC I, MHC II, CD86, CD40 or CIRE when DCs were cultured with nanoparticles fabricated combinatorially or conventionally (Supplementary Figure 10.1).

Statistical and informatics analyses

Statistical analysis was performed using JMP® statistical software. Two-group comparisons were made using a student’s T-test whereas multiple-group comparisons were made with Tukey’s HSD in which case the data was log transformed.

Principal Component Analysis (PCA), a dimensionality reduction technique, was employed to provide a straightforward and parsimonious description of the covariance structure \(^{24,25}\). The principal components (PCs) are linear combinations of the original variables, and present new axes that represent the directions with maximum variance. The projection of the original multi-dimensional data to the two-dimensional space constructed by the first and second PCs provides a means of data visualization and interpretation and can uncover unknown relationships, thereby enabling new data interpretations \(^{24,25}\). In this work, PCA was used to uncover the latent features of the DC activation data and explain the relationships between
polymer chemistry, cell marker expression, and cytokine production. The PCA analysis for DC activation (Figure 10.4F) used double positive populations based upon all combinations of MHC II, CD40, CD86, IL-6 and IL-12p40 data. Partial least squares (PLS) regression connected the descriptor data set with the results of the PC analyses. By projecting the data onto a high-dimensional hyperplane defined by the PCA analysis of training data, the impact of the descriptors on the property were identified, while taking co-linearity into account [26].

10.4 Results

_Nanoparticle internalization is required for enhanced CD40 expression and cytokine production but not for enhanced expression of MHC II and CD86_

Bacterial internalization by APCs is an important step in cellular activation and immune signaling [27-29]. The CPH:SA and CPTEG:CPH nanoparticles were, therefore, tested for their ability to be internalized by DCs. Incubation of CPTEG-rich and SA-rich chemistries with DCs resulted in an average of ~6% and ~30% nanoparticle-positive cells, respectively (Figure 10.1). The observed positive correlation between nanoparticle internalization and decreasing hydrophobicity is consistent with that observed for cell surface marker expression and cytokine secretion (Supplementary Figures 10.1 and 10.2). Moreover, these particle chemistry trends are in agreement with previous work from our laboratories assessing internalization of particles by THP-1 human monocytes and murine DCs via confocal microscopy [12, 23].

Previous studies from our laboratory have demonstrated that DC activation is dependent upon nanoparticle chemistry [12, 18], however, these studies did not directly assess the
activation of DCs relative to the presence of internalized nanoparticles. Thus, we sought to determine the effect of nanoparticle internalization and chemistry on DC activation. The addition of SA- or CPTEG-rich nanoparticles to DC cultures provided a greater enhancement of cell surface marker expression (Figure 10.2) and cytokine production (Figure 10.3) as compared to CPH-rich nanoparticles. Strikingly, nanoparticle internalization was necessary for increasing surface CD40 expression and the production of the cytokines IL-6 and IL-12p40 (Figures 10.2E, 10.2F and 10.3). In contrast, enhancement of MHC II and CD86 surface expression on DCs was not dependent on nanoparticle internalization, as evidenced by no significant difference in expression levels between nanoparticle-negative and -positive populations (Figure 10.2A-D).

Amphiphilic 50:50 CPTEG:CPH nanoparticles mimic LPS by creating DC populations that are similar in activation phenotype

To date, analysis of DC activation has focused on single parameters (i.e., MHC II or IL-6 expression). However, expression of antigen presenting and co-stimulatory molecules and secretion of cytokines by DCs act simultaneously to orchestrate an adaptive immune response, including activation of T cells and differentiation of B cells into plasma cells. To study the relationship between nanoparticle chemistry and DC activation, CD11c-positive cell populations were analyzed for their simultaneous production of cytokines and expression of cell markers. This analysis focused on the three treatment groups (LPS, poly(SA), and 50:50 CPTEG:CPH) that induced the most significant increases in DC activation and produced the largest percentages of cells that were positive for surface markers and cytokines. The other treatment groups (CPH-rich and non-stimulated) were not included in
this analysis because their double positive DC populations were too small. We studied only DCs that were double positive for two of the following parameters: MHC II, CD86, CD40, IL-12p40, or IL-6 (Figure 10.4A-E). The analysis was performed by singling out cells positive for one event (i.e. MHC II) and within that population, identifying cells also positive for another event (i.e. CD86, CD40, IL-12p40, or IL-6). The results revealed remarkably similar activation trends between DCs treated with the amphiphilic 50:50 CPTEG:CPH nanoparticles and LPS (Figure 10.4). In contrast, the activation phenotype of DCs treated with poly(SA) nanoparticles was dissimilar to the phenotype created by LPS stimulation. PCA of this data confirmed that 50:50 CPTEG:CPH is significantly more similar to LPS than poly(SA) and that poly(SA) and LPS are not similar in terms of DC activation (Figure 10.4F). Together, these data suggest that while both nanoparticle groups stimulate DCs, the amphiphilic 50:50 CPTEG:CPH nanoparticles activate the DCs in a similar manner to LPS while poly(SA) nanoparticles do not.

*Use of informatics analysis to identify descriptors responsible for the pathogen-mimicking behavior of the amphiphilic 50:50 CPTEG:CPH nanoparticles*

Additional informatics analyses were performed to assess the similarities between the molecular descriptors for LPS and 50:50 CPTEG:CPH nanoparticles that are important for DC activation. Specifically, PLS analysis was used to identify which aspects of the molecular structure of 50:50 CPTEG:CPH led to the LPS-like behavior. The relationship between nanoparticle chemistries (50:50 CPTEG:CPH and poly(SA)) and LPS was quantitatively defined by comparing a list of molecular descriptors with PCA positions of the chemistries, whereby PCA served as a parameterization of the chemistries based on DC activation. Figure
10.5A shows the descriptors that were studied and Figure 10.5B shows the relationships between the descriptors and DC activation data. The descriptor library combined experimentally measured descriptors with those defined by Bicerano\textsuperscript{[31]}. The descriptors with negative impact values in Figure 10.5B make the formulations less similar to LPS, while descriptors with higher impact values make the formulations more likely to be pathogen-mimicking. Based on this analysis, number of backbone oxygen moieties, percent of hydroxyl end groups, polymer hydrophobicity, and number of alkyl ethers were identified as the key descriptors responsible for the pathogen-mimicking activation of DCs by the amphiphilic 50:50 CPTEG:CPH nanoparticles.

**10.5 Discussion**

In this work, nanoparticle chemistry and hydrophobicity were found to play an integral role in particle internalization by DCs (Figure 10.1). Once internalized, these properties continued to influence cell surface marker expression (Figure 10.2) and cytokine production (Figure 10.3). The least hydrophobic polymer chemistries, poly(SA) and 60:40 CPTEG:CPH, exhibited the greatest internalization by DCs (Figure 10.1); however, it is unlikely that hydrophobicity alone dictated this cellular response. These results are consistent with previous reports demonstrating that SA-rich polymer chemistries are efficiently taken up by APCs via both phagocytosis and endocytosis whereas CPH-rich nanoparticles are solely taken up by endocytosis and to a lesser degree\textsuperscript{[23]}. We therefore hypothesize that cellular internalization of the nanoparticles may be a function of other polymer-associated molecular patterns and descriptors. The patterns found in conserved, molecular signatures on pathogens are important signals of ‘danger’ to APCs, and include molecules such as LPS and flagella.
It is interesting to note that Seong and Matzinger have proposed that one of these ‘danger signals’ could be hydrophobicity. It is known that hydrophobic polystyrene particles are readily taken up by APCs; however, they induce very little activation of APCs (data not shown), which indicates that to achieve activation, a compilation of chemical and physical signals is required. A hydrophobic homopolymer like poly(SA) contains SA repeat units, whereas the amphiphilic 50:50 CPTEG:CPH copolymer presents alternating repeat units of CPTEG and CPH, together with a large number of backbone oxygen moieties and hydroxyl end groups. The reactivity ratios of the monomers in the 50:50 copolymer lead to alternating patterns of oxygen-rich CPTEG and CPH monomers that are presented to the cell. The presence of such patterns in these amphiphilic copolymers may explain why they are readily internalized. Poly(SA) particles may mimic a hydrophobicity-related ‘danger signal’, while amphiphilic CPTEG-rich particles may be internalized due to their ethylene glycol backbone that enables rapid degradation of the anhydride bonds in an aqueous environment and exposure of hydroxyl end groups to the APCs. Of note, hydroxyl end group and oxygen backbone moieties are also found associated with many components of pathogens and are known to influence pathogen internalization.

When designing vaccine adjuvants to enhance the immune response and to release encapsulated antigens, it is desirable for the adjuvant to be capable of mimicking the immune activation of DCs induced by a pathogen or natural infection. This includes upregulation of adhesion receptors (for migration to draining lymph nodes), antigen presentation machinery, and T cell co-stimulatory molecules. However, it is important that the adjuvants induce limited production of pro-inflammatory cytokines to prevent immune-mediated host tissue
damage. The polyanhydride nanoparticle adjuvants, 50:50 CPTEG:CPH and poly(SA), demonstrated the ability to enhance surface expression of MHC I, MHC II, CD86 and CIRE to levels greater than or equal to that induced by stimulation with the known PAMP, LPS (Supplementary Figure 10.1). These nanoparticle formulations were also able to enhance cytokine production in a chemistry dependent manner (Supplementary Figure 10.2). These data support our previous studies demonstrating that polyanhydride nanoparticles are biocompatible and induce significantly less cytokine secretion as compared to LPS \cite{12, 18}. In contrast to “off-the-shelf” materials like poly(lactic-co-glycolic acid) (PLGA), alginate, or chitosan, the SA- and CPTEG-rich nanoparticles were capable of enhancing expression of CIRE, MHC I, MHC II, and IL-12p40 production over non-stimulated cells \cite{35, 36}.

Further analyses revealed that nanoparticle internalization was required for the enhanced expression of CD40 and production of IL-12p40 and IL-6 whereas expression of CD86 and MHC II was found to be independent of nanoparticle internalization (Figure 10.2 and 10.3). This “bystander” effect observed for enhanced MHC II and CD86 expression by nanoparticle-negative DCs indicates that these molecules can be indirectly upregulated, possibly through communication with nanoparticle-positive cells. Our observations are consistent with previous studies describing that upregulation of surface MHC II and CD86 expression is less dependent or even completely independent of bacterial association and internalization as compared to expression of CD40 \cite{27, 28}. In addition, IL-12p40 and IL-6 production has been shown to be inhibited by preventing the association of DCs with bacterial cells \cite{27-29}. While dependent on bacterial association, production of IL-12p40 \cite{29} and IL-6 \cite{27} can be independent of phagocytosis. Together, our data demonstrate that CD40
signaling and production of IL-12p40 and IL-6 are controlled by particle internalization, which reveals an important pathogen-mimicking characteristic of polyanhydride nanoadjuvants.

Upon detection of a pathogen, APCs upregulate expression of antigen presentation and costimulatory molecules as well as secrete cytokines in order to efficiently activate naïve T cells and promote the differentiation of B cells [30]. In this work, the combinatorial approach enabled the identification of two nanoparticle adjuvants, poly(SA) and 50:50 CPTEG:CPH, which possess the ability to markedly enhance cell surface expression and cytokine production. Thus, these two adjuvants were investigated further for their influence on DC cell surface marker expression and cytokine production using a differential, population-based analysis. Both the amphiphilic 50:50 CPTEG:CPH nanoparticles and LPS induced surface expression of CD86 on DCs that were also positive for MHC II, CD40, IL-12p40 or IL-6 (Figure 10.4). These treatments also induced surface expression of MHC II on DCs that were also expressing CD86, CD40, IL-12p40 or IL-6 (Figure 10.4). Alternatively, poly(SA) nanoparticles stimulated CD40 expression on DCs positive for MHC II, CD86, IL-12p40 or IL-6, as well as induced IL-6 production from DCs that were also positive for MHC II, CD86, CD40 or IL-12p40 (Figure 10.4). These observations are consistent with previous work demonstrating that CD40 triggering induced maintenance of high levels of MHC II and upregulation of CD58, CD80, and CD86 [37]. Moreover, the phenotype MHC II\textsuperscript{high}, costimulatory molecule\textsuperscript{high}, and cytokine\textsuperscript{high} is indicative of fully mature DCs [38]. Together, these data indicate that polyanhydride nanoparticles are capable of inducing a mature DC phenotype.
The results presented herein indicate that while both SA-rich and CPTEG-rich polymer chemistries stimulate DCs, they are likely doing so through different pathways. In contrast to SA-rich nanoparticles, the amphiphilic 50:50 CPTEG:CPH nanoparticles activated DCs in a manner similar to that observed for LPS. These findings were validated with PCA (Figure 10.4F), which confirmed the similarities between 50:50 CPTEG:CPH nanoparticles and LPS and their dissimilarity to poly(SA) nanoparticles. Toll-like receptor agonists, such as LPS, have hydroxyl end groups and oxygen moieties in their backbone structure \[^{[3, 39]}\], which may be responsible for the similarities in DC activation observed between LPS and 50:50 CPTEG:CPH nanoparticles. LPS is an amphiphilic molecule containing lipid A (hydrophobic and hydrophilic regions), a variable hydrophilic O-polysaccharide, and core polysaccharide portions \[^{[40]}\]. The number, length, pattern, and location of the acyl chains \[^{[34, 40]}\] as well as structural changes to these chains alter the bioactivity of the lipid A \[^{[34, 40, 41]}\]. These results exemplify the importance of LPS structure, specific molecular oxygen patterns, and carbon chains to its bioactivity. Additionally, LPS and bacteria have thermal properties comparable to that of 50:50 CPTEG:CPH, which may also contribute to the observed similar cellular interactions \[^{[42, 43]}\]. Lower phase transition temperatures between the liquid crystalline and gel state of LPS have been shown to increase its endotoxic activity \[^{[44]}\]. Previous investigations indicate that structural properties are capable of influencing cellular behavior, such as the oxygen content in the polymer backbone dictating cellular growth \[^{[45]}\] and nanoparticle hydroxyl end group chemistry activating DCs via the complement pathway \[^{[46]}\].

The informatics analysis employed in this work identified specific molecular descriptors of
polyanhydrides that were responsible for the LPS-like activation of DCs. Specifically, number of backbone oxygen moieties, percent of hydroxyl end groups, hydrophobicity, and number of alkyl ethers were identified as the key properties influencing this pathogen-mimicking behavior (Figure 10.5). These analyses indicate that polymer chemistry markedly influences cytokine production and expression of cell surface markers in a pathogen-mimicking manner. The high throughput approach used in these studies to investigate a library of polyanhydride nanoparticles on DC activation resulted in the identification of amphiphilic polyanhydride nanoparticles with similar DC activation properties as LPS without the toxic side effects. This methodology has important implications for rational design of adjuvants for use in next-generation vaccines against emerging and re-emerging diseases.

### 10.6 Conclusions

The combinatorial approach developed in this work enabled the rapid investigation of the effects of polymer chemistry on nanoparticle internalization and activation of DCs. Our studies identified amphiphilic polyanhydride nanoparticles that possess pathogen-mimicking properties with respect to their capacity to activate DCs. Chemistry-dependent trends were observed, with the least hydrophobic chemistries (SA and CPTEG-rich) promoting the greatest internalization of nanoparticles and the production of cytokines by DCs. The same chemistry-dependent trends were observed for elevated expression of antigen presenting, T cell co-stimulatory, and migration-associated molecules. This work indicates that the signals associated with activating APCs are not just limited to hydrophobicity and are more likely to vary in complexity (e.g., presentation of oxygen-rich molecular patterns to cells) and elicit
unique forms of cellular activation. Other parameters, such as amphiphilicity, surface composition, thermal properties, and structural properties may also play key roles in innate immune activation.

10.7 Acknowledgments

The authors would like to thank the ONR-MURI Award (NN00014-06-1-1176) and the U.S. Army Medical Research and Materiel Command (Grant No. W81XWH-10-1-0806) for financial support. We would also like to thank Dr. Aaron Clapp of the Department of Chemical and Biological Engineering (CBE) at Iowa State University for providing cadmium-selenide quantum dots and Ms. Ashley Yeager of the CBE department at Iowa State University for assistance in dendritic cell culture and flow cytometry. Supporting Information is available online from Wiley InterScience or from the author.

10.8 References


43. Rodriguez-Torres A, Ramos-Sanchez MC, Orduna-Domingo A, Martin-Gil FJ, Martin-Gil J. Differential scanning calorimetry investigations on LPS and free lipids A of the

### 10.9 List of Figures

![Figure 10.1](image)

Figure 10.1: SA- and CPTEG- rich polyanhydride nanoparticles are internalized by dendritic cells. The percentage of nanoparticle-positive DCs after incubation with: A) CPH:SA nanoparticles or B) CPTEG:CPH nanoparticles. Data are represented as the mean ± SEM. N= 6 of three separate experiments. Treatments with different letters are significantly different from one another at p-value < 0.049.
Figure 10.2: Nanoparticle internalization by DCs is important for CD40 expression but not CD86 or MHC II expression. Percent of DC populations that internalized (nanoparticle+) or did not internalize (nanoparticle−) (A) CPH:SA nanoparticles or (B) CPTEG:CPH nanoparticles that were also positive for MHC II. Percent of DC populations that did or did not internalize (C) CPH:SA nanoparticles or (D) CPTEG:CPH nanoparticles that were also positive for CD86. Percent of DC populations that did or did not internalize (E) CPH:SA nanoparticles or (F) CPTEG:CPH nanoparticles that were also positive for CD40. Data are represented as the mean ± SEM. N=4 of three separate experiments. * represents a statistically significant difference between nanoparticle+ and nanoparticle− groups within a group at p-value < 0.04.
Figure 10.3: Nanoparticle internalization by DCs is important for cytokine production and is dependent upon nanoparticle chemistry. Percent of DC populations that internalized (nanoparticle\(^+\)) or did not internalize (nanoparticle\(^-\)) (A) CPH:SA nanoparticles or (B) CPTEG:CPH nanoparticles that were also positive for IL-12p40. Percent of DC populations that did or did not internalize (C) CPH:SA nanoparticles or (D) CPTEG:CPH nanoparticles that were also positive for IL-6. Data are represented as the mean ± SEM. N=4 of three separate experiments. * represents a statistically significant difference between nanoparticle\(^+\) and nanoparticle\(^-\) groups within a treatment at p-value < 0.026.
Figure 10.4: LPS or 50:50 CPTEG:CPH nanoparticle treatment of DCs revealed similar populations of double positive DCs while different double positive DC populations were generated following treatment with poly(SA) nanoparticles. Data are graphed as the percentage of CD11c⁺ DCs positive for (A) MHC II, (B) CD86, (C) CD40, (D) IL-12p40 or (E) IL-6 that were also positive for a second surface marker or cytokine. Data are represented as the mean ± SEM. N=6 of three separate experiments. Treatments with different letters are significantly different from one another at p-value < 0.044. F) PCA plot of the double positive DC population data validating that LPS and 50:50 CPTEG:CPH were similar to one another while poly(SA) was different from those two treatments.
A) DESCRIPTORS

<table>
<thead>
<tr>
<th>Descriptor</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Water Contact Angle</td>
</tr>
<tr>
<td>2</td>
<td>Aliphatic C</td>
</tr>
<tr>
<td>3</td>
<td>Aromatic C</td>
</tr>
<tr>
<td>4</td>
<td>Backbone O</td>
</tr>
<tr>
<td>5</td>
<td>Molecular Weight</td>
</tr>
<tr>
<td>6</td>
<td>% OH Endgroup</td>
</tr>
<tr>
<td>7</td>
<td>Atomic index1 (0yx)</td>
</tr>
<tr>
<td>8</td>
<td>Atomic index2 (0yv)</td>
</tr>
<tr>
<td>9</td>
<td>Connectivity Index1 (1yx)</td>
</tr>
<tr>
<td>10</td>
<td>Connectivity Index2 (1yv)</td>
</tr>
<tr>
<td>11</td>
<td>The number of carbon atoms</td>
</tr>
<tr>
<td>12</td>
<td>The number of oxygen atoms</td>
</tr>
<tr>
<td>13</td>
<td>The number of hydrogen atoms</td>
</tr>
<tr>
<td>14</td>
<td>Number of backbone -COO- non-conjugated (N ester_n)</td>
</tr>
<tr>
<td>15</td>
<td>Number of backbone -COO- one-sided conjugation with aromatic ring (N ester_c)</td>
</tr>
<tr>
<td>16</td>
<td>The number of O- in a polymeric repeat unit (N ether)</td>
</tr>
<tr>
<td>17</td>
<td>N ester=2<em>N ester+3</em>N ether</td>
</tr>
<tr>
<td>18</td>
<td>The number of ether (R-O-R') linkages between two units R and R' both of which are connected to the alkyl carbon atom (N alkyl ether)</td>
</tr>
<tr>
<td>19</td>
<td>N group=-N alkyl ether+7<em>N CO+2</em>N ether C=O</td>
</tr>
<tr>
<td>20</td>
<td>The total number of rotational degrees of freedom parameter</td>
</tr>
<tr>
<td>21</td>
<td>The number of atoms in the shortest path across the backbone of a polymeric repeat unit</td>
</tr>
<tr>
<td>22</td>
<td>The number of aromatic rings in a polymeric repeat unit</td>
</tr>
<tr>
<td>23</td>
<td>The number of CH3 in a polymeric repeat unit</td>
</tr>
</tbody>
</table>

B) DESCRIPTORS

- Alkyl ether
- %OH Endgroup
- Backbone O
- Hydrophobicity

Less Pathogen Mimicking | More Pathogen Mimicking
Figure 10.5: Identification of –OH end groups, oxygen content, alkyl ether, and hydrophobicity as important pathogen-mimicking structural descriptors. A) Molecular descriptors (1-23) for the enumeration of the features of polyanhydrides and LPS. The structure descriptors are defined by Bicerano [31]. B) Partial least squares analysis was used to identify the polymer descriptors with the most pathogen-mimicking impact on dendritic cell activation.
10.10 Supporting Information

A) **Combinatorial**

50:50 CPH:SA  
50:50 CPTEG:CPH

**Conventional**

50:50 CPH:SA  
50:50 CPTEG:CPH

---

B) 

<table>
<thead>
<tr>
<th></th>
<th>MHC I MFI</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Combinatorial</td>
<td>3000</td>
<td>Conventional</td>
<td>2500</td>
</tr>
<tr>
<td></td>
<td>2500</td>
<td></td>
<td>2000</td>
</tr>
<tr>
<td></td>
<td>2000</td>
<td></td>
<td>1500</td>
</tr>
<tr>
<td></td>
<td>1500</td>
<td></td>
<td>1000</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td></td>
<td>500</td>
</tr>
</tbody>
</table>

C) 

<table>
<thead>
<tr>
<th></th>
<th>MHC II MFI</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Combinatorial</td>
<td>8000</td>
<td>Conventional</td>
<td>7000</td>
</tr>
<tr>
<td></td>
<td>7000</td>
<td></td>
<td>6000</td>
</tr>
<tr>
<td></td>
<td>6000</td>
<td></td>
<td>5000</td>
</tr>
<tr>
<td></td>
<td>5000</td>
<td></td>
<td>4000</td>
</tr>
<tr>
<td></td>
<td>4000</td>
<td></td>
<td>3000</td>
</tr>
</tbody>
</table>

D) 

<table>
<thead>
<tr>
<th></th>
<th>CD86 MFI</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Combinatorial</td>
<td>7000</td>
<td>Conventional</td>
<td>6000</td>
</tr>
<tr>
<td></td>
<td>6000</td>
<td></td>
<td>5000</td>
</tr>
<tr>
<td></td>
<td>5000</td>
<td></td>
<td>4000</td>
</tr>
<tr>
<td></td>
<td>4000</td>
<td></td>
<td>3000</td>
</tr>
<tr>
<td></td>
<td>3000</td>
<td></td>
<td>2000</td>
</tr>
</tbody>
</table>

E) 

<table>
<thead>
<tr>
<th></th>
<th>CD40 MFI</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Combinatorial</td>
<td>10000</td>
<td>Conventional</td>
<td>9000</td>
</tr>
<tr>
<td></td>
<td>9000</td>
<td></td>
<td>8000</td>
</tr>
<tr>
<td></td>
<td>8000</td>
<td></td>
<td>7000</td>
</tr>
<tr>
<td></td>
<td>7000</td>
<td></td>
<td>6000</td>
</tr>
<tr>
<td></td>
<td>6000</td>
<td></td>
<td>5000</td>
</tr>
</tbody>
</table>

F) 

<table>
<thead>
<tr>
<th></th>
<th>CD16 MFI</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Combinatorial</td>
<td>2500</td>
<td>Conventional</td>
<td>2000</td>
</tr>
<tr>
<td></td>
<td>2000</td>
<td></td>
<td>1500</td>
</tr>
<tr>
<td></td>
<td>1500</td>
<td></td>
<td>1000</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td></td>
<td>500</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td></td>
<td>0</td>
</tr>
</tbody>
</table>
Supplementary Figure 10.1: CPTEG- and SA-rich nanoparticles synthesized combinatorially activate DCs similarly to conventionally fabricated nanoparticles. A) SEM photomicrographs of combinatorially (black bars) and conventionally (white bars) fabricated nanoparticles demonstrate similar size distributions and surface morphology. Mean fluorescence intensity (MFI) of (B) MHC I, (C) MHC II, (D) CD86, (E) CD40 and (F) CIRE on DCs incubated with combinatorially or conventionally fabricated CPH:SA and CPTEG:CPH nanoparticles. Non-stimulated (NS) cells were the negative control and LPS-stimulated cells were the positive control. Data are represented as the mean ± SEM. N=6 of three separate experiments. There were no statistically significant differences between the two groups for expression of any markers.

Validation of combinatorial synthesis and nanoparticle fabrication

Previous work from our laboratories has demonstrated that conventionally synthesized polyanhydride nanoparticles enhance cell surface expression of markers associated with DC activation as well as induce DCs to produce pro-inflammatory cytokines \[12, 18\]. In this work, polyanhydride nanoparticles were synthesized using a combinatorial methodology \[8, 9, 18\] and validated against those synthesized conventionally (i.e., using “one-sample-at-a-time” nanoprecipitation) using platforms for physicochemical characterization and DC activation. The \(^1\)H NMR characterization indicated that the combinatorially synthesized copolymers resulted in identical polymer peaks and showed similar molecular weights to those synthesized conventionally (~10,000-18,000 g/mol) \[6, 7, 11, 12, 16, 21-23\]. Scanning electron microscopy was employed to make visual assessments between conventionally and combinatorially fabricated nanoparticles. The average size was found to be ~200 nm, once
again consistent with the size range of nanoparticles fabricated conventionally (Supplementary Figure 10.1A)\textsuperscript{[16, 23]}.

Immune activation studies were also performed to validate the similarities in cell surface marker expression between the DCs treated with combinatorial and conventional nanoparticles. No significant differences were observed in DC expression of MHC I, MHC II, CD86, CD40 or CIRE when cells were cultured with nanoparticles fabricated by either method (Supplementary Figure 10.1). Also in agreement with previously published data\textsuperscript{[12, 18]}, combinatorially synthesized SA- and CPTEG-rich nanoparticles induced the greatest expression of cell surface markers. Specifically, stimulation with SA-rich copolymers enhanced DC expression of CD40 and MHC I whereas treatment with the amphiphilic CPTEG-rich copolymers were best at enhancing the expression of MHC II, CD86 and CIRE. Similar to the hydrophobicity trends observed for surface marker expression, the percentage of DCs positive for secretion of the cytokines IL-6 and IL-12p40 increased with decreasing nanoparticle hydrophobicity (Supplementary Figure 10.2).

Supplementary Figure 10.2: Cytokine production is induced by nanoparticles co-incubated
with DCs in a chemistry-dependent manner. Percentage of DCs positive for either IL-12p40 and IL-6 when cultured with (A) CPH:SA nanoparticles, (B) CPTEG:CPH nanoparticles, and (C) control treatments (LPS = positive control and NS = negative control). Data are represented as the mean ± SEM. N=6 of three separate experiments. * indicates statistical significance from NS (p-value < 0.001).
Chapter 11: High Throughput Evaluation of *In vivo* Biodistribution of Polyanhydride Nanoparticles

A paper to be submitted for publication in *Small*

Latrisha K. Petersen¹, Lucas Huntimer², Katherine Walz¹, Amanda Ramer-Tait², Michael J. Wannemuehler², and Balaji Narasimhan¹*

**Keywords:** Adjuvants, polyanhydride nanoparticles, *in vivo*, biodistribution, depot

¹Department of Chemical and Biological Engineering, Iowa State University, Ames, IA 50011
²Department of Veterinary Microbiology and Preventive Medicine, Iowa State University, Ames, IA 50011
11.1 Abstract

Several challenges are associated with current vaccine strategies some of which include repeated immunizations, which can restrict patient compliance, and limited immunization routes, which could hinder immune protection when applied differently than the pathogen infection route. Thus, there is a need for vaccine adjuvants capable of multi-route administration and prolonged antigen release through mucoadhesion to the respiratory tract or by providing a depot within tissue. In this work, we utilized a combinatorial platform to investigate the in vivo biodistribution, depot effect, and mucoadhesion of polyanhydride nanoparticles as dictated by nanoparticle chemistry and administration route (parenteral and intranasal). This was achieved by employing a live animal imaging system with near infrared fluorescent markers to simultaneously investigate these parameters. All nanoparticle chemistries resulted in rapid dispersal when delivered intranasally and provided a long-term depot when administered parenterally. Chemistry-dependent trends were identified; specifically, intranasally administered amphiphilic and hydrophobic polyanhydride nanoparticles demonstrated superior mucoadhesive properties, enabling prolonged residence in lung tissue. Parenterally administered amphiphilic nanoparticles demonstrated the longest injection site residence time. These results provide new chemistry- and route-dependent insight into the biodistribution and tissue-specific targeting of these nanoparticle-based vaccine adjuvants for improved antigen delivery, depot, and dispersion.

11.2 Introduction

Biodegradable polymers have the advantage of delivering biological molecules at controlled rates, limiting the need for repeated treatments [1-5]. This characteristic has beneficial
implications in vaccine delivery, as it promotes continual antigen exposure and the subsequent development of immunological memory. Many conventional vaccination methods include frequent administrations of the antigen to achieve protective immunity against pathogens. When these administrations are performed via injections, they are often painful and lead to poor patient compliance. It has been suggested that the best way to achieve vaccine efficacy is by vaccinating through the same route that the pathogen uses to infect the host. In this regard, intranasal delivery has several advantages over parenteral routes for vaccination against respiratory pathogens. This needle-free approach does not require trained personnel, has increased patient compliance, and is capable of enhancing both mucosal and systemic immune responses. The major drawback of this administration method is the relatively poor immunogenicity of the protein antigens that are used in non-adjuvanted vaccines. Often there is rapid mucociliary clearance of these antigens, which can result in short respiratory tract residence times and minimal immune response. Thus, there is a need for biocompatible, mucoadhesive, vaccine adjuvants that have administration route versatility and control over antigen release, ultimately enabling more effective disease prevention and treatment.

Polyanhydrides are a class of biodegradable, non-toxic, non-mutagenic materials that are capable of encapsulating and delivering biological molecules in vivo. These polymers are capable of being formulated into nanoparticles enabling administration intramuscularly or intranasally. Nanoparticles based upon copolymers of sebacic acid (SA), 1,6-bis-(p-carboxyphenoxy hexane) (CPH), and 1,8-bis-(p-carboxyphenoxy)-3,6-dioxaoctane (CPTEG) have been shown to exhibit tunable properties, including high protein loading
efficiency, tailored protein release kinetics, and immunomodulatory adjuvant behavior\textsuperscript{[14-28]}. Amphiphilic copolymers based on CPTEG and CPH have also demonstrated the ability to enhance cell surface marker expression similar to lipopolysaccharide (LPS), a pathogen associated molecular pattern (PAMP) which is found on the outer membrane of Gram-negative bacteria, without the toxic side effects associated with extreme levels of cytokine production\textsuperscript{[15,24,27,28]}. They have been shown to possess (non-specific) “pathogen-mimicking” properties, making them ideal vaccine adjuvants\textsuperscript{[14,27,28]}. These properties are essential for targeting the correct areas of the immune system and enabling proper exposure of antigen to promote immunological memory. Recently, long-term protection against a lethal challenge of \textit{Yersinia pestis} was achieved with a single-dose intranasal vaccine composed of antigen-encapsulated polyanhydride nanoparticles\textsuperscript{[5]}. Additionally, polyanhydrides have shown the capability to preserve the functionality of encapsulated proteins upon release\textsuperscript{[17-22,25,29]}. Many of these characteristics are controlled by copolymer chemistry and therefore by selecting the polymer chemistry, these properties can be modulated to fit the desired application.

While a significant amount of work has been done \textit{in vitro} to assess polyanhydride nanoparticle performance in terms of protein release, protein stability, cellular uptake, and cellular activation, little work to date has investigated the biodistribution or trafficking of these nanoparticles \textit{in vivo} as a function of administration route and nanoparticle chemistry. To design efficacious vaccine delivery systems capable of mimicking the infection route of a pathogen, knowledge of the distribution and dispersal of this delivery system is essential. Additionally, the residence time of these nanoparticles \textit{in vivo} remains largely unknown and is integral to understand the depot and delivery capabilities of this platform. We present data
herein defining the *in vivo* biodistribution and residence times of polyanhydride nanoparticles administered by various routes. These studies were performed using a high throughput, combinatorial approach with a live animal imaging system and near infrared fluorescent markers to simultaneously investigate the effect of several chemistries or administration routes in individual mice.

11.3 Materials and Methods

**Materials**

The chemicals utilized in the monomer synthesis include: 4-*p*-fluorobenzonitrile, purchased from Apollo Scientific (Cheshire, UK); 1-methyl-2-pyrrolidinone, 1,6-dibromohexane, triethylene glycol, 4-hydroxybenzoic acid, 4-*p*-- and 1,6-dibromohexane; these were purchased from Sigma Aldrich (St. Louis, MO); and dimethyl formamide (DMF), toluene, sulfuric acid, acetonitrile, and potassium carbonate were obtained from Fisher Scientific (Fairlawn, NJ). Chemicals for the polymer synthesis and nanoparticle fabrication pentane, methylene chloride, acetic anhydride and chloroform; all purchased from Fisher Scientific. Deuterated chemicals for NMR analysis included chloroform and dimethyl sulfoxide (Cambridge Isotope Laboratories, Andover, MA). Fluorescent dyes for *in vivo* imaging included: Texas Red®-X succinimidyld ester (TR) was purchased from Invitrogen (Carlsbad, CA) and VivoTag 680 (VT680) and VivoTag 800 (VT800) were purchased from Perkin Elmer. SKH1-E mice were obtained from Charles River (Wilmington, MA) and housed under specific pathogen-free conditions. Animal procedures were conducted with the approval of the Iowa State University Institutional Animal Care and Use Committee.
Combinatorial polymer synthesis, nanoparticle fabrication, and characterization

SA monomer was purchased from Sigma Aldrich. 1,6-bis(p-carboxyphenoxy)hexane (CPH) monomer and 1,8-bis(p-carboxyphenoxy)-3,6-dioxaocatane) (CPTEG) were synthesized as described previously [30]. CPTEG:CPH and CPH:SA copolymers were combinatorially synthesized as described before [23,27,29,31]. The polymers were dissolved in methylene chloride, fluorescent dye was added to the dissolved polymer, the dye-polymer solution was dispersed by sonication at 40 Hz for 30 s, and rapidly precipitated into a non-solvent (pentane) for the formation of multiple dye-loaded nanoparticle chemistries. They were dried using rapid filtration and stored under dry conditions at -20 °C until use in vivo. The polymers were characterized by 1H nuclear magnetic resonance (NMR) spectroscopy and gel permeation chromatography (GPC) and the nanoparticles were imaged using scanning electron microscopy (SEM).

Nanoparticle administration in vivo and image capture of mice and organs

Nanoparticles were suspended in sterile saline and sonicated at 15 Hz for 30 s to uniformly disperse the particles. SKH-1 mice were anesthetized with isoflurane and administered 50 μL of the desired treatment (dye-loaded nanoparticles, dye only, or saline). In this study, three routes of administration were studied: subcutaneous (SC; in the nape of the neck), intramuscular (IM; in the inner thigh) and intranasal (IN, in the nares). The nanoparticle chemistries studied were 50:50 CPTEG:CPH, 50:50 CPH:SA, 20:80 CPTEG:CPH, and 20:80 CPH:SA. In the route-dependent study, 50:50 CPTEG:CPH and 50:50 CPH:SA nanoparticles were encapsulated with TR for SC administration, VT800 for IN administration, and VT680 for IM administration. For the chemistry-dependent IN study, 50:50 CPTEG:CPH and 50:50
CPH:SA were loaded with VT680 and 20:80 CPTEG:CPH and 20:80 CPH:SA with VT800. A total of 166.66 μg of dye-loaded nanoparticles were administered per route. Control groups were included for each combinatorial study, which consisted of mice that only received one treatment. Additionally, dye only and saline controls were administered for each route. X-ray and fluorescent images of both the anterior and posterior sides of each mouse were obtained using the *In vivo* Multispectral FX Pro (Carestream, Rochester, NY) at 3 h, 6 h, 12 h, 24 h, 3 days, 7 days, and 14 days. The TR, VT680, and VT800 dyes were imaged at excitation wavelengths of 540nm, 670nm, and 760nm and emission wavelengths of 600nm, 750nm, and 830nm respectively. On day 14, the mice were imaged and euthanized. *Ex vivo* tissue analysis of the liver, spleen, kidneys, lungs, and injection sites was performed to determine nanoparticle presence. Briefly, the tissues were removed, washed with ethanol, and imaged. Both sides of the organs were imaged utilizing the same methods as described for imaging live animals.

*Image analysis*

Sixteen-bit mouse and organ tiff images were analyzed with Image J (NIH, Bethesda, MD). Images were inverted and background was subtracted based upon a rolling ball radius of 50, 150, and 150 from each fluorescent image for TR, VT680, and VT800, respectively. Images were stacked and circular regions of interest (ROIs) created for the mouse around the head, neck, chest, lower abdomen, and leg injection sites and outlined ROIs were created around each organ. Mean fluorescence intensity (MFI) was recorded for each site for each fluorescent image. Image overlays were created by stacking the images, creating a color composite, and adjusting each channel to the desired color. Macros were created and utilized
for all images when determining ROI MFIs and for creating image overlays.

**Data and statistical analysis**

All data was normalized to the saline group. JMP software (SAS Institute, Cary, NC) was used to make comparisons between treatments and the negative (saline) control using the student $t$-test and comparisons between different treatments (route or chemistry) were performed using a model ANOVA with Tukey’s HSD.

**11.4 Results**

**Polymer and nanoparticle characterization**

$^1$H NMR was used to determine the molecular weight and chemical structure of the combinatorially synthesized polyanhydrides for this work. The polymers were found to be within the expected molecular weight range (10,000 to 18,000 Da), which is in agreement with previously published work $^{[18-20,30]}$. SEM imaging of the dye-loaded nanoparticles revealed results consistent with previous studies, with average particle sizes of ~200 nm and uniform surface morphology across chemistries (data not shown) $^{[5,24,29,32]}$. The chemical and structural characterization of the dye-loaded nanoparticles was also consistent across chemistries and batches and with previous work $^{[23,29]}$.

**Biodistribution and depot effect of nanoparticles is influenced by route of administration**

In these studies, 50:50 CPTEG:CPH or 50:50 CPH:SA nanoparticles were administered via three different routes, SC, IM, or IN. Nanoparticles for each route were labeled with a different fluorescent dye TR (SC), VT680 (IM), and VT800 (IN). Figure 11.1 presents
representative time course images of mice administered 50:50 CPTEG:CPH nanoparticles (M1) or 50:50 CPH:SA nanoparticles (M2) via three different routes. Nanoparticles administered IN remained detectable in the mouse for approximately 24 h, whereas nanoparticles administered SC or IM resided in the mice at least 14 days (Figure 11.1). When compared with the dye only control group for IM or SC administration, the nanoparticles exhibited a significantly longer residence time at the site of injection (data not shown). Separated images for each fluorescent channel are shown in Supplementary Figure 11.1A. The nanoparticles delivered intranasally dispersed throughout the body of the mouse. Alternatively, little fluorescence was detected in the tissue at any other site other than the injection site for nanoparticles administered SC or IM. Figure 11.2, which is a graphical representation of the images in Figure 11.1, compares different injection sites for 50:50 CPTEG:CPH (Figure 11.2A) and 50:50 CPH:SA (Figure 11.2B) nanoparticles. Nanoparticles injected IM deposited fluorescent signal the longest at the injection site while the nanoparticles injected SC appeared to diminish in fluorescence intensity the fastest (Figure 11.2). At the parenteral administration sites (i.e., SC and IM), 50:50 CPTEG:CPH nanoparticles lingered longer than the 50:50 CPH:SA particles. Both nanoparticles chemistries were found to behave similarly when administered IN, moving rapidly through the body of the mouse with minimal nanoparticle fluorescence remaining after 24 h (Figures 11.2C and D). Importantly, control mice with nanoparticles injected by only one route revealed similar biodistribution trends as mice administered nanoparticles via all three routes (Supplementary Figure 11.1).

Despite the fact that fluorescence was below detection levels in the chest area of the mouse
after 24 h, examination of the excised organs indicates that nanoparticles are still present in the lung tissue after 14 days (Figure 11.3 and Supplementary Figure 11.2). It is important to note that the lack of fluorescence in the whole mouse images when the nanoparticles were delivered intranasally (Figures 11.1 and 11.2) is likely due to the blockage of fluorescent signal in deep tissue. Additionally, injection sites for each administration route indicate that while similar levels of fluorescence were determined in the whole mouse images, actual fluorescence in the injection site tissue is greatest for IM-administered nanoparticles. These findings indicate that the IM administration of nanoparticles provides the longest depot of residing nanoparticles; however, nanoparticles administered via SC or IN routes dissipate rapidly and distribute to distal tissues of the body.

_Nanoparticle chemistry dictates lung tissue adhesion and clearance from the body_

Figure 11.4 presents representative time course images of mice administered 20:80 and 50:50 CPTEG:CPH nanoparticles (M1) or 20:80 and 50:50 CPH:SA nanoparticles (M2) for the chemistry dependent study. As seen in Figure 11.4, CPTEG:CPH and CPH:SA nanoparticles appear to move rapidly throughout the mouse body as observed with particles administered IN in the first study (Figure 11.1). These images again reveal that most nanoparticles fall below detection levels after 24 h. Supplementary Figure 11.3 shows each fluorescent image observed separately, which provides a clearer visualization of the biodistribution of each nanoparticle chemistry. Figure 11.5 indicates that 20:80 CPH:SA was distributed throughout the body the most rapidly and was detectable at significantly higher levels than the other nanoparticle chemistries in the lower abdomen. The other nanoparticle chemistries appeared to similarly distribute throughout the body, moving from the head to lower abdomen within
the first 24 h. Control mice administered only one nanoparticle chemistry per mouse demonstrated similar biodistribution patterns to the mice administered multiple chemistries (Supplementary Figure 11.3B).

*Ex vivo* analysis of the organs 14 days post IN immunization revealed fluorescence in the lungs, despite the inability to detect fluorescence *in vivo* after 24 h (Figure 11.6 and Supplementary Figure 11.4), indicates that all formulations of nanoparticles are retained in the lungs for at least 14 days.

**11.5 Discussion**

The use of polyanhydride particle systems as vaccine adjuvants provides many advantages over current options because they eliminate the need for frequent administrations. Moreover, they provide an antigen depot and can adjuvant the immune system through non-specific stimulation of antigen presenting cells. To further explore their use *in vivo*, we utilized a combinatorial approach for the simultaneous investigation of the effect of route and chemistry on *in vivo* biodistribution, trafficking, and residence time of polyanhydride nanoparticles. This approach facilitated multiple studies to be carried out per mouse, which reduced the number of experimental subjects, time, and cost. The high throughput studies revealed that polyanhydride nanoparticles provide a depot for antigen release and are retained in deep tissue after immunization. Many of these nanoparticles (50:50 CPTEG:CPH and 50:50 CPH:SA) were also identified to possess unique mucoadhesive properties, making them ideal for intranasal vaccination against respiratory pathogens. These two chemistries were found to optimal for long-term presence at the IM or SC injection sites or within deep
lungenparenchym nach IN-Verabreichung. Diese kombinatorische in vivo-Ansatz zur Untersuchung der Nanopartikelverteilung im Körper bereitstellt einen Rahmen für die Optimierung polymerer Verformulierungen und der Übertragungswege für individuelle Anwendungen.

Abhängig von dem Infektionspathogen sind Impfungen möglicherweise auf verschiedene Übertragungswege effektiver. Zusätzlich kann die Verwendung eines anpassbaren Übertragungssystems, das eine über lange Zeit kontrollierte Wirksamkeitsfreisetzung ermöglicht, die Notwendigkeit der Wiederholung von Applikationen eliminieren. Unsere Arbeit zeigt, dass polyanhydrid-Nanopartikel im Einspritzbereich oder im Schleimhautmembranen mindestens zwei Wochen (Abbildungen 11.3 und 11.6) nach subkutaner, intramuskulärer oder intranasaler Verabreichung residiert, was darauf hindeutet, dass sie für die gleiche Dauer von aufgenommenem Antigen freigeben. Abbildung 11.1 suggeriert, dass wenn in weniger fliessfähigem Gewebespace (intramuskulär oder subkutan vs. intranasal) injiziert werden, die Partikel eingeschränkter in diesem Raum sind, was weniger Bewegung bewirkt, das eine längere Zeitdauer als Antigendepot. Externe Aufnahmen der Mäuse zeigen, dass parenteral applizierten Nanopartikeln ähnliche Dauern von bis zu 14 Tagen nach intramuskulärer oder subkutaner Verabreichung, und laufende Studien zeigen, dass sie nach 2 Monaten erstaunlich bleiben (Abbildungen 11.1 und 11.2 und Daten nicht gezeigt). Jedoch, wenn man sich die Einspritzbereichsgewebe untersucht, ist die Anwesenheit von Nanopartikelfluoreszenz signifikant höher für intramuskuläre Verabreichung (Abbildung 11.3). Diese Beobachtungen deuten darauf hin, dass Nanopartikel tatsächlich verschiedene Dauern haben, je nach parenteraler Einspritzungsort. Da es bekannt ist, dass diese Partikel ihre Ladung über Zeiträume von einer Woche bis zu einigen Monaten [30,34,35] freigeben können, demonstrieren diese Ergebnisse die wertvolle Fähigkeit der Kontrolle der Antigenerfreisetzung sowohl durch Nanopartikelchemie und Administrationsweg. Studien haben auch gezeigt, dass wenn Antigen in Partikeln geladen und parenteral verabreicht wird, höhere Antikörper...
titers were observed than when the antigen is delivered alone \[^4\]. Not only could the prolonged release time of a drug delivery device reduce the dose required for immunization, it may also eliminate the need for multiple administrations. Additional studies have shown that polyanhydride particles induce low levels of inflammation \[^36\] which suggests minimal post-administration side effects for the recipient. These improved characteristics would likely increase patience compliance and minimize antigen dose, ultimately leading to more cost-effective vaccine strategies.

IN vaccination is thought to be an advantageous delivery method for respiratory pathogens, including seasonal influenza, group A streptococcus, and inhaled anthrax. The relatively poor immunogenicity of non-adjuvanted antigens and high probability of mucociliary clearance often render this vaccination route ineffective \[^11,37,38\]. Thus, vaccination against respiratory pathogens with subunit vaccines is primarily administered parenterally. However, a limitation with parenteral vaccination is that only serum IgG is induced against the vaccine antigen with little production of IgA in the respiratory mucosa, leaving the upper airways unprotected \[^11\]. The polyanhydride nanoparticles investigated in this work demonstrated prolonged residence in deep lung tissue at least 14 days following IN administration (Figures 11.3 and 11.6). The nanoparticles delivered IN dispersed throughout the body of the mouse; an observation seen only after IN administration (Figures 11.1 and 11.2). This phenomenon likely promotes an interaction between the particles and the mucosal membranes of the upper and lower respiratory tract, in addition to facilitating creation of a depot for antigen release and development of immunological memory. Based upon the close association between the nanoparticles and the mucosal lung tissue (Figures 11.3 and 11.6), it is predicted that these rapidly dispersed nanoparticles are primarily adhering to the tissues of the respiratory system.
Nanoparticles that do not adhere are likely passed through the digestive tract and excreted (Figures 11.4 and 11.5).

This work has also demonstrated that nanoparticle chemistry plays an integral role in tissue residence time and biodistribution of nanoparticles when delivered IN (Figures 11.4-11.6). Nanoparticles made of 20:80 CPH:SA dispersed throughout the body most rapidly, while the other nanoparticle formulations resided longer in the respiratory tract (Figures 11.4 and 11.5). This may be a result of the lower hydrophobicity, higher $T_g$ (Table 11.1), and faster degradation properties of 20:80 CPH:SA. These properties make 20:80 CPH:SA nanoparticles decrease in size more rapidly, rendering them more compatible with the internal aqueous environment of the mouse. Moreover, they would move more rapidly throughout the body without being stalled in mucosal surfaces or membranes. Previous reports indicate that polystyrene particles less than 500 nm have the ability to diffuse through the mucin layer in a mucous membrane, indicating that all nanoparticles tested in this work would be sufficiently small enough for diffusion $^{[39]}$. Particle hydrophobicity is also thought to play a role in mucosal transport, with the least hydrophobic particles having the highest translocation permeability $^{[39]}$. Work by Szentkuti reported that hydrophobic latex nanoparticles 415 nm and smaller rapidly penetrated the mucosal layer attaching to the apical membranes of the surface cells, indicating that they are cell-adhesive rather than muco-adhesive $^{[40]}$. Therefore, nanoparticle size, $T_g$, and hydrophobicity (Table 11.1) may all influence particle transport and mucosal adhesion. The least hydrophobic nanoparticles with lower $T_g$s, such as 50:50 CPTEG:CPH and 50:50 CPH:SA, may be the best option for treatment or vaccination in the respiratory system because of their structure and superior
adhesive properties to the mucosal membrane of the lungs (Figures 11.4 and 11.6). The low $T_g$ nanoparticles are more flexible and can change shape as dictated by the environment [27,28]. It is interesting to note that pathogens and their surface molecules (i.e. LPS (lipopolysaccharide)) have similar thermal properties [41,42] to these low $T_g$ nanoparticles. Additionally, these nanoparticles, like pathogens, possess mucoadhesive properties, enabling their association with the respiratory system. Therefore, vaccination with antigen-loaded nanoparticles based upon these polymers would be able to follow a very similar route and associate with similar tissues in which the pathogen infects.

When administered IN, these polyanhydride nanoparticles demonstrate equivalent or greater residence times at the nasal administration site as other mucoadhesive polymers such as N,N,N-trimethylchitosan (TMC), chitosan microspheres, and starch microspheres [37,43]. The polyanhydride nanoparticle system may be more effective for vaccination against respiratory pathogens because of their ability to associate with lung tissue, a location where TMC was not identified [37]. The presence of hydroxyl end groups is predicted to influence bioadhesive interactions to mucosal surfaces by increasing the hydrogen bonding with components of the mucosal surface (hydroxyl end groups) [44]. As CPTEG:CPH nanoparticles enter an aqueous environment, they degrade and expose high levels of hydroxyl end groups which, as suggested, may promote hydrogen bonding, resulting in a very intimate interaction between the mucosal surface and nanoparticles. This would then make the nanoparticles more accessible to APC uptake, activation, migration to the draining lymph nodes. In addition to the mucoadhesive properties demonstrated in this work, 50:50 CPTEG:CPH nanoparticles also have inherent non-specific “pathogen-like” stimulation of the immune system [15,24,27,28].
Recent work from our laboratories has shown that IN vaccination against pneumonic plague with antigen encapsulated into and adjuvanted with 50:50 CPTEG:CPH nanoparticles protected mice from lethal *Y. pestis* challenge better than the antigen alone or antigen adjuvanted with MPLA in single dose regimens at 23 weeks post vaccination \(^5\). Clearly, the amphiphilic 50:50 CPTEG:CPH nanoparticles encompass unique mucoadhesive properties enabling prolonged antigen exposure for protection against respiratory pathogens.

The advantages of the nanoparticle delivery system for vaccine delivery can be translated to drug delivery applications such as treatment of respiratory diseases, internal wound healing complications, or control over bacterial and cellular behavior \(^6,11,37\). Anti-microbials, growth factors, or cell-specific adhesion molecules can be delivered more effectively to tissue by biodegradable nanoparticles for localized control of bacterial and cellular behavior \(^45,46\). Antibiotics, antivirals, or immune stimulating cytokines (IL-12) may also be more effective for post-infection treatment of respiratory pathogens when delivered IN \(^47\) which could be improved with the use of a prolonged release, mucoadhesive nanoparticle-based delivery system. Several treatment strategies could be improved upon with a nanoparticle based drug delivery system capable of providing long-term, targeted therapy in a single administration.

**11.6 Conclusions**

The combinatorial *in vivo* studies described in this work have demonstrated the chemistry- and route-dependent biodistribution and depot effect of polyanhydride nanoparticles. This approach enabled a rapid investigation of nanoparticle dispersion, depot, and residence time as dictated by administration route and nanoparticle chemistry. Amphiphilic 50:50
CPTEG:CPH nanoparticles demonstrated the longest residence time at parenteral injection sites and would be expected to provide a long-term antigen depot \textit{in vivo}, allowing for the development of immunological memory. Additionally, both 50:50 CPTEG:CPH and 50:50 CPH:SA nanoparticles demonstrated superior mucoadhesive properties, enabling prolonged residence in lung tissue. These mucoadhesive polymer nanoparticles provide new opportunities for their use as adjuvants in intranasal and parenteral vaccination. The insights gained from these studies will enable the rational design of vaccine and therapeutic drug delivery systems for targeted and localized delivery of their payloads over extended time periods.

11.7 Acknowledgments

The authors would like to acknowledge financial support from the ONR-MURI Award (NN00014-06-1-1176) and the U.S. Army Medical Research and Materiel Command (Grant No. W81XWH-09-1-0386). This material is based upon work supported by the National Science Foundation under Grant No. EEC 0851519 to B.N.

11.8 References

5. Ulery BD, Kumar D, Ramer-Tait A, Metzger DW, Wannemuehler MJ, Narasimhan B.


11.9 List of Figures

Figure 11.1: Representative mouse images showing differential persistence at the injection site of nanoparticles administered via three routes over a two week period. Persistence of IM particles > 14 days, persistence of SC particles ~14 days and persistence of IN particles < 1 day. M1 = mouse administered 50:50 CPTEG:CPH nanoparticles, M2 = mouse administered 50:50 CPH:SA nanoparticles, and M3 = mouse administered saline. Blue indicates TR-loaded particles administered SC, red indicates VT680-loaded particles administered IM, and yellow indicates VT800-loaded particles administered IN.

Figure 11.2: Nanoparticles administered via three routes persisted at the injection site longest
when administered IM; however, IN particles rapidly disseminated throughout the body. ROI analysis of mice depicted in Figure 11.1. Mice administered 50:50 CPTEG:CPH nanoparticles (A) persist at SC and IM injection site longer than that for mice administered 50:50 CPH:SA nanoparticles (B). 50:50 CPTEG:CPH (C) and 50:50 CPH:SA nanoparticles (D) rapidly disperse through the mouse and terminate in the chest area with very little particle fluorescence in the lower abdomen area. n = 5 for 50:50 CPTEG:CPH and n=3 for 50:50 CPH:SA. Letters indicate statistical significance between each treatment group and the asterisk indicates statistical significance from the saline control (p-value < 0.05).

Figure 11.3: Nanoparticles were retained in the IM injection site tissue longer than at the other two sites. Also, nanoparticles administered IN are targeted to the lungs and retained there after 14 days for both A) 50:50 CPTEG:CPH (A) and 50:50 CPH:SA (B) nanoparticles. ROI analysis of the mouse organs after 14 days. No nanoparticle fluorescence was observed in the liver, spleen, or kidneys. n = 5 for 50:50 CPTEG:CPH and n=3 for 50:50 CPH:SA. Letters indicate statistical significance between each treatment group and the asterisk indicates statistical significance from the saline control (p-value < 0.05).
Figure 11.4: Representative mouse images indicating that IN administration of two nanoparticle chemistries, 50:50 and 20:80 CPH:SA or 50:50 and 20:80 CPTEG:CPH, resulted in rapid dispersion of the particles throughout the body within the first 24 hours after administration. However, CPTEG:CPH nanoparticles tended to localize in different areas than CPH:SA nanoparticles, indicating a differential effect on biodistribution based upon nanoparticle chemistry. M1 = mouse administered VT680-loaded 50:50 CPTEG:CPH nanoparticles IN (red) and VT800-loaded 20:80 CPTEG:CPH nanoparticles (yellow), M2 = mouse administered VT680-loaded 50:50 CPH:SA nanoparticles IN (red) and 20:80 CPH:SA VT800-loaded nanoparticles IN (yellow), and M3 = mouse administered saline IN.
Figure 11.5: Chemistry played an important role in nanoparticle distribution throughout the body. ROI analysis of mice depicted in Figure 11.4 for A) head, B) neck, C) chest, and D) lower abdomen. The 20:80 CPH:SA nanoparticles show significantly greater fluorescence in the lower abdomen at initial time points. n = 3 for all groups. Letters indicate statistical significance between each treatment group and the asterisk indicates statistical significance from the saline control (p-value < 0.05).
Figure 11.6: Even though no longer visible in the whole mouse images, all nanoparticle chemistries administered IN were targeted to the lungs and retained there for at least 14 days. No nanoparticle fluorescence was observed in the liver, spleen, or kidneys. ROI analysis of the mouse organs at 14 days post-vaccination. n = 3 for all groups. Letters indicate statistical significance between each treatment group and the asterisk indicates statistical significance from the saline control (p-value < 0.05).

11.10 List of Tables

Table 11.1: Thermal properties and contact angles of polyanhydrides \cite{18, 22, 30, 31}.

<table>
<thead>
<tr>
<th>Chemistry</th>
<th>T_g (°C)</th>
<th>T_m (°C)</th>
<th>Contact Angle (°)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50:50 CPTEG:CPH</td>
<td>8</td>
<td>None</td>
<td>45</td>
</tr>
<tr>
<td>20:80 CPTEG:CPH</td>
<td>18</td>
<td>None</td>
<td>45</td>
</tr>
<tr>
<td>50:50 CPH:SA</td>
<td>11</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>20:80 CPH:SA</td>
<td>50</td>
<td>67</td>
<td>50</td>
</tr>
</tbody>
</table>
Supplementary Figure 11.1: Administration of nanoparticles via three different routes simultaneously to the same mouse resulted in similar nanoparticle distribution patterns as compared to administration of nanoparticles via each route independently. A) Representative mouse images from Figure 11.1 separated by filter channel. M1 = mouse administered 50:50 CPTEG:CPH nanoparticles, M2 = mouse administered 50:50 CPH:SA nanoparticles, and M3 = mouse administered saline. B) Images of mice that received administration of nanoparticles via only one route. M4 = mouse administered 50:50 CPTEG:CPH SC, M5 = mouse administered 50:50 CPTEG:CPH IM, M6 = mouse administered 50:50 CPTEG:CPH IN, M7 = mouse administered 50:50 CPH:SA SC, M8 = mouse administered 50:50 CPH:SA IM, and M9 = mouse administered 50:50 CPH:SA IN. Blue indicates TR-loaded particles administered SC, red indicates VT680-loaded particles administered IM, and yellow indicates VT800-loaded particles administered IN.
Supplementary Figure 11.2: Representative 14 day organ images corresponding to Figure 11.3 separated by filter channel which indicate that IN administered nanoparticles (yellow) are easily observed in the lung tissue while IM (red) and SC (blue) administered nanoparticles are easily observed in the injection site tissue and fluorescence in the each tissue is indicative of nanoparticle persistence. M1 = mouse administered 50:50 CPTEG:CPH nanoparticles, M2 = mouse administered 50:50 CPH:SA nanoparticles, and M3 = mouse administered saline. Blue indicates TR-loaded particles administered SC, red indicates VT680-loaded particles administered IM, and yellow indicates VT800-loaded particles administered IN.
Supplementary Figure 11.3: Each mouse administered nanoparticles of two different chemistries results in similar nanoparticle distribution compared to separate mice administered nanoparticles of each chemistry independently. A) Representative mouse images from Figure 11.4 separated by filter channel which indicate that the nanoparticle of CPTEG:CPH chemistries may disperse differently than those of CPH:SA throughout the mouse body when administered IN. M1 = mouse administered VT680-loaded 50:50 CPTEG:CPH nanoparticles IN (red) and VT800-loaded 20:80 CPTEG:CPH nanoparticles (yellow) and M2 = mouse administered VT680-loaded 50:50 CPH:SA nanoparticles IN (red) and 20:80 CPH:SA VT800-loaded nanoparticles IN (yellow). B) Images of mice that received administration of nanoparticles with only one chemistry. M3 = mouse administered 50:50 CPTEG:CPH IN (red), M4 = mouse administered 20:80 CPTEG:CPH IN (yellow), M5
Supplementary Figure 11.4: Representative 14 day organ images corresponding to Figure 11.6 separated by filter channel which indicate that IN administered nanoparticles are easily observed in the lung tissue even though they are no longer detectable in the whole mouse images. M1 = mouse administered VT680-loaded 50:50 CPTEG:CPH nanoparticles IN (red) and VT800-loaded 20:80 CPTEG:CPH nanoparticles (yellow) and M2 = mouse administered VT680-loaded 50:50 CPH:SA nanoparticles IN (red) and 20:80 CPH:SA VT800-loaded nanoparticles IN (yellow).
Chapter 12: Conclusions

While pathogenic microorganisms continue to mutate at a rapid rate and new diseases are diagnosed every day, there is an immediate need to develop faster approaches for the discovery and development of therapeutic devices to enhance, target, and stabilize protein-based drugs. This work has focused on creating and utilizing high throughput approaches for the rapid optimization of polyanhydrides for use as drug delivery vehicles and vaccine adjuvants. A combinatorial framework has been developed to rapidly synthesize and characterize libraries of these biomaterials and simultaneously investigate multiple protein/polymer, cell/polymer and host/polymer interactions. Using this framework, several phenomena were monitored at high throughput and key relationships between polymer chemistry and structure, temperature, pH, device geometry, protein release kinetics, protein stability, cytotoxicity, immune activation, cellular differentiation, cellular adhesion, in vivo biodistribution, and in vivo mucosal adhesion were discovered and validated.

Protein release kinetics from polyanhydride nanoparticles were assessed with a novel fluorescent based technique in which decreasing protein release was correlated with increasing polymer hydrophobicity and acidic pH release conditions. Protein stability for the protective antigen for anthrax (PA) and bovine serum albumin (BSA) was determined upon encapsulation, storage, and release from polymer nanoparticles in which CPH-rich (CPH:SA) and all CPTEG:CPH chemistries were able to preserve protein structure of BSA under all conditions tested. While 50:50 CPTEG:CPH proved most capable of preserving PA activity upon encapsulation and release; 50:50 CPTEG:CPH and 20:80 CPH:SA demonstrated
excellent storage of PA at elevated temperatures (40 °C) for 2 months.

Immune activation and adjuvant properties were investigated by assessing cytokine production and cell surface marker expression of dendritic cells (DCs), which are the primary antigen presenting cells of the immune system. This work revealed trends demonstrating ↑ cell surface marker expression with ↓ nanoparticle hydrophobicity and ↑ cytokine production with ↑ oxygen content in polymer backbone. With the use of informatics tools, 50:50 CPTEG:CPH was found to possess pathogen-mimicking properties and the specific properties responsible for the pathogen-mimicking behavior are hydrophobicity, number of backbone oxygen groups, percent of hydroxyl end groups, and number of alkyl ethers.

High throughput, near-red infrared in vivo imaging, enabled rapid identification of the superior mucoadhesive properties of 50:50 CPTEG:CPH and 50:50 CPH:SA nanoparticles. Biodistribution and adjuvant depot effect were also readily identified, in which route and chemistry influenced these properties with intranasally administered 20:80 CPH:SA nanoparticles moving the most rapidly through the mouse body and intramuscularly administered 50:50 CPTEG:CPH nanoparticles residing in tissue the longest (i.e, depot effect).

Taken together these studies will enable the rapid design of polyanhydrides as drug delivery devices and vaccine adjuvants. The high throughput methodologies developed in this doctoral thesis hold promise to enhance and design new therapeutic treatments and vaccines that are necessary to keep up with the rapidly advancing forms of disease.
Chapter 13: Future Work

This research has demonstrated that polyanhydrides are a versatile biodegradable platform for drug delivery applications from vaccine adjuvants to delivering therapeutics to tissue engineering constructs. The versatility of these materials provides a basis for further research. Since these polymers provide protein and cell conducive environments suitable for many tissue engineering applications, further studies investigating the polymer mechanical integrity and ability to be constructed into a 3D scaffold network are necessary. In addition to tissue engineering applications, the use of these polymers as nanoparticle adjuvants for vaccines has shown much promise. While many properties such as concentration, chemistry, hydrophobicity, oxygen-content, etc., have been investigated for their effect on antigen presenting cells (APCs) of the immune system, little work to date has investigated the effect of polyanhydride particle shape and size on immune activation. Future work in this area many help design vaccine delivery vehicles that better mimic the shape of bacteria or better facilitate cellular internalization. Finally, future work combining the broad database of knowledge gathered through these combinatorial investigations could be utilized to design intranasal, single-dose, dose-sparing vaccine strategies for anthrax, pneumonia and influenza.

13.1 CPTEG:CPH Films: Applications in Drug Delivery and Tissue Engineering

Multi-component implants, drug-eluting stents, and tissue engineering constructs all rely on cellular acceptance by the host and in most cases require the ability to deliver therapeutic drugs, cell promoting growth factors, or immunosuppressant cytokines\cite{1-5}. This has been investigated as described in the previous chapters (4, 7, 8 and 9) with polyanhydride films
composed of CPH:SA and CPTEG:CPH copolymers \cite{6-9}. While several desirable protein/material and cell/material characteristics have been identified including preservation of protein structure, controlled release rate, low immune activation, enhanced cellular adhesion and differentiation \cite{6-8, 10}, the mechanical integrity and 3D scaffold design has not been demonstrated. Polymeric mechanical properties can be investigated using the combinatorial approach as described previously \cite{11}. Both the mechanical integrity and scaffold design are dictated by the tissue in which the scaffold is being injected or implanted \cite{5}. Hard tissue such as bone requires stiff polymeric structures; in contrast, soft tissue like nerves requires malleable polymers. Similarly, elastomeric tissue such as the skin or blood vessels requires flexible polymer scaffolds \cite{5}. In addition to mechanical integrity, a 3D scaffold design is necessary for cellular integration into the construct. Several different approaches to 3D scaffold design have been reported including porous (Figure 13.1A), cell-cleavable, and electro-spun networks (Figure 13.1B), nerve guiding conduits (Figure 13.1C), and elaborate grey and white matter track for spinal cord regeneration \cite{5, 12-19}. Hence, it is of interest to investigate the mechanical properties of CPTEG:CPH and CPH:SA polymers in a combinatorial format and study their ability to be constructed into 3D networks. Further studies can then be conducted in high throughput to investigate protein/scaffold and cell/scaffold interactions as described in Chapters 4-10 and by other researchers \cite{6-11, 20-27} and with the use of targeted strategies \cite{28}.
13.2 Polyanhydride Particle Size and Shape Dependency on Cytotoxicity and Activation of Primary Murine Dendritic Cells

A major focus of current research on polymeric adjuvants has been to investigate the use of nano- and microparticle adjuvants and how they influence the activation of APCs [7,28-34]. Chapters 8 and 10 provided an in depth discussion of the adjuvant properties of polyanhydride nanoparticles composed of CPH:SA and CPTEG:CPH copolymers [7,27]. While many of the mechanisms of particle internalization and activation are still unknown there appear to be several variables (particle size, roughness, shape, chemistry, etc.) that are likely to affect internalization by DCs and the subsequent immune response [7,27-30,35-42]. While much of the previous work has alluded to the effect of polymer chemistry on immune modulation [7,27,29,30], particle size [42-44] and shape [41,45] are known to play an integral role in uptake and are thus hypothesized to also play a role in immune activation. It has been reported previously that particles <10 µm but > 500 nm undergo internalization by phagocytosis, particles <500 nm but > 200 nm are internalized through endocytosis and are trafficked to early endosomal compartments with a pH of 6.0, and particles < 200 nm are internalized through endocytosis and are directed to late endosomal/lysosomal compartments with a pH of 4.5–5.0 [42]. Additionally, shape and orientation of PLGA and PS particles has been reported to affect uptake by macrophages and suggest internalization similarities between particles and bacteria of the same shape [41]. All reports of polyanhydride particle/cell interactions have been investigated with polydisperse polymer particles as seen in the SEM images and size distribution plots in several reports [7-9,35,46]. This makes it difficult to discern the effect of particle size and shape on immune activation.
Monodisperse poly(SA) particles have been fabricated by Berkland and co-workers utilizing precision particle fabrication technology \(^{[47]}\). An image of monodisperse poly(SA) microparticles fabricated with the precision particle fabrication technology \(^{[47]}\) is shown in Figure 13.2. Additionally, shape varying particles based upon poly(lactic-co-glycolic) acid and polystyrene have been fabricated by Mitragotri and co-workers utilizing particle stretching technology employing PDMS \(^{[41]}\). These methods could be easily amenable to fabricate polyanhydride nanoparticles based upon CPH:SA and CPTEG:CPH copolymers varying in size and shape. It is therefore of great interest to investigate the effect of using monodisperse polyanhydride particles of various shapes on the internalization mechanisms of DCs and the subsequent immune response in a combinatorial format. Knowledge of the effect of particle size, particle shape, and polymer chemistry upon immune activation will allow for the rational design and development of more effective vaccine adjuvant/delivery vehicles able to better target intended immune responses.

**13.3 50:50 CPTEG:CPH Nanoparticle Adjuvants for the Single-dose Vaccination Against Anthrax, Pneumonia, and Influenza**

A major focus of research has been on the design of biocompatible polymeric adjuvants capable of controlling antigen release, providing a long-term antigen depot, and enhancing the immune response. Chapters 4 and 6 demonstrated that these polymer nanoparticles are capable of releasing encapsulated proteins in a chemistry dependent manner for weeks to months \(^{[8, 48]}\), while Chapter 11 validated these findings *in vivo* \(^{[49]}\). Additionally, Chapter 11 revealed mucoadhesive properties of 50:50 CPTEG:CPH and 50:50 CPH:SA nanoparticles and demonstrated that both CPTEG:CPH and CPH:SA nanoparticles provide a long-term
antigen depot in vivo. Chapters 5 and 6 revealed the effective protein stabilization capabilities of these polymer nanoparticles for several proteins \cite{9, 48}. Several cell/materials interactions were investigated in Chapters 7, 8, 10, and 11 which indicate that these polymers are both biocompatible and immunomodulatory. Thus, it would be of interest to take this diverse knowledge of protein/polymer, cell/polymer, and host/polymer interactions for the design and optimization of respiratory vaccine adjuvants against pathogens such as anthrax, pneumonia, and influenza. Recently, long-term protection against a lethal challenge of \textit{Yersinia pestis} was achieved with a single-dose intranasal vaccine composed of antigen-encapsulated 50:50 CPTEG:CPH nanoparticles \cite{50}. Based upon these findings and the superior mucoadhesive \cite{49}, protein stabilization \cite{46, 48, 51}, and adjuvant properties \cite{7, 27, 30} of 50:50 CPTEG:CPH nanoparticles, future studies designing respiratory vaccines would benefit by utilizing this adjuvant. Preliminary studies have indicated that elevated antibody titers against the pneumonia antigen, PspA, are achieved with the use of these nanoparticle adjuvants in a single dose administered subcutaneously, as shown in Figure 13.3. These studies provide insights for vaccination against respiratory pathogens including anthrax, pneumonia, and influenza with polyanhydride nanoparticles. Further studies could be conducted by varying the amount of soluble and encapsulated antigen to optimize antibody production and avidity and possibly reduce the amount of antigen required for protection. Additionally, since many of these pathogens infect via inhalation and replicate in the respiratory tract, it may be advantageous to deliver these nanoparticle adjuvanted vaccines intranasally \cite{52-54}. Development of a single-dose, dose-sparing vaccine adjuvant capable of being delivered intranasally would provide a novel strategy for overcoming respiratory pathogen threats to public health in a patient-compliant and cost effective manner.
13.4 References


**13.5 List of Figures**

![Figure 13.1](image1.png)

Figure 13.1: Images of A) porous and B) electro-spun polymer networks and C) nerve regeneration conduits [5].

![Figure 13.2](image2.png)

Figure 13.2: SEM image of 20 μm monodisperse poly(SA) particles [47].
Figure 13.3: Total IgG anti-PspA concentrations from the serum of mice immunized with blank 50:50 CPTEG:CPH nanoparticles (blank nano), 1 μg soluble PspA + 2 μg encapsulated PspA into 50:50 CPTEG:CPH nanoparticles (soluble PspA + PspA nano), 1 μg PspA conjugated to alum (PspA + alum), and saline. Mice administered blank and PspA-loaded nanoparticles were only immunized once but mice administered PspA + alum were immunized 3 times. Error bars represent standard deviation and n=6.