

Engineering Double-Walled Nanospheres for More Effective Oral Drug Delivery:
Quantitative Analysis of Uptake, Biodistribution, Bioadhesion and
Nanoencapsulation

Daniel Youngwhan Cho
A.B., Sc.B., Brown University, 2007
Sc.M., Brown University, 2012

Thesis

Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy
in the Division of Biology and Medicine at Brown University

PROVIDENCE, RHODE ISLAND
MAY 2012

Copyright © 2012 Daniel Youngwhan Cho

AUTHORIZATION TO LEND AND REPRODUCE THE THESIS

As the sole author of this thesis, I authorize Brown University to lend it to other institutions or individuals for the purpose of scholarly research.

Date: _____

Daniel Youngwhan Cho, Author

I further authorize Brown University to reproduce this thesis by photocopying or other means, in total or in part, at the request of other institutions or individuals for the purpose of scholarly research.

Date: _____

Daniel Youngwhan Cho, Author

This thesis by Daniel Youngwhan Cho is accepted in its present form by the Department of Molecular Pharmacology, Physiology and Biotechnology in the Division of Biology and Medicine as satisfying the requirements for the degree of Doctor of Philosophy.

Date: _____

Edith Mathiowitz, Ph.D., Advisor

Date: _____

Jeffrey R. Morgan, Ph.D., Committee Chair

Date: _____

Eric M. Darling, Ph.D., Committee Member

Date: _____

G. Tayhas R. Palmore, Ph.D., Committee Member

Date: _____

Beth A. Zielinski, Ph.D., Committee Member

Date: _____

Joshua J. Reineke, Ph.D., External Reader

Approved by the Graduate Council

Date: _____

Peter Webber, Ph.D., Dean of the Graduate School

Vita

DANIEL YOUNGWHAN CHO

Education

- 2015 BROWN UNIVERSITY. PROVIDENCE, RI.
Doctor of Medicine, May 2015. (anticipated).
- 2012 BROWN UNIVERSITY. PROVIDENCE, RI.
Doctor of Philosophy in Medical Science, May 2012.
- 2012 BROWN UNIVERSITY. PROVIDENCE, RI.
Master of Science in Biology, May 2012.
- 2007 BROWN UNIVERSITY. PROVIDENCE, RI.
Bachelor of Science in Biochemistry with Honors, May 2007.
- 2007 BROWN UNIVERSITY. PROVIDENCE, RI.
Bachelor of Arts in Visual Arts with Honors, May 2007.

Research Experience

- 2006–12 *Research Assistant*, Mathiowitz Lab. Department of Molecular Pharmacology, Physiology and Biotechnology. Brown University. Providence, RI.
- 2005 *Research Assistant*, Creton Lab. Department of Molecular Biology, Cell Biology and Biochemistry. Brown University. Providence, RI.
- 2003–04 *Research Assistant*, Moulton Lab. Department of Chemistry. Brown University. Providence, RI.

Work Experience

- 2012–13 *Senior Scientist: Polymer Chemistry*, NuLabel Technologies, Inc. Providence, RI.
- 2011–12 *Consultant*, NuLabel Technologies, Inc. Providence, RI.
- 2010–12 *Consulting Surgeon*, Formative Center for the Evaluation of Environmental Impacts on Fetal Development. Brown University/Women & Infants Hospital. Providence, RI.
- 2009–11 *Consultant*, Angiogenesis Biology. Bio-Tree Systems, Inc. Providence, RI.

Publications

R. Patel*, **D.Y. Cho***, C. Tian, A. Chang, K. Estrellas, D. Decoteau, S. Furtado, E. Mathiowitz. *Sustained Delivery of Small Hydrophilic Drugs Using PLGA Micro- and Nanospheres Prepared by Solvent Removal*. Journal of Microencapsulation. In Press.

R. Patel, **D.Y. Cho**, C. Tian, A. Chang, D. Decoteau, E. Mathiowitz. *A Novel W/O/O Double-Emulsion Solvent Removal Method for the Nanoencapsulation of Doxycycline*. Scientifically Speaking, Controlled Release Society Newsletter Vol. 28, No. 2 (2011).

* these authors contributed equally to this work

Patents

B.D. Lux, **D.Y. Cho**, D.A. Sonshine, H.M. Munnely, P. So, K. Tabis. *Solution Activatable Multi-Layer Adhesive Compositions for Liner-Free Labels and Methods of Activation Thereof*. USSN 13/339,898. December 2011

B.D. Lux, **D.Y. Cho**, D.A. Sonshine. *Fluid Activatable Adhesives and Fluids for Activating Same for use with Liner-Free Labels*. USSN 13/294,438. November 2011.

B.D. Lux, **D.Y. Cho**, F. Shvartzman. *Fluid Activatable Adhesives and Fluids for Activating Same for Use with Liner Free Labels*. USSN 13/294,545 November 2011.

D.Y. Cho, J. Reineke, E. Mathiowitz, B. Laulicht. *Nanoparticle Compositions and Methods for Improved Oral Delivery of Active Agents*. USSN 13/179,205. July 2011.

R. Patel, **D.Y. Cho**, E. Mathiowitz. *Dual Delivery System of Genetically Modified Cells and Polymeric Microspheres*. USSN 61/436,064. March 2011.

D.Y. Cho, J. Reineke, E. Mathiowitz, B. Laulicht. *Methods for Preparing Multi-Walled Nanoparticles by Sequential Phase Inversion Nanoencapsulation*. USSN 61/362,615. July 2010.

Conference Proceedings

L. Quattrochi, **D.Y. Cho**, N. Chavez, Z. Pfeiffer, E. Oancea, E. Mathiowitz. *In Vitro Assessment of Lipid-Polymer Hybrid Melanosomes in Human Neonatal Epidermal Melanocytes*. 2011 Annual Meeting of the Biomedical Engineering Society. Hartford, CT. October 2011

D.Y. Cho, C. Tian, K.M. Estrellas, Y.T. Liu, J.J. Reineke, S. Furtado, E. Mathiowitz. *Bioadhesive Double-Walled Nanospheres as an Oral Insulin Delivery Platform*. 38th Annual Meeting and Exposition of the Controlled Release Society. Baltimore, MD. August 2011.

L. Quattrochi, **D.Y. Cho**, K.M. Estrellas, N.L. Wicks, E. Oancea, E. Mathiowitz. *Development of an Artificial Melanosome Utilizing a Lipid-Polymer Hybrid Microparticle*. 38th Annual Meeting and Exposition of the Controlled Release Society. Baltimore, MD. August 2011.

D. Decoteau, **D.Y. Cho**, L. Zhang, R. Stefani, R. Patel, E. Mathiowitz. *Single Step Fabrication of Wet Spun Binary Phase Composite Microfibers*. 38th Annual Meeting and Exposition of the Controlled Release Society. Baltimore, MD. August 2011.

D.Y. Cho, C. Tian, J.J. Reineke, E. Mathiowitz. *Progress in the development of an oral insulin formulation for diabetes mellitus*. The Joint Meeting of the American Society for Clinical Investigation and the Association of American Physicians. Chicago, IL. April 2011.

D.Y. Cho. *Polymeric Micro- and Nanoencapsulation of Therapeutic Agents for Regenerative Medicine Applications*. Center for Biomedical Engineering: Repair and Regeneration Seminar. Brown University. Providence, RI. December 2010.

D.Y. Cho, L. Patel, E. Mathiowitz. *Fabrication of PEG-ylated Double-Walled Nanospheres by Solvent Evaporation*. 2010 Annual Meeting of the Biomedical Engineering Society. Austin, TX. November 2010.

D.Y. Cho, C. Tian, K.M. Estrellas, J. Reineke, E. Mathiowitz. *Development of a Rapid Release Oral Insulin Formulation for Diabetes Mellitus*. 2010 Annual MD/PhD Program Retreat. Brown University. Providence, RI. September 2010.

C. Tian, S. Azad, **D.Y. Cho**, B.D. Lux, T. Peak, S. Furtado, E. Mathiowitz. *Comparing the Effects of Fabrication Parameters on Micro- and Nanospheres for Drug Delivery Applications*. 2010 Brown University Summer Research Symposium for the Humanities, Sciences and Social Sciences. Providence, RI. August 2010.

D.Y. Cho, K.M. Estrellas, C. Tian, J. Reineke, E. Mathiowitz. *A Novel Method for the Fabrication of Double-Walled Nanospheres by Sequential Phase Inversion Nanoencapsulation*. 37th Annual Meeting and Exposition of the Controlled Release Society. Portland, OR. July 2010.

D.Y. Cho, C. Tian, K.M. Estrellas, E. Mathiowitz. *Effect of Encapsulation Method on Protein Loaded Nanospheres Fabricated by Phase Inversion Nanoencapsulation*. 37th Annual Meeting and Exposition of the Controlled Release Society. Portland, OR. July 2010.

R. Patel, **D.Y. Cho**, C. Tian, A. Chang, D. Decoteau, J. Morgan, E. Mathiowitz. *A Novel W/O/O Double Emulsion Solvent Removal Method for the Nanoencapsulation of Doxycycline*. 37th Annual Meeting and Exposition of the Controlled Release Society. Portland, Or. July 2010.

D.Y. Cho, J. Reineke, E. Mathiowitz. *Quantitative Analysis of Bioadhesive Nanoparticle Uptake From the Small Intestine for Oral Drug Delivery Applications*. The Joint Meeting of the American Society for Clinical Investigation and the Association of American Physicians. Chicago, IL. April 2010.

J. Reineke, **D.Y. Cho**, Y. Liu, E. Mathiowitz. *Quantitative and Mechanistic Analysis of Bioadhesive Microparticle Uptake for Oral Drug Delivery*. 2009 Annual MD/PhD Program Retreat. Brown University. Providence, RI. September 2009.

J. Reineke, **D. Cho**, P. Cheifetz, Y. Liu, E. Mathiowitz. *Bioadhesion of Microspheres Correlates with In Vivo Intestinal Uptake*. 36th Annual Meeting and Exposition of the Controlled Release Society. Copenhagen, Denmark. July 2009.

J. Reineke, A. Morello, Y. Liu, **D. Cho**, E. Mathiowitz. *Microsphere Uptake Kinetics and Biodistribution from Isolated Intestinal Regions*. 36th Annual Meeting and Exposition of the Controlled Release Society. Copenhagen, Denmark. July 2009.

Peer Review Activities

2012– Journal of Microencapsulation

Teaching Experience

2011 *Instructor*, Summer and Continuing Studies. Brown University. Providence, RI.
CEEN0905: Engineering Biomedical Systems

2010–11 *Teaching Assistant*. Brown University. Providence, RI.
BIOL2110: Drug and Gene Delivery
BIOL2140: Principles of Experimental Animal Surgery

2010–12 *Instructor*. The Alpert Medical School of Brown University. Providence, RI.
BIOL3645: General Pathology

2008–12 *Teaching Assistant*. The Alpert Medical School of Brown University. Providence, RI.
BIOL3640A: IMS-1 Scientific Foundations of Medicine
BIOL3640D: IMS-1 General Pathology
BIOL3650A: IMS-2 Brain Sciences
BIOL3640B: IMS-2 Microbiology/Infectious Diseases
BIOL3650C: IMS-2 Endocrine Sciences
BIOL3662: IMS-3 Cardiovascular
BIOL3664: IMS-3 Renal
BIOL3663: IMS-3 Pulmonary
BIOL3665: IMS-3 Supporting Structures
BIOL3672: IMS-4 Hematology
BIOL3673: IMS-4 Gastroenterology
BIOL3674: IMS-4 Human Reproduction, Growth and Development
BIOL3666/3675: IMS-3/4 Systemic Pathology
BIOL3667/3676: IMS-3/4 System-Based Pharmacology
BIOL5510: Basic Science Building Blocks
BIOL5700: Bridging the Bench and Bedside

2008–11 *Guest Lecturer*. Brown University. Providence, RI.
BIOL3640A: IMS-1 Scientific Foundations of Medicine: Genetics
“Introduction to Mitosis and Meiosis”
BIOL920-3B: Engineering Biomedical Systems
“Tissue Engineering”
“Biomaterials”
“Drug Delivery”
“Gene Therapy”
BIOL2110: Drug and Gene Delivery
“Bioadhesive Oral Drug Delivery Systems”

2008–09 *Teaching Assistant*, Summer and Continuing Studies. Brown University. Providence, RI.
BI920-3B: Engineering Biomedical Systems

2008–09 *Teaching Fellow*, Teaching Academy. The Alpert Medical School of Brown University. Providence, RI.
BIOL3540C: Human Anatomy

- 2005–08 *Faculty*, Department of Chemistry. University of Rhode Island. Kingston, RI.
 CHM102: Laboratory for Chemistry 101 (General Chemistry I Lecture)
 CHM105: Laboratory for Chemistry 103 (Introductory Chemistry Lecture)
 CHM114: Laboratory for Chemistry 112 (General Chemistry II Lecture)
- 2005–08 *Teaching Assistant*, Department of Chemistry. Brown University. Providence, RI
 CHEM0330: Equilibrium, Rate and Structure
 CHEM0350: Organic Chemistry I

Research Trainees

- 2010–11 Lakir Patel. Sc.B. in Biology (2013, anticipated).
- 2010–11 Taylor Peak. Sc.B. in Biology (2012, anticipated). Recipient of Summer Undergraduate Teaching and Research Award (UTRA), 2011.
- 2010–11 Stephanie Cheung. “*Comparative Analysis of DNA Nanoencapsulation by Phase Inversion.*” Sc.B. in Biology with Honors.
- 2010–11 Marissa Anto. Special Student. B.A. Art History, Harvard University. M.P.H., London School of Economics.
- 2009–11 Shara Azad. Sc.B. in Biology (2012, anticipated). Recipient of Summer Undergraduate Teaching and Research Award (UTRA), 2010. Recipient of Spring Undergraduate Teaching and Research Award (UTRA), 2011.
- 2009–11 Cheng (Cindy) Tian. “*Comparative Analysis of Protein Nanoencapsulation by Phase Inversion.*” Sc.B. in Biology with Honors. Recipient of Summer Undergraduate Teaching and Research Award (UTRA), 2010. Recipient of Senior Prize in Biology for Academic Excellence, 2011.
- 2010 Alexis Mancini. “*Synthesis and Characterization of Novel Bioadhesive Phenylalanine-, Tyrosine- and DOPA-conjugated Polymers for Oral Drug Delivery Applications.*” Sc.B. in Biomedical Engineering with Honors. Recipient of the Outstanding Student in Biomedical Engineering Award, 2010. Recipient of the Pierre M. Galletti Prize, 2010.
- 2009–10 Kenneth Estrellas. “*Fabrication of Double-Walled Nanospheres by Sequential Phase Inversion Nanoencapsulation.*” Sc.B. in Biology with Honors. “*Synthesis and Evaluation of Time-Dependent Mucoadhesion of Novel Bioadhesive Polymers.*” Sc.M. in Biology.
- 2009–10 Ashley Pender. “*An Implantable Polymeric Nanosphere-based Drug Delivery System for the Prevention of Suture Line Fibrocalcification.*” Sc.B. in Biology with Honors.

Service

- 2009–11 Admissions Interviewer, MD/PhD Program. The Alpert Medical School of Brown University. Providence, RI.
- 2008–11 Admissions Interviewer, Brown University. Providence, RI.
- 2008–10 MD/PhD Representative, Medical Student Senate. The Alpert Medical School of Brown University. Providence, RI.
- 2008–09 Co-President, Surgery Interest Group. The Alpert Medical School of Brown University. Providence, RI.

- 2008–09 Co-President, Wilderness Medicine Interest Group. The Alpert Medical School of Brown University. Providence, RI.
- 2004–07 Student Advisor, Meiklejohn Advising Program. Brown University. Providence, RI.

Honors

- 2011 Recipient of the Controlled Release Society Outstanding Oral Drug Delivery Award. Baltimore, MD.
- 2008 Exhibitor in Physician Artist’s Exhibit, RIMWA. Dryden Gallery. Providence, RI.
- 2007 Sigma Xi.
- 2007 “Lookism: An Interactive Installation”, Senior Honors Visual Art Show. Brown University. Providence, RI.
- 2007 Recipient of the Lealyn B. Clapp Prize for Outstanding Senior Honors Thesis in Biochemistry. Brown University. Providence, RI.
- 2007 Recipient of the William Weston Award for Fine Arts. Brown University. Providence, RI.

Professional Societies

American Association for the Advancement of Science (AAAS)

American Physician Scientists Association (APSA)

American Medical Association (AMA)

Controlled Release Society (CRS)

Sigma Xi

Preface and Acknowledgements

This work would not have been possible without the help and support of the many kind people who have been a part of my graduate experience.

I would first like to thank my advisor, Dr. Edith Mathiowitz, for introducing me to the field of drug delivery and allowing me to complete my undergraduate, Master's and PhD research in her lab. I remember looking through the course catalogue my sophomore year and seeing a Biology course entitled "Polymer Science for Biomaterials". This course seemed out of place in the list of the more traditional Biology courses so out of curiosity, I decided to show up to the first day of class and met Edith, who spent the lecture comparing polymers to spaghetti and demonstrating the strange behaviors that polymers exhibit. Little did I know that 8 years later, I would have taken all the courses in the department and be completing my third degree with Edith as my mentor. I am forever grateful to her for teaching me how to be a creative thinker, pushing me to be a better scientist and encouraging me to collaborate with others and explore my interests while always reminding me to find balance in my life so I can have time to enjoy myself. I know I leave her lab ready to finish my medical training and prepared to pursue a career as a physician scientist.

I would like to thank Dr. Jeffrey Morgan, Dr. Eric Darling, Dr. G. Tayhas R. Palmore and Dr. Beth Zielinski for their mentorship and advice during my graduate years as well as for serving on my committee. Even through the toughest times, their encouragement and support helped me to complete this thesis.

I am forever indebted to Dr. Joshua Reineke for allowing me to work as an undergraduate research assistant under his guidance when I joined the Mathiowitz lab after a terrible experience working in a molecular biology lab in which I was supposed to complete my Honors thesis in Biochemistry. It was through his mentoring and patience that I learned

the basics of nanosphere fabrication, polymer chemistry and animal surgery while realizing my desire to pursue a career in research. I am glad to have had the opportunity to continue to collaborate with his lab at Wayne State and to have him as the external reader on my committee.

I would also like to thank all the members of the Mathiowitz Lab, past and present. The lab has been able to accumulate an immense body of work that has served as an inspiration for my own research. I am especially grateful for Dr. Stacia Furtado, Roxanne Burrill, Yu-Ting Liu, Danya Lavin, Bryan Laulicht, Lauren Quattrochi, Hai-Tao Qian, A. Peter Morello, Peter Cheifetz and Allison Kwong for their assistance with my research and their help around the lab. Additionally, I have had the opportunity to work with and mentor a group of phenomenal undergraduate students, all of whom have been essential to the work in this dissertation. I would like to thank Kenneth Estrellas, Cheng (Cindy) Tian, Alexis Mancini, Stephanie Cheung, Lakir Patel, Marissa Anto, Shara Azad and Taylor Peak for their dedication to the lab and all their hard work. In particular, Ken and Cindy were my first and best undergraduates; I was extremely fortunate to have had the opportunity to work with them and I wish them the best of luck as they pursue their own research careers. Most importantly, I would like to thank Roshni Patel and Benjamin Lux for their friendship and encouragement during my graduate years; I hope to continue to collaborate with each of them as we continue on with our careers.

I would like to thank Dr. James Harper, Dr. Moses Goddard and the Animal Care Facility for their assistance with my animal work. I also need to acknowledge Geoff Williams and Anthony McCormick with their assistance with the microscopes as well as Paula Weston for sharing her expertise with transmission electron microscopy. I would like to thank Lauren Quattrochi and the Oancea Lab for collaborating with me on the Artificial Melanosome project as well as the Marshall Lab for donating their plasmids for the gene delivery project that I was working on. I would also like to thank Alexander Leydon for help with Adobe Illustrator, his patience and time at the confocal microscope trying to get images of my nanospheres which wouldn't stop moving and his thoughtful questions and conversations regarding my work. I am forever indebted to him for his support and friendship.

I am grateful to all the assistance I received from the administrative staff in the Depart-

ment of Molecular Pharmacology, Physiology and Biotechnology as well as the Division of Biology and Medicine. I am forever thankful to Carol Folan and Cheryl Pariseau for all their help throughout the years with all the administrative things I had no idea how to deal with. I would like to thank the MD/PhD program for providing me with the opportunity to train as a physician scientist and for providing me with funding for my graduate studies. Additionally, I would like to thank Dr. Juli Ip for continuing to be my advisor even though I am no longer a PLME undergraduate.

I would like to thank my friends and colleagues in the Biotechnology and Biomedical Engineering graduate programs, the MD/PhD program and from the medical school as well as my coworkers from NuLabel Technologies and my teammates and friends from Broomstones Curling Club for their interest in my work. The many conversations I have had with them about my research has helped me to better communicate my work and made me realize how much of a nerd I really am.

There are a countless number of other people that I would like to thank but obviously, I cannot name them all. To those of you who have been an important part of my life these past few years, thank you.

Lastly, I would like to thank and dedicate this thesis to my family. I am forever indebted to my mother, father and sister for supporting me through this long and arduous endeavor.

Contents

1	Introduction	2
1.1	Drug Delivery Technologies	2
1.2	Polymeric Micro-/Nanospheres	3
1.2.1	Micro-/Nanoencapsulation Technologies	3
1.2.1.1	Solvent Evaporation	5
1.2.1.2	Solvent Removal	7
1.2.1.3	Phase Inversion Nanoencapsulation (PIN)	9
1.2.1.4	Double-Walled Microspheres	11
1.3	Oral Drug Delivery	14
1.3.1	Advantages of Oral Drug Delivery	14
1.3.2	Oral Drug Delivery Technologies	15
1.4	Particle Uptake from the Small Intestine	17
1.4.1	Anatomy and Histology of the Gastrointestinal System	17
1.4.2	Principles of Particle Translocation	20
1.4.3	Particle Uptake Mechanisms	22
1.4.4	Bioadhesion	24
1.5	Experimental Goals	26
1.6	References	28
2	Intestinal Microsphere Uptake: Quantitative Analysis of Uptake, Mechanisms and Biodistribution of Microspheres as Potential Oral Drug Delivery Systems	35
2.1	Abstract	35
2.2	Introduction	36
2.3	Materials and Methods	38
2.3.1	Materials	38
2.3.2	Isolated Loop	39
2.3.3	Oral Gavage	39
2.3.4	Tissue Processing and Polymer Detection	40
2.3.5	Transmission Electron Microscopy	41
2.3.6	Confocal Microscopy	41
2.3.7	Statistical Analyses	42
2.4	Results and Discussion	42
2.4.1	Electron Microscopy of Microsphere Uptake in the Jejunum	42
2.4.2	Confocal Microscopy of Microsphere Uptake in the Jejunum	44
2.4.3	Effect of Microsphere Size and Intestinal Region on Uptake	45
2.4.4	Organ Distribution Following Uptake	48

2.4.5	Kinetics of Microsphere Uptake	50
2.4.6	Organ Distribution Kinetics	53
2.4.7	Effect of Mechanism-Specific Endocytosis Inhibition on Microsphere Uptake in the Jejunum	53
2.4.8	Effect of Mechanism-Specific Phagocytosis and Endocytosis Inhibition on Microsphere Uptake in the Ileum	56
2.5	Conclusions	58
2.6	Supplemental Material	60
2.6.1	Supplemental Material & Methods	60
2.6.1.1	Cytotoxicity of Uptake Inhibitors	60
2.6.2	Supplemental Results & Discussion	60
2.6.2.1	Cytotoxicity of Uptake Inhibition	60
2.7	References	63
3	Time-Dependent Mucoadhesion of Conjugated Bioadhesive Polymers	67
3.1	Abstract	67
3.2	Introduction	68
3.3	Materials and Methods	71
3.3.1	Polymer Synthesis	71
3.3.2	Dissolution Testing	71
3.3.3	Probe Preparation	72
3.3.4	Tissue Preparation	72
3.3.5	Bioadhesion Testing	73
3.3.6	Data Analysis	74
3.4	Results	75
3.4.1	Polymer Dissolution	75
3.4.2	Probe Images	76
3.4.3	Control Testing	76
3.4.4	Bioadhesion Testing of Polyacrylic Acid (PAA)	78
3.4.5	Bioadhesion Testing of PBMA-Derivatives	80
3.4.6	Bioadhesion Testing of PEMA-Derivatives	82
3.5	Discussion	84
3.6	Conclusions	87
3.7	Supplemental Material	88
3.7.1	Materials and Methods	88
3.7.1.1	Fourier Transform Infrared Spectroscopy (FTIR)	88
3.7.1.2	Differential Scanning Calorimetry (DSC)	88
3.7.2	Results and Discussion	88
3.7.2.1	Fourier Transform Infrared (FTIR) Spectroscopy Analysis	88
3.7.2.2	Thermal Analysis	90
3.8	References	90
4	Can Bioadhesive Nanoparticles Allow For More Effective Particle Uptake from the Small Intestine?	93
4.1	Abstract	93
4.2	Introduction	94
4.3	Materials and Methods	96
4.3.1	Materials	96

4.3.2	Solvent Evaporation	97
4.3.3	Phase Inversion Nanoencapsulation	97
4.3.4	Scanning Electron Microscopy	97
4.3.5	Particle Size Analysis	98
4.3.6	Light Microscopy	98
4.3.7	PMMA Loading Assay	98
4.3.8	Texture Analyzer Bioadhesion Assay	99
4.3.9	Everted Sac Bioadhesion Assay	99
4.3.10	In Vivo Dosage Preparation	100
4.3.11	Isolated Loop	100
4.3.12	Tissue Processing and Polymer Detection	101
4.3.13	Statistical Analysis	102
4.4	Results and Discussion	102
4.4.1	Nanosphere Characterization	103
4.4.2	Bioadhesion Testing	104
4.4.3	Total Uptake and Biodistribution	106
4.5	Conclusions	108
4.6	References	109
5	Fabrication of Double-Walled Polymeric Nanospheres by Sequential Phase Inversion Nanoencapsulation	112
5.1	Abstract	112
5.2	Introduction	113
5.3	Materials and Methods	114
5.3.1	Materials	114
5.3.2	Cloud Point Testing	115
5.3.3	Sequential Phase Inversion Nanoencapsulation	116
5.3.4	Characterization	117
5.3.4.1	Particle Size Analysis	117
5.3.4.2	Fourier Transform Infrared Spectroscopy	117
5.3.4.3	Differential Scanning Calorimetry	117
5.3.4.4	Scanning Electron Microscopy	118
5.3.4.5	Confocal Microscopy	118
5.4	Results and Discussion	118
5.4.1	Sequential Phase Inversion Nanoencapsulation (sPIN) Rationale	118
5.4.2	Cloud Point Testing	120
5.4.3	Nanosphere Preparation	121
5.4.4	Nanosphere Characterization	122
5.4.4.1	Particle Size Analysis	122
5.4.4.2	Scanning Electron Microscopy	124
5.4.4.3	FTIR Analysis	126
5.4.4.4	Thermal Analysis	127
5.4.4.5	Polymer Localization	129
5.5	Conclusions	129
5.6	References	130

6	Comparative Analysis of Insulin Nanoencapsulation by Phase Inversion	132
6.1	Abstract	132
6.2	Introduction	133
6.3	Materials and Methods	135
6.3.1	Materials	135
6.3.2	Insulin Encapsulation	135
6.3.3	Particle Size Analysis	138
6.3.4	Differential Scanning Calorimetry	138
6.3.5	Scanning Electron Microscopy	138
6.3.6	Encapsulation Efficiency	138
6.3.7	Release Study	139
6.3.8	Transmission Electron Microscopy	139
6.4	Results and Discussion	139
6.4.1	Nanosphere Preparation	139
6.4.2	Nanosphere Characterization	141
6.4.3	Encapsulation Efficiencies	142
6.4.4	Effect of Loading Method on Insulin Release from 1% Loaded Formulations	144
6.4.5	Effect of Salt Excipient on Insulin Release from 1% Loaded Formulations	146
6.4.6	Effect of Total Protein Loading on Insulin Release	147
6.4.7	Transmission Electron Microscopy	151
6.5	Conclusions	154
6.6	References	154
7	Conclusions	157
7.1	Particle Uptake from the Small Intestine	157
7.2	Bioadhesion	158
7.3	Uptake of Bioadhesive Nanospheres	159
7.4	Sequential Phase Inversion Nanoencapsulation (sPIN)	160
7.5	Protein Nanoencapsulation	161
7.6	Potential Oral Drug Delivery System	162
7.7	Future Directions	164
7.8	Conclusions	166
7.9	References	166
A	Development of an Artificial Melanosome by Engineering a Lipid-Polymer Hybrid Microparticle	168
A.1	Introduction	168
A.2	Materials and Methods	169
A.2.1	Materials	169
A.2.2	Melanin Synthesis	169
A.2.3	Melanin Micronization by Phase Inversion	170
A.2.4	Microsphere Preparation	170
A.2.5	Dynamic Light Scattering	173
A.2.6	Fourier Transform Infrared Spectroscopy	173
A.2.7	Differential Scanning Calorimetry	173
A.2.8	Scanning Electron Microscopy	174

A.2.9	Lecithin Coating & In Vitro Studies	174
A.3	Results and Discussion	174
A.3.1	Preparation of Melanin for Microencapsulation	174
A.3.1.1	Encapsulation of Melanin by Solvent Evaporation	178
A.3.2	Lecithin Coating to form Artificial Melanosomes	179
A.3.3	In Vitro Cytocompatibility	181
A.4	Conclusions	181
A.5	References	182
B	PEGylated Superparamagnetic Double-Walled Nanospheres for Micromagnetic-Microfluidic Dialysis as a Novel Contrast Imaging Modality	183
B.1	Introduction	183
B.2	Materials and Methods	185
B.2.1	Nanosphere Preparation	185
B.2.2	Particle Size Analysis	185
B.2.3	Fourier Transform Infrared Spectroscopy	185
B.2.4	Differential Scanning Calorimetry	186
B.2.5	Scanning Electron Microscopy	186
B.3	Results and Discussion	186
B.3.1	Modified Solvent Evaporation Method	186
B.3.2	Control Nanosphere Characterization	187
B.3.3	PEGylated Superparamagnetic Double-Walled Nanospheres	189
B.4	Conclusions	190
B.5	References	190
C	Abstracts and Conference Proceedings	192

List of Tables

3.1	Solubility of pBMA- and pEMA-derivative polymers	75
3.2	Bioinspired bioadhesive polymer side chain attachment efficiencies and glass transition temperatures.	90
5.1	Cloud point testing of 2% w/v core polymer solutions in THF with shell polymer solvent, ethanol	121
5.2	Thermal analysis of double-walled nanosphere formulations prepared by sPIN with different shell polymers and PMMA cores	127
5.3	Thermal analysis of double-walled nanosphere formulations prepared by sPIN with pBMAD-shells and different core polymers	127
6.1	BZI-loaded PLGA PIN formulations, encapsulation efficiencies, theoretical and actual loadings, particle size and thermal properties.	140
A.1	Particle size and thermal properties of melanin lipid-polymer hybrid microparticles.	180

List of Figures

1.1	Potential configurations of microcapsules and microspheres	4
1.2	Schematic of the solvent evaporation method.	5
1.3	Loss of hydrophilic drugs to aqueous bath during solvent evaporation.	7
1.4	Schematic of the solvent removal method.	8
1.5	Phase separation diagram for a polymer/solvent/non-solvent system	10
1.6	Phase separation diagram for a polymer/polymer/solvent system	12
1.7	Possible configurations for the two-polymer system: complete engulfing, partial engulfing and non-engulfing	13
1.8	Challenges presented by the gastrointestinal tracts that prevent the oral administration of many classes of therapeutic agents.	14
1.9	Rationale for polymeric microencapsulation	15
1.10	Low-power micrograph of ileum	18
1.11	Follicle of mucosa-associated lymphoid tissue (MALT) present in a Peyer's patch	19
1.12	Mechanisms of particle uptake from the small intestine include the paracellular cellular pathway, endocytotic mechanisms and phagocytosis	20
1.13	Mechanisms of phagocytotic and endocytotic particle internalization.	22
1.14	Schematic diagram of clathrin pit formation, cargo capture, scission and clathrin recycling	23
2.1	TEM images of 200 nm and 500 nm polystyrene microspheres after local delivery to the jejunum	43
2.2	LSM images of a single rat jejunum villus after 1 hour local delivery of PC-red fluorescent 500 nm PS microspheres.	44
2.3	Quantitative uptake of 500 nm, 1, 2 and 5 μ m polystyrene microspheres	45
2.4	Organ distribution of polystyrene microspheres following uptake after administration to the jejunum, ileum and orally	49
2.5	Kinetics of polystyrene microsphere uptake	51
2.6	Effect of chemical inhibitors on uptake of polystyrene microspheres	54
2.7	Cytotoxicity of uptake inhibitors	62
3.1	Structures of PBMA- and PEMA-derivative polymers	70
3.2	Schematics of texture analyzer bioadhesion testing apparatus and polymer-tissue tensile interface	73
3.3	Photographs of PBMA and PEMAD probes following bioadhesion testing	77
3.4	Fracture strength and tensile work values of PAA, PBMA- and PEMA-derivative polymers	79

3.5	Comparison of the fracture strength values for the conjugated bioadhesive polymers	81
3.6	Comparison of the tensile work values for the conjugated bioadhesive polymers.	83
3.7	FTIR spectra of PBMA-derivative and PEMA-derivative polymers	89
4.1	3-D representation of critical in vitro and in vivo bioadhesion parameters for PLGA, PLA, PFASA and PS.	95
4.2	Scanning electron and light microscopy of PS, PMMA, and PBMA/PMMA nanospheres	103
4.3	Bioadhesion testing of PMMA, PS and PBMA	105
4.4	Total uptake and biodistribution of PMMA, PS and PBMA/PMMA nanosphere formulations	107
4.5	Correlation between texture analyzer (in vitro), everted sac bioadhesion (ex vivo) and isolated loop uptake studies (in vivo)	108
5.1	Schematic of the sequential phase inversion nanoencapsulation (sPIN) process for the PBMA/PLGA (75:25) double-walled formulation.	116
5.2	Hypothesized mechanism of particle formation via the sequential phase inversion nanoencapsulation (sPIN) process	120
5.3	Coulter laser particle size analysis of prepared by sPIN with different shell polymers and PMMA cores.	123
5.4	Scanning electron micrographs of double-walled nanosphere formulations prepared by sPIN with PBMA-shells and different core polymers	124
5.5	Scanning Electron Micrographs of double-walled nanosphere formulations prepared by sPIN with different shell polymers and PMMA cores	125
5.6	Fourier transform infrared spectroscopy (FTIR) analysis of PLGA, PBMA and PBMA-shell/PLGA (75:25)-core nanosphere	126
5.7	High magnification images of PBMA/PLGA-FITC insulin nanospheres . . .	128
5.8	Light and fluorescence microscopy of PBMA/PLGA-FITC insulin nanospheres	129
6.1	Schematic of the nanoencapsulation methods used for the preparation of insulin-loaded PLGA nanospheres.	136
6.2	Scanning electron micrographs of BZI PIN formulations	142
6.3	Theoretical loading vs. encapsulation efficiency for BZI PIN formulations . .	143
6.4	Cumulative % release of insulin from emulsion, cryogenic emulsion and nanonized-BZI formulations	145
6.5	Initial 8-hour, 7-day and 42-day cumulative percent release and 42-day cumulative mass release of insulin from nano-BZI formulations	148
6.6	42-day cumulative percent release of insulin normalized to theoretical loading from nanonized-BZI formulations	150
6.7	Transmission electron micrograph of 9% nanonized-BZI-loaded nanosphere cross-section	152
6.8	Transmission electron micrographs of 19% nanonized-BZI-loaded nanospheres	153
7.1	Schematic of potential oral drug delivery system based on bioadhesive and biodegradable double-walled nanospheres.	163
A.1	Schematic of melanin synthesis.	169

A.2	Schematic of melanin phase inversion micronization.	171
A.3	Schematic of artificial melanosome preparation.	172
A.4	DOPA and synthetic melanin	175
A.5	FTIR spectral comparison of commercial melanin to the lab-created melanin.	175
A.6	UV-VIS spectral analysis of commercial, synthetic and natural melanin	176
A.7	Scanning electron micrograph of melanin-PLLA spheres	177
A.8	FTIR spectra of melanin-PLLA microspheres, blank spheres and synthetic melanin	178
A.9	Microscopy of artificial melanosomes with human neonatal melanocytes	181
B.1	FTIR spectra of the PEG:PLGA/PLLA double-walled nanospheres formulation, bulk PEG:PLGA and bulk PLLA.	187
B.2	SEM images of the PEG:PLGA/PLLA nanospheres and PLLA nanospheres	188
B.3	FTIR spectra of the unloaded and SPIO-loaded PEG:PLGA/PLLA formulations.	189

Abstract of *Engineering Double-Walled Nanospheres for More Effective Oral Drug Delivery: Quantitative Analysis of Uptake, Biodistribution, Bioadhesion and Nanoencapsulation*, by Daniel Youngwhan Cho, Ph.D., Brown University, May 2012.

The field of medicine relies heavily on the use of therapeutic agents for the treatment of various illnesses but many of these drugs have chemical or physical properties that prevent them from being administered orally, despite this being the most convenient method with the highest rates of patient comfort and compliance. The use of polymeric microspheres and nanospheres shows significant promise due to their ability to protect the drug from the harsh GI environment while allowing for controlled drug release kinetics. The ultimate goal of this work is to provide a foundation for the development of more effective oral drug delivery platforms based on the knowledge obtained from systematic evaluations of particle uptake mechanisms, bioadhesion, nanosphere preparation and protein encapsulation. In vivo studies revealed an inverse relationship between particle size and uptake from the small intestine while implicating endocytotic mechanisms by enterocytes as the predominant pathway of particle internalization. A series of bioinspired bioadhesive polymers capable of generating and sustaining strong bioadhesive interactions with the small intestinal mucosa were developed and used to engineer bioadhesive and biodegradable double-walled nanospheres via a sequential phase inversion nanoencapsulation method for use as carriers in oral drug delivery. Evaluation of these nanospheres in vivo revealed an 11.5-fold enhancement of particle uptake through the incorporation of a bioadhesive polymer shell to non-adhesive nanospheres suggesting that bioadhesion is an effective strategy for achieving efficient uptake of orally administered particulate drug carriers. Additionally, the systematic evaluation of protein encapsulation by phase inversion revealed that high actual drug loadings, reduced initial burst release and sustained release kinetics can be achieved over a 42 day period to deliver physiologically relevant levels of insulin from 500 nm poly(lactic-co-glycolic acid) nanospheres. Through the systematic evaluation of particle uptake and tissue distribution, the synthesis and characterization of novel bioadhesive polymers, the development of novel nanoencapsulation methods and systematic evaluation of protein nanoencapsulation by phase inversion, we have provided a framework upon which to develop more effective oral drug delivery platforms.

Chapter 1

Introduction

1.1 Drug Delivery Technologies

The obvious advantage and need for controlled release technologies for drug delivery stems from the need to achieve specific drug concentrations in the target tissues for prolonged periods of time to maximize their therapeutic effects. Traditional methods of drug delivery, while effective for many applications, allow for neither rate-control nor targeted or localizable delivery [1,2].

Thus, much research has been focused over the past 30 years on the use of degradable polymeric microspheres for controlled drug delivery applications. The idea of controlled release from polymers originated in the 1960s with the use of non-degradable polymers, such as silicone rubber by J. Folkman in 1964 [3,4] and polyethylene by S.J. Desai in 1965 [1,5-11]. While the notion of controlled drug delivery from polymeric devices was a huge step towards the development of the field of drug delivery, these early devices had their obvious limitations – most notably, the need for surgical removal due to their biopersistence. The concept of polymeric microspheres was also introduced in the 1960s by Chang et al [12,13], followed by the use of biodegradable polymeric materials for drug delivery in the 1970s [14,15], which would preclude the need for the surgical removal of these drug delivery devices. Since then, the field of controlled drug delivery has proliferated, resulting in the development of a variety of different drug delivery technologies, some of which have successfully made it through the FDA approval process and are even in clinical use today.

1.2 Polymeric Micro-/Nanospheres

1.2.1 Micro-/Nanoencapsulation Technologies

Micro- and nanoencapsulation are technologies focused on the entrapment of solids, liquids or gases inside one or more polymeric coatings to form compositions with micro- or nano-scale geometries [1,5]. This can be achieved via two different approaches: chemical or physical. Chemical methods involve polymerization during the encapsulation process and include interfacial and in situ polymerization techniques. In contrast, physical methods rely on the controlled precipitation of polymers to entrap the desired cargo; these techniques include solvent evaporation, coacervation, solvent removal, hot melt and many others. Since this field originated in the 1970s, an enormous number of encapsulation technologies have been developed, especially in recent years with the surge of interest in biotechnology, biomedical engineering and nanotechnology. The potential applications for micro- and nanoencapsulation technologies are numerous and include fields such as drug delivery, tissue engineering, cosmetics and food science.

Two general structures exist for encapsulated products, microcapsules and microspheres. A microcapsule is a system that consists of a core of the entrapped solid, liquid or gaseous material surrounded by a polymeric phase; these systems are often referred to as reservoir systems since the encapsulated material is localized in a collection in the core. In contrast, a microsphere consists of a continuous phase of polymer in which the encapsulated material is dispersed; often referred to as a matrix system, these systems have no well-defined localized core of encapsulated material or shell of polymer. These two configurations of drug delivery particles can be achieved depending on the encapsulation method. However, some methods, such as solvent evaporation, can yield either configuration depending on total drug loading as well as any specific processing parameters applied to achieve the desired characteristics [5,16].

Described below are micro- and nanoencapsulation techniques commonly used in the encapsulation of therapeutic agents for biomedical applications, specifically those that can be used to produce small diameter sub-micron sized microspheres and nanospheres, which are of particular relevance to the work described in this thesis.

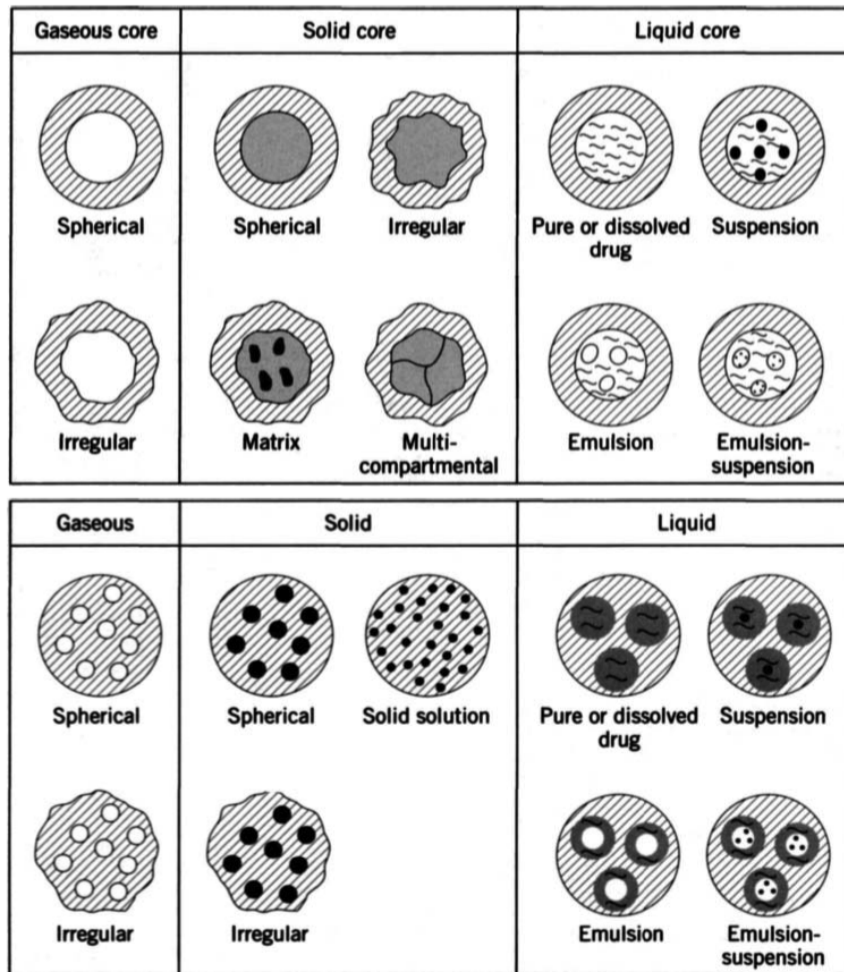


Figure 1.1: Potential configurations of microcapsules and microspheres. Figure from [5].

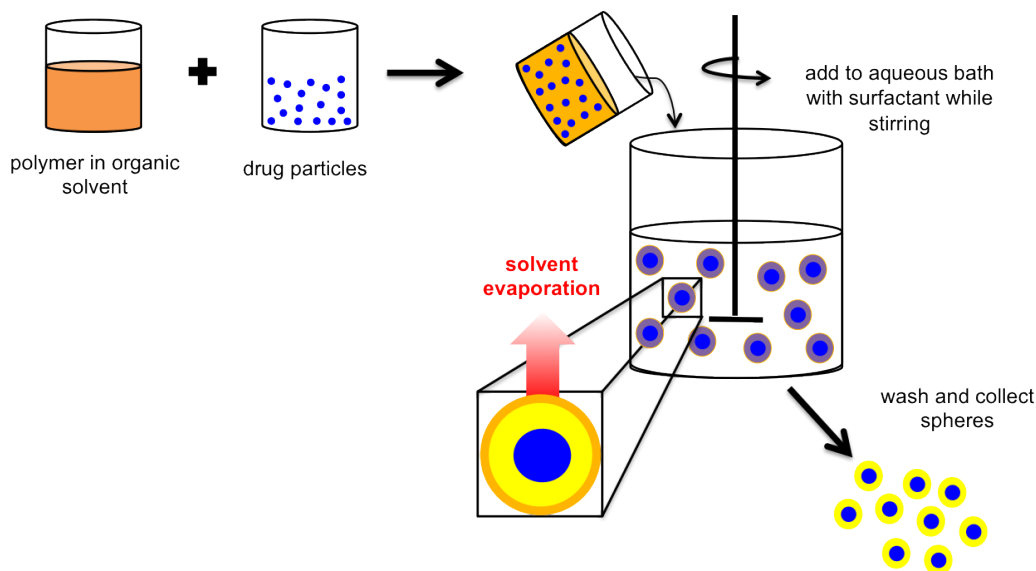


Figure 1.2: Schematic of the solvent evaporation method.

1.2.1.1 Solvent Evaporation

Solvent evaporation is the oldest and most commonly utilized method for encapsulating drugs in biodegradable polymeric matrices [17-20]. Traditionally, this method relies on the emulsification of a polymeric solution into an aqueous phase containing a surfactant, most commonly polyvinyl alcohol (PVA), to yield an oil-in-water (o/w) emulsion; as this emulsion is stirred, the evaporation of the organic solvent results in the precipitation of the polymer and entrapment of the drug in each droplet to form particles, which can then be collected by centrifugation, ultracentrifugation, filtration or dialysis [2,21,22]. While advantageous for hydrophobic drugs soluble in organic solvents, this method presents a variety of challenges for hydrophilic drugs. In order to entrap a hydrophilic drug within the polymeric matrix, a double-emulsion method is most commonly employed [19,22,23]. This method requires the drug to be dissolved in an aqueous phase and emulsified into the polymer solution to form the primary water-in-oil (w/o) emulsion, which is then emulsified into a PVA solution to form the secondary water-in-oil-in-water (w/o/w) emulsion; similar to traditional solvent evaporation, this emulsion is stirred to allow for sphere formation (Figure 1.4).

Although the concept of solvent evaporation is straightforward, there are a variety of variables that affect the properties of the microspheres produced by this method. These include: polymer molecular weight and concentration, polymer crystallinity, chemical characteristics

of the drug, physical state of drug during encapsulation, selection of organic solvent, surfactant selection for emulsion stabilization, temperature and shear rate [5,18,24-26]. While the effect of each of these manufacturing conditions must be determined experimentally for each system, some trends have been established for this process. Most importantly, the organic solvent for the polymer must be selected to be water immiscible with a high volatility, to allow for emulsion formation and solvent evaporation, respectively; these characteristics make methylene chloride an excellent solvent for this method though other volatile organic solvents such as chloroform, methyl ethyl ketone (MEK), ethyl acetate (EtAc) can be used. Additionally, the major determinant of particle size is the shear rate used to create the emulsion, with minor contributions from polymer molecular weight, surfactant selection, solution viscosity and other parameters. The characteristics of the polymer and the drug used in the process determine solvent selection, drug loading method and other variables.

Through the incorporation of high shear rates, polymeric nanospheres are commonly prepared by the solvent evaporation method. However, high-energy homogenization or sonication is often required to reduce the particle size of the emulsion droplets to yield nanospheres [5,27]. While these techniques are suitable for the encapsulation of small molecule drugs, most proteins are likely to denature in the high shear environment [5,16,17,19,20,22,28-30], a significant concern due to the high cost of certain protein therapeutics. Thus, the encapsulation of sensitive therapeutics requires the addition of excipients or modifications to the emulsification process to preserve bioactivity.

As expected, the hydrophilic nature of many drugs, including proteins, may result in leaching of the drug into the aqueous continuous phase during sphere formation (Figure 1.3), which can result in drug losses and decreased entrapment efficiencies, particularly for nanoparticles which have high surface area to volume ratios. Modifications have been made to this method to overcome the loss of drug to the aqueous secondary phase during fabrication through a variety of methods, including the addition of excipients to decrease drug solubility [31-33] or adjustment of pH [34]. However, even with these strategies, the encapsulated drug is often left trapped at or near the polymer surface, yielding poor release kinetics in addition to low efficiencies of encapsulation [22]. Despite these advantages, solvent evaporation remains a mainstay in the field of microencapsulation, particularly in the

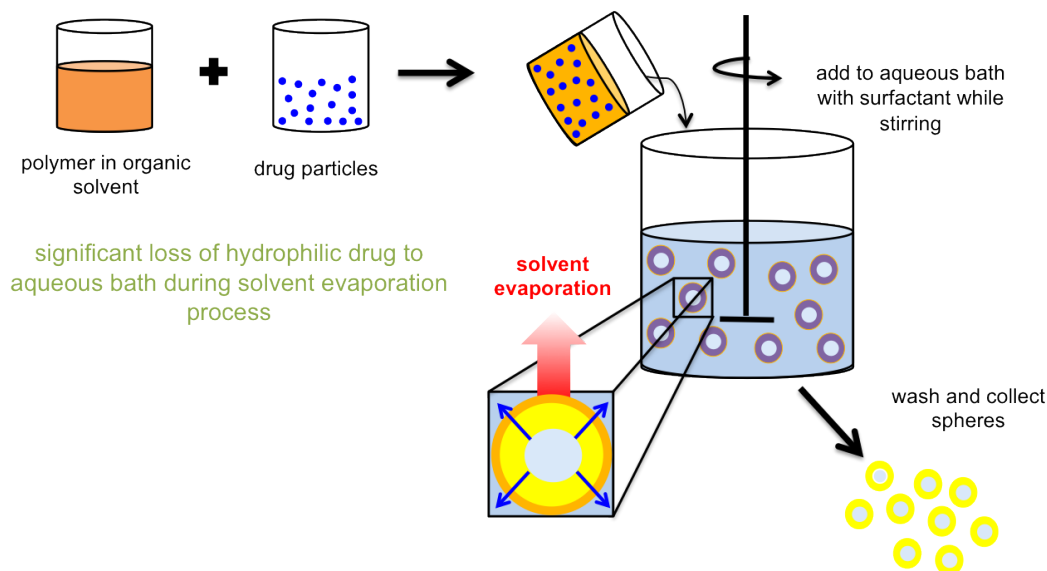


Figure 1.3: Loss of hydrophilic drugs to aqueous bath during solvent evaporation.

preparation of polymeric nanospheres for drug delivery.

1.2.1.2 Solvent Removal

Solvent removal is a modification of organic phase separation, also known as coacervation, originally developed as an alternative to solvent evaporation for the encapsulation of therapeutics within polyanhydrides due to the extreme lability of the anhydride bond in the presence of water as a way to preserve chemical integrity of the polymer by replacing the aqueous bath with an oil phase [35]. With advancements in the field of microencapsulation, many similar techniques have been developed resulting in the confusing nomenclature, which includes terms such as solvent extraction and solvent diffusion.

Organic phase separation relies on the dissolution of the polymer in the continuous phase, followed by the formation of a coacervate upon the addition of a phase inducer resulting in sphere formation and drug encapsulation [36]. In contrast, solvent removal relies on the oil-in-oil (o/o) emulsification of a polymer solution into a continuous phase. The extraction of the organic solvent into this continuous phase causes a decrease in the local solubility of the polymer in the polymer-rich droplets resulting in particle formation, a more rapid process compared to many other encapsulation techniques (Figure 1.4).

Since the solvent removal process relies on the diffusion of the polymer solvent into the

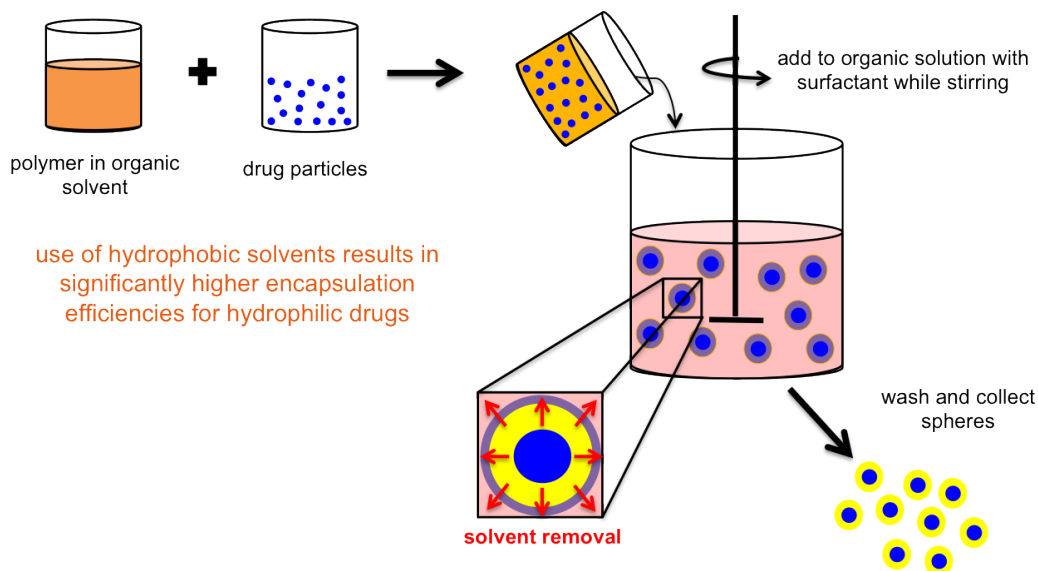


Figure 1.4: Schematic of the solvent removal method.

continuous phase, it must be selected such that it is a non-solvent for the polymer that is miscible with the polymer solvent; thus, hydrophobic oils are most commonly used. Upon dispersion of the polymer solution in the continuous phase, the outer shell of each polymer solution droplet precipitates followed by the extraction of the solvent through this polymeric membrane into the continuous phase typically resulting in the formation of spheres with a dense outer shell and a porous core, especially with less crystalline polymers [5]. Studies have shown that this rate of solvent extraction plays a role in determining the porosity of the spheres, with faster solvent removal rates associated with a decrease in porosity and higher encapsulation efficiencies [37,38]. Similar to solvent evaporation, the size of the polymer solution droplets in the continuous phase immediately prior to solvent extraction determines the final particle size; while high shear rates can be used to produce nanospheres, they can have detrimental effects on sensitive therapeutics.

The use of this method has been focused on the encapsulation of proteins - studies have confirmed the best correlations between theoretical and actual loadings were achieved when encapsulating hydrophilic proteins by solvent removal because the microencapsulation process occurs in a hydrophobic environment [35,39]. Additionally, the rapidity of the polymer precipitation reduces the duration of protein exposure to organic solvents, which may help to preserve bioactivity.

1.2.1.3 Phase Inversion Nanoencapsulation (PIN)

Phase inversion nanoencapsulation (PIN) is a novel method developed by the Mathiowitz lab that can be used to spontaneously encapsulate therapeutic agents in microspheres and nanospheres in the range of 200 nm and 30 μ m [5,40]. This method is a relatively simple process in which a dilute polymer solution containing the target drug is rapidly added to an excess of non-solvent. The PIN method relies on the polymeric phase separation due to the reduction in polymer solubility in the environment of a non-solvent (Figure 1.5). With the addition of a non-solvent to a polymer solution past the critical point, a two-phase system is formed with one polymer rich phase and another with relatively little polymer. The polymer in the solution undergoes a transition from a homogenous solution to an unstable two-phase mixture in which spherical droplets of non-solvent or drug in the polymer rich phase serve as nucleation sites for the precipitation of the polymer upon the diffusion of the polymer solvent into the excess non-solvent, similar to the solvent removal method [40]. Although the exact mechanism of particle formation is not yet clearly understood, one likely theory postulates a nucleation and growth mechanism by the precipitation of the polymer around a core micellar structure. An alternative hypothesis supports the formation of primary particles during the initial precipitation resulting from solvent removal followed by their coalescence to form secondary particles of the final microsphere diameter. It is plausible that the nucleation and growth hypothesis is the mechanism of particle formation in solutions with the more dilute polymer concentrations suitable for PIN while the coalescence theory may apply to more concentrated polymer solutions with higher viscosities as are often used in formation of porous polymer membranes and hollow fibers [40].

In contrast to many of the traditional microencapsulation techniques, such as solvent evaporation and solvent removal, which often require multiple steps and often expose therapeutic agents to denaturing conditions, PIN is advantageous in that it is a one-step encapsulation process that is nearly instantaneous and relatively gentle on the target drug. The only requirement for the successful fabrication of microspheres by PIN is the appropriate selection of solvent and non-solvent: the solvent and non-solvent must be miscible for particle formation with a minimal solvent:non-solvent ratio of 1:40, though a ratio of 1:100 is opti-

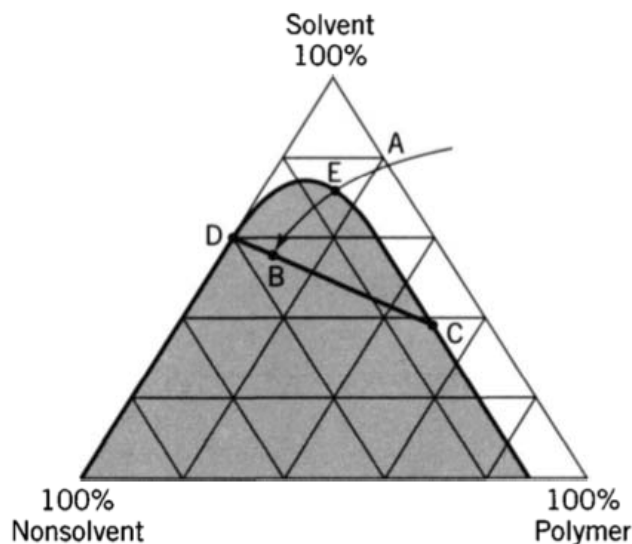


Figure 1.5: Phase separation diagram for a polymer/solvent/non-solvent system. Figure from [5].

mal. Furthermore, the diameter of the microparticles produced by PIN is dependent on the viscosity of the initial polymer solution rather than the mixing rate of the initial emulsion as in traditional techniques. Using initial polymer concentrations in the range of 1 – 2 % (w/v) and solution viscosities of 1 – 2 cps generates particles of 100 – 500 nm while polymer concentrations of 2 – 5 % (w/v) with viscosities of 2 – 3 cps result in particles between 500 – 3000 nm [40]. As is the case with solvent removal, the hydrophobic conditions used in the preparation of the nanospheres allows for the minimal loss of the therapeutic agent during the encapsulation process.

PIN is a versatile process that has significant potential for use in oral drug delivery because it can be used to encapsulate small molecules, proteins and genetic material [20,30,40-42]. The therapeutic agent can be loaded into microspheres by adding it as a micronized solid or by emulsification into the polymer solution before phase inversion. As a micronized solid, the drug acts as a core nucleus around which the polymer solution can precipitate. When an aqueous solution of the drug is emulsified into the polymer solution, the emulsion droplet serves as the nucleus around which the polymer precipitates to form microspheres, which can then be lyophilized to precipitate the drug as the emulsion is dehydrated. In both cases, PIN offers high encapsulation efficiency while maintaining the bioactivity of the

encapsulated therapeutic agent.

1.2.1.4 Double-Walled Microspheres

Although traditional microsphere-based drug delivery systems show significant promise for drug delivery, many of the current methods of fabrication result in an initial “burst” effect caused by the release of drug particles trapped on the microsphere surface. A solution to overcoming this problem is the use of double-walled or multilayered microspheres, which can not only overcome the “burst” effect but can allow for the sequential release of multiple therapeutic agents. An additional benefit is that the chemical and physical properties of different polymers can be used to tailor double-walled microspheres for different applications, whether it be the controlled release of drugs or the manipulation of surface chemistry to modify their interactions with biological environments.

Earlier methods of preparing double-walled microspheres involved many steps in which single-walled microspheres were fabricated then coated with a second polymer via pan-, dip-coating or spray drying, etc [1]. Unfortunately, these methods result in incomplete or uneven deposition of the coating polymer while adding additional steps to the manufacturing process, which can result in quality control issues and lower yields [3]. Furthermore, as microencapsulation techniques become more sophisticated and yield smaller diameter micro- and nanoparticles, the technical challenges of coating them make a single-step fabrication process not only desirable but also requisite.

Our group has previously developed a novel one-step process for preparing large-diameter double-walled microspheres using both modified solvent evaporation and solvent evaporation processes that rely on the phenomenon of phase separation in polymer/polymer/solvent systems (Figure 1.6) combined with emulsion theory [1,5,7-11]. By adding a two-polymer solution to a continuous aqueous phase and forming a stable o/w emulsion, the evaporation of the organic solvent results in the phase separation of the polymers in each droplet. Under the right conditions with the appropriate interfacial tensions and evaporation rate, phase separation occurs such that one polymer engulfs the other to form highly uniform double-walled microspheres. Further work in our lab has recently led to the modification of our previous method to fabricate double-walled nanospheres using this polymer-polymer phase

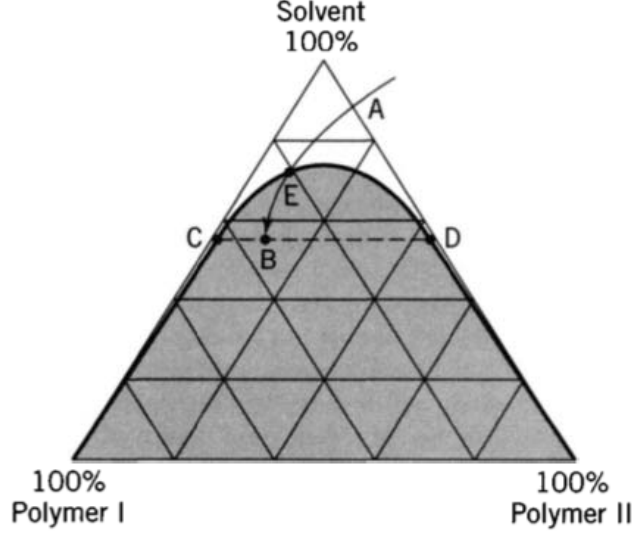


Figure 1.6: Phase separation diagram for a polymer/polymer/solvent system. Figure from [5].

separation.

When two chemically different polymers are mixed in solution at high concentrations, the positive free energy of mixing ($\Delta G_m > 0$) results in the inability of the two polymers to mix causing their separation into two phases due to polymer-polymer incompatibility. The free energy of mixing can be described by:

$$\Delta G_m = \Delta H_m - T \Delta S_m$$

For two polymers, the enthalpy of mixing (ΔH_m) is generally > 0 , indicative of endothermic mixing, while the entropy of mixing (ΔS_m) is very small. Therefore, if the ΔH_m for the two polymers is positive, then polymer-polymer incompatibility will occur. The immiscibility of two polymers in solution can be predicted by the difference in the pure component solubility parameters for each polymer [12]:

$$\delta = \frac{\rho \sum G}{M}$$

where δ is the solubility parameter, ρ is the density of the polymer and M is the molecular

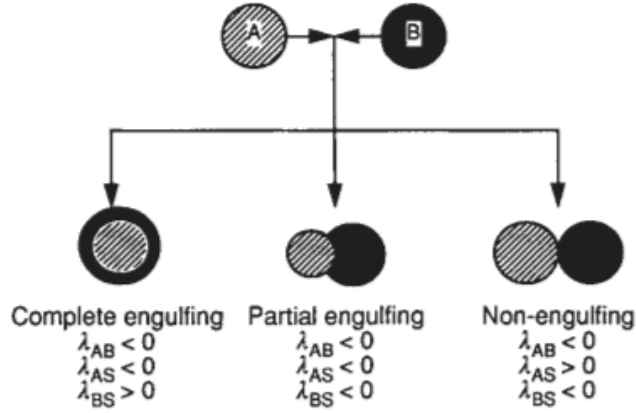


Figure 1.7: Possible configurations for the two-polymer system: complete engulfing, partial engulfing and non-engulfing as predicted by the values of the three spreading coefficients for the system. Figure from [1].

weight of the monomer unit [14]. A greater difference in the solubility parameter correlates with a more positive value for ΔH_m and a higher likelihood that the two polymers will be immiscible. Though this inherent characteristic of polymers is a challenge that must be overcome when a homogenous polymer blend is desired, this incompatibility provides the basis for the fabrication of double-walled microspheres in one step.

Although phase separation is the basis for this technique, the interaction of the two dissimilar polymer phases must be considered since this is what actually determines the localization of each polymer within the microspheres. This can be represented by Harkin's equation, which describes the spreading coefficient (λ_{AB}) as determined by the interfacial tensions (γ) between the solvent (S) and polymers A and B:

$$\lambda_{AB} = \gamma_{BS} - \gamma_{AS} - \gamma_{AB}$$

For positive values of λ_{AB} , polymer A spontaneously spreads onto polymer B; as seen in figure 1.7, depending on the values of the three possible spreading coefficients, the result can be complete engulfment, partial engulfment and non-engulfment [1].

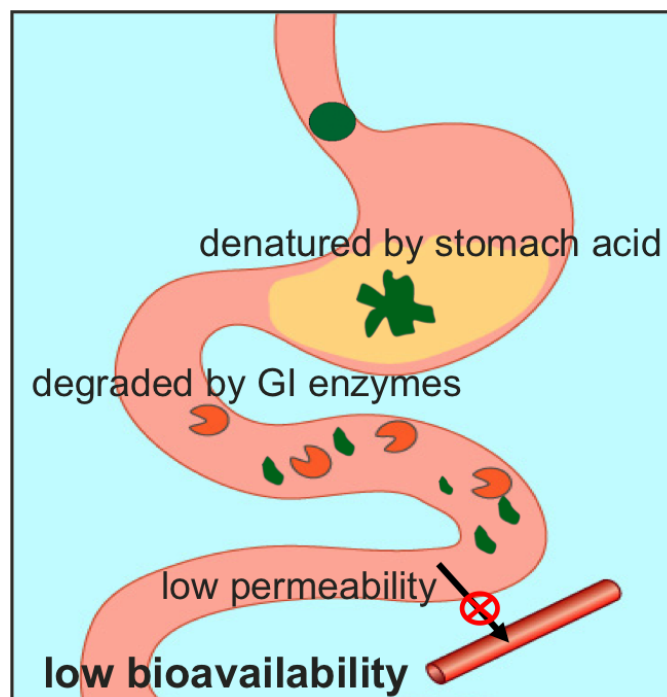


Figure 1.8: Challenges presented by the gastrointestinal tracts that prevent the oral administration of many classes of therapeutic agents. Image courtesy of Yu-Ting Liu.

1.3 Oral Drug Delivery

1.3.1 Advantages of Oral Drug Delivery

The field of medicine relies heavily on the use of therapeutic agents for the treatment of various illnesses but many of these drugs have chemical or physical properties that prevent them from being administered orally, despite this being the most convenient method. The many recent advances in the field of biotechnology and pharmaceutical science have led to the development of a variety of protein- and DNA-based therapeutics. The oral administration of drugs is the easiest and most convenient method because of the high rates of patient acceptance and compliance when a multiple dosing schedule is required [16]. Unfortunately, the gastrointestinal (GI) tract presents a variety of physical, chemical and enzymatic barriers that prevent many classes of drugs such as peptides, proteins, macromolecules, hydrophobic drugs and DNA from being administered orally in their natural form [17,19,20]. These barriers result in the low bioavailability of these drugs when delivered orally, which could be overcome with the use of microsphere drug delivery systems for efficient oral administration

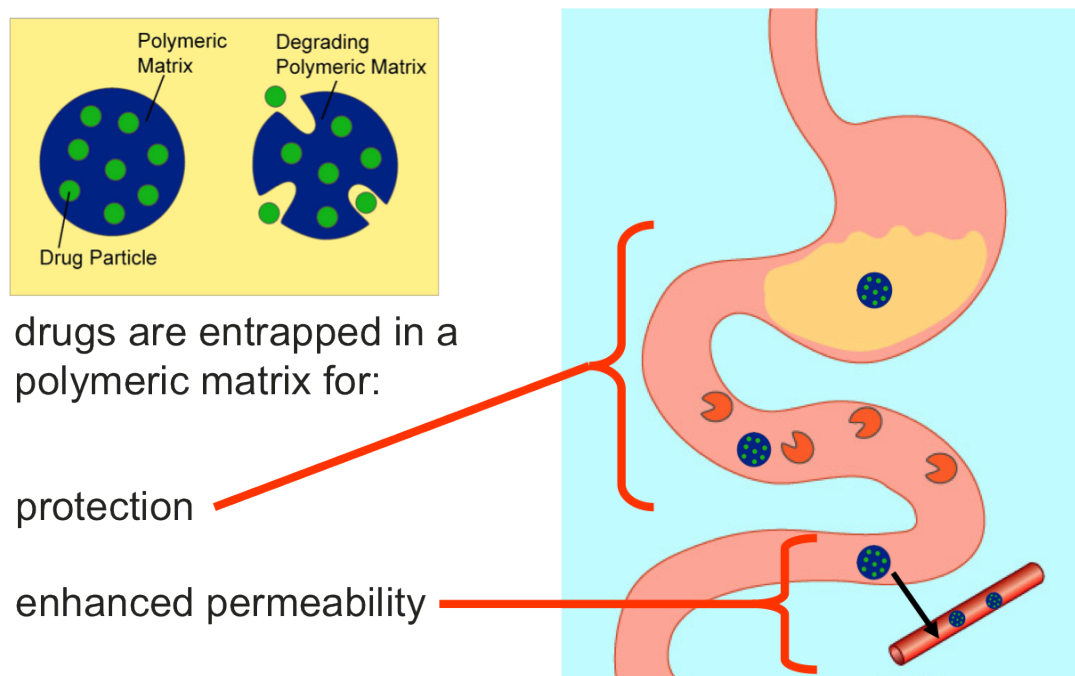


Figure 1.9: Rationale for polymeric microencapsulation. Image courtesy of Yu-Ting Liu.

(Figure 1.8).

While many different strategies have been evaluated for the oral administration of these therapeutics, each has been plagued with obstacles that have prevented the development of successful oral drug delivery systems for these classes of drugs. The use of polymeric microspheres and nanospheres shows significant promise due to their ability to protect the drug from the harsh GI environment while allowing for controlled drug release kinetics (Figure 1.9). However, the major obstacle for this approach is the low level of uptake by small intestinal enterocytes. In recent years, a significant amount of research has focused on the design and fabrication of drug delivery systems that overcome the obstacles faced in the delivery of drugs for therapeutic applications.

1.3.2 Oral Drug Delivery Technologies

Previous research with polymers has resulted in their dominant role as carrier molecules in drug delivery systems since their use for the controlled release of drugs was initially introduced in the 1960s [2,21].

A variety of different technologies have been developed for the oral delivery of thera-

peutic agents. Most traditionally include tablets and capsules, many of which have been designed with excipients and coatings to allow for some control of drug release kinetics. Enteric coatings, such as the Eudragit polymers, have been used to protect drug molecules from gastric degradation due their pH-dependent solubilities [19,23]; polymers have been developed for use in these applications with dissolution behaviors at a wide range of pH values to target specific regions of the gastrointestinal tract. Other more specialized technologies used in oral drug delivery include osmotic pumps [24,26], which consist of a drug in a rigid, semi-permeable membrane that is released through a small opening due to osmotic pressure in aqueous environments, as well as hydrogels, which are three-dimensional water-swellaable but insoluble cross-linked hydrophilic polymers in which a drug is entrapped. However, these technologies only control the release of the drug from the system into the gastrointestinal tract and provide neither full protection throughout gastrointestinal transit nor permeation enhancement.

Oral microsphere drug and gene delivery systems have been developed in which drugs or genetic material are entrapped in a polymeric matrix that enhances protection and permeability while increasing GI transit time [5]. Furthermore, oral microsphere delivery systems can be designed to have specific controlled release profiles to result in an effective increase in the bioavailability of the encapsulated therapeutic agents [5,16,17,19,20,22,29,30].

Encapsulation in a polymeric matrix is effective in overcoming the physical, chemical and enzymatic barriers in the delivery of drugs and genes presented by the GI tract. The polymeric matrix protects the drug or gene from the stomach acid and digestive enzymes responsible for degradation and loss of biological activity [22]. If the polymeric matrix possesses the right physicochemical surface properties, the translocation across the GI epithelium can be greatly improved [43]. The increased uptake results in higher bioavailability and therefore activity.

As described in Nature, our lab has previously demonstrated the oral delivery and biological activity of hydrophobic drugs, insulin and plasmid DNA using polymeric microspheres; the oral bioavailability of insulin was 5% in both rats and dogs [30] but we believe this number can be significantly improved via our systematic evaluation of particle-based drug delivery systems, reduction in particle size and use of more bioadhesive polymers.

1.4 Particle Uptake from the Small Intestine

1.4.1 Anatomy and Histology of the Gastrointestinal System

Though the stomach and colon have some absorptive activity, the small intestine is the primary absorption site for orally administered microparticles. The small intestine is composed of three distinctive sections: the duodenum, the jejunum and the ileum – each with different cellular compositions and physiological functions; the jejunum and the ileum are the main sites of absorption [44]. Chyme first enters the small intestine at the duodenum, where food breakdown continues after leaving the stomach. The duodenum is distinguishable by the presence of Brunner’s glands, which are responsible for the secretion of alkaline mucus to neutralize acids and inactivate pepsin [44]. The jejunum and the ileum are the main sites of absorption. The jejunum, which lacks Brunner’s glands, has longer villi to increase the surface area available for absorption and very small numbers of Peyer’s patches [45]. The ileum, which follows the jejunum, contains a high number of Peyer’s patches and is conventionally thought to be a better target for oral microparticle delivery (Figure 1.10)[45].

The first sites implicated in macromolecular uptake were the Peyer’s patches that are present on the anti-mesenteric side of the gut wall. Peyer’s patches were originally implicated as the site of lymphatic particulate entry in 1926 when oral administration of colloidal dyes in rats resulted in the intense accumulation of dye particles in these regions. Previous work showing the presence of micron-sized particles in the lymph of dogs after oral intake suggested a link between uptake and the lymphatic system. The most obvious structure present in a Peyer’s patch is the follicle, which contains a mixture of lymphocytes, macrophages and dendritic cells near the gut lumen (Figure 1.11). Peyer’s patches are encased by a specialized epithelium lacking mucus producing goblet cells to allow close interaction with particulate antigens. It is thought that the abundance of M-cells in this epithelium is responsible for the phagocytic activity associated with uptake of microparticulates. [45]

M-cells are lymphoepithelial cells that have irregular shaped ‘microfolds’ similar to the villi on adjacent enterocytes. These cells are responsible for the active transport of a variety of inert materials from the gut lumen towards the follicles of the mucosa-associated lymphoid tissue (MALT). From there, these particles can migrate to the blood via the mesentery lymph

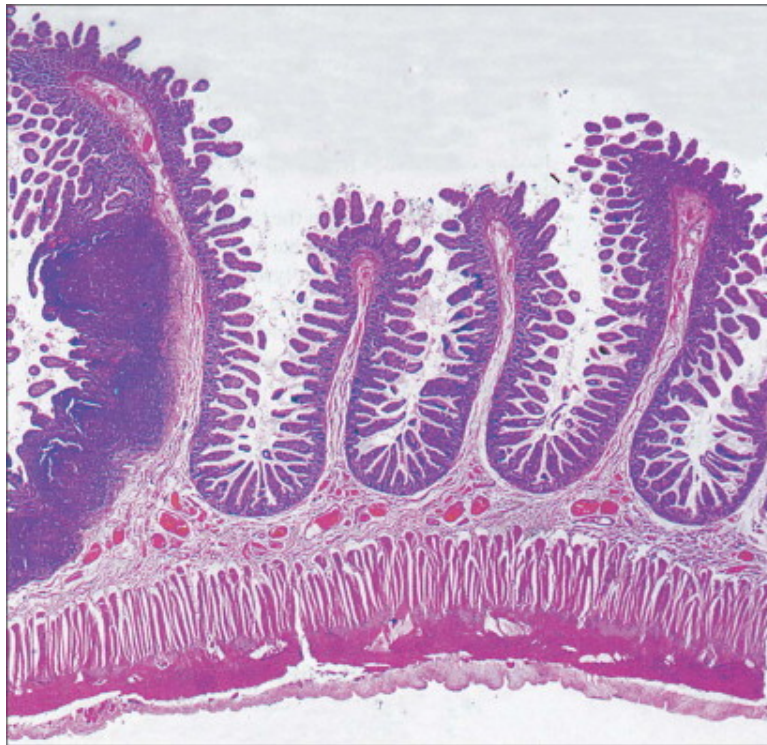


Figure 1.10: Low-power micrograph of ileum. The microfolds of the intestine are covered with villi projecting into the lumen. A Peyer's patch is present on the left side of the image. Image from Wheater's Functional Histology (2000).

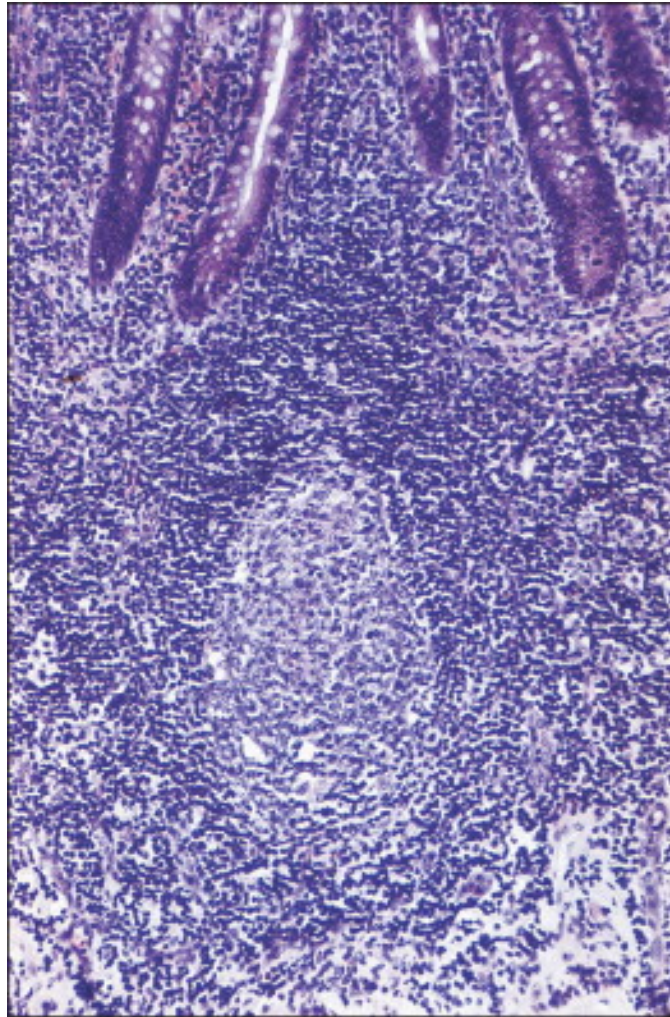


Figure 1.11: Follicle of mucosa-associated lymphoid tissue (MALT) present in a Peyer's patch. Image from Gray's Anatomy (2008).

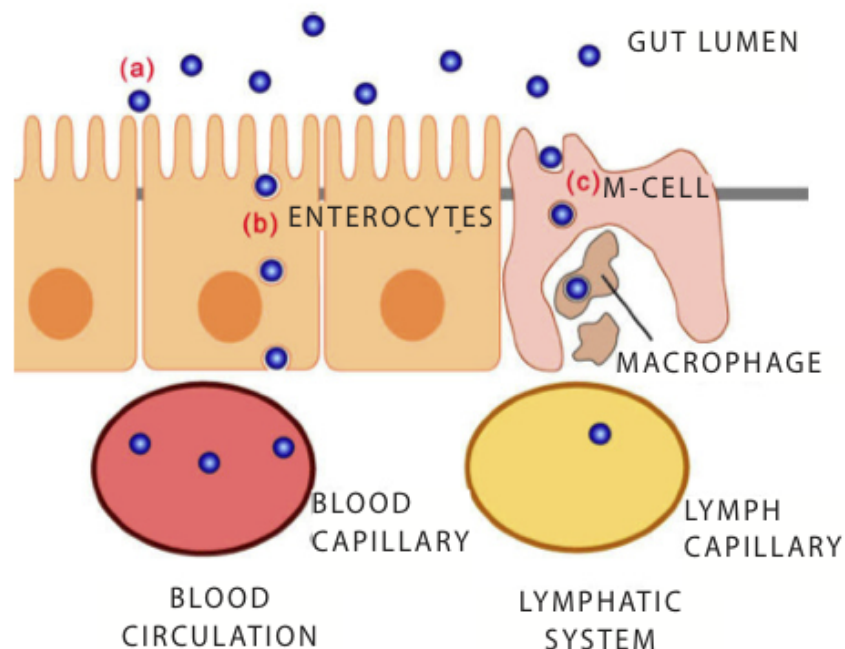


Figure 1.12: Mechanisms of particle uptake from the small intestine include the (a) paracellular cellular pathway, (b) endocytotic mechanisms and (c) phagocytosis. Image courtesy of Yu-Ting Liu.

nodes and the thoracic duct. M-cells are also implicated as the body's immune surveillance mechanism for actively sampling the contents of the GI tract for potentially harmful antigens, which are presented to underlying follicular B- and T- cells. [45]

Although Peyer's patches were originally implicated in the uptake of particles from the GI tract, enterocytes have also been shown to be sites of particle entry. Currently, there are thought to be three mechanisms of particle uptake, each dependent on particle size (Figure 1.12). Macromolecules that are smaller than 50 nm in size diffuse through the space between intestinal epithelial cells. In the endocytotic or transcellular pathway, particles that are smaller than 200 nm are absorbed by enterocytes through endocytosis. The M-cells, described above, are responsible for phagocytosis of particles smaller than 5 μm .

1.4.2 Principles of Particle Translocation

The earliest evidence for microparticle uptake from the GI tract was cited in 1844 when Herbst observed starch granules in the blood and lymph vessels of dogs after they had been

fed orally [45]. Since then, dozens of studies have shown proof of microparticle uptake from the GI tract through intestinal lymphatics with various particles that range in size from nanometers to hundreds of microns. The few studies in humans have demonstrated the uptake of a variety of different microparticles 2 μm in diameter.

Much of the early research of intestinal particle uptake focused on clusters of lymphoid follicles in the small intestine, called Peyer’s patches (PP), where M-cells are clustered because it was believed that these were the only sites for the cellular uptake of particles. Studies of M-cell phagocytosis have shown that a wide range of particles including poly(lactic-co-glycolic acid) (PLGA) and polystyrene (PS) as large as 3 to 10 μm can be internalized by this mechanism [46-48]. Although endocytosis by enterocytes is thought to contribute minimally to intestinal particle uptake, studies have suggested that the internalization of polymeric particles as large as 5 μm can be mediated by enterocytes [49,50]. However, due to the historical disbelief in the potential for the translocation of particles of this size range by endocytosis, there have been no systematic evaluations of these observations to date.

The uptake of micro- and nanoparticles from the GI tract has been evaluated using a variety of microscopy techniques (e.g. light microscopy/histology, fluorescence and scanning electron microscopy), isotopic tracers and drug release; however, these methods are mostly qualitative and often fail to provide information about the fate of the particles in the body. A significant number of studies have evaluated particle uptake in vitro using Caco-2 cells derived from a human colon adenocarcinoma cell line that differentiates to mimic small intestinal enterocytes when grown in culture on Transwell membranes [22,51-54]. This model is widely used in the pharmaceutical field to study the transepithelial transport of small molecule drugs but several groups have used these cells to study the absorption of starch, PS, poly(acrylic acid) (PAA) and PLGA particles by intestinal cells [51]. Co-cultures of Caco-2 cells with HT29-H cells, another adenocarcinoma cell line that expresses the characteristics of goblet cells, have been used to better approximate the small intestine with the incorporation of a mucous layer [55]. However, these models may not be suitable for the analysis of particle internalization [22,51]; they provide limited information about translocation, fail to provide any insight into the final fate of the particles in the body and have undetermined in vitro-in vivo correlations (IVIVC).

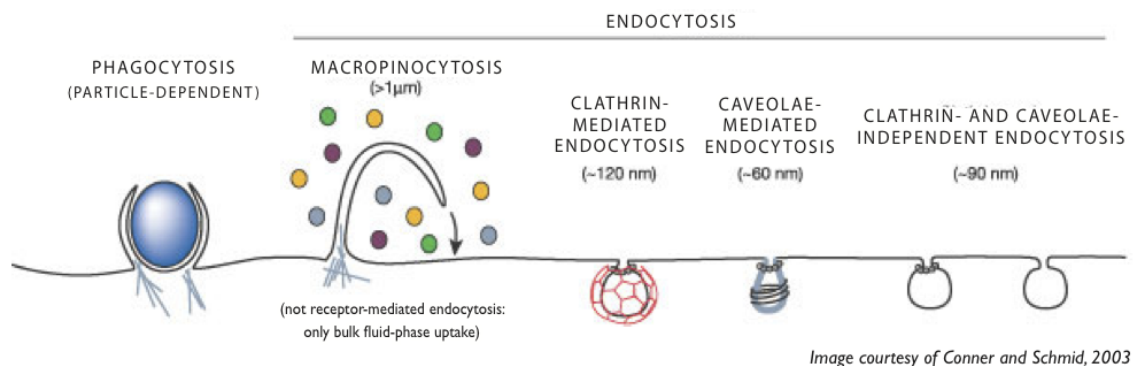


Figure 1.13: Mechanisms of phagocytotic and endocytotic particle internalization.

Most importantly, none of the previous studies of particle uptake for drug delivery applications have systematically evaluated the effect of different particle characteristics on uptake and tissue distribution.

1.4.3 Particle Uptake Mechanisms

Currently, there are thought to be three predominant mechanisms of particle uptake, each dependent on particle size. The paracellular pathway is a non-phagocytic, non-endocytotic mechanism by which small molecules diffuse through the tight junctions of intestinal cells; tight junctions form a strong barrier and if intact, serve as a significant obstacle to particle uptake in the intestines [56]. In contrast, the diffusive pathway is a non-vesicular, transcytotic pathway by which small particles (<50 nm) can passively migrate through cellular pores [57]. Illustrated in figure 1.13 are the two primary mechanisms by which we believe the uptake of sub-micron sized particles is mediated: phagocytosis and endocytosis.

Phagocytosis by M-cells is believed to be a major contributor to particle uptake in the ileum, where it serves the purpose of immune surveillance by delivering small quantities of intestinal contents to the GALT for presentation to the immune system by antigen presenting cells. While it had been previously suggested that clathrin mediates all cellular endocytotic pathways, recent studies have elucidated other clathrin-independent mechanisms (i.e. caveolae-mediated endocytosis (lipid rafts), macropinocytosis, etc.) confirming that endocytosis is a diverse and complex method of particle internalization that we are just beginning to understand; thus, further research must be done to gain a better understanding of cellular

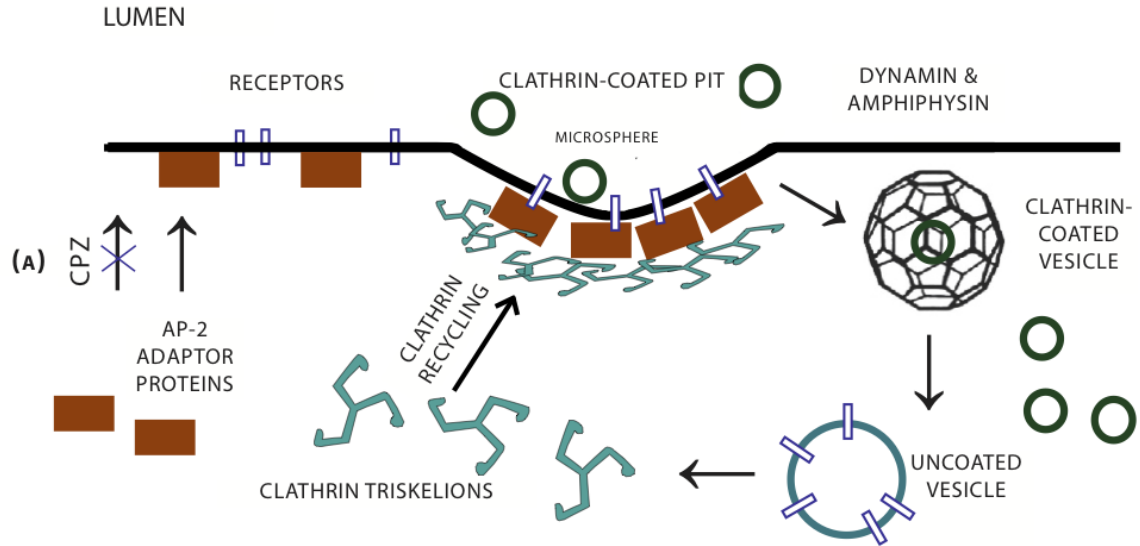


Figure 1.14: Schematic diagram of clathrin pit formation, cargo capture, scission and clathrin recycling. (A) Chlorpromazine (CPZ) prevents clathrin recycling and clathrin-dependent endocytosis by blocking the recruitment of AP-2 adaptor proteins to the cell membrane. Image courtesy of Allison Kwong.

trafficking through endocytotic mechanisms.

Clathrin is a triskellion-shaped protein that self assembles its light and heavy chains into a geodesic lattice when recruited by adapter proteins to form the AP complex resulting in the invagination of the cell membrane as clathrin-coated pits which pinch off by the GTPase dynamin [58-61]. Following endocytosis, the clathrin-coat disassembles from the newly formed vesicle in an ATP-dependent process followed by recycling of the clathrin and AP complexes to the cell surface for further internalization (Figure 1.14).

Clathrin triskelions are made of three legs with each consisting of a 190 kDa heavy chain and an associated ~25 kDa light chain [62]. Recruitment of clathrin by adaptor proteins to the cell surface at the start of the endocytotic process can form extended clathrin lattices of varying shapes and sizes [63]. Studies of clathrin-mediated endocytosis have demonstrated its role in the transport of a variety of biological molecules including transferrin, reovirus and low-density lipoprotein (LDL) [62]; furthermore, clathrin has been well established as an essential part of many cellular processes, such as synaptic vesicle endocytosis and cell signaling. A leader in the field of clathrin-mediated endocytosis, the Kirchhausen lab has

developed many cell lines expressing fluorescently labeled clathrin to allow for the real-time visualization of clathrin assembly and small biological molecule internalization a variety of advanced microscopy techniques. Using these models, many groups have begun to study the interactions of clathrin and its cargo as well as to identify the proteins involved in this pathway. However, most biologists in this field strongly believe that the uptake of particles by this process is limited to small biological molecules (< 100 nm) and thus are not pursuing research into the transport of micron and sub-micron sized particles. Results from our lab have demonstrated that polystyrene microspheres of up to $2\text{ }\mu\text{m}$ in diameter can be internalized by clathrin-dependent endocytosis, though at lower rates than their smaller counterparts. This is of particular importance since non-phagocytic enterocytes compose 98% of the surface area of the small intestine; the presence of an uptake mechanism for micro- and nanoparticles by enterocytes suggests that the entirety of the small intestine can be involved in the internalization of particle-based drug delivery systems rather than the $< 1\%$ of the surface area comprised of Peyer’s patches as originally believed.

1.4.4 Bioadhesion

A bioadhesive has been defined as a synthetic or biological material, which is capable of adhering to a biological substrate or tissue [64]. In the context of drug delivery, the development and testing of materials demonstrating bioadhesive behavior, specifically to membranes of the intestine, is of interest. The bonding mechanism between the polymer and biological tissue remains an area of debate. The bioadhesive polymer may bond with the cell layer, the mucus layer, or a combination of both. The bonds are believed to be of generally of two categories: mechanical or chemical [56,65]. Mechanical bonds are the result of a physical entanglement of polymer and tissue, the strength of which is directly proportional to the depth of penetration and degree of entanglement of the polymer chains into the mucus [66,67]. Chemical bonds between the polymer and tissue, on the other hand, include strong primary covalent bonds, as well as secondary forces such as ionic bonds, Van der Waals interactions, and hydrogen bonds [68]. Although these forces are relatively weak, strong adhesion can result from to a large number of interaction sites. Polymers with a greater concentration of polar carboxyl and hydroxyl groups and, thus, higher hydrophilicity tend

to develop more intense mucoadhesive bonds [69,70].

Bioadhesive drug delivery systems can consist of microspheres or nanospheres composed entirely of a bioadhesive polymer or with an outer coating of the bioadhesive polymer. The polymers used in the fabrication of bioadhesive nanospheres influence their properties and their behavior in vivo. The greatest barrier in the use of nanosphere-based drug delivery systems has been the low level of particle translocation from the small intestinal lumen to the bloodstream. In the case of bioadhesive microspheres, an adhesive interaction may form with the epithelial cell layer, the GI mucosa or a combination of the two; because the adhesion to cell surfaces involves highly specific receptor-mediated interactions it is thought that the bioadhesion of microspheres is to the mucosal lining of the GI tract; thus, it is often referred to as mucoadhesion [44,45,71]. However, the varying turnover time and composition of mucus, the differing bioadhesiveness of polymers in different pH ranges and changes in physiology as a result of disease may cause the bioadhesion of microspheres to the GI mucosa to behave unpredictably [45]. The use of bioadhesive polymers in oral nanosphere drug delivery systems is expected to enhance uptake by increasing GI transit time and decreasing the distance the particles must diffuse to reach the cells responsible for uptake [44,65,68,71]. The enhancement of uptake with the use of bioadhesive nanospheres depends on the direct contact between the nanospheres and the mucosal lining of the small intestine. Furthermore, the differences in the surface chemistries of the bioadhesive polymers used to fabricate the nanospheres are thought to alter their biodistribution following uptake. Much work with these polymers has been done in our lab including the synthesis of bioadhesive polymers, development of various methods to fabricate and evaluate bioadhesive nanospheres.

A challenge in bioadhesion testing is developing a standard method to characterize the bioadhesiveness of a polymer. Traditionally, bioadhesion has been a relative measurement. Nonetheless, the strength of the bioadhesive bond can be measured in a laboratory setting. Two common in vitro techniques are the Wilhelmy plate technique, which provides a dynamic contact-angle measurement using a tensiometer, and a shear test, which measures the force required to separate two glass slides coated with polymer and separated by a thin layer of mucus [72]. The Mathiowitz lab has been at the forefront of developing techniques for the evaluation of bioadhesion between polymers and intestinal tissue. Previously, our lab

has developed a method to study the bioadhesion of single microsphere and intestinal tissue using a CAHN electronic microbalance [73], which has further resulted in our more recent development of a modified Texture Analyzer technique to record the force required to break the adhesive bond between a polymer-coated pinhead and a sample of rat intestinal tissue. Additionally, an everted sac bioadhesion assay has been developed to study bioadhesion in the micro- and nanoscale, to determine the adhesion of microspheres to freshly excised intestinal tissue in a passive test that more closely mimics the in vivo environment than previous techniques [74]. Furthermore, this technique allows for the evaluation of nanospheres, which are extremely difficult to manipulate for active tests of bioadhesion.

1.5 Experimental Goals

The goals of this work are to provide the fundamental framework for the development of an oral drug delivery platform based on bioadhesive double-walled nanospheres, particularly for protein therapeutics. This will be accomplished through the systematic analyses of particle uptake and tissue distribution from the small intestine, bioadhesion, nanosphere fabrication and protein nanoencapsulation.

Aim 1: Quantitative analysis of particle uptake and biodistribution from the small intestine.

Using an isolated loop model, in vivo studies of particle uptake will be performed using polystyrene micro- and nanospheres. The effects of particle size, small intestinal location and uptake kinetics on total translocation and tissue distribution will be evaluated. Furthermore, using known inhibitors of cellular uptake pathways, the mechanisms involved in the translocation of particulate drug delivery systems will be probed. This data will provide insight into the optimal particle size as well as other crucial information about the behavior of these delivery systems in vivo.

Aim 2: Characterization of novel bio-inspired catechol-functionalized bioadhesive polymers.

Using a series of bioadhesive polymers prepared through the ring-opening conjugation reaction of L-phenylalanine, L-tyrosine and L-DOPA to low molecular weight poly(butadiene-maleic anhydride) and high molecular weight poly(ethylene-maleic anhydride), the time-dependent mucoadhesive behavior of these polymers with fresh rodent small intestinal tissue will be determined via tensile testing methods using a modified texture analyzer apparatus. This will allow for the identification of an effective bioadhesive polymer for use in the development of our delivery system.

Aim 3: Quantitative analysis of bioadhesive nanoparticle uptake and biodistribution from the small intestine.

Using non-bioadhesive polymethyl methacrylate (PMMA), moderately bioadhesive polystyrene (PS) and bioadhesive poly(butadiene-maleic anhydride-co-DOPA), the effect of bioadhesion on the total uptake and biodistribution from the jejunum will be evaluated in vivo. Furthermore, the bioadhesive behavior of these polymers will be characterized using both an in vitro texture analyzer method and an ex vivo everted sac method. This data will provide justification for the use of bioadhesion as a potentially effective strategy for the enhancement of particle uptake from the small intestine.

Aim 4: Development of a method for the fabrication of bioadhesive and biodegradable double-walled nanospheres.

Using the phase inversion nanoencapsulation (PIN) method combined with polymer phase separation phenomena, a method will be developed for the preparation of double-walled nanospheres in a single step based on the sequential phase inversion of a biodegradable polymer followed by a bioadhesive polymer. A variety of polymer combinations will be evaluated to produce bioadhesive nanospheres in the desired size range for optimal drug delivery.

Aim 5: Development and evaluation of methods for the encapsulation of therapeutic agents by phase inversion nanoencapsulation (PIN).

Although the PIN method has been used in our lab for the encapsulation of therapeutic agents in polymeric micro- and nanospheres, analyses of the effects of different drug-loading methods has not yet been performed. Using a water-in-oil (w/o) emulsion, a w/o cryogenic emulsion and a solid-in-oil (s/o) dispersion loading methods, the effect of these manufacturing parameters on protein loading and release from PLGA nanospheres will be evaluated.

The results of the studies in Aims 1 and 3 will provide insight into the uptake mechanisms involved the translocation of particles from the small intestine and provide the motivation for the use of bioadhesives and the desired particle size to maximize particle uptake to enhance bioavailability. The studies of bioadhesion in Aims 2 and 3 will help elucidate the physical and bioadhesive characteristics of our novel polymers and identify those that are most suitable for use in oral drug delivery. Combining the results of Aims 1, 2 and 3, bioadhesive double-walled nanospheres in the desired size range with the optimal polymer composition can be prepared using sequential phase inversion nanoencapsulation in Aim 4, which can be loaded with protein using the methods and loadings determined in Aim 5 to be most effective. By combining the results of these experimental goals, a more effective oral protein delivery platform based on double-walled nanospheres can be engineered.

1.6 References

- [1] K.J. Pekarek, J.S. Jacob, E. Mathiowitz, Double-walled polymer microspheres for controlled drug release., *Nature*. 367 (1994) 258–260.
- [2] S. Freiberg, X. Zhu, Polymer microspheres for controlled drug release., *International Journal of Pharmaceutics*. 282 (2004) 1–18.
- [3] E.L. Parrott, *Pharmaceutical technology; fundamental pharmaceutics*, Burgess Pub. Co., Minneapolis, Minn., 1970.
- [4] J. Folkman, D. Long, The use of silicone rubber as a carrier for prolonged drug therapy, *J. Surg. Res.* 4 (1964) 139–142.
- [5] E. Mathiowitz, M.R. Kretiz, L. Brannon-Peppas, *Microencapsulation*, 1st ed. John Wiley & Sons, Inc., New York ,NY, 1999.

- [6] S. Desai, A. Simonelli, W. Higuchi, Investigation of factors influencing release of solid drug dispersed in inert matrices., *J. Pharm. Sci.* 54 (1965) 1459–1464.
- [7] K.J. Leach, E. Mathiowitz, Degradation of double-walled polymer microspheres of PLLA and P(CPP:SA)20:80. I. In vitro degradation., *Biomaterials.* 19 (1998) 1973–1980.
- [8] K.J. Leach, S. Takahashi, E. Mathiowitz, Degradation of double-walled polymer microspheres of PLLA and P(CPP:SA)20:80. II. In vivo degradation., *Biomaterials.* 19 (1998) 1981–1988.
- [9] K. Pekarek, M. Dyrud, K. Ferrer, Y. Jong, E. Mathiowitz, In vitro and in vivo degradation of double-walled polymer microspheres, *Journal of Controlled Release.* 40 (1996) 169–178.
- [10] K. Leach, K. Noh, E. Mathiowitz, Effect of manufacturing conditions on the formation of double-walled polymer microspheres., *Journal of Microencapsulation.* 16 (1999) 153–167.
- [11] N.A. Rahman, E. Mathiowitz, Localization of bovine serum albumin in double-walled microspheres., *J Control Release.* 94 (2004) 163–175.
- [12] S. Cooper, G. Estes, A.C.S.D.O.P. Chemistry, Multiphase Polymers, Amer Chemical Society, 1979.
- [13] T. Chang, Semipermeable Microcapsules., *Science.* 146 (1964) 524–525.
- [14] L.H. Sperling, Introduction to Physical Polymer Science, LibreDigital, 2006.
- [15] R. Jalil, J.R. Nixon, Biodegradable poly(lactic acid) and poly(lactide-co-glycolide) microcapsules: problems associated with preparative techniques and release properties, *Journal of Microencapsulation.* 7 (1990) 297–325.
- [16] H. Chen, R. Langer, Oral particulate delivery: status and future trends., *Advanced Drug Delivery Reviews.* 34 (1998) 339–350.
- [17] A. Fasano, Novel approaches for oral delivery of macromolecules., *J. Pharm. Sci.* 87 (1998) 1351–1356.
- [18] S. Freitas, H.P. Merkle, B. Gander, Microencapsulation by solvent extraction/evaporation: reviewing the state of the art of microsphere preparation process technology, *Journal of Controlled Release.* 102 (2005) 313–332.
- [19] G. Carino, E. Mathiowitz, Oral insulin delivery., *Advanced Drug Delivery Reviews.* 35 (1999) 249–257.

- [20] C.G. Thanos, Z. Liu, J. Reineke, E. Edwards, E. Mathiowitz, Improving relative bioavailability of dicumarol by reducing particle size and adding the adhesive poly(fumaric-co-sebacic) anhydride., *Pharm Res.* 20 (2003) 1093–1100.
- [21] R. Bodmeier, J. Siepmann, *Nondegradable Polymers for Drug Delivery, ... Drug Delivery*, 1999.
- [22] F. Delie, M.J. Blanco-Príeto, Polymeric particulates to improve oral bioavailability of peptide drugs., *Molecules.* 10 (2005) 65–80.
- [23] D. Dupeyrón, M. González, V. Sáez, J. Ramon, J. Rieumont, Nano-encapsulation of protein using an enteric polymer as carrier, *IEE Proc., Nanobiotechnol.* 152 (2005) 165–168.
- [24] S. Herrlich, S. Spieth, S. Messner, R. Zengerle, Osmotic micropumps for drug delivery., *Advanced Drug Delivery Reviews.* (2012).
- [25] M. Li, O. Rouaud, D. Poncelet, Microencapsulation by solvent evaporation: state of the art for process engineering approaches., *International Journal of Pharmaceutics.* 363 (2008) 26–39.
- [26] K. Takada, H. Yoshikawa, *Oral Drug Delivery, Traditional*, John Wiley & Sons, New York, NY, n.d.
- [27] C.P. Reis, R. Neufeld, A. Ribeiro, Nanoencapsulation I. Methods for preparation of drug-loaded polymeric nanoparticles, (2006).
- [28] S.C. Yadav, A. Kumari, R. Yadav, Development of peptide and protein nanotherapeutics by nanoencapsulation and nanobioconjugation., *Peptides.* 32 (2011) 173–187.
- [29] G.P. Carino, J.S. Jacob, E. Mathiowitz, Nanosphere based oral insulin delivery., *J Control Release.* 65 (2000) 261–269.
- [30] E. Mathiowitz, J.S. Jacob, Y.S. Jong, G.P. Carino, D.E. Chickering, P. Chaturvedi, et al., Biologically erodable microspheres as potential oral drug delivery systems., *Nature.* 386 (1997) 410–414.
- [31] K. Pistel, T. Kissel, Effects of salt addition on the microencapsulation of proteins using W/O/W double emulsion technique, *Journal of Microencapsulation.* 17 (2000) 467–483.
- [32] K. Fu, R. Harrell, K. Zinski, C. Um, A. Jaklenec, J. Frazier, et al., A potential approach for decreasing the burst effect of protein from PLGA microspheres., *J. Pharm. Sci.* 92 (2003) 1582–1591.

- [33] X.M. Deng, X.H. Li, M.L. Yuan, C.D. Xiong, Z.T. Huang, W.X. Jia, et al., Optimization of preparative conditions for poly-DL-lactide- polyethylene glycol microspheres with entrapped *Vibrio cholera* antigens, *J Control Release*. 58 (1999) 123–131.
- [34] M. Blanco-Prieto, C. Lecaroz, M. Renedo, J. Kunkova, C. Gamazo, In vitro evaluation of gentamicin released from microparticles, *Int J Pharm*. 242 (2002) 203–206.
- [35] E. Mathiowitz, W.M. Saltzman, A. Domb, P. Dor, R. Langer, Polyanhydride microspheres as drug carriers. II. Microencapsulation by solvent removal, *J. Appl. Polym. Sci*. 35 (1988) 755–774.
- [36] N. Nihant, C. Grandfils, R. Jérôme, P. Teyssie, Microencapsulation by coacervation of poly(lactide-co-glycolide) IV. Effect of the processing parameters on coacervation and encapsulation, *Journal of Controlled Release*. 35 (1995) 117–125.
- [37] W. Li, K. Anderson, R. Mehta, P. DeLuca, Prediction of solvent removal profile and effect on properties for peptide-loaded PLGA microspheres prepared by solvent extraction/evaporation method, *Journal of Controlled Release*. 37 (1995) 199–214.
- [38] Y. Yeo, K. Park, Control of encapsulation efficiency and initial burst in polymeric microparticle systems., *Arch. Pharm. Res*. 27 (2004) 1–12.
- [39] E. Mathiowitz, D. Kline, R. Langer, Morphology of polyanhydride microsphere delivery systems., *Scanning Microsc*. 4 (1990) 329–340.
- [40] J.J. S, M. Edith, A Novel Mechanism for Spontaneous Encapsulation of Active Agents: Phase Inversion Nanoencapsulation, in: *ACS Symposium Series CY -*, American Chemical Society, Washington, DC, 2011: pp. 214–223–223.
- [41] S. Furtado, D. Abramson, R. Burrill, G. Olivier, C. Gourd, E. Bubbers, et al., Oral delivery of insulin loaded poly(fumaric-co-sebacic) anhydride microspheres., *International Journal of Pharmaceutics*. 347 (2008) 149–155.
- [42] S. Furtado, D. Abramson, L. Simhkay, D. Wobberkind, E. Mathiowitz, Subcutaneous delivery of insulin loaded poly(fumaric-co-sebacic anhydride) microspheres to type 1 diabetic rats, *European Journal of Pharmaceutics and Biopharmaceutics*. 63 (2006) 229–236.
- [43] A. Andrianov, L. Payne, Polymeric carriers for oral uptake of microparticulates., *Advanced Drug Delivery Reviews*. 34 (1998) 155–170.
- [44] M. Helliwell, The use of bioadhesives in targeted delivery within the gastrointestinal tract, *Advanced Drug Delivery Reviews*. 11 (1993) 221–251.

- [45] N. Hussain, V. Jaitley, A.T. Florence, Recent advances in the understanding of uptake of microparticulates across the gastrointestinal lymphatics., *Advanced Drug Delivery Reviews*. 50 (2001) 107–142.
- [46] T. Ermak, E. Dougherty, H. Bhagat, Z. Kabok, J. Pappo, Uptake and transport of copolymer biodegradable microspheres by rabbit Peyer’s patch M cells, *Cell and Tissue Research*. 279 (1995) 433–436.
- [47] M. Doyle-McCullough, S. Smyth, S. Moyes, K. Ccarr, Factors influencing intestinal microparticle uptake in vivo, *International Journal of Pharmaceutics*. 335 (2007) 79–89.
- [48] N. Foster, M.A. Clark, M. Jepson, *Ulex europaeus* 1 lectin targets microspheres to mouse Peyer’s patch M-cells in vivo, *Vaccine*. (1998).
- [49] C. Damgé, M. Aprahamian, H. Marchais, J.P. Benoit, M. Pinget, Intestinal absorption of PLAGA microspheres in the rat., *J. Anat.* 189 (Pt 3) (1996) 491–501.
- [50] G. Hodges, E. Carr, R. Hazzard, Uptake and Translocation of Microparticles in the Small Intestine, *Digestive Diseases and* (1995).
- [51] F. Delie, Evaluation of nano- and microparticle uptake by the gastrointestinal tract., *Advanced Drug Delivery Reviews*. 34 (1998) 221–233.
- [52] M. Desai, V. Labhasetwar, E. Walter, R. Levy, The mechanism of uptake of biodegradable microparticles in Caco-2 cells is size dependent, *Pharmaceutical* (1997).
- [53] A.T. Florence, Issues in Oral Nanoparticle Drug Carrier Uptake and Targeting, *Journal of Drug Targeting*. 12 (2004) 65–70.
- [54] H. Hillaireau, P. Couvreur, Nanocarriers’ entry into the cell: relevance to drug delivery, *Cell. Mol. Life Sci.* 66 (2009) 2873–2896.
- [55] K. Khanvilkar, M.D. Donovan, D.R. Flanagan, Drug transfer through mucus., *Advanced Drug Delivery Reviews*. 48 (2001) 173–193.
- [56] G. Ponchel, J.M. Irache, Specific and non-specific bioadhesive particulate systems for oral delivery to the gastrointestinal tract, *Advanced Drug Delivery Reviews*. (1998).
- [57] M. Geiser, B. Rothen-Rutishauser, N. Kapp, S. Schürch, W. Kreyling, H. Schulz, et al., Ultrafine particles cross cellular membranes by nonphagocytic mechanisms in lungs and in cultured cells., *Environ. Health Perspect.* 113 (2005) 1555–1560.
- [58] J.E. Heuser, R.G. Anderson, Hypertonic media inhibit receptor-mediated endocytosis by blocking clathrin-coated pit formation., *The Journal of Cell Biology*. 108 (1989) 389–400.

- [59] C.J. Smith, N. Grigorieff, B.M. Pearce, Clathrin coats at 21 Å resolution: a cellular assembly designed to recycle multiple membrane receptors., *Embo J.* 17 (1998) 4943–4953.
- [60] B. Pearce, Clathrin: a unique protein associated with intracellular transfer of membrane by coated vesicles, in: *Proceedings of the National Academy of ...*, 1976.
- [61] T. Kirchhausen, Adaptors for clathrin-mediated traffic, *Annual Review of Cell and Developmental ...* (1999).
- [62] T. Kirchhausen, W. Boll, A. van Oijen, Single-molecule live-cell imaging of clathrin-based endocytosis, *Biochemical ...* (2005).
- [63] M. Ehrlich, W. Boll, A. van Oijen, R. Hariharan, K. Chandran, M.L. Nibert, et al., Endocytosis by Random Initiation and Stabilization of Clathrin-Coated Pits, *Cell.* 118 (2004) 591–605.
- [64] N.A. Peppas, P.A. Buri, Surface, interfacial and molecular aspects of polymer bioadhesion on soft tissues, *Journal of Controlled Release.* 2 (1985) 257–275.
- [65] J. Woodley, Bioadhesion: new possibilities for drug administration?, *Clinical Pharmacokinetics.* 40 (2001) 77–84.
- [66] Y. Huang, W. Leobandung, A. Foss, N.A. Peppas, Molecular aspects of muco- and bioadhesion: tethered structures and site-specific surfaces., *J Control Release.* 65 (2000) 63–71.
- [67] N.A. Peppas, J.J. Sahlin, Hydrogels as mucoadhesive and bioadhesive materials: a review., *Biomaterials.* 17 (1996) 1553–1561.
- [68] C. Santos, Evaluation of anhydride oligomers within polymer microsphere blends and their impact on bioadhesion and drug delivery in vitro, *Biomaterials.* 24 (2003) 3571–3583.
- [69] G.P. Andrews, T.P. Laverty, D.S. Jones, Mucoadhesive polymeric platforms for controlled drug delivery., *Eur J Pharm Biopharm.* 71 (2009) 505–518.
- [70] F. Madsen, K. Eberth, J.D. Smart, A rheological assessment of the nature of interactions between mucoadhesive polymers and a homogenised mucus gel., *Biomaterials.* 19 (1998) 1083–1092.
- [71] J. Vasir, Bioadhesive microspheres as a controlled drug delivery system, *International Journal of Pharmaceutics.* 255 (2003) 13–32.
- [72] M. Davidovich-Pinhas, H. Bianco-Peled, Mucoadhesion: a review of characterization techniques., *Expert Opin Drug Deliv.* 7 (2010) 259–271.

- [73]** D. Chickering, E. Mathiowitz, Bioadhesive microspheres: I. A novel electrobalance-based method to study adhesive interactions between individual microspheres and intestinal mucosa, *Journal of Controlled Release*. 34 (1995) 251–261.
- [74]** C.A. Santos, J.S. Jacob, B.A. Hertzog, B.D. Freedman, D.L. Press, P. Harnpicharnchai, et al., Correlation of two bioadhesion assays: the everted sac technique and the CAHN microbalance., *J Control Release*. 61 (1999) 113–122.

Chapter 2

Intestinal Microsphere Uptake: Quantitative Analysis of Uptake, Mechanisms and Biodistribution of Microspheres as Potential Oral Drug Delivery Systems

2.1 Abstract

The uptake and internalization of polymeric nano- and microparticulates from the intestinal tract is well established. However, the in vivo pathways involved and systemic distributions following uptake are not well understood. We report here the quantitative uptake and organ distribution of polystyrene microspheres from isolated intestinal regions. Uptake was highest from the jejunum for the smallest size tested 500 nm at 45.8 ± 8.6 % of total dose with distribution primarily to the liver. Administration of PS MS along with pathway inhibitors indicates that clathrin-mediated endocytosis may be the primary route of internalization. Further, when this pathway is inhibited the organ distribution shifts away from liver to many other compartments including systemic blood, lung and spleen. This work illustrates the high potential of polymeric nano- and micro-particle systems for oral delivery and indicates

Joshua J. Reineke*, Daniel Y. Cho*, Yu-Ting Liu, A. Peter Morello, Jules Jacob, Chris G. Thanos and Edith Mathiowitz

*both authors contributed equally to this work

potential uptake pathways to target and ways to alter final organ fate of particulate systems for applications in targeted delivery.

2.2 Introduction

The internalization of microspheres, has historically been heavily debated and controversial. Work done in the mid-1990's provided concrete evidence of microsphere internalization following per os (PO) administration but the first indication that microparticles are internalized via intestinal cells was presented as early as 1968 by Volkheimer et al. [1] as a rare phenomenon.

Since micro-fold cells (M-cells) of the follicle-associated epithelium of Peyer's patches are known to phagocytose intraluminal protein antigens [2, 3], viruses [4, 5] and micro-organisms [6, 7], M-cells were naturally the center of initial (and many current) investigations into microparticle uptake. In fact, the majority of studies on the intestinal uptake of microparticles have focused on M cells, including a large emphasis on targeting to M cells [8-17].

However, there have been a handful of studies that have shown internalization by intestinal enterocytes [18]. This route of uptake would be highly advantageous since M-cells only comprise $< 1\%$ of the intestinal surface area. Although a number of studies have provided clear evidence of microparticle uptake by enterocytes in vitro and in vivo [8, 13-17, 19-22], non-Peyer's patch uptake by intestinal enterocytes is currently thought to be a minimal contributor to microparticle uptake in vivo [23, 24] with only a single literature review in the past decade recognizing the importance of the active role enterocytes may play in microparticle uptake [25].

The majority of microparticle uptake studies utilize qualitative or semi-quantitative methods including electron microscopy, fluorescent microscopy, polarized light microscopy, fluorescence associated cell sorting (FACS), fluorometry, gradient centrifugation and radioactive analysis. Quantitative results are necessary to better evaluate and understand the potential of oral microsphere drug delivery systems. The first truly quantitative method was developed by Jani et al. [26] and relies gel permeation chromatography to quantify the uptake of polymeric particles.

There is an absence of information regarding microsphere uptake in specific intestinal regions with the majority of studies focused on M cells of Peyer's patches. Apart from evidence indicating the role of M cells and enterocytes in uptake, little is known about the specific cellular pathway of microsphere internalization. Knowledge of microparticle uptake would be important for the design of more efficient and 'smart' oral drug delivery systems that could exploit specific pathways. There are three prevalent routes proposed in the literature: (1) intracellular uptake by absorptive epithelium (enterocytes) via endocytosis believed to be limited to particles < 50 nm, (2) paracellular transit between tight junctions of absorptive epithelium, although, it is not considered a likely route without the use of permeation enhancers, and (3) phagocytosis by M cells of Peyer's patches, believed to be the most important pathway involved [8, 22, 27-29]. Despite the large number of microparticle uptake studies showing evidence of microparticles in various regions or cell types, very little research has been done to understand the specific cellular mechanisms involved or the relative importance of these mechanisms.

Mechanistic studies can be carried out in vitro by way of cell culture. However, the predominant cell models (primarily Caco-2 cell monolayers) vary greatly in morphology [30], which may change in the presence of particulates [31-33]. Additionally, the relevance of an in vitro cell culture uptake model to the dynamic in vivo environment (gastrointestinal mobility, fluid flow, mucus secretion, cell turnover, etc.) is unknown. A number of cell culture studies investigate uptake trends, but do not look at specific cellular mechanisms.

Moving beyond the discussion of microparticle internalization via intestinal cells one may inquire about the organ fate of the microparticles. Organ fate results are important for accurate interpretation of pharmacokinetic and toxicology studies and may even enlighten the potential for organ targeting. Microparticle fate is dependent upon in vivo microparticle stability, intestinal epithelial tissue interactions, cellular uptake and translocation, and destination stability [34, 35]. There is still a lack of quantitative organ distribution data from oral administration studies of particle uptake.

In order to systematically evaluate particle uptake from the small intestine for oral drug delivery applications, polystyrene microspheres were administered to isolated loops of jejunum and ileum in rats. Particle translocation by enterocytes was visualized using trans-

mission electron microscopy and confocal microscopy. Particle uptake and distribution to target tissues were systematically evaluated using a quantitative gel permeation chromatography method for polymer detection. The effect of particle size and uptake location on total uptake and biodistribution from the jejunum, ileum and oral administration were compared. Additionally, the kinetics of particle uptake and tissue distribution was evaluated at 1, 3 and 5 hours. Utilizing the isolated loop in vivo model and quantitative uptake analysis allowed for the determination of the cellular mechanism(s) of microsphere uptake in the jejunum and ileum through the co-administration of the uptake inhibitors chlorpromazine, phorbol 12-myristate 13-acetate, colchicine, and cytochalasins B and D as well as the determination of the organ distribution profiles of microsphere drug delivery systems resulting from specific uptake mechanisms following intestinal administration.

2.3 Materials and Methods

2.3.1 Materials

Polystyrene (PS) beads were purchased from Polysciences, Inc. (Warrington, PA) in specific bead diameters of 500 nm, 1, 2 and 5 μm and stored at 4°C until use. Suspensions were used as supplied in concentrations of approximately 25 mg/mL and were used as received in all uptake experiments. A dosage volume of 1 mL was used in all studies and resulted in a total dose of 25 mg.

All uptake inhibitors were purchased from Sigma-Aldrich (St. Louis, MO) and stored as indicated until use. Dosing concentrations and dosing periods were selected for each inhibitor based on material safety data sheets and well-described in vitro cell culture protocols from the literature [36-43]. Chlorpromazine (CPZ) was dissolved into phosphate buffer solution at a concentration of 50 $\mu\text{g/mL}$ immediately prior to administration. A 0.1 mM concentration of colchicine in phosphate buffer solution was prepared immediately prior to administration. Cytochalasin B (CytB), cytochalasin D (CytD) and phorbol 12-myristate 13-acetate (PMA) were first dissolved at a high concentration into dimethyl sulfoxide and stored at -80°C until use. Immediately prior to administration, dimethyl sulfoxide and inhibitor solutions were thawed and diluted down to concentrations of 60 μM , 4 μM and 2 μM solutions with

phosphate buffered saline for cytochalasin B, cytochalasin D and phorbol 12-myristate 13-acetate, respectively.

2.3.2 Isolated Loop

For the direct administration of formulations to a specific gastrointestinal region an isolated loop technique was employed. Male, Sprague-Dawley rats, weighing 200 – 250 g were anesthetized with 3 % isoflurane inhalation. A 3 cm midline abdominal incision was made and the desired intestinal section was identified. The jejunum was identified proximally by the ligament of Trietz and distally by increased gut associated lymphoid tissue (GALT). The ileum was identified proximally by increased GALT and distally by the cecum. Once the desired intestinal section was identified, a 6 cm length was isolated by suture ligation with monofilament silk 0-0 sutures at each end taking care not to disrupt blood flow from mesenteric arteries. A suspension of the microspheres of interest was then injected directly into the isolated region. The isolated loop was returned to the abdomen, which was closed to maintain body temperature and moisture. Following a specified incubation period, the following samples were collect in order: 1 mL blood from portal vein, 1 mL blood from celiac artery, lungs, heart, spleen, kidneys, liver, isolated loop, rinse of isolated loop, and brain. All tissues were stored at -18°C until further processing. Studies were ran in animal groups of n=4 for each study.

For the mechanistic studies an initial dose of inhibitor was administered by direct injection into the isolated loop. After a 30-minute period to allow inhibitor action, a suspension of the microspheres of interest was injected directly into the isolated region. At repeated periods of 0.5, 1, 1.5, 2 and 3 hours, chlorpromazine, phorbol 12-myristate 13-acetate, cytochalasin D, cytochalasin B and colchicine, respectively, were administered to maintain drug action.

2.3.3 Oral Gavage

Oral administration of microsphere formulations was performed by way of gastric gavage needle in male Sprague-Dawley rats weighing 200 – 250 g. Rats were anesthetized with 3 % isoflurane inhalation and microsphere suspensions were delivered by way of gastric gavage.

Rats were allowed to recover and had access to standard chow and water ad libidum. During the entire term of the study, all fecal matter and urine was collected for each animal. At the specified time, the animal was anesthetized a second time with 3 % isoflurane inhalation. A midline abdominal incision was made and the following samples were collected in order: 1 mL blood from portal vein, 1 mL blood from celiac artery, lungs, heart, spleen, kidneys, liver, stomach, rinse of stomach, duodenum, rinse of duodenum, jejunum, rinse of jejunum, ileum, rinse of ileum, cecum, rinse of cecum, colon, rinse of colon, and brain. Additionally, all urine was aspirated from the bladder and all feces removed from rectum and added to previously collected urine and fecal matter samples, respectively. All tissues were stored at -18°C until further processing. Studies were ran in animal cohort sizes of n=4 for each study.

2.3.4 Tissue Processing and Polymer Detection

All tissue samples were processed and quantitatively analyzed for polymer content by gel permeation chromatography. Tissue samples were physically cut into small pieces, added to ~5 mL PBS and homogenized on a Cole-Palmer Ultrasonic Homogenizer CV26 with a high gain Q horn and extender set at 40 % amplitude for 30 seconds. Homogenized samples were lyophilized for 48 hours resulting in a powdered tissue digest. An extraction of the marker polymer was then performed by the addition of ~10 mL chloroform with mixing on an end-over-end mixer for 96 hours. Extractions were then filtered through 0.1 μ m PTFE filters to remove non-soluble debris. Filtered extractions were lyophilized for 24 hours and stored at -18°C until analysis.

Lyophilized extractions were reconstituted in 1 mL of chloroform by mixing on an end-over-end mixer for 1 hour. Each solution was filtered a final time through a 0.1 μ m PTFE syringe filter and ran on a Shimadzu GPC equipped with Waters Styragel HR5E and HR4E columns and a Shimadzu RID-10A refractive index detector. A specific peak for the marker polymer was identified and the area under the curve was calculated and related to the polymer concentration by comparison to a linear standard curve for PS with R^2 values of 0.9993. Since the sample volume was known, the exact amount of polymer was calculated from the detected concentrations. Positive controls resulted $99.8 \pm 1.3\%$ recovery and negative

controls resulted in 0% recovery.

The resulting data was computed to yield quantitative information regarding the uptake and tissue distribution by the following calculations. Percent of uptake was calculated by taking the sum of all amounts detected in each tissue (excluding isolated loop and loop rinse samples) divided by the total dose administered and multiplied by 100. Tissue distribution was directly compared for each tissue across study groups. Additionally, the sum of all amounts detected in every tissue sample was divided by the total administered dose to determine mass balance.

2.3.5 Transmission Electron Microscopy

Following a 1-hour isolated loop experiment of 500 nm PS microspheres in the rat jejunum isolated loop sections were then harvested, washed in PBS, fixed in a 20% paraformaldehyde/20% glutaraldehyde in sodium cacodylate buffer for 2 hours at 37°C. Samples were then rinsed with 0.2 M sodium cacodylate buffer and post-fixed in 1% osmium tetroxide in 0.2 M sodium cacodylate for 3 hours at room temperature then stained with 1% uranyl acetate overnight at room temperature. Samples then underwent a series of ethanol dehydrations, embedded in LR White embedding medium and allowed to polymerize for three to five days. Blocks were sectioned at a thickness of 85 nm on a Reichert Ultra microtome with a diamond knife. Sections were placed on copper grids and viewed on a Phillips 410 transmission electron microscope equipped with an Advantage HR CCD camera. Images were acquired with Advanced Microscopy Techniques imaging software.

2.3.6 Confocal Microscopy

Following a 1-hour isolated loop experiment of fluorescent 500 nm PS microspheres in the rat jejunum isolated loop sections were then harvested, washed in PBS, fixed in 75% formaldehyde for 1 hour. Prior to imaging, intestinal sections were stained with FM1-23 fluorescent membrane staining (ex. @ 488 nm and em. @ 600 nm) and DAPI fluorescent nuclei staining (ex @ 358 nm and em. @ 461 nm). The PS microspheres used in this study were fluorescently tagged with PC-red (ex. @ 377 nm and em. @ 479 nm). Tissues were embedded into optimal cutting temperature (OCT) media for sectioning, and cut into 20 μ m sections with a

Leica cryostat in preparation for imaging. Samples were imaged on a Leica TCS SP2 AOBS spectral confocal microscope with UV excitation, at a fluorescence emission of 450 nm, and the resulting images were analyzed by OpenLab software for quantitative comparison of the samples' fluorescence intensity. Multiple optical sections of each sample were taken.

2.3.7 Statistical Analyses

Standard errors were calculated and a 1-way ANOVA was performed with Microcal Origin Graphical Software (Northampton, MA). Significance was determined at $p \leq 0.05$.

2.4 Results and Discussion

2.4.1 Electron Microscopy of Microsphere Uptake in the Jejunum

TEM was used to visualize microspheres being internalized into enterocytes on the cellular level following a 1-hour isolated loop study in the rat jejunum. Initially, smaller 200 nm PS microspheres were used to increase the likelihood of obtaining images of particle uptake. Representative TEM images of 200 nm particles within intestinal enterocytes are shown in figure 2.1A-C. The 200 nm spheres were observed to first enter the cell through the lateral membrane near the apical region (Figure 2.1A, arrow). Many 200 nm spheres were found within vesicles in the cytosol (Figure 2.1B, arrow) and in the Golgi apparatus (figure 2.1C, arrows) indicating an endocytotic pathway followed by packaging for exocytosis through the basal membrane. However, no exocytosis events were seen and may be as a result of the short 1-hour period consistent with the kinetic results showing times of greater than 1 hour needed to traverse to other organs (Figure 2.4).

Imaging of 500 nm PS microspheres after a 1-hour incubation in an isolated loop by TEM indicated that the same pathway shown for 200 nm microspheres is utilized for the larger microspheres. Representative TEM images of 500 nm spheres within intestinal enterocytes are shown in figure 2.1D-E. The 500 nm PS spheres first enter enterocytes through the lateral membrane near the apical cell boundary (Figure 2.1D, arrow). After only 1 hour many 500 nm spheres can be seen in endocytotic vesicles within the cell cytosol (Figure 2.1E, arrows). These results provide evidence for the endocytosis of 500 nm microspheres by enterocytes

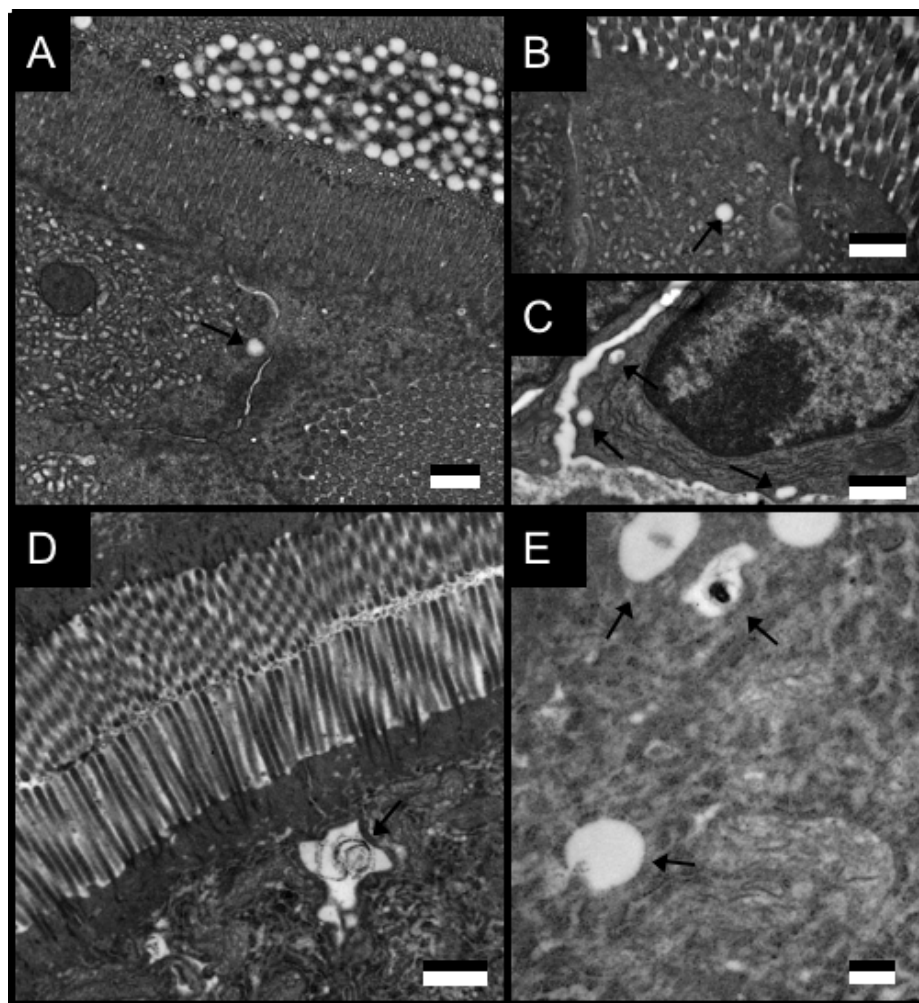


Figure 2.1: TEM images of 200 nm (A-C) and 500 nm (D-E) PS microspheres after 5 minutes of local delivery in the rat jejunum. Arrows indicate the location of microspheres. All scale bars are 500nm.

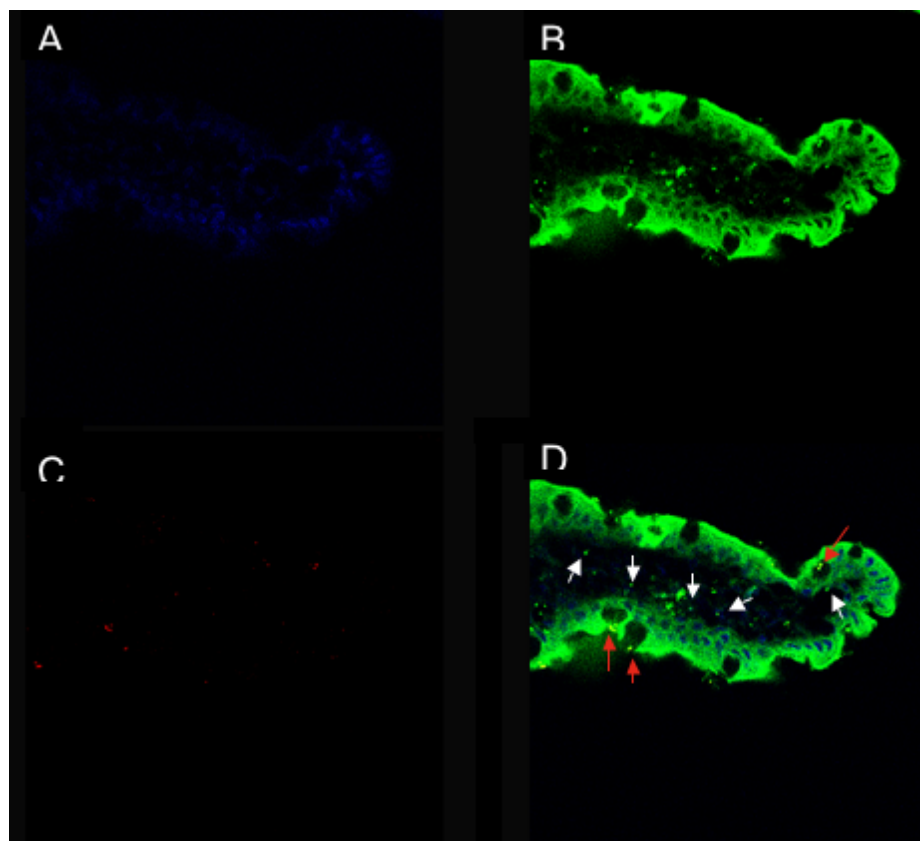


Figure 2.2: LSM images of a single rat jejunum villus after 1 hour local delivery of PC-red fluorescent 500 nm PS microspheres. Three channels were used to display DAPI nuclei staining (A), FM1-23 membrane staining (B) and PC-red microspheres. Also shown is an overlay of all three channels (D). Red arrows indicate microspheres present within the mucosal void of goblet cells and white arrows indicate microspheres that have penetrated beyond the epithelial cell layer.

in the rat jejunum.

2.4.2 Confocal Microscopy of Microsphere Uptake in the Jejunum

Confocal Laser Scanning Microscopy (LSM) following a 1-hour isolated loop experiment of 500 nm PS microspheres in the rat jejunum was used to visualize particle uptake on the tissue level. Figure 2.2 consists of a representative LSM image of a villus in the jejunum with DAPI staining for cell nuclei (A), FM1-23 membrane staining (B), fluorescent PS microspheres (C), and an overlay of panels A, B and C shown in panel D with arrows indicating the location of microspheres. Microspheres can be seen traversing from the intestinal lumen through the enterocytes into the villus core, which contains the vasculature that allows for absorbed

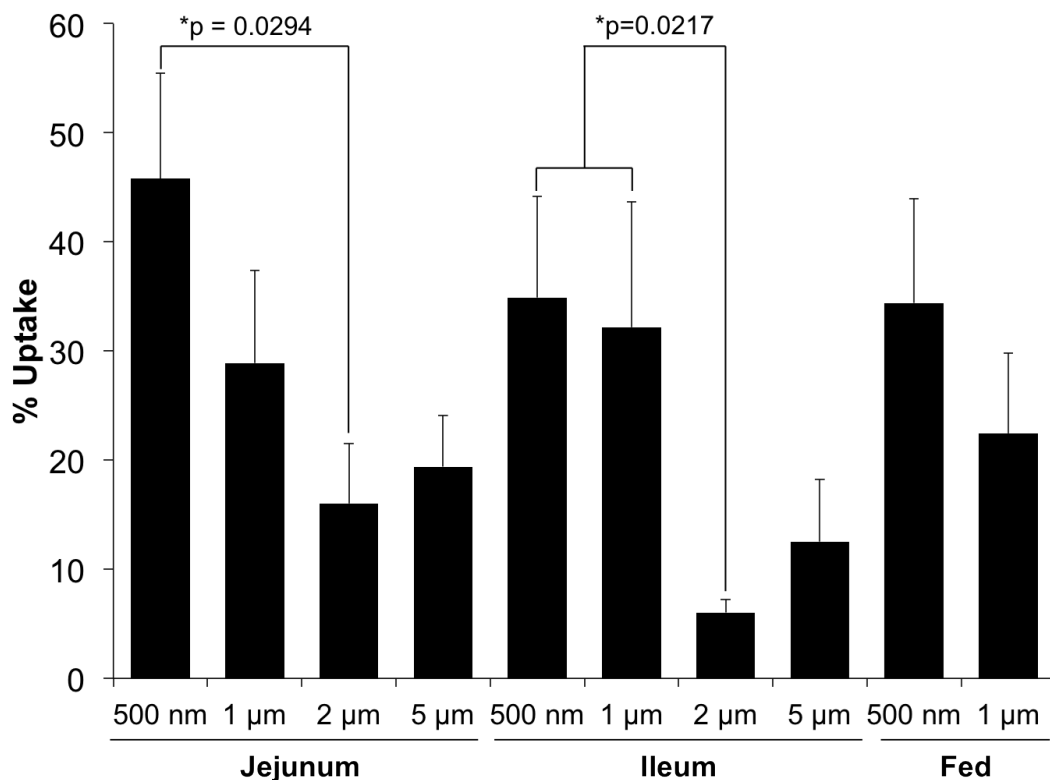


Figure 2.3: Quantitative uptake expressed as a percentage of dose for PS microspheres of 500 nm, 1, 2 and 5 μm in diameter. Uptake is shown after five hours post local delivery to the jejunum or ileum or after being fed orally in a rat model. $n = 4$.

materials to enter the circulation. These images indicated another possible route of particle entry through goblet cells during and/or following mucus secretion. Three-dimensional fluorescence analysis was performed on each microsphere within a z-stack to ensure that the vertical position of the microsphere actually lay within the tissue and was not a sectioning artifact (data not shown).

2.4.3 Effect of Microsphere Size and Intestinal Region on Uptake

To evaluate particle uptake on the organismal level, PS microspheres of 500 nm, 1, 2 and 5 μm were locally administered to 6 cm sections of jejunum or ileum and the amount of uptake five hours after administration was quantified. Additionally, 500 nm and 1 μm PS microspheres were fed by way of oral gavage followed by uptake quantification after five hours. The percentage of intestinal uptake for all sizes after 5 hours of incubation in an isolated loop in either the jejunum or ileum and 5 hours after oral gavage are shown in the

bar graph in figure 2.3. Consistent with results found by others [26,44, 45-48], microsphere uptake increased as the microsphere diameter decreased. This result was independent of the location of delivery (Figure 2.3).

Uptake is conventionally considered to occur primarily by route of microfold cells residing in Peyer’s patches. However, we observed a higher uptake of 500 nm PS microspheres in the Peyer’s patch-free region of the jejunum, $45.8 \pm 8.6 \%$, than was found in the Peyer’s patch-rich region of the ileum, $34.9 \pm 9.3 \%$, indicating an alternative mechanism to microfold cell phagocytosis that is size-dependent (Figure 2.3). Intestinal enterocytes lack the ability of phagocytosis indicating an endocytotic pathway. The endocytosis of particles larger than 1 μm has not been described in the literature and only a single report by Rejman et. al [49] has shown endocytosis of 500 nm particles. Therefore, results of high uptake in non-Peyer’s patch jejunum for 500 nm and 1 μm beads, shown in figure 2.3, motivated the mechanistic studies that will be discussed later on.

Comparing the size dependency of uptake in the jejunum to the size dependency in the ileum we see two different trends emerge. Uptake in the jejunum decreases with each increase in microsphere size in a stepwise fashion. For example, uptake was highest with 500 nm particles ($45.8 \pm 8.6 \%$) and decreased as the microsphere size increased to 1 μm ($28.9 \pm 8.4 \%$), decreasing further to $16.0 \pm 5.5 \%$ for 2 μm microspheres. This is much different than the binary-like behavior of a size cut-off observed in the ileum where 500 nm and 1 μm microspheres have the same amount of uptake (34.9 ± 9.3 and $32.2 \pm 11.5 \%$, respectively) with a dramatic decrease to $6.0 \pm 1.2 \%$ when the microsphere size is increased to 2 μm . In the case of the ileum, the mechanism is most likely the same for 500 nm and 1 μm microspheres with a rate independent of size. The phagocytotic uptake mechanism of the ileum, known to occur by M-cells, most likely accounts for the observed size dependency uptake profile in the ileum. In the Peyer’s patch-free jejunum the phagocytotic pathway is not present and may result in the different size dependency of uptake observed in the jejunum. Since uptake in the jejunum experiences a size dependency, a non-phagocytotic pathway could be taking place. Regions of jejunum free of Peyer’s patches were selected by design, yet it is impossible to assure that no phagocytotic cells are present in the region. However, the importance of these observed differences must be emphasized, as this, in

our view, is the first indication of a non-phagocytotic pathway having a significant role in microsphere uptake.

The results in the jejunum and ileum are maximal due to the fact that a small portion of each region is subjected to a virtually infinite microsphere population. While this method is great in determining the potential of oral microsphere delivery systems, and in understanding regional differences, it may not be directly physiologically relevant. An oral gavage administration was also utilized in studying particle uptake. The oral administration mimics a typical dosing regimen that is more clinically relevant and theoretically results in lower local dose concentrations (non-infinite exposure) as well as possible microsphere uptake by all GI regions simultaneously. The amount of uptake was indeed reduced for both 500 nm and 1 μ m PS microspheres administered orally compared to isolated loop experiments in both the jejunum and ileum (Figure 2.3). However, there were still significant levels of uptake after oral gavage with 34.4 ± 9.5 and 22.5 ± 7.4 % of total uptake for 500 nm and 1 μ m PS microspheres, respectively. This result is encouraging for the prospects of microsphere drug delivery systems for oral administration.

Interestingly the size dependency of uptake following oral administration is similar to what was observed with local delivery in the jejunum (Figure 2.3). For example, 500 nm PS beads had a higher uptake after oral administration, 34.4 ± 9.5 %, than 1 μ m PS microspheres, 22.5 ± 7.4 %. This is similar to the resulting size dependency after local delivery to the jejunum where the uptake was 45.8 ± 8.6 and 28.9 ± 8.4 % for 500 nm and 1 μ m microspheres, respectively, and dissimilar to resulting size dependency after local administration to the ileum where 500 nm and 1 μ m microspheres had almost the same level of uptake (34.9 ± 9.3 and 32.2 ± 11.5 %, respectively). It may be that the mechanism responsible for microsphere uptake in the Peyer's patch-free jejunum is the most relevant mechanism to oral administration, despite being the least studied. However, this conclusion cannot be firmly made as there may be differences in the GI transit of each microsphere population as a result of the size differences. Additionally, this may be an artifact of the rat model whose jejunum accounts for approximately ninety percent of the small intestine.

2.4.4 Organ Distribution Following Uptake

Organ distribution of 500 nm PS microspheres is dependent on the location of delivery as can be seen in figure 2.4A, B and C where the percentage of microspheres detected in each tissue is shown for local delivery to the jejunum, local delivery to the ileum and oral administration. When delivered locally to the jejunum, 36.7 ± 9.9 % of the total dose was present in the liver. However, when the same microspheres were delivered locally to the ileum the organ distribution was spread amongst many organs including the heart (3.7 ± 3.0 %) and spleen (3.7 ± 2.6 %). Regardless, the majority of the microspheres were still found in the liver (26.3 ± 14.2 %). This shift in organ distribution further supports the theory that there may be two different mechanisms occurring in the two intestinal regions. Peyer's patches are often referred to as gut associated lymphoid tissue, or GALT, due to the fact that lymph nodes often occupy the mucosal layer immediately beneath Peyer's patches. Increased distribution to the heart and spleen may be a result of phagocytosis of the microspheres by microfold cells in Peyer's patch regions followed by passage into the lymphatic circulation. Lymph travels through the lymphatic ducts and returns into the systemic circulation through the thoracic duct rather than going directly to the liver as in the venous 'first-pass' pathway. Therefore, when 500 nm PS microspheres are delivered to the ileum, some may potentially bypass the liver via lymphatic circulation. When the same microspheres are delivered to the Peyer's patch-free jejunum, distribution to the spleen and heart diminish due to an absence of GALT. Rather, they enter the hepatic circulation and are directed to the liver.

For 1 μm PS microspheres, the dependence of organ distribution on the location of delivery is even more dramatic as can be seen in figure 2.4A. After local delivery to the jejunum there is nearly an equal percentage found in the liver and lungs with 9.0 ± 3.3 % and 8.5 ± 6.0 % of total dose, respectively. One explanation for this may be that the smaller 500 nm microspheres are able to be absorbed by hepatocytes within the liver and that the larger 1 μm microspheres cannot be absorbed and continue in the circulation until being lodged in the microvasculature of the lung. This reasoning is substantiated by Ogarwara et al [47] who have shown that Kupffer cells of the liver have the ability to adsorb high amounts of 500 nm PS spheres after intravenous injection. PS microspheres 2 and 5 μm

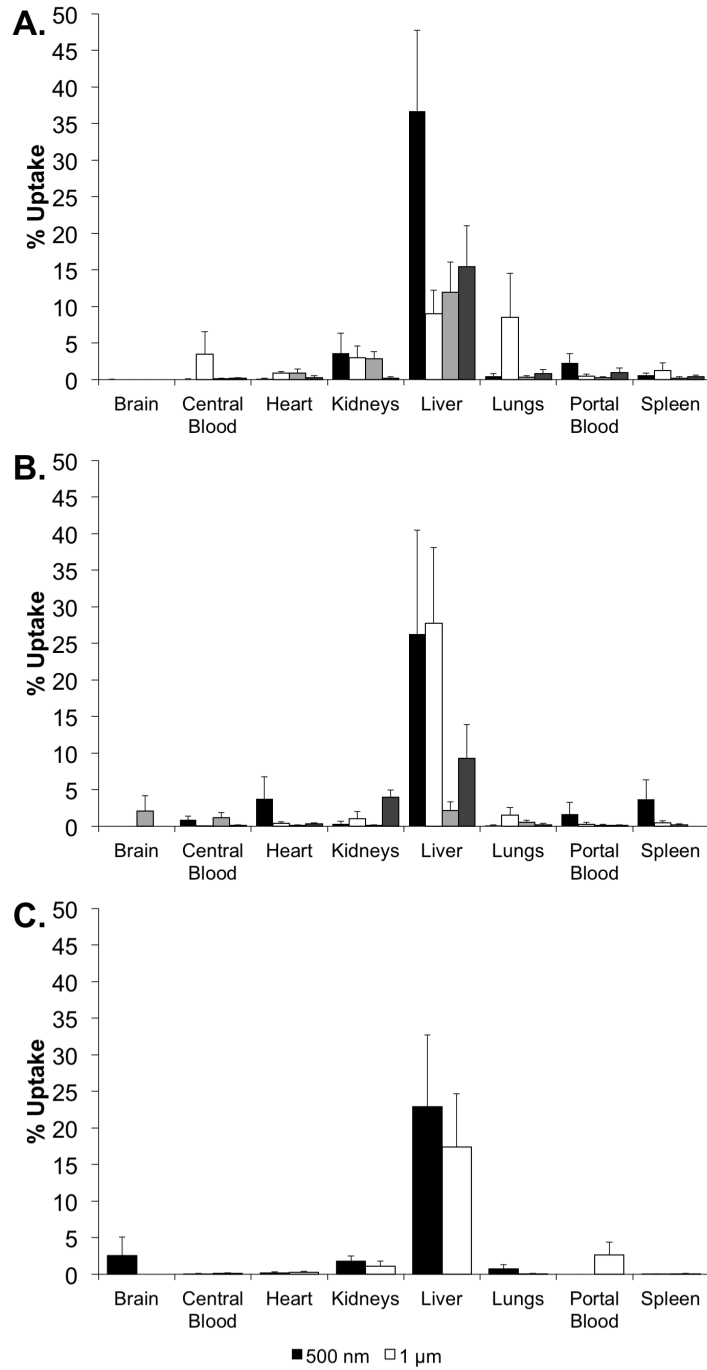


Figure 2.4: Organ distribution of 500 nm, 1, 2 and 5 μ m PS microspheres following 5 hours of local administration to rat jejunum (A) and ileum (B) as well as 500 nm and 1 μ m administered orally (C). n = 4.

in diameter had similar organ distribution as was observed with the 500 nm microspheres. However, due to the low total uptake of these larger sized beads, shown in figure 2.3, these results are less meaningful.

When the same 1 μm microspheres are delivered locally to the ileum the organ profile shifts with the majority of the spheres localizing to the liver, $27.8 \pm 10.4 \%$ (Figure 2.4B). Organ distribution after local administration to the ileum had no evident size dependency with the majority of the microspheres localizing to the liver (Figure 2.4B). This further supports the idea that the same uptake mechanism could be responsible for the absorption of 500 nm and 1 μm PS microspheres in the ileum. Therefore, for microspheres of 500 nm and 1 μm delivered locally to the ileum, neither the uptake (Figure 2.3) or organ distribution (Figure 2.4B) is size dependent.

Organ distribution after oral administration is not size dependent as shown in figure 2.4C where the majority of the particles are found in the liver for both 500 nm and 1 μm microspheres. Oral administration of 500 nm PS microspheres results in similar organ fate as when the same microspheres are delivered to the jejunum. The resulting liver-centric organ distribution may further indicate that the mechanism of uptake utilized within the jejunum could well also be the primary mechanism involved after an oral administration. Oral administration of 1 μm PS microspheres (Figure 2.4C) is similar to local delivery of the same microspheres to the ileum indicating that the mechanism involved in the ileum is dominant when 1 μm microspheres are delivered orally also contrary to what was found with 500 nm microspheres. These results make it evident that the uptake mechanism of microspheres is not only region dependent, but also size dependent in each region.

2.4.5 Kinetics of Microsphere Uptake

A final parameter that was investigated by way of quantitative uptake and organ distribution was time. By ending the isolated loop studies at various times after microsphere administration, information on uptake and organ distribution kinetics can be gained. For these studies two models were chosen. The first model chosen was 500 nm PS microspheres locally administered to the jejunum since this model yielded the highest total amount of uptake in addition to raising many questions on microsphere uptake mechanisms in non-

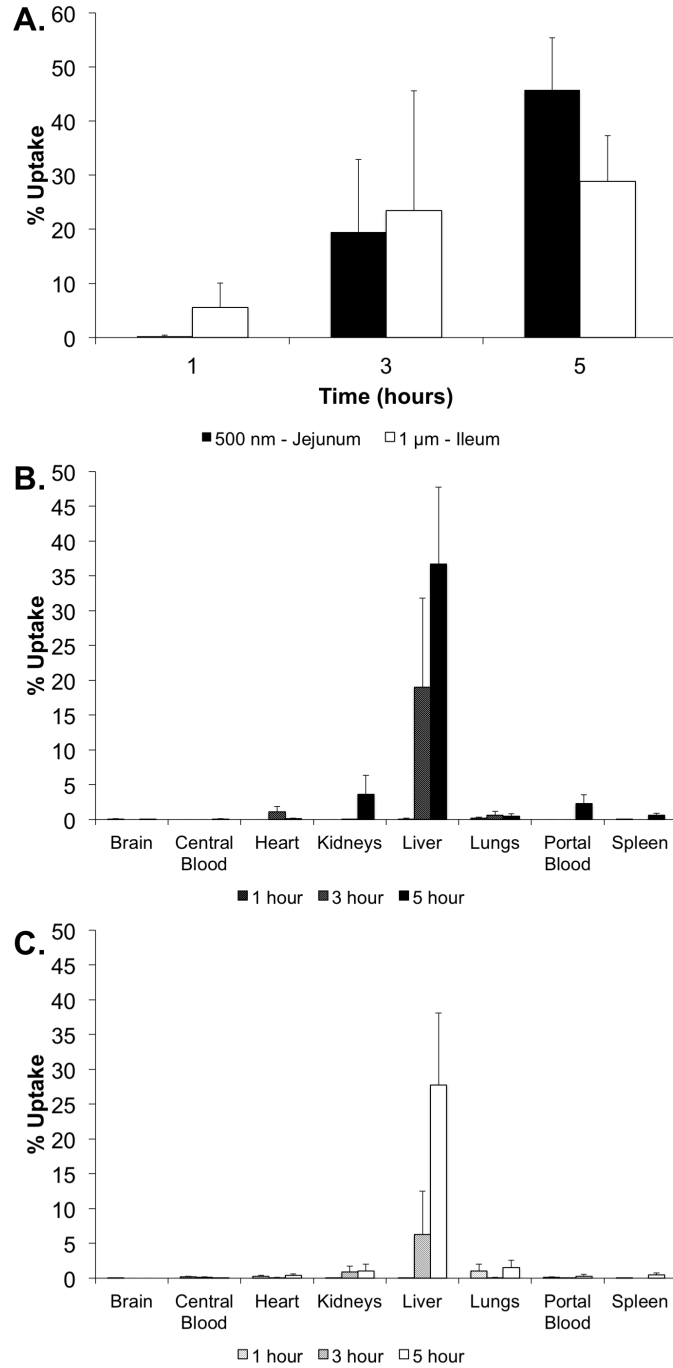


Figure 2.5: Quantitative uptake (A) after local delivery of 500 nm PS microspheres in the jejunum and 1 μ m PS microspheres in the ileum as a function of time in a rat model; organ distribution of 500 nm PS microspheres at 1, 3 and 5 hours after local delivery to the jejunum (B) and 1 μ m PS microspheres in a rat model at 1, 3 and 5 hours after local delivery to the ileum (C). $n = 4$.

Peyer's patch regions. The second model chosen was 1 μm PS microspheres delivered locally to the ileum mostly to compare mechanism kinetics in the ileum versus jejunum. Since there is no significant difference in 500 nm and 1 μm PS microspheres in terms of uptake in the ileum, using 1 μm PS microspheres were thought to both yield comparable results in terms of uptake in addition to yielding information on organ distribution over time as a function of size.

In both the jejunum and ileum, uptake steadily increased over time up to five hours (Figure 2.5A). Interestingly, the manner in which they increased was slightly different in that uptake in the jejunum increased linearly over time and uptake in the ileum seemed to plateau. Additionally, there was virtually no uptake evident in the jejunum at 1 hour (0.26 ± 0.13 %) compared to 5.58 ± 4.48 % of the total dose being absorbed after only 1 hour in the ileum. Again, these differences point to different methods of microsphere uptake occurring in the two intestinal regions. The mechanism involved in the jejunum seems to have a lag time, perhaps for initial interactions or cellular signaling response, followed by a linear increase in uptake indicating a constant absorption rate that does not saturate over the five-hour period. The mechanism(s) involved in the ileum immediately initiates absorption and increases over time with a slowing rate of microsphere absorption indicating saturation at some point in the process.

Early in the uptake kinetics study, size actually favors the smaller 500 nm microspheres as prior experiments (described above) and research by other groups indicate higher uptake with decreased microsphere size. However, despite this 'advantage', the 1 μm microspheres in the ileum have a higher initial uptake than the 500 nm microspheres in the jejunum and, if anything, strengthen the evidence of differing uptake mechanisms.

At later periods in the kinetic study, one may argue that the lower uptake seen in the ileum is as result of the larger 1 μm microsphere size and not of a saturation of the uptake mechanism. However, as is shown in figure 2.3, at the five-hour time point, 500 nm microspheres did not have a statistically different amount of uptake in the ileum than was observed with 1 μm microspheres. Therefore, this potential pitfall of the study actually further substantiates the conclusion that different mechanisms must be occurring in the two intestinal regions.

2.4.6 Organ Distribution Kinetics

Kinetics of organ distribution of 500 nm PS microspheres delivered locally to the jejunum are shown in figure 2.5B. There is virtually no uptake after 1 hour, but by 3 hours the microspheres begin to localize to the liver only. After five hours, the majority of the microspheres are still located in the liver with a small amount present in the kidneys. This points to the fact that at all times, microspheres traverse directly to the liver and after an extended period they begin to move to the kidney as is typical of an elimination pathway. The kinetics of organ distribution of 1 μ m microspheres delivered locally to the ileum is more complicated and can be seen in figure 2.5C. In the first hour, microspheres distribute mostly to the lungs without any presence in the liver. This may indicate phagocytosis in Peyer's patch regions resulting in delivery to the lymphatic system, which bypasses the liver delivering the microspheres to the lungs. This may also explain why no uptake occurs in the first hour after delivery to the Peyer's patch-free jejunum. However, at three hours virtually no microspheres are present in the lungs, but there is a spike in the amount detected in the liver with a small amount also detected in the kidneys. A second mechanism may be implicated to occur in the ileum by these results. Perhaps the phagocytotic pathway saturates, but the same mechanism present in the jejunum is also present in regions of the ileum surrounding the Peyer's patches resulting in the observed liver and kidney elimination pathway discussed earlier. By five hours, microspheres are most evident in the liver but are also present in the kidneys and lungs indicating both mechanisms occurring simultaneously.

2.4.7 Effect of Mechanism-Specific Endocytosis Inhibition on Microsphere Uptake in the Jejunum

Chemical blockers were first used to study the uptake mechanism of 500 nm PS microspheres in the jejunum. Uptake results for 500 nm PS microspheres in the jejunum with co-administration of CPZ, PMA and CytD are compared to the control (no drug) in figure 2.6A. Every endocytosis blocker used (CPZ, PMA and CytD) had dramatic effects on the total uptake indicating a multitude of mechanisms involved.

CPZ had the largest inhibitory effect on the uptake of 500 nm PS microspheres with

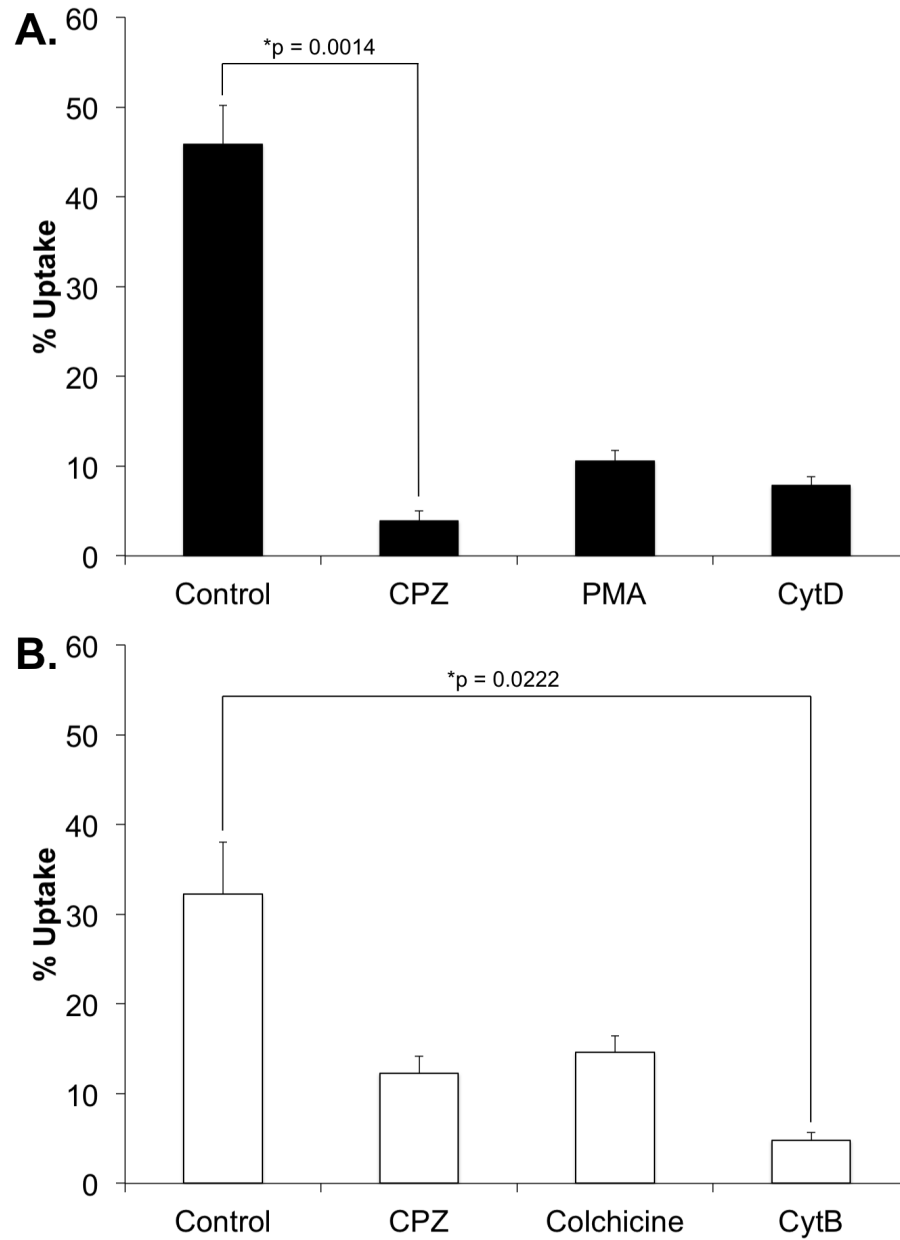


Figure 2.6: (A) Uptake expressed as percent of dose for 500 nm PS microspheres delivered locally to the jejunum without any drug (control), with CPZ, PMA and CytD in a rat model; (B) uptake of 1 μ m PS microspheres after local delivery to the ileum for 5 hours without (control) and with co-administration of CPZ, colchicine and CytB in a rat model. $n = 4$.

3.60 ± 2.08 % uptake of the total dose compared to 45.8 ± 8.6 % uptake when no inhibitor was used. CPZ prevents the recycling of clathrin resulting in a blockade of clathrin-coated vesicles, thus having a high specificity to inhibition of clathrin-mediated endocytosis only. Clathrin coated vesicles are likely a critical pathway of 500 nm PS microspheres into epithelial cells of the rat jejunum. While there is certainty in the role of clathrin being critical to microsphere uptake in the jejunum, the remaining 3.6 ± 2.08 % uptake that occurs may be of the result of a second mechanism or the result of incomplete inhibition of the clathrin pathway. Additionally, the low amount of uptake may be a result of clathrin-mediated endocytosis that occurred prior to the exhaustion of clathrin since CPZ prevents the recycling of clathrin only and not the initial use of clathrin.

Another well known endocytotic pathway involves the formation of caveolae, or cholesterol rich membrane regions, that can easily invaginate, due to a localized increase in membrane flexibility, and bud off from the membrane resulting in endocytosis of extracellular content. PMA specifically binds to and phosphorylates caveolin, a necessary protein in the formation of caveolae, and inhibits caveolae-mediated endocytosis. The inhibition of the caveolar pathway resulted in a decreased uptake of 500 nm PS microspheres in the jejunum to 10.32 ± 2.32 % uptake of the total dose compared to 45.8 ± 8.6 % uptake of the total dose when no inhibition was used. This inhibition obviates the critical role played by caveolae in uptake of 500 nm microspheres. If caveolae-mediated endocytosis were to occur independently of clathrin-mediated endocytosis, we would have expected to see the amount of uptake inhibition by PMA to be equal to or less than the amount of uptake in the presence of CPZ. For example, total inhibition with PMA would be ≤ 3.6 % uptake of the total dose subtracted from 45.8 % uptake of the total dose without any inhibition resulting in uptake of ~ 42.2 % uptake of the total dose. However, PMA has a much larger inhibitory effect on microsphere uptake indicating that both clathrin- and caveolar-mediated endocytosis cooperatively work in the endocytosis of 500 nm PS microspheres. CPZ is observed to have a higher amount of inhibition than PMA indicating that some clathrin-mediated endocytosis may occur without the cooperation of caveolae, ~ 6.72 % of total dose, or that there is a partial inhibition of caveolar-mediated endocytosis. These results leave open the possibility that a minor, secondary uptake mechanism may occur.

Inhibition of endocytosis independent of a specific mechanism was performed by the administration of CytD. CytD inhibits the polymerization of actin filaments and, in the case of enterocytes, changes the brush border morphology inhibiting all endocytotic pathways occurring at the apical cell membrane. CytD effectively inhibited uptake of 500 nm PS microspheres in the jejunum resulting in only 7.50 ± 2.00 % uptake of the total dose adsorbed in contrast to 45.8 ± 8.6 % uptake without any inhibition. This is further verification of the results found with the inhibition of clathrin- and caveolar-mediated endocytosis pathways and points to the possibility that most (perhaps all) of the endocytosis occurs at the apical membrane. This cannot be definitively stated as changes in brush border morphology may further block access to lateral membranes inhibiting endocytosis at locations other than those on the apical cell membrane. Furthermore, CytD effects the mucus secretion and may have an impact on particulate uptake. Since uptake was not entirely inhibited a minor secondary method may be involved or there was only a partial inhibition.

Since all three blockers effectively inhibited the majority of uptake, analysis of biodistribution revealed only small amounts of the microspheres are detected in each of the tissues. However, the pattern of organ distribution changes when there is inhibition with presence of microspheres in nearly all tissues as opposed to primarily the liver when there is no inhibition. In fact, in all four of the animals administered CPZ to inhibit clathrin, the liver contained no trace of the spheres. This further substantiates the possibility of a minor secondary mechanism of microsphere uptake in the jejunum. The secondary mechanism appears to enable a bypass of the liver resulting in a more systemic distribution. This is consistent with the results discussed in the kinetics of uptake of 500 nm microspheres in the jejunum in the previous section.

2.4.8 Effect of Mechanism-Specific Phagocytosis and Endocytosis Inhibition on Microsphere Uptake in the Ileum

Evaluating the mechanisms of uptake in the ileum is somewhat more complex due to the presence of various cell types; most notably the enterocytes and microfold cells of Peyer's patches. However, it is also the location of choice for the majority of uptake studies due to well-known phagocytotic internalization of large particles such as microspheres. Quantitative

uptake results of 1 μm PS microspheres in the ileum in the presence of CPZ, colchicine and CytB are shown in figure 2.6B and compared to the control study group (no blocker). The complex nature of microsphere uptake in this region is evident from in vivo quantitative uptake results in the presence of the specific uptake blockers. Information gained from the use of each blocker will be discussed in detail below.

As discussed above, CPZ specifically inhibits clathrin-mediated endocytosis. A decrease in uptake of 1 μm PS microspheres in the jejunum to 12.11 ± 3.57 % uptake of the total dose in the presence of CPZ in comparison to 32.2 ± 11.5 % uptake without any blockers indicates a strong role for clathrin-mediated endocytosis in the ileum. However, the overall percent reduction in uptake observed in the ileum is less than what is seen in the jejunum revealing that it does not play as critical of a role in the ileum as in the jejunum.

Colchicine acts on microtubules preventing the trafficking of endocytotic vesicles, thus inhibiting all endocytosis with little inhibition of phagocytotic pathways. When 1 μm microspheres are administered locally to the rat ileum in the presence of colchicine, uptake is reduced to 14.44 ± 3.57 % uptake of the total dose from the 32.2 ± 11.5 % uptake when no inhibitor is present. Colchicine and CPZ results in similar uptake inhibitions with 14.44 ± 3.57 % and 12.11 ± 3.57 % uptake of the total dose absorbed. A similar trend was seen in the jejunum when comparing inhibition with CPZ and PMA (Figure 2.6B), indicating that a similar cooperative mechanism of clathrin- and caveolar-mediated endocytosis may occur in the ileum as well. If the small difference in inhibition between CPZ and colchicine were to be considered, it would be indicative of a clathrin-mediated pathway independent of caveolae, similar, again, to what was observed in the jejunum.

Inhibition of both phagocytosis and endocytosis, with the use of CytB, further decreases the uptake of 1 μm microspheres delivered locally to the ileum to 4.52 ± 1.61 % uptake of the total dose compared to 32.2 ± 11.5 % or 14.44 ± 3.57 % uptake of the total dose in the absence of inhibitors or after inhibition of endocytosis only, respectively. CytB inhibits both phagocytosis and endocytosis by preventing the polymerization of actin filaments needed for vesicle trafficking, in the case of endocytosis, and for membrane extension, in the case of phagocytosis. When these two pathways are inhibited, nearly all uptake is inhibited. The small amount of uptake remaining may be indicative of a minor tertiary pathway, such as

transcellular uptake, or may be an artifact of partial inhibition.

Comparing inhibition of endocytosis by colchicine to inhibition of both endocytosis and phagocytosis by CytB reveals the relative importance of phagocytosis in the ileum. Uptake of 14.44 ± 3.57 % and 4.52 ± 1.61 % uptake of the total dose by colchicine and CytB, respectively, differ by ~ 9.9 % uptake of the dose. This value would be the predicted amount of uptake if phagocytosis were inhibited individually. However, there is no known inhibitor of phagocytosis that does not also inhibit endocytosis and this is the only possible indication of the importance of phagocytosis. It is important to note that there may be partial inhibition by colchicine (skewing results favoring the contribution of phagocytosis) and/or CytB (skewing results unfavorably to the contribution of phagocytosis). Therefore, caution should be taken when interpreting data regarding the role of phagocytosis.

Following inhibition by CPZ or colchicine, the organ distribution of 1 μm microspheres distributes to all organs as may be expected. Endocytosis is inhibited by both CPZ and colchicine resulting in uptake of microspheres primarily by phagocytosis, most likely in Peyer's patch regions adjacent to lymphatic tissue, effectively shunting the transit away from the liver where microspheres are observed the most when no inhibitors are present. CytB also exhibits expected behavior in that the amount in all organs is reduced as both phagocytosis and endocytosis are inhibited. If inhibition of only phagocytosis were possible, it would be expected that microspheres would be shunted to the liver, appearing very similar to the organ distribution profile observed without the presence of inhibitors.

2.5 Conclusions

In summary, quantitative uptake, biodistribution and uptake kinetics of microspheres are dependent on microsphere size and intestinal location. Corresponding to quantitative microsphere uptake results found by others [26, 36, 37-40], there was an inverse relationship between microsphere uptake and microsphere size in all regions. A second milestone reached in this work that has not been shown in the literature was the potential for high levels of uptake in intestinal regions absent of Peyer's patches. This high amount of uptake illustrates a great potential for oral microsphere drug delivery systems not only because of the

amount, but also because it is the first time that significant uptake has been shown to occur in Peyer's patch-free regions of the small intestine. This work indicates that a mechanism other than phagocytosis by M-cells may potentially be responsible for significant uptake of microsphere drug delivery systems.

Currently, no research in the area of microsphere drug delivery systems has been published that systematically investigated the mechanisms of intestinal uptake *in vivo*. Utilizing an *in vivo* isolated loop technique for mechanistic studies has the advantage of isolating a specific intestinal region and the advantage to deliver specific chemical uptake inhibitors locally in a controlled manner. Our data suggests that the primary mechanism of microsphere uptake in the rat small intestine is via clathrin-mediated endocytosis in synergy with caveolar-mediated endocytosis. Phagocytosis in the ileum was also a significant pathway, but not the primary pathway as had been previously thought.

Although the data from these studies revolutionize current dogma in the area of oral microsphere delivery systems, these are the first quantitative and systematic *in vivo* uptake mechanism studies performed and have not yet yielded a full understanding of the mechanisms involved. However, the primary role of endocytosis by intestinal enterocytes in the uptake of microspheres lends new life to the potential of oral microsphere delivery systems. Previous studies have focused on optimizing microsphere uptake by M-cell phagocytosis and have suffered the criticism of being unable to deliver high enough doses as M-cells comprise of $< 1\%$ of the intestinal surface area. By taking advantage of endocytotic pathways, efficacious delivery by oral microsphere systems may be achieved. In addition, it appears that the mechanism of uptake influences the final organ fate of the microspheres opening the potential of organ directed therapies by exploiting specific uptake pathways.

2.6 Supplemental Material

2.6.1 Supplemental Material & Methods

2.6.1.1 Cytotoxicity of Uptake Inhibitors

To assess the cytotoxicity of uptake inhibitors a blue-LIVE/DEAD fixable dead cell stain kit from Molecular Probes, Inc. (Eugene, OR) was used. For each inhibitor used (CPZ, PMA, CytB, CytD and colchicine) an isolated loop with 5.5 hours of inhibitor incubation was performed as described above without the administration of microspheres. Additionally, a solution of phosphate buffer saline and a 70 % solution of ethanol were used for negative and positive controls, respectively. Following the 5.5-hour incubation period 5 μ L of the blue-fluorescent reactive dye solution was mixed with 195 μ L of phosphate buffer solution, injected into the isolated loop and incubated for an additional 30 minutes. The intestinal section was then harvested and washed with fresh phosphate buffer solution and placed in 37 % paraformaldehyde for 1 hour. Fixed and stained intestinal samples were then embedded in optimal cutting temperature media and sectioned into 50 μ m sections with a Leica cryostat for imaging.

Samples were imaged on a Leica TCS SP2 AOBS spectral confocal microscope with ultraviolet excitation. Emission was read at 450 nm at multiple optical sections for each sample. OpenLab software was used to quantitatively compare the relative intensity of staining in all samples.

2.6.2 Supplemental Results & Discussion

2.6.2.1 Cytotoxicity of Uptake Inhibition

As was mentioned in the introduction, no in vivo studies have been published to systematically investigate uptake mechanisms and pathways for the purpose of studying polymeric translocation. Using chemical blockers allows inhibition of specific pathways and when used in conjunction with quantitative uptake studies, allows investigation of specific pathways. Although the blockers have been chosen for their specificity, each inhibitor has the potential for cytotoxicity. To ensure that any observed changes in uptake are a result of pathway

inhibition, a study was performed to evaluate the cytotoxicity in the in vivo environment. A LIVE/DEAD fluorescent staining assay for in vivo cell viability was utilized for this purpose. The assay described in the methods section was successful in yielding quantitative results regarding in vivo cell viability. Results are shown in figure 2.7 with fluorescent images of a representative sample for each study group shown in blue, corresponding intensity maps shown in red and a bar graph of average intensity for each image shown in the bottom right.

A negative control of fresh intestinal tissue had a low level of fluorescent intensity (Figure 2.7A). This low level detection is expected as intestinal epithelium has a high turn-over (<24 hours) and a basal amount of cell death would be expected. As a positive control, 70% ethanol was administered to an isolated loop. High intensities of samples exposed to ethanol, indicating high cytotoxicity (Figure 2.7C), demonstrated the success of the fluorescent staining assay. Additionally, the isolated loop section with ethanol appeared shrunken and dark purple to black in color. This observed change was seen only in tissue exposed to ethanol, while all other isolated loops included in the study had no observed change in appearance. Although the isolated loop technique, and many variations of it, has been implemented for many years, no previous study has investigated cell viability with the technique. In an isolated loop administered phosphate buffer solution, a moderate amount of intensity was evident (Figure 2.7B) indicating that there is an increased amount of cytotoxicity with the isolated loop procedure itself. However, results from the previous chapter illustrated that this level of cytotoxicity does not interfere with the uptake process. Moreover, this can be used as a baseline for the cytotoxicity of the drugs since this is what was experienced in all previous isolated loop studies.

Performing the fluorescent cytotoxicity assay on isolated loops administered chemical blockers demonstrated a low level of toxicity that was comparable to, and never exceeded, the amount of cytotoxicity seen with the isolated loop control (Figure 2.7). Therefore, quantitative uptake results gained by the use of these chemical blockers can be considered effects of blocking the specific pathways and not as the result of cell toxicity.

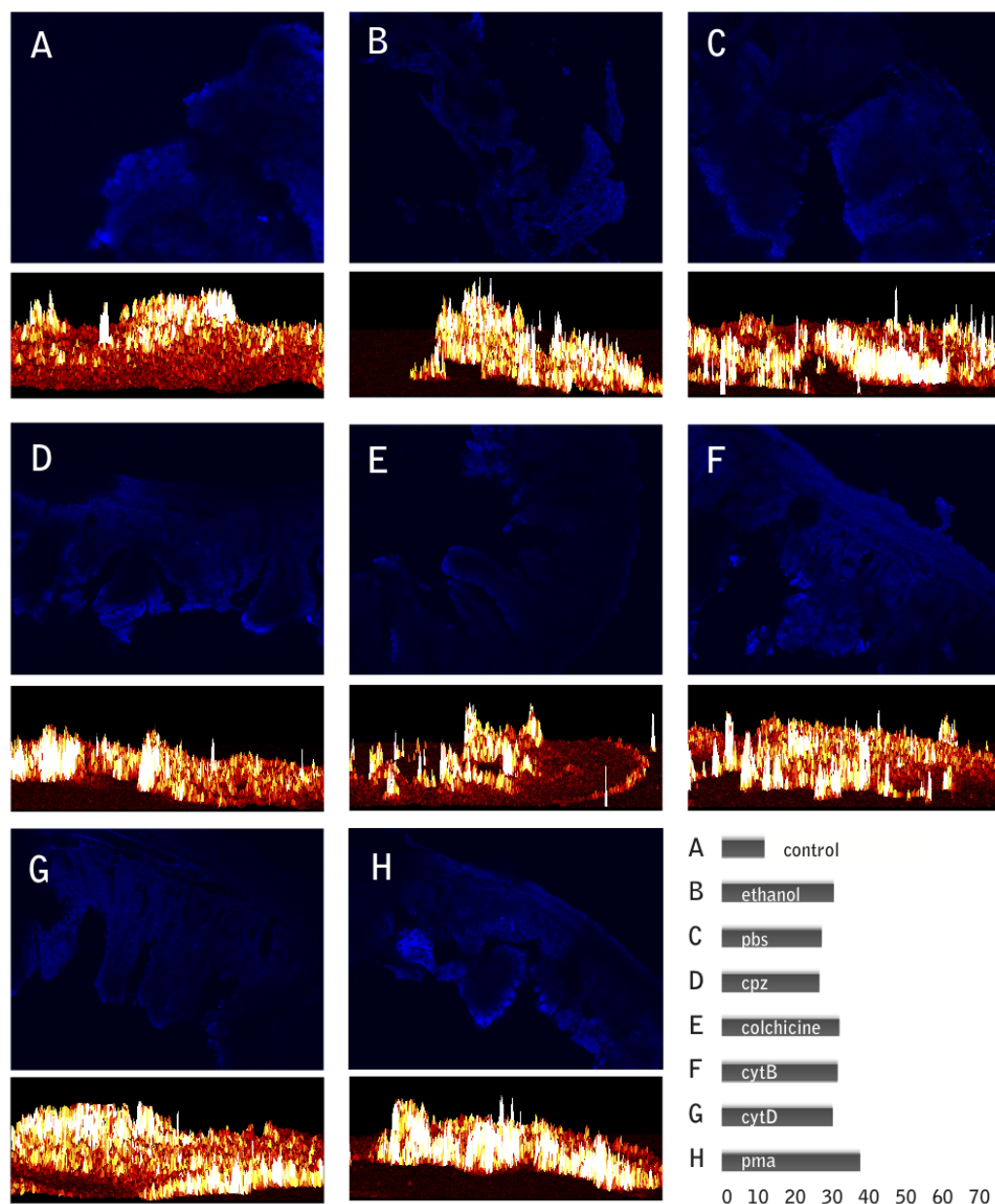


Figure 2.7: Uptake inhibitors were evaluated for cytotoxicity to ensure that previous results for uptake quantitation were not as a result of cytotoxicity. A LIVE/DEAD fluorescent stain was used on isolated loops for each drug, with a 5.5-hour incubations. Intensity maps are shown in red and the bar graph in the bottom right of the figure plots the average relative intensities (20 images analyzed for each group, max intensity 255); (A) control, (B) PBS, (C) 70% ethanol, (D) chlorpromazine, (E) colchicine, (F) cytochalasin B, (G) cytochalasin D and (H) PMA.

2.7 References

- [1]. Volkheimer, G. and F. Schulz, The phenomenon of persorption. *Digestion*, 1968. 1: p. 213-218.
- [2]. Owen, R.L., Sequential uptake of horseradish peroxidase by lymphoid follicle epithelium of Peyer's patches in the normal unobstructed mouse intestine: an ultrastructural study. *Gastroenterology*, 1977. 72(3): p. 440-51.
- [3]. Bockman, D. and M. Cooper, Pinocytosis by epithelium associated with lymphoid follicles in the bursa of Fabricius, appendix and Peyer's patches. An electron microscopic study. *American Journal of Anatomy*, 1973. 136: p. 455.
- [4]. Amerongen, H.M., et al., Transepithelial transport of HIV-1 by intestinal M cells: a mechanism for transmission of AIDS. *J Acquir Immune Defic Syndr*, 1991. 4(8): p. 760-5.
- [5]. Lapenta, C., et al., Human intestinal lamina propria lymphocytes are naturally permissive to HIV-1 infection. *Eur J Immunol*, 1999. 29(4): p. 1202-8.
- [6]. Wolf, J.L., et al., Intestinal M cells: a pathway for entry of reovirus into the host. *Science*, 1981. 212(4493): p. 471-2.
- [7]. Owen, R.L., et al., M cell transport of *Vibrio cholerae* from the intestinal lumen into Peyer's patches: a mechanism for antigen sampling and for microbial transepithelial migration. *J Infect Dis*, 1986. 153(6): p. 1108-18.
- [8]. Ponchel, G. and J.-M. Irache, Specific and non-specific bioadhesive particulate systems for oral delivery to the gastrointestinal tract. *Advanced Drug Delivery Reviews*, 1998. 34(2-3): p. 191-219.
- [9]. Norris, D., N. Puri, and P. Sinko, The effect of physical barriers and properties on the oral absorption of particulates. *Advanced Drug Delivery Reviews*, 1998. 34(2-3): p. 135-154.
- [10]. Ermak, T. and P. Giannasca, Microparticle targeting to M cells. *Advanced Drug Delivery Reviews*, 1998. 34(2-3): p. 261-283.
- [11]. Lavelle, E., et al., The importance of gastrointestinal uptake of particles in the design of oral delivery systems. *Advanced Drug Delivery Reviews*, 1995. 18(1): p. 5-22.
- [12]. Yeh, P.-Y., H. Ellens, and P. Smith, Physiological considerations in the design of particulate dosage forms for oral vaccine delivery. *Advanced Drug Delivery Reviews*, 1998. 34(2-3): p. 123-133.
- [13]. Thomas, N., et al., Particle uptake and translocation across epithelial membranes. *Journal of Anatomy*, 1996. 189(3): p. 487-490.
- [14]. Kreuter, J., Nanoparticles and microparticles for drug and vaccine delivery. *Journal of Anatomy*, 1996. 189(3): p. 503-505.

- [15]. Andrianov, A. and L. Payne, Polymeric carriers for oral uptake of microparticulates. *Advanced Drug Delivery Reviews*, 1998. 34(2-3): p. 155-170.
- [16]. Chen, H. and R. Langer, Oral particulate delivery: status and future trends. *Advanced Drug Delivery Reviews*, 1998. 34(2-3): p. 339-350.
- [17]. Hussain, N., V. Jaitley, and A. Florence, Recent advances in the understanding of uptake of microparticulates across the gastrointestinal lymphatics. *Advanced Drug Delivery Reviews*, 2001. 50(1-2): p. 107-142.
- [18]. Florence, A., et al., Factors affecting the oral uptake and translocation of polystyrene nanoparticles: histological and analytical evidence. *Journal of Drug Targeting*, 1995. 3(1): p. 65-70.
- [19]. Ermak, T., et al., Uptake and transport of copolymer biodegradable microspheres by rabbit Peyer's patch M cells. *Cell and Tissue Research*, 1995. 279(2): p. 433-436.
- [20]. Seifert, J., B. Haraszti, and W. Sass, The influence of age and particle number on absorption of polystyrene particles from the rat gut. *Journal of Anatomy*, 1996. 189(3): p. 483-486.
- [21]. Hoshi, S., et al., Uptake of orally administered polystyrene latex and poly(D,L-lactic/glycolic acid) microspheres into intestinal lymphoid tissues in chickens. *Veterinary Immunology and Immunopathology*, 1999. 70(1-2): p. 33-42.
- [22]. O'Hagan, D., Intestinal translocation of particulates - implications for drug and antigen delivery. *Advanced Drug Delivery Reviews*, 1990. 5: p. 265-285.
- [23]. Damge, C., et al., Intestinal absorption of PLGA microspheres in the rat. *Journal of Anatomy*, 1996. 189(3): p. 491-501.
- [24]. O'Hagan, D., The intestinal uptake of particles and the implications for drug and antigen delivery. *Journal of Anatomy*, 1996. 189(3): p. 477-482.
- [25]. Delie, F., Evaluation of nano- and microparticle uptake by the gastrointestinal tract. *Advanced Drug Delivery Reviews*, 1998. 34(2-3): p. 221-233.
- [26]. Jani, P., et al., Nanoparticle uptake by the rat gastrointestinal mucosa: quantitation and particle size dependency. *Journal of Pharmacy and Pharmacology*, 1990. 42: p. 821-826.
- [27]. Kreuter, J., Peroral administration of nanoparticles. *Advanced Drug Delivery Reviews*, 1991. 7: p. 71-86.
- [28]. Chabot, S., et al., TLRs regulate the gatekeeping functions of the intestinal follicle-associated epithelium. *Journal of Immunology*, 2006. 176(7): p. 4275-4283.
- [29]. Pappo, J. and T. Ermak, Uptake and translocation of fluorescent latex particles by rabbit Peyer's patch follicle epithelium: a quantitative model for M cell uptake. *Clinical and Experimental Immunology*, 1989. 76(1): p. 144-148.
- [30]. Herold, G., et al., Morphology of CaCo-2 cells varies in different cell batches. *In Vitro Cellular & Developmental Biology*, 1994. 30(5): p. 289-291.

- [31]. Bjork, E., et al., Starch microspheres enhance insulin absorption across epithelial cells by affecting the integrity of tight junctions. *Pharmaceutical Research*, 1992. 9: p. S215.
- [32]. Kriwet, B. and T. Kissel, Poly(acrylic acid) microparticles widen the intercellular spaces of Caco-2 cell monolayer: an examination by confocal laser scanning microscopy. *Eur J Pharm Biopharm*, 1996. 42: p. 233-240.
- [33]. Edman, P., E. Bjork, and L. Ryden, Microspheres as nasal delivery system for peptide drugs. *Journal of Controlled Release*, 1992. 21: p. 165-172.
- [34]. Florence, A. and P. Jani, Particulate delivery: the challenge of the oral route, in *Pharmaceutical Particulate Carriers*, A. Rolland, Editor. 1993, Marcel Dekker: New York. p. 65-107.
- [35]. Couvreur, P. and F. Puisieux, Nano- and micro-particles for the delivery of polypeptides and proteins. *Advanced Drug Delivery Reviews*, 1993. 10: p. 141-162.
- [36] Guasch, R.M., C. Guerri, and J.E. O'Connor, Flow cytometric analysis of concanavalin A binding to isolated Golgi fractions from rat liver. *Exp Cell Res*, 1993. 207(1): p. 136-41.
- [37] Guasch, R.M., C. Guerri, and J.E. O'Connor, Study of surface carbohydrates on isolated Golgi subfractions by fluorescent-lectin binding and flow cytometry. *Cytometry*, 1995. 19(2): p. 112-8.
- [38] Kirchhausen, T., et al., Clathrin light chains LCA and LCB are similar, polymorphic, and share repeated heptad motifs. *Science*, 1987. 236(4799): p. 320-4.
- [39] DeTulleo, L. and T. Kirchhausen, The clathrin endocytic pathway in viral infection. *Embo J*, 1998. 17(16): p. 4585-93.
- [40] Fukuyama, K., et al., Apoptosis induced by ischemia-reperfusion and fasting in gastric mucosa compared to small intestinal mucosa in rats. *Dig Dis Sci*, 2001. 46(3): p. 545-9.
- [41] Crittenden, M.R., et al., Intratumoral immunotherapy: using the tumour against itself. *Immunology*, 2005. 114(1): p. 11-22.
- [42] Addison, C.L., et al., Intratumoral coinjection of adenoviral vectors expressing IL-2 and IL-12 results in enhanced frequency of regression of injected and untreated distal tumors. *Gene Therapy*, 1998. 5: p. 1400-1409.

- [43] Gambotto, A., et al., Cancer Gene Therapy, 1999. 6(1): p. 45-53.

- [44]. Jani, P., D. McCarthy, and A. Florence, Nanosphere and microsphere uptake via Peyer's patches: observation of the rate of uptake in the rat after a single oral dose. International Journal of Pharmaceutics, 1992. 86(2-3): p. 239-246.

- [45]. Zauner, W., N. Farrow, and A. Haines, In vitro uptake of polystyrene microspheres: effect of particle size, cell line and cell density. Journal of Controlled Release, 2001. 71(1): p. 39-51.

- [46]. Griese, M. and D. Reinhardt, Smaller sized particles are preferentially taken up by alveolar type II pneumocytes. Journal of Drug Targeting, 1998. 5: p. 471-479.

- [47]. Ogawara, K.-i., et al., Hepatic uptake of polystyrene microspheres in rats: Effect of particle size on intrahepatic distribution. Journal of Controlled Release, 1998. 59(1): p. 15-22.

- [48]. Moghimi, S., et al., Non-phagocytic uptake of intravenously injected microspheres in rat spleen: Influence of particle size and hydrophilic coating. Biochemical and Biophysical Research Communications, 1991. 177(2): p. 861-866.

- [49]. Rejman, J., et al., Size-dependent internalization of particles via the pathways of clathrin- and caveolae-mediated endocytosis. Biochemical Journal, 2004. 377: p. 159-169.

Chapter 3

Time-Dependent Mucoadhesion of Conjugated Bioadhesive Polymers

3.1 Abstract

The time-dependent bioadhesive performance of a series of polymers prepared by the conjugation of L-phenylalanine, L-tyrosine and L-DOPA to a low molecular weight poly(butadiene-maleic anhydride) and a high molecular weight poly(ethylene-maleic anhydride) were evaluated with freshly excised rat small intestinal tissue using a texture analyzer apparatus. Characterizing the bioadhesive forces of these polymers as a function of time relative to polycarbophil, a slightly cross-linked poly(acrylic acid)-derivative, revealed different fracture strengths as well as tensile work values for the two polymer backbones and each of three amino acid side chains. While the poly(acrylic acid) showed a rapid and significant loss of bioadhesion over the testing period, the newly developed synthetic polymers were able to maintain their bioadhesive performance over 91 minutes with the overall magnitude of bioadhesion depending on the hydrogen bonding potential of the side chains. These results shed light on the potential of these polymers for use in the development of more effective bioadhesive drug delivery systems.

Kenneth Estrellas^{*}, Daniel Y. Cho^{*}, Alexis Mancini, Bryan Laulicht and Edith Mathiowitz
^{*}both authors contributed equally to this work

3.2 Introduction

Bioadhesion is of particular interest in the development of drug delivery systems due to the fact that intimate contact between a delivery vehicle and the absorptive epithelium can result in enhanced effectiveness and efficiency of the therapeutic cargo [1]. Bioadhesion refers to the adhesion between a biological material and an artificial substrate while mucoadhesion is a specific type of bioadhesion to mucus, which is a common target for drug delivery [1-3]. Mucoadhesion can occur through a variety of different mechanisms but the two most commonly studied include mechanical and/or chemical interactions [4]; for most polymers, a combination of these two mechanisms are responsible for bioadhesive phenomena [2]. In the case of many bioadhesive hydrogels, the physical interpenetration and entanglement of polymer chains with mucin strands can result in the generation of bioadhesive interactions [5,6]. In contrast, chemical interactions can be the result of strong primary bonds, such as covalent bonds, and weaker secondary forces (i.e. ionic/electrostatic interactions, Van der Waals interactions and hydrogen bonds) [7]. The presence of sialic acid, sulfates, and other groups associated with mucin strands lend the protein polyelectrolytic characteristics at physiologic pH [8], allowing for these types of interactions. Covalent bonding can occur, but these bonds may become permanent, inhibiting uptake of the carrier, which may be desired for certain applications [9]. Hydrogen bonding is more likely to be the driving force for the mucoadhesion of polymers with hydrophilic functional groups [10], such as hydroxyls, thiols, and carboxylic acids. These interactions tend to be weaker and transient, but more prominent; in practice, hydrophilic polymers which possess a large number of potential hydrogen bonding sites have shown high levels of bioadhesion due to their greater hydrogen bonding activity [11].

A variety of in vitro methods have been used to quantify the bioadhesive performance of polymers [12,13]. Although no widely-used procedure has been established as standard [10], a number of direct testing methods have been used to obtain measurements of bioadhesive performance through determination of factors such as the force required to break a polymer-tissue bond [12]. Although drug delivery vehicles are more likely to experience a combination of tensile (axial) and shear (tangential) forces in vivo [14], most in vitro assays measure only

one of these parameters. Materials testing equipment such as texture analyzers are often used to measure and/or administer the force required to adhere and detach a polymer sample from a substrate [15]. The choice of substrate has a marked effect on test results; although synthetic alternatives are more readily available, fresh mucosa from an animal source remains the most accurate option [12]. The maximum detachment force measured during these tests is often used to calculate the fracture strength of the bond, while the total work of adhesion is determined by integration of the load vs. deformation curve yielded by tensile testing [12].

DOPA has been identified as a major component of mollusk adhesive mucus in byssal fibers, which are able to generate strong adhesive forces in nature due to the ability of DOPA to complex with metal ions in aqueous environments; thus, there has been much interest in the use of DOPA in the development of bioadhesives [16-20]. Previous work by our group has demonstrated the extremely bioadhesive behavior of polyanhydrides that results from the generation of carboxylic acid groups upon hydrolysis [7,21]. By combining the polyanhydride backbone chemistry with the DOPA functionality, our group has developed novel bioadhesive polymers in which two maleic anhydride co-polymer backbones, a low molecular weight poly(butadiene-maleic anhydride) (PBMA) and a high molecular weight poly(ethylene-maleic anhydride) (PEMA), were conjugated to catechol-precursor amino acids with increasing numbers of hydroxyl group substitutions from the unsubstituted phenylalanine to tyrosine to L-DOPA (Figure 3.1). The conjugation of the amino acid to the backbone required the hydrolytic ring opening of the maleic anhydride to form maleic acid followed by the subsequent conjugation of the amino acid through an amide bond to one of the liberated carboxyl groups. The grafting of these catechol-precursor amino acids introduces another free carboxylic acid in addition to an aromatic functionality for the phenylalanine yielding the addition of both hydrophobic and hydrophilic moieties; the tyrosine and DOPA conjugates further include single and double hydroxyl substitutions on the aromatic ring to further enhance hydrophilicity and hydrogen bonding potential. This reaction generates a significant number of free carboxyl and hydroxyl groups on the polymer from both the backbone and the amino acid conjugates, all of which can serve as hydrogen bonding sites with mucin to generate bioadhesive behavior.

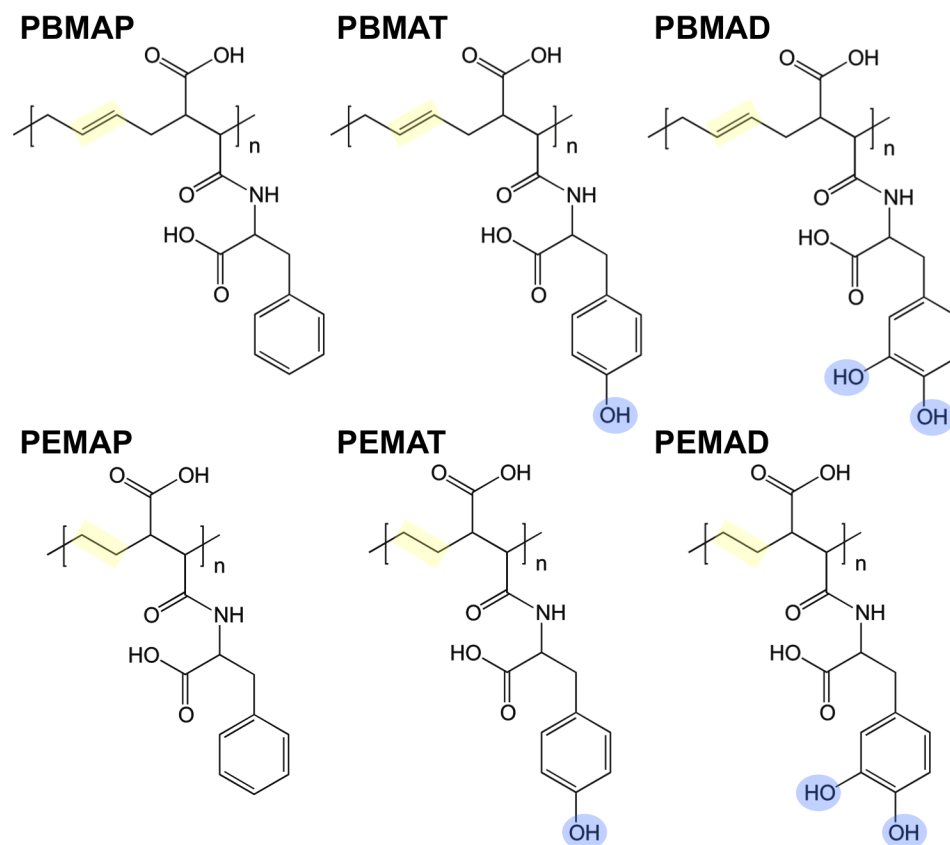


Figure 3.1: Structures of PBMA- and PEMA-derivative polymers; the butadiene (B) and ethylene (E) bonds are highlighted in yellow while the hydroxyl substitutions on the phenylalanine (P), tyrosine (T) and DOPA (D) moieties are highlighted in blue.

These polymers allow for the systematic evaluation of bioadhesive properties as a function of molecular weight, hydroxyl content on the side chains and duration of interaction with the mucosal tissue. In previous studies, we have found that there is an approximately 15 minute delay in the development of the adhesive forces of these polymers upon contact with small intestinal tissue; the purpose of this work is to further evaluate this time-dependent behavior with various contact times using a texture analyzer method and evaluate the fracture strength and tensile work of each of the bioadhesive polymers.

3.3 Materials and Methods

3.3.1 Polymer Synthesis

Each conjugated bioadhesive was synthesized and characterized according to previously established methods [22]. The two backbone polymers, poly(butadiene-maleic anhydride) (PBMA) (MW=10,000 - 15,000 Da) and poly(ethylene-maleic anhydride) (PEMA) (MW=400,000 Da) were purchased from Polysciences, Inc (Warrington, PA). All of the amino acids used in the conjugation process, L-phenylalanine (MW=165.19 g/mol), L-tyrosine (MW=181.19 g/mol), and 3,4-dihydroxy-L-phenylalanine (L-DOPA) (MW=197.19 g/mol) were purchased from Sigma Chemical Company (St. Louis, MO). Dimethyl sulfoxide was purchased from Mallinckrodt Baker, Inc. (Paris, KY). Snakeskin® pleated dialysis tubing from Thermo Scientific (Rockford, IL) was used to purify the polymer.

Briefly, the backbone polymer and sufficient amino acid to react with all available reaction sites were dissolved in DMSO and heated to 70°C and stirred for 24 hours to allow for the conjugation reaction to occur. The resulting polymer solution was poured into dialysis tubing and dialyzed for 96 hours, flash frozen in liquid nitrogen and lyophilized for 72 hours to yield purified conjugated polymer in powder form.

3.3.2 Dissolution Testing

Phosphate buffered saline (PBS) was reconstituted using tablets from MP Biomedicals, LLC (Solon, OH); the pH of the saline was adjusted using HCl to prepare PBS solutions with pH values of 1.0, 3.0, 5.0 and 7.4. 20 mg of each polymer was added to 2 mL of each pH buffered

solution and placed on a shaker table to evaluate polymer dissolution over 24 hours.

3.3.3 Probe Preparation

Glass-headed pins with mean head diameter ~ 2.8 mm were obtained from Dritz Quilting (Spartanburg, SC). Glass-headed pins were dip-coated into various 5% w/v solutions of each bioadhesive polymer in acetone in order to achieve a spherical, uniform coating. Poly(acrylic acid) probes were prepared by dip-coating glass-headed pins into a 1% w/v solution of Noveon AA-1 polycarbophil (Lubrizol Corporation, Wickliffe, OH) in deionized water. 8-10 pins were prepared for each test. The pins were dipped eight times, and mounted between coatings onto an inverted Styrofoam block to allow the solution to dry uniformly around the pinhead. Electronic calipers were used to measure the diameter of each pin prior to tensile testing to ensure uniformity among samples.

3.3.4 Tissue Preparation

In order to more closely mimic in vivo conditions, segments of rat intestinal tissue were harvested and used as a substrate during evaluation of the novel bioadhesives. Male Sprague-Dawley rats (Charles River Laboratories, Wilmington MA) with approximate mean weight of 300 grams were sacrificed by asphyxiation in a carbon dioxide chamber. After death was confirmed, the jejunum was removed, placed into PBS, then cut into 3 cm segments. Each segment was placed into a 20 mL scintillation vial filled with ~ 20 mL PBS (pH 7.4), and stored on ice prior to testing, a maximum of five hours.

When ready for use, each segment was flushed with 1 mL PBS in order to remove the intestinal contents, cut along the anti-mesenteric border, and mounted into a custom fabricated tissue holder with the luminal surface exposed, allowing for contact between the tissue mucosa and the polymer-coated probe. ~ 2.0 mL PBS was added to the tissue holder in order to fully submerge the tissue and probe to keep the tissue hydrated and prevent interference from the surface tension at the air/liquid interface. Temperature was maintained at 37°C with a temperature-controlled circulating water bath connected to the tissue holder.

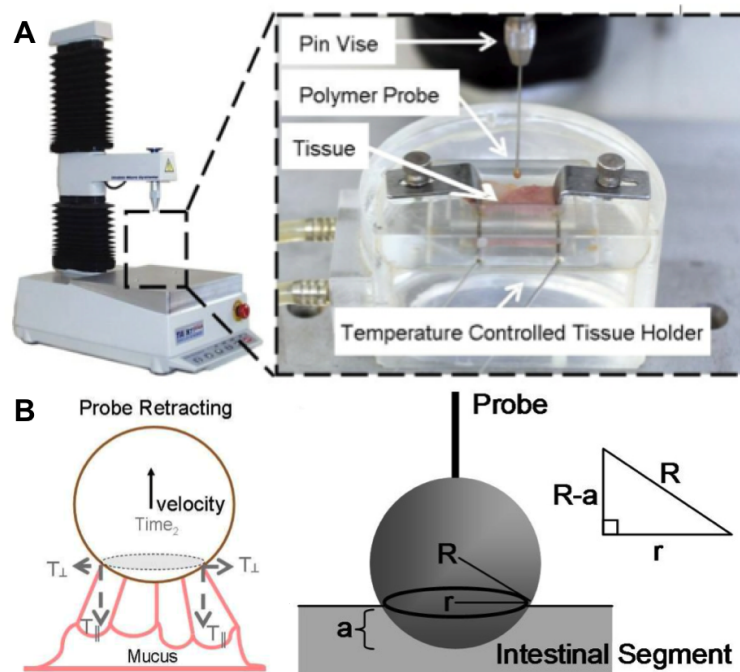


Figure 3.2: (A) Schematic of Texture Analyzer bioadhesion testing apparatus; (B) schematic of polymer-tissue tensile interface and calculation of cross sectional area based on compressive deformation.

3.3.5 Bioadhesion Testing

A TA.XTPPlus Texture Analyzer (Texture Technologies, Scarsdale NY) was used during the tensile tests (Figure 3.2A). Prior to each test, a pin vise was used to mount each polymer probe onto the measuring arm of the texture analyzer. The arm was positioned ~ 3.0 cm above the PBS/tissue interface before each test began, as shown in figure 3.2. The pin was then lowered at a constant rate of 0.5 mm/s until a compressive force of 5 grams (0.0490 N) was reached. The penetration depth of each pin into the tissue was measured during this point. Contact between the polymer-coated probe and the tissue was maintained at six different durations starting at 7 minutes and proceeding at 7 minute intervals up to 42 minutes. Longer contact times of 70 and 91 minutes were also tested. Additionally, two sets of blank pins were measured and tested at full time course as a control.

After the contact time elapsed for each test, the probe was raised away from the interface at a constant rate of 0.5 mm/s, returning to the initial position and breaking the bond in the process. Tensile forces were measured during detachment to produce a load vs. deformation

curve, which was used with the measured diameter and penetration depth of each probe to determine fracture strength and tensile work at each time point. Both the probe and tissue were replaced between tests. Finally, in order to study the dissolution patterns of the polymer probes over the full time course, one set of PBMA and PEMA pins were imaged immediately after each test with a Leica Wild M420 light microscope (Leica Microsystems GmbH, Wetzlar, Germany) and a Nikon CoolPix 990 digital camera (Nikon Corporation, Tokyo, Japan) set to zero zoom.

3.3.6 Data Analysis

Measurements obtained from the Texture Analyzer were used to produce a load vs. deformation curve, which was used to calculate a number of bioadhesion parameters for each of the polymers. To assess bioadhesive performance, fracture strength and tensile work were calculated for each test and averaged (n=4). Tensile work was calculated as the area under the portion of the load-deformation curve while fracture strength is defined by the following equation:

$$Fracture\ Strength = \frac{Peak\ Tensile\ Load}{Cross - Sectional\ Area\ of\ Pin}$$

Since only vertical forces are measured by the texture analyzer, the horizontal cross-section of each pin was used to calculate the area of the interface between polymer and tissue (Figure 3.2B), allowing for the determination of the fracture strength of the bond during each test. Since pin diameters were measured for each test, the radius of the pin was used in conjunction with the value of compressive deformation given by the Texture Analyzer to determine the projected surface area of the sphere at the calculated penetration depth:

$$A = \pi R^2 - \pi(R - a)^2$$

where R is the radius of the probe and a is the measured penetration depth (Figure 3.2B). This parameter will be designated fracture strength – deformation. Notably, despite

pH	PBMAP	PBMAT	PBMAD	PEMAP	PEMAT	PEMAD
1.0	×	×	×	×	×	×
3.0	×	×	×	×	×	×
5.0	×	×	×	×	×	×
7.4	○	○	○	○	○	○

Table 3.1: Solubility of pBMA- and pEMA-derivative polymers in phosphate buffered saline at various pH values over the course of 24 hours; ×: insoluble, ○: soluble.

the presence of multiple peaks, only the final peak value prior to the point of failure was used to calculate the fracture strength of the bond. The presence of multiple peaks, with some registering force values at hundreds of mN, was not taken into account when determining fracture strength. Both fracture strength-deformation and tensile work data are presented.

3.4 Results

The various polymers evaluated in our work are synthesized from two backbones, a low molecular weight poly(butadiene-maleic anhydride) (PBMA) and a high molecular weight poly(ethylene-maleic anhydride) (PEMA), with three side chain chemistries, L-phenylalanine (P), L-tyrosine (T) and L-DOPA (D). The structures of each polymer are shown in figure 3.1.

3.4.1 Polymer Dissolution

To determine the properties of the polymers, we first evaluated their solubility at different pH values. The results of the polymer dissolution testing are presented in table 3.1. All of the PBMA- and PEMA-derivative polymers exhibit unique pH-sensitive solubility characteristics. Each of the polymers were readily soluble at pH 7.4, with all samples completely dissolving within an hour; the PBMA-polymers dissolved more rapidly than the PEMA-derivatives, likely as a result of the differences in molecular weights between the two backbones (10 – 15 kDA and 400 kDA, respectively). All polymers were insoluble in the more acidic solutions (pH 1.0, 3.0 and 5.0). The high concentration of carboxylic acid residues on the polymers may serve as an explanation for their pH-sensitive dissolution profiles. In acidic environments, the carboxylic acid residues remain in their unionized form

due to the high local concentration of protons rendering the polymer insoluble; however, in basic environments, the decrease in local proton concentration results in the ionization of the carboxylic acid residues which causes electrostatic repulsion of the side chains and the dissolution of the polymers.

3.4.2 Probe Images

Prior to tensile testing, each glass-headed pin was dip coated eight times to achieve a uniform coating of the bioadhesive polymer (Figure 3.3). Images of the probes at the end of each run allow for the gross evaluation of polymer coating resulting from exposure to a simulated intestinal fluid (pH = 7.4, 37°C) and the intestinal mucosa.

The lower molecular weight PBMA-derivatives (10 – 15 kDa) exhibited higher degrees of dissolution than the high molecular weight PEMA-derivatives (400 kDa) at each time point, regardless of the amino acid conjugation. Most notably, comparison of the probes reveals a more rapid rate of polymer dissolution for the PBMA relative to the PEMA. This is probably the effect of lower molecular weight and increased hydrophilicity of the PBMA, suggesting that the higher molecular weight polymers can withstand longer hydration times and maintain polymer integrity despite similar side chain chemistries (Figure 3.3).

It was evident from the side chain structure that the more hydrophilic nature of the L-DOPA functional group results in the fastest rate of polymer dissolution from the probes, followed by the tyrosine and phenylalanine-conjugated polymers for both the PBMA and PEMA backbones (data not shown). Phenylalanine is the most hydrophobic of the amino acid conjugates with no hydroxyl substitutions and thus was found to dissolve at the slowest rate; in contrast, progression through the catechol biosynthetic pathway results in the hydroxylation of phenylalanine resulting in one hydroxyl substitution on tyrosine and two on DOPA (Figure 3.1) associated with increasing hydrophilicities, more rapid dissolution in basic aqueous environments as well as the different bioadhesive behaviors described below.

3.4.3 Control Testing

Testing of blank glass pins (n=2) at each time point yielded fracture strength and tensile work values of zero; the deformation to failure curve was nonexistent, and no visible adhesion

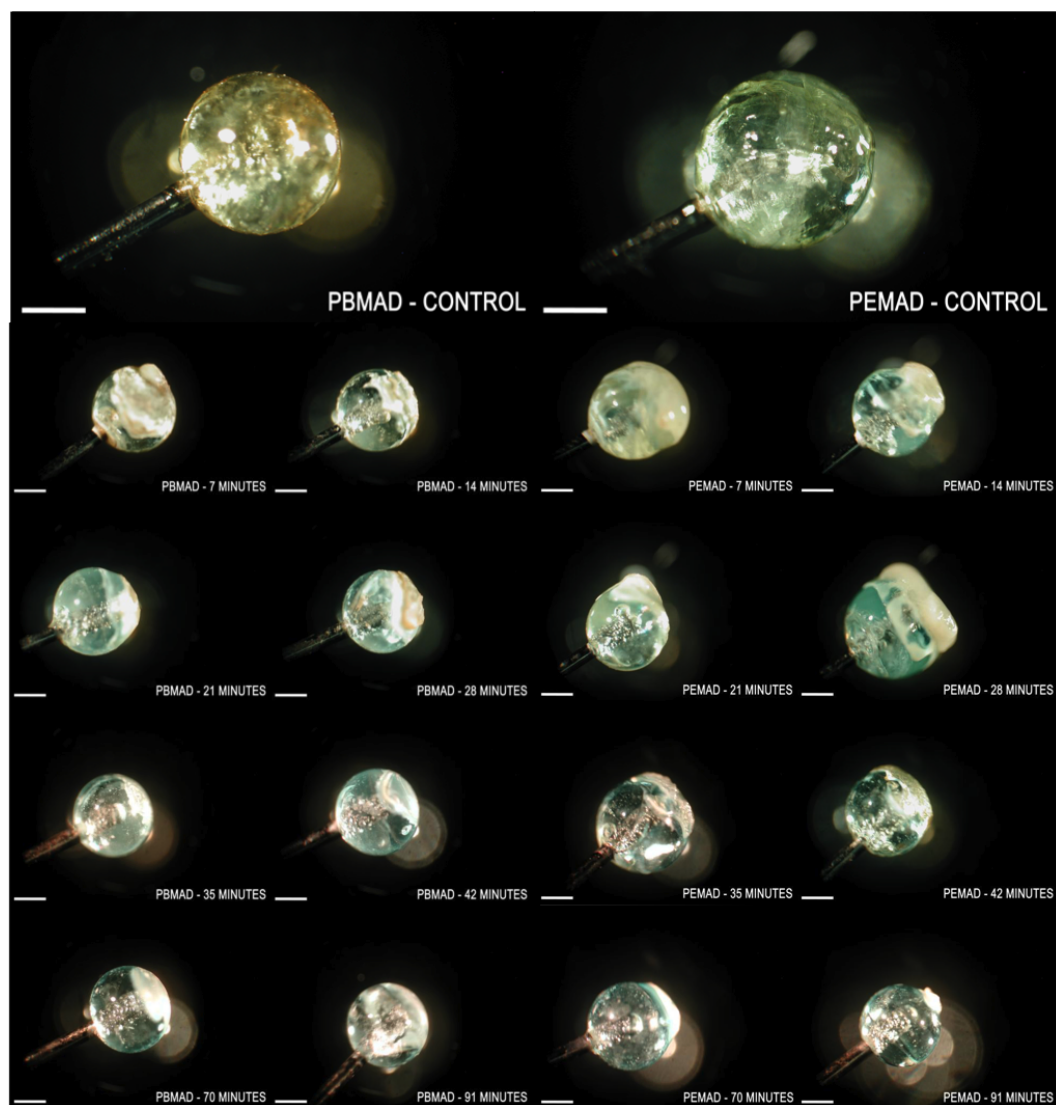


Figure 3.3: Photographs of PBMA and PEMAD probes following bioadhesion testing. All images were taken within 60 seconds of the completion of each test; 20x magnification, scale bar = 1 mm.

was seen during detachment of each pin from the tissue, confirming all data from tensile testing is the result of the bioadhesive polymer coating and not the glass pin head itself.

The fracture strength values observed from bioadhesion testing for all the polymers tested throughout the 91 minute time points are presented in figure 3.4A while the tensile work values are displayed in figure 3.4B. To allow for a closer analysis of this data, the fracture strength and tensile work of each of the polymers organized by backbone and side-chain at each time point are plotted in figures 3.5 and 3.6, respectively.

3.4.4 Bioadhesion Testing of Polyacrylic Acid (PAA)

Polycarbophil, a calcium-salt of poly(acrylic acid) cross-linked with divinyl glycol commercially used as a bioadhesive in pharmaceutical applications, was initially evaluated due to the structural similarities between it and the new bioadhesive polymers. Notably, polyacrylic acid consists of a carbon backbone with a high degree of carboxylic acid side chain substitutions which can confer strong bioadhesive properties due to hydrogen bonding; polycarbophil is a slightly cross-linked form of the polymer that may have a reduced amount of carboxylic acid moieties, but is still sufficient to display strong adhesive forces.

The fracture strength results (Figures 3.4A and 3.5) show the rapid generation of high bioadhesive forces over the initial 14 minutes with the small intestinal tissue to reach a peak value of 491.46 ± 250.66 mN/cm², followed by a steady loss in bioadhesion over the 91 minutes. In contrast, the tensile work data (Figures 3.4B and 3.6) shows a peak at the earliest time point (7 minutes) of 40612.00 ± 19349.98 nJ followed by a gradual drop by 42 minutes with minimal adhesion at the 70 and 91 minute time points (5601.5 ± 4453.82 nJ and 292.75 ± 124.91 nJ, respectively).

This data indicate that the PAA shows a rapid onset of bioadhesion but is unable to maintain high levels of bioadhesion over the time frame of our experiments. This behavior is likely due to the fact that PAA is a very lightly cross-linked hydrogel, which exhibits rapid swelling behavior upon exposure to aqueous environments. This allows for some physical interaction of the polymer chains as well as hydrogen bonding of the carboxylic acid side chains with intestinal mucin, but the rapid hydration of the polymer results in the loss of these interactions.

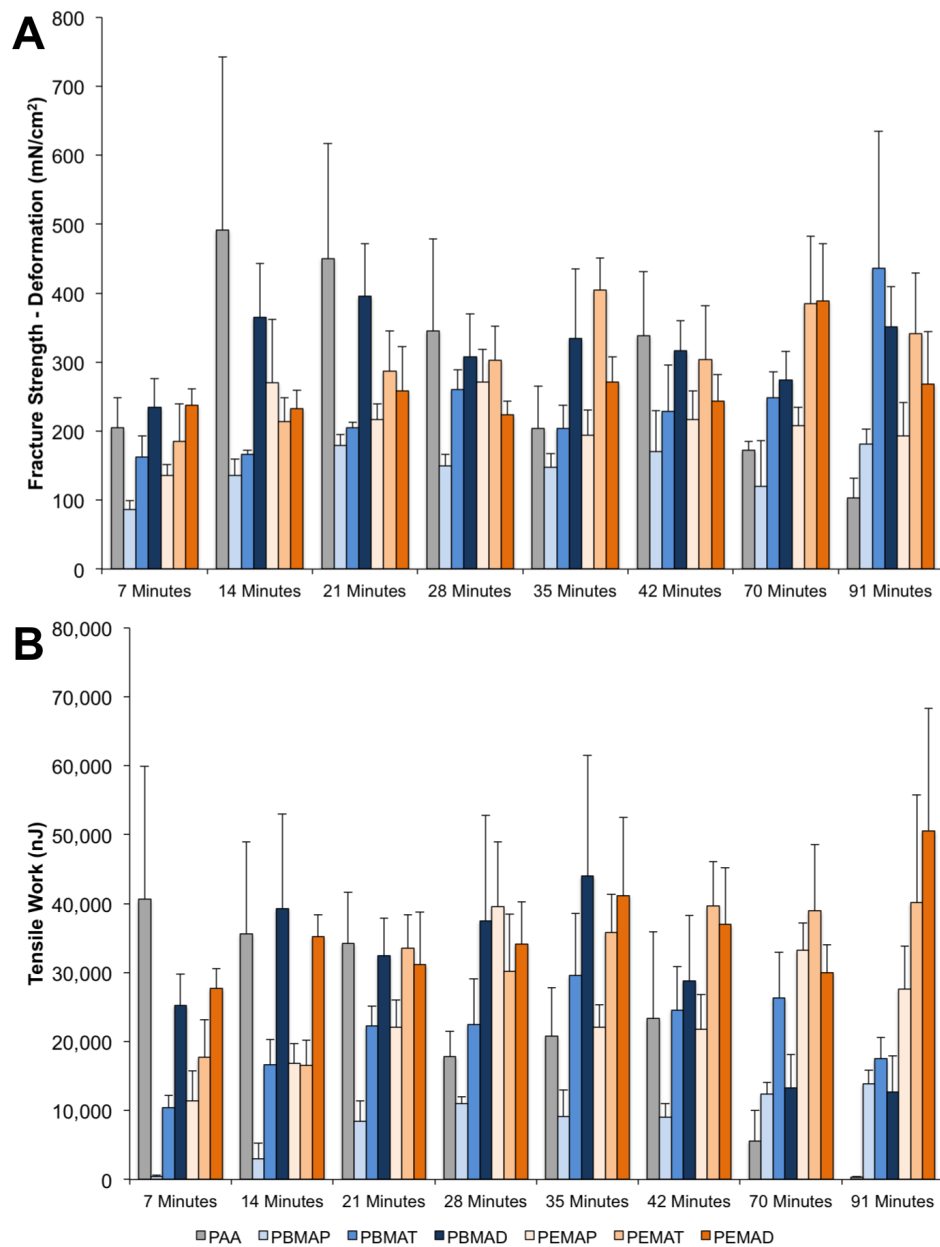


Figure 3.4: (A) Fracture strength of PAA, PBMA- and PEMA-derivative polymers from tensile tests with fresh rat small intestinal tissue over 91 minutes; (B) Tensile work of PAA, PBMA- and PEMA-derivative polymers from tensile tests over 91 minutes. The error bars represent the standard deviation of the measurements, $n = 4$.

3.4.5 Bioadhesion Testing of PBMA-Derivatives

The poly(butadiene-maleic anhydride) (PBMA)-derivative polymers conjugated with phenylalanine (PBMAP), tyrosine (PBMAT) and DOPA (PBMAD) are a series of low molecular weight (15,000 Da) polymers that were developed with a hydrophobic backbone and side chains of increasing hydrophilicity and hydrogen bonding potential. Across the 91 minute testing intervals, average fracture strength values of 146.28 ± 32.36 mN/cm², 238.79 ± 87.16 mN/cm² and 322.34 ± 51.49 mN/cm² were obtained for the low molecular weight PBMAP, PBMAT and PBMAD polymers, respectively. Likewise, the maximum fracture strength values achieved by each of the polymers were determined to be 181.55 ± 21.43 mN/cm², 436.29 ± 198.15 mN/cm² and 395.40 ± 75.93 mN/cm² (Figure 3.4A).

The average tensile work values for each of the polymers over 91 minutes were calculated to be 8415.09 ± 4570.96 nJ, 21225.13 ± 6105 nJ, 29153.56 ± 11600.72 nJ (Figure 3.4B). In comparison, the PAA testing revealed an average tensile work of 22283.91 ± 14356.82 nJ indicating that with the exception of PBMAP, the novel bioadhesive polymers had stronger bioadhesive interactions with intestinal mucosa (corresponding to 0.38, .95 and 1.31 times the average work observed with PAA) over a period of 91 minutes (Figures 3.4B and 3.6).

With the increase in the number of hydroxylations seen from phenylalanine to tyrosine to DOPA, we see a stepwise increase in the average tensile work values obtained with each of these polymers. Interestingly, comparison of the total number of potential hydrogen bonding sites on the repeat unit of each polymer and the observed average tensile work values revealed a strong correlation with an R² value of 0.9807 for the PBMA polymers, suggesting that hydrogen bonding may be a predominant mechanism in the mucoadhesive interactions between these polymers and intestinal tissue.

While the fracture strength-deformation data revealed almost constant values in the entire 91 minute study period (Figure 3.4A), the time-dependent behavior of each polymer is more interesting when looking at the tensile work data (Figure 3.4B). This is due to the fact that tensile work takes the entire area under the deformation to failure curve into account, which may render it a more comprehensive measure of bioadhesion for these polymers. Evaluation of the bioadhesive profile over time revealed interesting trends attributable to

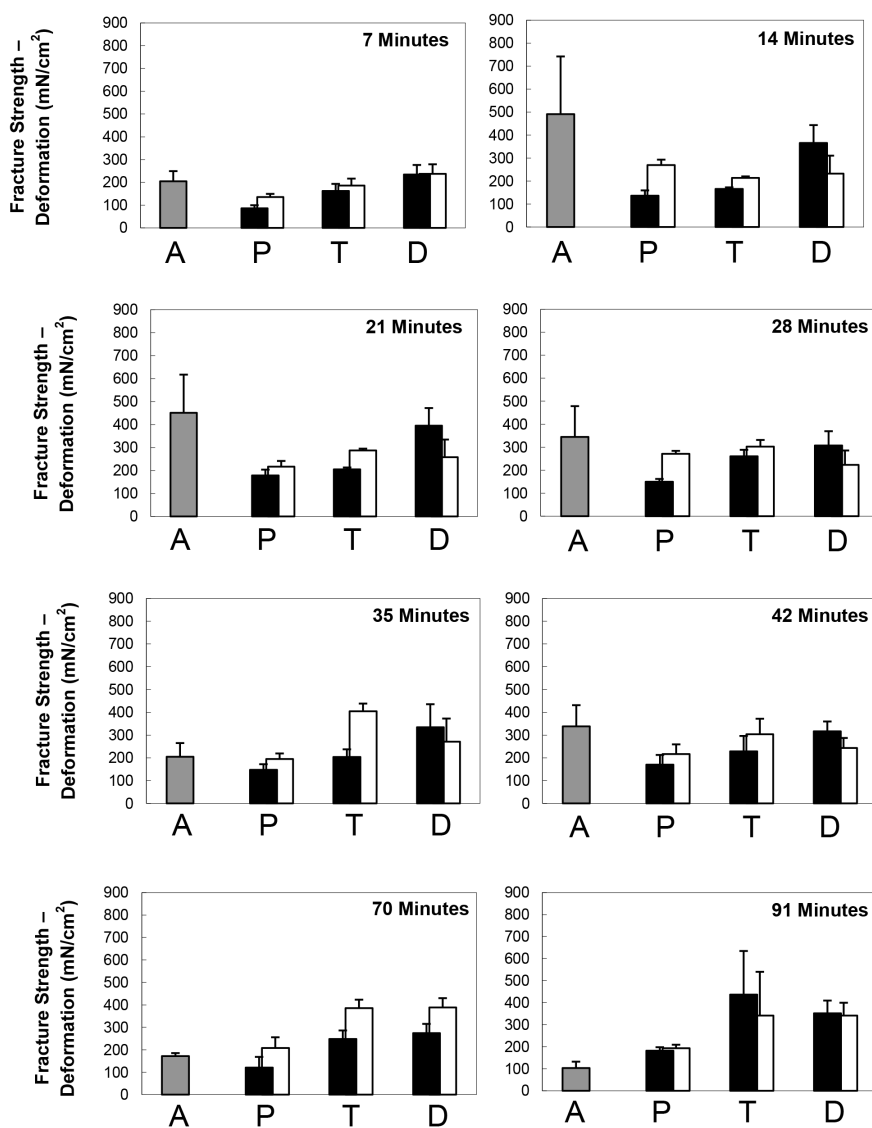


Figure 3.5: Fracture strength values for each of the conjugated bioadhesive polymers. Each set of polymers is ordered by side chain hydroxyl group content, and contact time; black bars: PBMA backbone, white bars: PEMA backbone; A: PAA, P: phenylalanine, T: tyrosine, D: DOPA. The error bars represent the standard deviation of the measurements, n=4.

the chemical characteristics of each these polymers (Figures 3.5 and 3.6). PBMAP shows a steady increase in tensile work values over the first 28 minutes (from 419 ± 173.04 nJ to 10975.75 ± 1010.36 nJ) followed by the maintenance of a steady level of bioadhesion at this level through the later time points; in contrast, the PBMAT shows a similar increase in bioadhesion over the earlier time points until it reaches peak tensile work values at 35 minutes (29565 ± 8975.63 nJ), followed by a marginal decrease through the later time points. The PBMA polymer shows a different pattern with the rapid onset of very high bioadhesive forces over the first 14 minutes (39288 ± 13681.07 nJ), which are maintained through 35 minutes (43996.25 ± 17529.48 nJ), followed by a rapid and significant decrease through 91 minutes (12665.25 ± 5217.40 nJ). The earlier onset and more rapid loss of bioadhesion seen with the PBMA and PBMAT relative to PBMAP is likely the result of the increased hydrophilicity seen with each of these polymers which allows for faster hydration of the side chains, thus creating faster hydrogen bonding with mucin while increasing the rate of polymer dissolution from the probe associated with a loss of bioadhesion, as seen in the dissolution testing. The ability of the PBMAP to maintain its bioadhesion throughout the 91 minute duration but at lower tensile work values may be a result of its relative hydrophobicity compared to the other polymers.

3.4.6 Bioadhesion Testing of PEMA-Derivatives

Similar to the PBMA-derivative polymers, a higher molecular weight (400,000 Da) poly(ethylene-maleic anhydride) polymer was used to develop a series of polymers conjugated to phenylalanine (PEMAP), tyrosine (PEMAT) and DOPA (PEMAD). The PEMA polymers have a lower molecular weight repeat unit, the M_R of the PBMA is 151 Da while the M_R of the PEMA is 126 Da. Thus, with the combination of the higher molecular weight and the smaller repeat unit, there are a greater number of substitutions in the polymer chains of the PEMA derivatives associated with a greater potential for hydrogen bonding relative to the PBMA polymers. Average fractures strength values of 213.21 ± 43.83 mN/cm², 303.10 ± 76.18 mN/cm² and 265.46 ± 52.56 mN/cm² were observed for the PEMAP, PEMAT and PEMAD polymers (Figure 3.4A). While the average tensile work values for the PEMAP, PEMAT and PEMAD were 24327.90 ± 8953.81 nJ, 31584.56 ± 9539.99 nJ and 35862.41

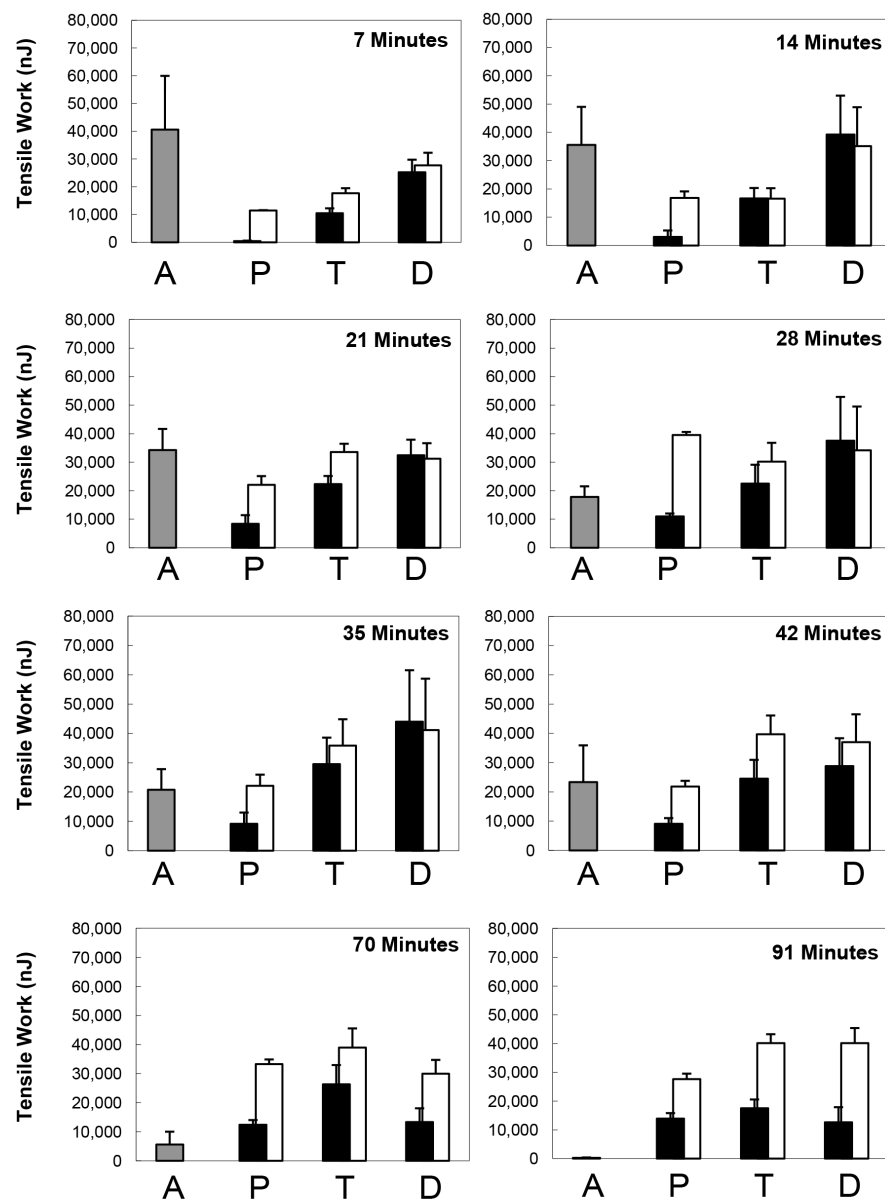


Figure 3.6: Tensile work values for each of the conjugated bioadhesive polymers. Each set of polymers is ordered by side chain hydroxyl group content, and contact time; black bars: PBMA backbone, white bars: PEMA backbone; A: PAA, P: phenylalanine, T: tyrosine, D: DOPA. The error bars represent the standard deviation of the measurements, n=4.

± 7289.20 nJ (Figure 3.4B), which represents 1.09, 1.41 and 1.61 times the average tensile work of PAA. Similar to the PBMA polymers, comparison of the total number of potential hydrogen bonding sites on the repeat unit of each polymer and the observed average tensile work values revealed a strong correlation ($R^2 = 0.9783$) for the PEMA polymers.

PEMAP shows a similar bioadhesion profile to PBMAP, but the tensile work values for the higher molecular weight polymer were shown to be higher (Figure 3.6), for example, tensile work calculations yielded values of 10975.75 ± 1010.36 nJ for the PBMAP and 39548.50 ± 9441.68 nJ for the PEMAP at 28 minutes; this is due to the increased hydrogen bonding potential of the PEMAP as described above. Unlike their PBMA-derivative counterparts, the PEMAT and PEMAD show increasing tensile work values through 21 and 14 minutes (33581 ± 4850.36 nJ and 35192.75 ± 3183.64 nJ) respectively, followed by the maintenance of a high level of bioadhesion through the rest of the 91 minute duration. This is in stark contrast to the lower molecular weight polymers as well as the PAA (Figure 3.6), which had a tendency to lose bioadhesion during these later time points. This ability of the PEMA-derived tyrosine and DOPA-conjugated polymers to maintain their bioadhesive performance at the later time points is likely the result of the higher molecular weight and increased side chain substitutions in comparison to their PBMA-derived equivalents, which allows for the polymer to maintain its integrity on the probe for longer durations. As before, there is evidence of rapid hydration and more rapid generation of bioadhesive forces with the DOPA conjugated polymers relative to tyrosine and phenylalanine due to the increased hydrophilicity of this side chain.

3.5 Discussion

While both the novel bioadhesive polymers and polycarbophil have similar general structures consisting of a hydrophobic backbone with carboxylic acid side groups, our bioadhesives are structurally more complex and include hydrophobic aromatic rings combined with an increasing number of hydrophilic carboxylic acid and hydroxyl moieties per repeat unit. This increased side chain length may serve to prolong dissolution times for our novel polymers due to the physical entanglement and hydrogen bonding of the side chains resulting in the pro-

longed bioadhesive behavior relative to PAA, which demonstrates strong initial performance but suffers from a rapid loss of bioadhesion behavior after the early time points.

Direct comparison of the polymers at each time point reveals distinct bioadhesion profiles for each backbone and side-chain (Figure 3.6). At the 7 minute time point, PAA exhibits the highest tensile work values (49951.67 ± 23966.33 nJ) while the novel bioadhesives exhibit much lower values. The PBMA polymers exhibit lower tensile work values relative to the PEMA polymers, and the phenylalanine conjugates have the lowest tensile work values followed by tyrosine then DOPA. At the 21 minute time point, a similar range of tensile work values is observed between the PAA, PBMA and PEMA polymers (in the 30000 nJ range), particularly the tyrosine and DOPA polymers while the phenylalanine polymers exhibit lower tensile work values. The 28 minute time point reveals a noticeable shift in the bioadhesive behavior of the polymers. The PAA tensile work values have dropped to 17794.75 ± 3716.32 nJ while the PBMAT, PEMAT, PBMA and PEMAD have achieved noticeably higher tensile work values (29565 ± 8975.63 nJ, 35831.25 ± 5484.09 nJ, 43996.25 ± 17529.48 nJ and 41121 ± 11382.10 nJ, respectively); the highest tensile work values for the PBMA are achieved at this time point. The PBMA and PEMAT values remain lower at 9174.5 ± 3816.21 nJ and 22102.25 ± 3267.40 nJ, similar to the tensile work seen at 21 minutes. At the 91 minute time point, the PAA exhibits negligible bioadhesive forces while the PEMA polymers maintain strong bioadhesion with tensile work values of 27609 ± 6253.65 nJ, 40168.25 ± 15600.84 nJ, 50585.5 ± 17740.15 nJ for the PEMAP, PEMAT and PEMAD, respectively. In contrast, the PBMA polymers exhibit lower values of 13896.5 ± 1930.31 nJ, 17497.24 ± 3074.36 nJ and 12665.25 ± 5217.40 nJ for the PBMA, PBMA and PBMA, respectively, but still show higher values than PAA. As evidenced by the tensile work data, the hydrophilic hydrogel nature of the PAA results in the rapid generation of high bioadhesive forces due to the rapid swelling and exposure of the carboxylic acid side chains, which can physically entangle and hydrogen bond with the small intestinal mucosa. However, as the polymer continues to swell, the bioadhesive forces are lost due to the inability of the polymer to maintain cohesive strength. In contrast, the novel catechol-conjugated polymers take longer to generate bioadhesive forces due to their hydrophobic backbones. The phenylalanine polymers exhibit the lowest bioadhesive forces among these

polymers while the highest bioadhesive forces are seen with the DOPA-conjugated polymers as a result of the increasing hydrogen bonding potential between the phenylalanine, tyrosine and DOPA. While the higher molecular weight PEMA polymers are able to maintain strong bioadhesive forces throughout the 91 minutes, the lower molecular weight PBMA polymers lose this property more quickly but maintain higher levels of bioadhesion than the PAA at the later time points.

Although it is a commonly used measurement for the evaluation of bioadhesion, there are limitations to the use of fracture strength values in this system; in particular, several peaks were recorded in the load-deformation curve during a single test in some instances but only the final peak recorded before ultimate failure of the tissue-probe interface was used as the peak tensile load to ensure consistency in the recorded measurements, resulting in a possible under-reporting of fracture strength values for some tests. PEMAD datasets in particular showed a higher incidence of initial peak tensile load spikes not taken into account when calculating fracture strength; this could have led to an under-reporting of the true fracture strength of the polymer and serve as a possible explanation of the deviation from the trend observed with PEMA^P and PEMA^T, which showed higher fracture strengths than their lower molecular weight counterparts - a trend that was surprisingly not observed for the PEMAD.

Our previous work with bioadhesive polymers focused on hard thermoplastic polymers or hydrogels; in contrast, some of the polymers evaluated in these studies are soluble under the testing conditions used here. This results in a change in the diameter of the probe over time due to polymer dissolution, which may alter the fracture strength values, which depend on contact area. Additionally, given the nature of the polymer-tissue interactions, the calculated contact area may not be the actual contact area but rather an approximation. Thus, for such a dynamic system using fresh intestinal tissue, tensile work may be a better indicator of bioadhesion than fracture strength.

3.6 Conclusions

A modified Texture Analyzer apparatus was used to test the bioadhesive performance of six conjugated bioadhesive polymers *in vitro* by measuring the force required to break the interactions between a polymer-coated probe and a freshly harvested segment of rat jejunum within an aqueous environment. The data was used to produce a load vs. deformation curve, which provided a wealth of information used to evaluate bioadhesive performance through the parameters of fracture strength and tensile work. Tensile work was determined to be a more comprehensive assessment of the bioadhesion of each polymer, since data from the entire deformation to failure segment of the load vs. deformation curve was taken into account. The data obtained from these studies demonstrate trends between molecular weight, functional group hydrophilicity, dissolution rate, and bioadhesive performance.

Our data suggest that an increase in the hydroxyl content of these polymers results in the generation of higher bioadhesive forces with intestinal mucosa; however, this comes at the cost of more rapid dissolution due to the increased hydrophilicity of the polymer. By increasing the molecular weight of the polymer backbone, the dissolution of the polymer can be controlled to allow for a longer duration of bioadhesion. An additional benefit is the pH-dependent solubility of these polymers observed from the dissolution testing, which is particularly advantageous for oral drug delivery applications. These polymers exhibit the ability to remain in their solid form in the more acidic environment of the stomach to protect any encapsulated pH-sensitive therapeutic cargo, such as proteins or genetic material, followed by the “activation” of the bioadhesive behavior of the polymer in the more basic small intestine to allow for increased GI transit time, enhanced interactions with the absorptive mucosal epithelium of the small intestine as well as pH-dependent controlled release characteristics for more effective drug delivery.

3.7 Supplemental Material

3.7.1 Materials and Methods

3.7.1.1 Fourier Transform Infrared Spectroscopy (FTIR)

All the polymers and amino acids were analyzed in lyophilized powder form using a Perkin Elmer Spectrum One FTIR with a Universal Attenuated Total Reflectance (ATR) Sampling Accessory and analyzed using built-in Spectrum software (Perkin-Elmer, Inc.; Waltham, MA).

3.7.1.2 Differential Scanning Calorimetry (DSC)

3-4 mg of each polymer was sealed into aluminum sample pans for DSC analysis using a DSC 7 equipped with controller model TAC 7/DX (Perkin-Elmer, Inc.; Waltham, MA). After equilibration at -25°C for 1 minute, samples were heated from -25 to 250°C, cooled to -25°C then reheated to 250°C at a rate of 10°C/min. All thermograms were analyzed using Perkin-Elmer Thermal Analysis software for the calculation of glass transition temperatures (T_g) from the second heat.

3.7.2 Results and Discussion

3.7.2.1 Fourier Transform Infrared (FTIR) Spectroscopy Analysis

FTIR analysis confirms NMR results demonstrating successful side chain grafting of the amino acid residues onto the PBMA and PEMA backbones (Figure 3.7). Comparison of the spectrum for PBMA with its bioinspired derivatives shows the loss of the anhydride peaks (1851.8 cm^{-1} and 1772.3 cm^{-1}) and the formation of peaks at $\sim 1700 \text{ cm}^{-1}$ and $\sim 1170 \text{ cm}^{-1}$ corresponding to the production of free carboxyl groups due to the hydrolysis of the maleic anhydride rings. Also, the amide bond formed from amino acid side chain grafting is seen in the bioinspired derivative polymers at $\sim 1520 \text{ cm}^{-1}$. Additionally, the increase in the area of the broad hydroxyl peak (3500 cm^{-1} - 2800 cm^{-1}) from the phenylalanine, to the tyrosine, to the DOPA-derived polymers is consistent with the ordering of the hydroxylation content of the grafted side chains.

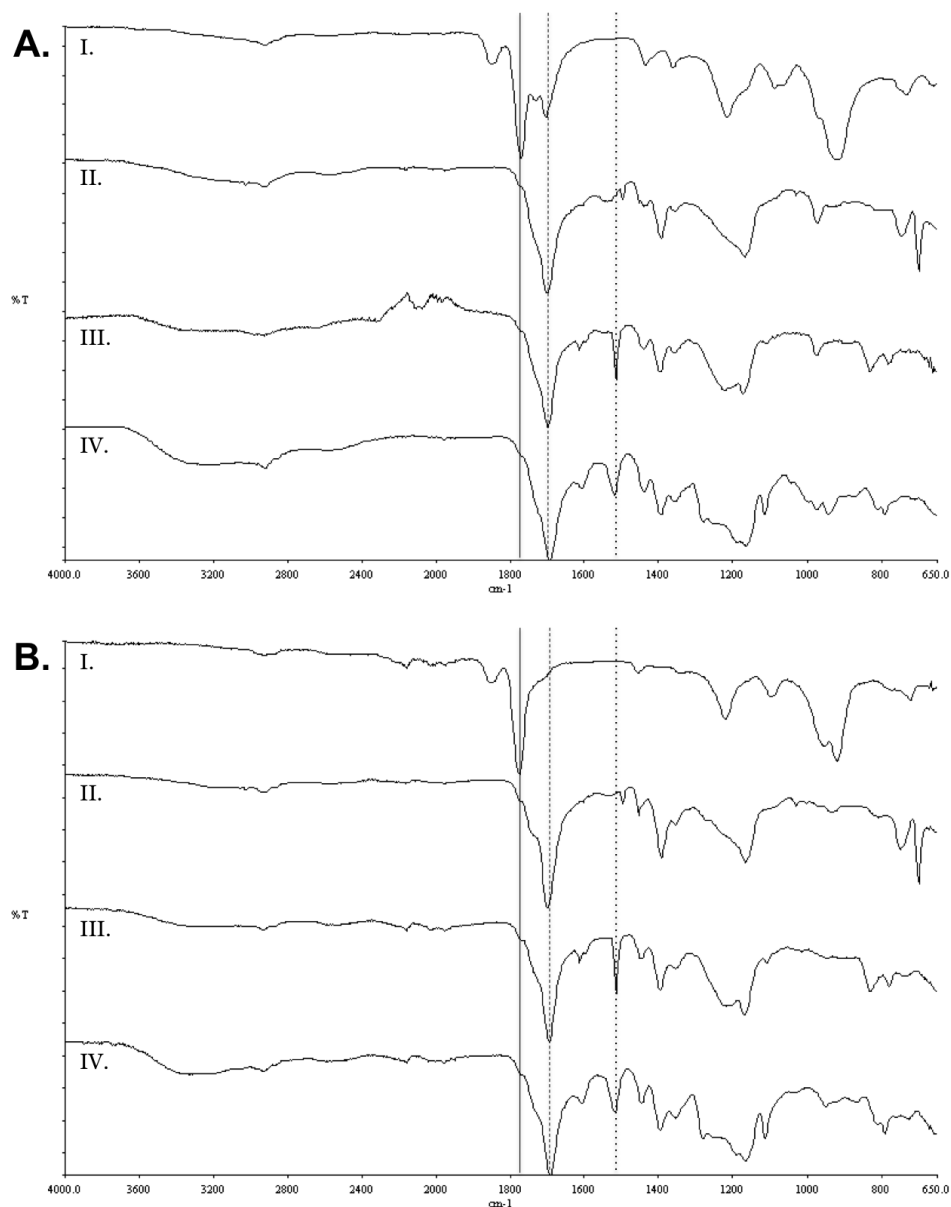


Figure 3.7: FTIR spectra of PBMA-derivative polymers (A) (I) PBMA, (II) PBMAP, (III) PBMAT, and (IV) PBMAAD and PEMA-derivative polymers (B) (I) PEMA, (II) PEMAP, (III) PEMAT, (IV) PEMAD. The solid vertical line indicates the anhydride peak ($\sim 1775 \text{cm}^{-1}$) of the backbone polymers, absent in the bioinspired derivatives. During synthesis, each anhydride ring opens to form a carboxylic acid group that corresponds to the dashed vertical line at $\sim 1700 \text{cm}^{-1}$ and attach the amino acid side group creating an amide bond that corresponds to the dotted vertical line at $\sim 1500 \text{cm}^{-1}$.

Polymer	Side Chain Attachment Efficiency	T _g (°C)
PBMA	-	94.86
PBMAP	79%	141.15
PBMAT	86%	169.24
PBMAD	75%	165.20
PEMA	-	139.35
PEMAP	98%	159.33
PEMAT	73%	197.54
PEMAD	91%	201.82

Table 3.2: Bioinspired bioadhesive polymer side chain attachment efficiencies and glass transition temperatures.

3.7.2.2 Thermal Analysis

Thermal analysis by DSC revealed glass transitions for all the bioinspired bioadhesive polymers in the temperature ranges scanned, summarized in table 3.2. Glass transition (T_g) temperatures of the higher molecular weight PEMA-derivative polymers (unreacted PEMA MW = 400 kDa) were higher than those of the lower molecular weight PBMA-derivatives (unreacted PBMA MW = 10 - 15 kDa). Additionally, side-chain modified polymers had higher glass transition temperatures than backbone polymers alone. The conjugation of more hydrophilic and bulky side chains resulted in increased the glass transition temperatures. The phenylalanine derivatives with the least hydrophilic side chains showed the lowest T_g values, while the bulkiest and most hydrophilic DOPA side chains had the highest T_g values. These increases in the glass transition temperatures could be due to increased hydrogen bonding between the side chains provided by the increase in hydroxylation of the aromatic rings, which could inhibit the mobility of the packed polymer chains required for the transition from the glassy to the amorphous state.

3.8 References

- [1] J. Woodley, Bioadhesion: new possibilities for drug administration?, Clinical Pharmacokinetics. 40 (2001) 77–84.
- [2] G. Ponchel, J.M. Irache, Specific and non-specific bioadhesive particulate systems for oral delivery to the gastrointestinal tract, Advanced Drug Delivery Reviews. (1998).

- [3] E. Mathiowitz, D. Chickering, J.S. Jacob, C. Santos, Bioadhesive Drug Delivery Systems, in: E. Mathiowitz (Ed.), *Encyclopedia of Controlled Drug Delivery*, 1st ed. John Wiley & Sons, Inc., New York, NY, n.d.: pp. 9–945.
- [4] R. Gurny, H. Junginger, Gurny: Bioadhesion: Possibilities and Future Trends, 1990.
- [5] Y. Huang, W. Leobandung, A. Foss, N.A. Peppas, Molecular aspects of muco- and bioadhesion: tethered structures and site-specific surfaces., *J Control Release*. 65 (2000) 63–71.
- [6] N.A. Peppas, J.J. Sahlin, Hydrogels as mucoadhesive and bioadhesive materials: a review., *Biomaterials*. 17 (1996) 1553–1561.
- [7] C. Santos, Evaluation of anhydride oligomers within polymer microsphere blends and their impact on bioadhesion and drug delivery in vitro, *Biomaterials*. 24 (2003) 3571–3583.
- [8] R. Capra, A. Baruzzi, L. Quinzani, Rheological, dielectric and diffusion analysis of mucin/carbopol matrices used in amperometric biosensors, *Sensors and Actuators B*. 124 (2007) 466–476.
- [9] K. Khanvilkar, M.D. Donovan, D.R. Flanagan, Drug transfer through mucus., *Advanced Drug Delivery Reviews*. 48 (2001) 173–193.
- [10] G.P. Andrews, T.P. Lavery, D.S. Jones, Mucoadhesive polymeric platforms for controlled drug delivery., *Eur J Pharm Biopharm*. 71 (2009) 505–518.
- [11] F. Madsen, K. Eberth, J.D. Smart, A rheological assessment of the nature of interactions between mucoadhesive polymers and a homogenised mucus gel., *Biomaterials*. 19 (1998) 1083–1092.
- [12] M. Davidovich-Pinhas, H. Bianco-Peled, Mucoadhesion: a review of characterization techniques., *Expert Opin Drug Deliv*. 7 (2010) 259–271.
- [13] C.A. Santos, J.S. Jacob, B.A. Hertzog, B.D. Freedman, D.L. Press, P. Harnpicharnchai, et al., Correlation of two bioadhesion assays: the everted sac technique and the CAHN microbalance., *J Control Release*. 61 (1999) 113–122.
- [14] Sau-Hung Spence Leung, J.R. Robinson, The contribution of anionic polymer structural features to mucoadhesion, *Journal of Controlled Release*. 5 (1988) 223–231.
- [15] N. Thirawong, J. Nunthanid, S. Puttipipatkachorn, P. Srimornsak, Mucoadhesive properties of various pectins on gastrointestinal mucosa: an in vitro evaluation using texture analyzer., *Eur J Pharm Biopharm*. 67 (2007) 132–140.
- [16] J.H. Waite, M.L. Tanzer, The bioadhesive of *Mytilus byssus*: a protein containing L-dopa., *Biochem. Biophys. Res. Commun*. 96 (1980) 1554–1561.
- [17] J. Waite, The DOPA Ephemera: A Recurrent Motif in Invertebrates, *The Biological Bulletin*. 183 (1992) 178–184.
- [18] L. Ninan, R. Stroshine, J. Wilker, Adhesive strength and curing rate of marine mussel protein extracts on porcine small intestinal submucosa, *Acta Biomater*. 3 (2007) 687–694.

- [19] H. Cha, D. Hwang, S. Lim, Development of bioadhesives from marine mussels, *Biotechnology Journal*. 3 (2008) 631–638.
- [20] J. Schnurrer, C.-M. Lehr, Mucoadhesive properties of the mussel adhesive protein, *International Journal of Pharmaceutics*. 141 (1996) 251–256.
- [21] J. Jacob, E. Mathiowitz, Poly(fumaric-co-sebacic) microspheres as oral drug delivery systems, *Biotechnology and . . .* (1996).
- [22] M. Schestopol, J. Jacob, R. Donnelly, T. Ricketts, A. Nangla, E. Mathiowitz, et al., Bioadhesive polymers with catechol functionality, U.S. Patent US 2005/0201974, 2004.

Chapter 4

Can Bioadhesive Nanoparticles Allow For More Effective Particle Uptake from the Small Intestine?

4.1 Abstract

There has been increasing interest in developing bioadhesive nanoparticles due to their great potential as carriers for therapeutics in oral drug delivery systems. Despite decades of research, such a system still has not been successfully implemented. This paper demonstrates the enormous potential of such engineered systems: the incorporation of a bioadhesive coating to non-bioadhesive nanospheres resulted in an enhancement of particle uptake in the small intestine from $5.8 \pm 1.9\%$ to $66.9 \pm 12.9\%$. Direct correlation was obtained between bulk tensile strength, in vitro binding to everted intestinal sacs and quantitative in vivo uptake; this data suggests that bulk bioadhesion properties of polymers can be used to predict their nanoscale bioadhesive behavior. The differential distribution of the nanospheres to various tissues following uptake suggests surface chemistry plays a significant role in their localization within the body. The results of these studies provide strong support for the use of bioadhesive polymers to enhance nanoparticle uptake from the small intestine for oral drug delivery.

Joshua J. Reineke^{*}, Daniel Y. Cho^{*}, Yu-Ting Liu, Peter Cheifetz, Bryan Laulicht and Edith Mathiowitz

^{*}both authors contributed equally to this work

4.2 Introduction

Micro- and nanosphere-based polymeric delivery systems have great potential for oral drug delivery due to their ability to protect encapsulated therapeutic agents from the harsh gastrointestinal tract and allowing for controlled drug release kinetics. Although a significant amount of effort has been focused on developing effective delivery systems for translation into clinical use, a major obstacle in the development of effective nanospheres for oral drug delivery is achieving high enough levels of uptake to reach therapeutic concentrations of the encapsulated drugs after administration. Thus, a variety of strategies have been evaluated for their potential to enhance the translocation of particles from the intestinal lumen, but most with limited success.

The phenomenon of bioadhesion or mucoadhesion, originally described by Park and Robinson, refers to the interactive forces between a synthetic or biological material and a mucosal surface [1]. When applied to oral drug delivery, bioadhesive polymers result in adhesion to the mucous membrane proximal to the intestinal cells allowing for enhanced interactions with the absorptive epithelium and increasing gastrointestinal transit time. There are several proposed mechanisms that mediate bioadhesion between the polymer and biological substrate; the most studied are mechanical and/or chemical interactions [2]. Mechanical bonds can be the result of the physical entanglement of mucin strands with polymer chains, as is the case for hydrogels, which are most often used for bioadhesive drug delivery [3,4]. Alternatively, chemical mechanisms can include strong primary bonds, which are not desired, as well as weaker secondary forces such as ionic bonds, Van der Waal's interactions and hydrogen bonds [3,5]. While the strengths of individual secondary forces are relatively weak, the high numbers of these interactions possible with polymeric materials can generate strong bioadhesion to mucous. Thus, polymers with high molecular weights and greater concentrations of reactive polar groups (-COOH, -OH) tend to develop more intense mucoadhesive bonds [2,5-11]. Studies have shown that thermoplastic polymers that are high in carboxyl groups can also produce intense bioadhesive interactions without physical entanglement [12-15] and be effective in improving the bioavailability of orally administered therapeutics [16,17].

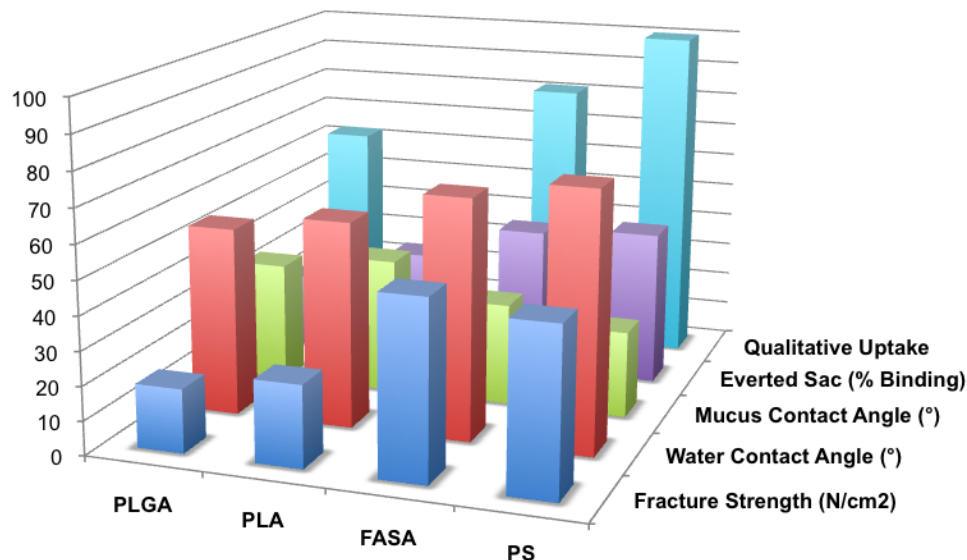


Figure 4.1: 3-D representation of critical in vitro and in vivo bioadhesion parameters for PLGA, PLA, PFASA and PS.

Nanoparticles made of bioadhesive polymers may, therefore, potentiate effective oral drug delivery due to the combination of their small size and high surface area [18-22]. Many reports have shown that the surface chemistry greatly affects the uptake of particles [23-28], which could be of particular interest given the high surface area found with nanoparticles. However, no systematic study has directly investigated the relationship between bioadhesive nanospheres and intestinal uptake.

For the last decade, work in our laboratory has focused on developing quantitative in vitro [2,12,29-33] and in vivo [29,32,34] methods to measure bioadhesion between microspheres and intestinal mucosal tissue. As part of this research, we have identified a series of quantitative techniques that helped us evaluate what we believe to be the critical parameters to select effective bioadhesive polymers and incorporate them into the preparation of nanoparticles that exhibit substantial uptake in vivo, as judged by optical, confocal and TEM microscopy. The results, as summarized in figure 4.1, clearly demonstrate tensile work measurements of the bulk bioadhesive properties of several polymers, of which polystyrene (PS, non-erodible) and poly(fumaric-co-sebacic) acid (PFASA, bioerodible) exhibited the strongest forces. Poly(lactic acid) (PLA, bioerodible) and poly(lactic-co-glycolic

acid) (PLGA, bioerodible) exhibited weaker bioadhesive forces. Studies of the contact angle of each polymer with water indicate that both PS and PFASA are very hydrophobic. The observation of a very high contact angle with water indicates a low affinity for aqueous solutions. In contrast, when contact angle was measured with mucus, it was clear that the first two polymers reveal the lowest contact angle indicating a strong affinity for mucus. This was further confirmed by additional measurements using an ex vivo everted sac assay, providing further evidence that the most adhesive polymers in the series to be PS and PFASA followed by PLGA then PLA. Interestingly, when we prepared nanospheres with each of these polymers and evaluated uptake from the small intestine using optical, confocal and transmission electron microscopy we found that PS and PFASA nanoparticles revealed qualitatively high uptake while PLGA and PLA had lower levels of uptake [29]. The results of this prior work provide a series of important parameters that allow for the identification and evaluation of potential bioadhesive polymers for potential use in drug delivery applications. Since PS is a non-erodible polymer, it can be used to quantitatively track the fate of nanoparticles administered in vivo following uptake.

Thus, the current study was directed to quantify the exact uptake of polymeric nanoparticles in vivo and to evaluate the role of bioadhesion on nanoparticle uptake and tissue distribution. To address this question we chose three different nanosphere formulations made of non-erodible polymers and characterized the tensile bioadhesive forces of the bulk polymer, bioadhesion of the nanospheres to everted intestinal sacs as well as uptake and biodistribution of the nanospheres from the small intestines.

4.3 Materials and Methods

4.3.1 Materials

Polystyrene beads (PS, 500 nm) were purchased from Polysciences, Inc. (Warrington, PA) and stored at 4 °C until use. Suspensions were used as supplied in concentrations of 25 mg/mL. Polymethyl methacrylate (PMMA, MW = 100 kDa) and polyvinyl alcohol (MW = 25 kDa, 88% hydrolyzed) were purchased from Polysciences, Inc. (Warrington, PA) and stored at room temperature. Poly(butadiene-maleic anhydride-co-L-DOPA) (PBMAD), was

provided by Spherics, Inc. (Mansfield, MA) and stored at room temperature. All solvents were of the highest commercial grade available.

4.3.2 Solvent Evaporation

Solvent evaporation was used to fabricate monodisperse PMMA nanospheres. PMMA was dissolved in chloroform at a concentration of 3.3 % w/v to comprise the organic phase. An aqueous phase of 1 % w/v polyvinyl alcohol (25 kDa, 88 % hydrolyzed) was prepared and mixed under a VirTis Cyclone IQ2 SENTRY microprocessor at 25,000 rpm with a straight open blade and VirTis baffled homogenizer flask. The organic phase with polymer was then added to the aqueous phase forming an o/w emulsion and continually mixed for 15 minutes. The emulsion was then added to a 1% w/v polyvinyl alcohol (25 kDa, 88% hydrolyzed) bath and mixed under an over-head stirrer for 12 hours at 3000 rpm to allow further solvent evaporation and nanosphere curing. This suspension was then centrifuged at 4000 rpm for 20 minutes and the supernatant poured off. The pellet was resuspended and washed in deionized water three times. Finally, the formulation was lyophilized for 48 hours and stored at room temperature until use.

4.3.3 Phase Inversion Nanoencapsulation

A modified version of phase inversion nanoencapsulation, originally described by Mathiowitz, et al [29,35], was utilized to fabricate spheres consisting of PMMA and PBMA. First, PMMA was dissolved in 5 mL of tetrahydrofuran (2 % w/v) and, in a separate vial, PBMA was dissolved in 10 mL of ethanol (2 % w/v). The polymer solutions were mixed and then rapidly added to a non-solvent bath of petroleum ether spontaneously forming nanospheres. Nanospheres were collected by positive-pressure filtration through PTFE filter paper with a pore diameter of 0.2 μm and lyophilized for 48 hours. Formulations were stored under vacuum-seal at room temperature until use.

4.3.4 Scanning Electron Microscopy

Morphology and particle size of formulations fabricated by solvent evaporation and phase inversion nanoencapsulation were analyzed by scanning electron microscopy. Formulations

were placed on a carbon-backed adhesive and sputter-coated with Au-Pd for 4 minutes at 20 mA and imaged on a Hitachi 2700 scanning electron microscope with an accelerating voltage of 8 kV.

4.3.5 Particle Size Analysis

Solvent removal and phase inversion nanoencapsulation formulations were reconstituted from powder form into an aqueous solution using 1 % w/v sodium lauryl sulfate (SLS)/1 % w/v polyvinylpyrrolidone (PVP) reconstitution media and bath sonication in 0.5 – 2 % w/v suspensions. Polystyrene nanosphere suspensions were diluted with deionized water down to ~1 % w/v solutions from the original supplied suspensions. A Beckman Coulter LS230 Laser Diffraction Particle Size Analyzer was used to evaluate size and size distribution of nanoparticle populations.

4.3.6 Light Microscopy

All formulations were imaged with light microscopy to evaluate morphology and aggregation. Formulations were suspended in 1 % w/v sodium lauryl sulfate (SLS)/1 % w/v polyvinylpyrrolidone (PVP) reconstitution media at a concentration of 0.5 % w/v. Drops of the suspension were placed on a slide and sealed under a cover slip with fast-drying nail polish. Slides were then imaged on a Zeiss Axiovert 200M microscope. To obtain bright field images a 40x water objective was used along with DIC and phase contrast techniques. Images were taken with a Zeiss AxioCam MRc5 digital color CCD camera.

4.3.7 PMMA Loading Assay

To determine the weight percent of PMMA in the PBMA/PMMA formulation fabricated by phase inversion nanoencapsulation, an extraction and chromatography detection method was used. First, a known amount (25-50 mg) of the PBMA/PMMA formulation was weighed into a vial and 5 mL of chloroform was added. The solution was mixed on an end-over-end mixer for 1 hour to ensure complete dissolution of PMMA. Then, a 1 mL sample of the solution was filtered through a 0.2 μ m PTFE syringe filter into a sample vial for gel permeation chromatography. The sample elution time was detected on a Shimadzu

RID-10A refractive index detector after flow (1 mL/min) through a column bank consisting of Waters Styragel HR4E and HR5E columns. The area under the distinctive PMMA peak on the refractive index versus elution time graph was calculated using Shimadzu software and compared to a linear standard curve of PMMA to directly yield the quantity of PMMA in the PBMA/PMMA formulation.

4.3.8 Texture Analyzer Bioadhesion Assay

Testing the bioadhesion of bulk polymeric materials to ex vivo rat jejunum was performed with a TA.XTplus Texture Analyzer (Texture Technologies Corp., Scarsdale, NY) equipped with a 1 kg load cell with 0.2 g sensitivity. Glass-headed pins with mean head diameter ~ 2.8 mm were dip-coated into various 5% w/v solutions of each polymer (PMMA and PS in methylene chloride, pBMA in acetone) and dried between coatings to achieve a uniform coating around the pinhead; the pins were dipped 8 times to ensure an adequate amount of polymer was coated onto each pin. Individual probes were then fitted onto the texture analyzer load arm and a biological sample chamber placed on the stage containing freshly excised rat jejunum submerged in phosphate buffer saline, pH 7.4 with luminal side facing upward at 37°C. The load arm descended at 0.5 mm/sec until a specified target force (5 g) between the sample probe and intestinal tissue was reached. This position was then held for 420 seconds followed by a rising of the load arm at 0.5 mm/sec.

Fracture strength was determined by taking the peak tensile load (found at the beginning of the fracture) and normalizing this value for the projected surface area (PSA) of the probe. Calculation of PSA by the relation $PSA = \pi(r^2 - [r - a]^2)$ where r is the radius of the sphere as measured by calipers and a is the depth of penetration. Tensile work was determined by taking the area under the load vs. deformation curve and normalizing for PSA.

4.3.9 Everted Sac Bioadhesion Assay

Utilizing an everted sac assay modified from Santos et al [30] the relative bioadhesion for each nanosphere formulation was determined. Male, Sprague-Dawley rats weighing 200 – 250 g were anesthetized with 3 % isoflurane prior to a midline abdominal incision. The

jejunum was removed, flushed with PBSG, and immediately immersed in fresh phosphate buffered saline containing 200 mg/dL glucose (PBSG). Segments of jejunum, 6 cm in length, were everted, using a stainless steel rod, and ligated at both ends with silk 0-0 monofilament sutures. The everted sac was then filled with ~2 mL PBSG and immersed in a nanosphere suspension prepared as follows. Pre-warmed (37 °C) PBSG was added to 60 mg of formulation (0.4 % w/v) and bath sonicated for 5 minutes. Once the isolated loop was added to the nanosphere suspensions, samples were placed on an end-over-end mixer at 37 °C for a 30-minute period. During this incubation period nanospheres are allowed to adhere spontaneously to the everted intestinal loop. Following incubation, the everted sac was removed and processed.

To quantify the amount of marker polymer (polystyrene or PMMA) in both the bound (everted sac tissue sample) and unbound (remaining nanosphere suspension) samples, gel permeation chromatography was used as described in further detail below. Results are presented as a binding ratio for each formulation with the binding ratio being defined as the ratio of the amount detected on the everted sac tissue to the amount detected in the suspended supernatant after removal of the everted sac tissue.

4.3.10 In Vivo Dosage Preparation

Suspensions of nanosphere formulations were utilized for all in vivo experiments. Polystyrene nanospheres are supplied as a 2.5 % w/v suspension and were used as received. For PMMA and PBMA/PMMA formulations the powder formulation was added to 1% sodium lauryl sulfate (SLS)/1 % w/v polyvinylpyrrolidone (PVP) to a final concentration of 2.5 % w/v. To adequately disperse the formulations, solutions were bath sonicated for 10 minutes immediately before administration. A dosage volume of 1 mL was used in all studies and resulted in a total dose of 25 mg.

4.3.11 Isolated Loop

For a controlled, direct administration of formulations to a specific gastrointestinal region an isolated loop technique was employed. Male, Sprague-Dawley rats, weighing 200 – 250 g were allowed access to standard chow and water ad libitum. Rats were anesthetized

with 3 % isoflurane inhalation. A 6 cm length of jejunum was isolated by suture ligation with monofilament silk 0-0 sutures at each end taking care not to disrupt blood flow from mesenteric arteries. Suspensions of the nanospheres were then injected directly into the isolated region's lumen. The isolated loop was returned to the abdomen, which was then closed to maintain body temperature and moisture. Following a 5 hour incubation period, the following samples were collected in order: 1 mL blood from portal vein, 1 mL blood from celiac artery, lungs, heart, spleen, kidneys, liver, isolated loop, rinse of isolated loop, and brain. All tissues were stored at -18 °C until further processing. Studies were run in animal cohort groups of 4 (n=4) for each study.

4.3.12 Tissue Processing and Polymer Detection

All samples from everted sac bioadhesion analysis and in vivo isolated loop experiments were processed and quantitatively analyzed for polymer content by gel permeation chromatography (GPC). Tissue samples were physically cut into small pieces, added to ~5 mL PBS and homogenized on a Cole-Palmer Ultrasonic Homogenizer CV26 with a high gain Q horn and extender set at 40 % amplitude for 30 seconds. Homogenized samples were then lyophilized for 48 hours resulting in a powdered tissue digest. An extraction of the marker polymer was then performed by the addition of chloroform and mixing on an end-over-end mixer for 96 hours. Extractions were then filtered through PTFE filters of 0.2 μ m pore diameter to remove non-soluble debris. Filtered extractions were lyophilized for 24 hours and stored at -18 °C until analysis.

Lyophilized extracts were reconstituted in 1 mL of chloroform by mixing on an end-over-end mixer for 1 hour. The solution was filtered a final time through a 0.1 μ m PTFE syringe filter and ran on a Shimadzu GPC equipped with Waters Styragel HR5E and HR4E columns and a Shimadzu RID-10A refractive index detector.

A specific peak for the marker polymer was identified and the area under the curve was calculated then related to the polymer concentration by comparison to a linear standard curve for PS and PMMA with R^2 values of 0.9993 and 0.9998, respectively. Sensitivity of the assay was tested over a range of 0.05 mg/mL to 25 mg/mL from doped tissue extracts. The linear relationship existed over the range of 0.2 mg/mL to 25 mg/mL. Below 0.8 mg/mL,

peaks had to be manually selected as they were below the peak detection threshold of the software. Since the sample volume is known, the exact amount of polymer can be calculated from the detected concentrations.

The resulting data was computed to yield quantitative information regarding the uptake and tissue distribution by the following calculations. Percent uptake was calculated by taking the sum of all amounts detected in tissues (excluding isolated loop and loop rinse samples) divided by the total dose administered and multiplied by 100. Tissue distribution could be directly compared for each tissue across study groups. However, further information may be gained by comparison of each tissue across study groups after normalizing for uptake. Mass balance was determined as the sum of all amounts detected in tissues, isolated loop and loop rinse samples divided by the total administered dose. Mass balance values of $49.54 \pm 0.78\%$, $102.96 \pm 7.78\%$ and $111.34 \pm 67.0\%$ for the PMMA, PS and PBMA experiments, respectively.

4.3.13 Statistical Analysis

Standard errors were calculated and a 1-way ANOVA was performed with Microcal Origin Graphical Software (Northampton, MA). Significance was determined at $p \leq 0.05$.

4.4 Results and Discussion

Commercially available PS nanospheres were used as a moderately bioadhesive formulation, and non-bioadhesive poly(methyl methacrylate) (PMMA) nanospheres were fabricated for comparison. Additionally, for a highly bioadhesive polymer model, non-adhesive PMMA nanoparticles were coated with poly(butadiene-maleic anhydride-co-L-DOPA) (PBMA), selected due to its high bioadhesivity and unique pH-dependent solubility in the gastrointestinal environment. Adhesion measurements of the bulk materials were compared to the everted sac measurements of each nanosphere formulation followed by quantification of the total uptake and biodistribution of the nanospheres after direct injection into isolated intestinal loops of rats *in vivo*.

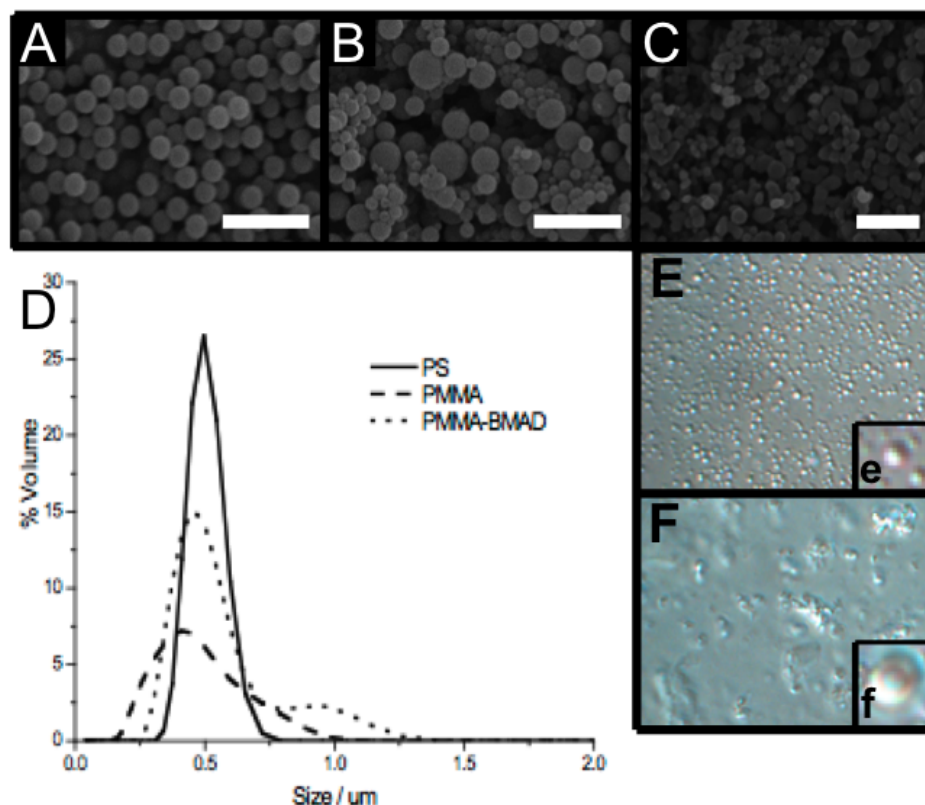


Figure 4.2: Scanning electron microscopy of PS (A), PMMA (B), and PBMA/PMMA (C) nanospheres. Scale bars = 2.0 μm . Size distribution profiles for each formulation as measured by laser diffraction are also shown (D). Optical microscopy of PMMA (E, e) and PBMA/PMMA (F, f) nanospheres at 40x (upper case) and 120x (lower case) magnification.

4.4.1 Nanosphere Characterization

PMMA and PBMA/PMMA nanospheres were spherical with a smooth external surface morphology. SEM images of each formulation are shown in figure 4.2A-C. Average diameters of PMMA and PBMA/PMMA nanospheres, as measured by laser diffraction, are 490 ± 550 and 540 ± 650 nm, respectively, which is comparable to the purchased 540 ± 80 nm PS nanospheres. Size distribution profiles of PS, PMMA and PBMA/PMMA nanospheres are shown in figure 4.2D.

Imaging with light microscopy showed a well-dispersed population of PMMA nanospheres (Figure 4.2E) that appear translucent (Figure 4.2e). PBMA/PMMA nanospheres had a tendency to aggregate into irregular clumps when in solution (Figure 4.2F) and appear yellow-brown due to optical properties of PBMA. When imaged under high magnification

(Figure 4.2f), the yellow-brown colored PBMA is observed to have coated the clear PMMA core. FTIR of the raw materials of PMMA and PBMA and the PBMA/PMMA formulation (data not shown) confirm that both polymers are present in the final formulation while DSC analysis (data not shown) confirmed the phase separation of the two polymers in the formulation.

4.4.2 Bioadhesion Testing

Bioadhesive measurements were performed on both the bulk materials and all nanosphere formulations. Adhesion measurements of the bulk materials, performed with a Texture Analyzer probe on freshly excised rat intestinal tissue, yielded statistically insignificant results for PMMA and PS, but PBMA had a significant increase in bioadhesion both in terms of fracture strength (103.6 ± 8.3 , 193.1 ± 31.4 and 242.6 ± 21.2 mN/cm², respectively), as shown in Figure 4.3A, and tensile work (0.327 ± 0.09 , 309.3 ± 28.6 and 20445.0 ± 5733.5 nJ, respectively).

Bioadhesion of the nanosphere formulations were directly measured with the use of an everted sac technique with rat intestinal sections followed by quantitative extraction of nanospheres and gel permeation chromatography analysis. Results are shown as a binding ratio (amount adhered to tissue:amount remaining in suspension) in Figure 4.3B for PS, PMMA and PBMA/PMMA nanospheres. PMMA nanospheres had the lowest bioadhesion with a binding ratio of 1.49 ± 0.82 , followed by PS nanospheres with a binding ratio of 2.51 ± 0.40 and PBMA/PMMA nanospheres had the highest bioadhesion with a binding ratio of 3.67 ± 2.38 . There was an increase in bioadhesion of the PBMA/PMMA nanospheres over the PMMA nanospheres with PS having an intermediate level of bioadhesion. The everted sac technique is useful in measuring the bioadhesion of the actual nanosphere formulations without an external force being applied; however, the variation with this method was high.

Comparison of the everted sac data for the bioadhesion of the nanospheres to the texture analyzer data for the bioadhesion of the bulk polymer revealed a strong direct correlation ($R^2 = 0.9608$) suggesting that the bioadhesive properties of the bulk polymer can be used to predict bioadhesive behavior on the nanoscale and vice versa (Figure 4.5A).

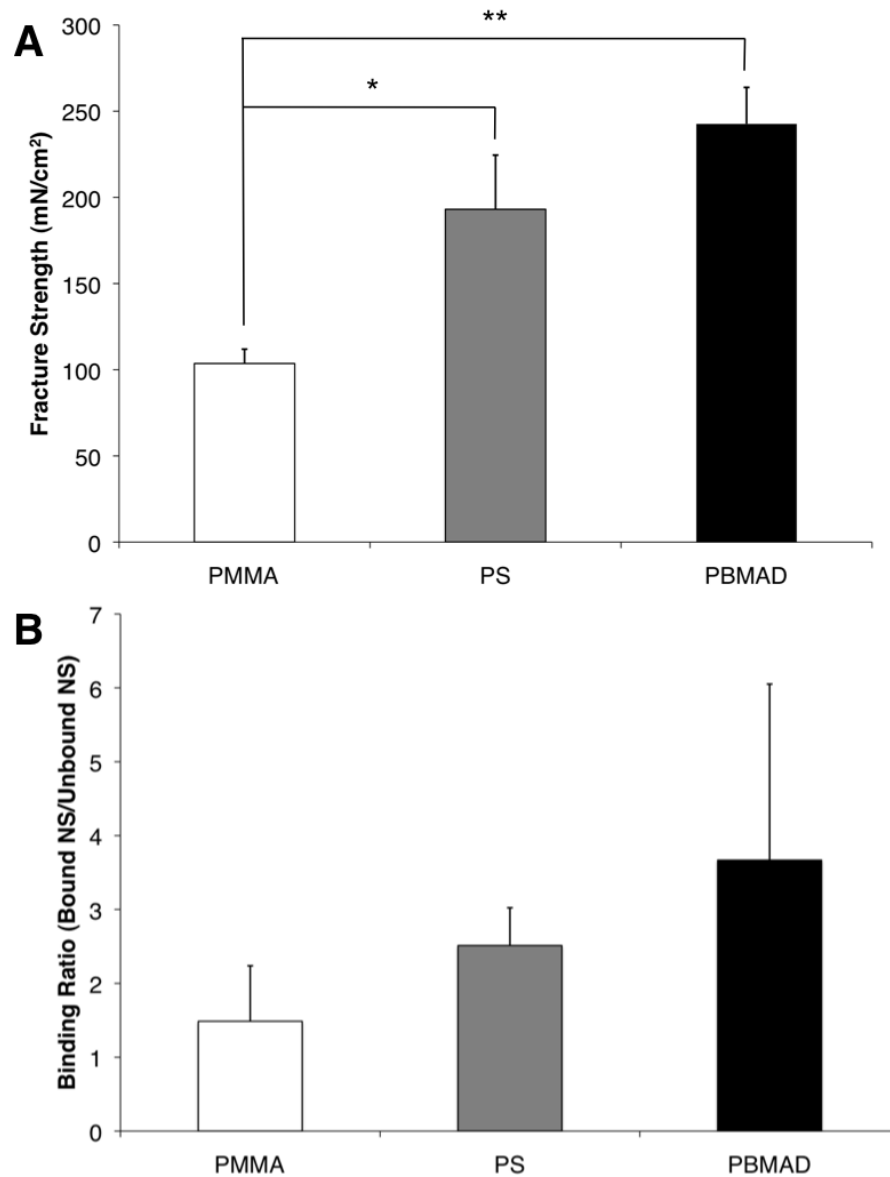


Figure 4.3: (A) In vitro bioadhesion: texture analyzer bioadhesion testing of bulk polymers: fracture strength of polymer probes with jejunum (n=6), * p < 0.05, ** p < 0.01; (B) ex vivo bioadhesion: everted sac assay using 500 nm nanospheres with the small intestines of rats (n=6).

4.4.3 Total Uptake and Biodistribution

Delivering 500 nm PS, PMMA and PBMA/PMMA nanospheres locally to the rat jejunum for a 5-hour period resulted in uptake of $45.8 \pm 8.6\%$, $5.8 \pm 1.9\%$ and $66.9 \pm 12.9\%$ the administered dose, respectively (Figure 4.4A). Most surprisingly, by adding a bioadhesive PBMA coating to the PMMA nanospheres, the uptake of these nanoparticles from the jejunum was increased substantially from $5.8 \pm 1.9\%$ to $66.9 \pm 12.9\%$, a significant improvement over the non-bioadhesive PMMA formulation. This provides strong evidence for the use of bioadhesive polymers as a means of enhancing particle uptake from the small intestine. This is significant not only due to the high amount of uptake of the bioadhesive nanospheres, but also because it occurred in the jejunum, tissue free of Peyer's patches. Therefore, uptake likely occurred via the absorptive epithelial comprising of $\sim 98\%$ of the surface of the small intestine. The likely mechanisms involved are a current area of further investigation in our laboratory.

The organ distribution following nanosphere uptake of the 500 nm PMMA, PS and PBMA/PMMA formulations differed by a great amount as is evident in figure 4.4B. The PS nanospheres distributed almost entirely to the liver with some evident in the kidneys as well. However, for PBMA/PMMA nanospheres an insignificant amount distributed to the liver, but instead distributed to many other tissues. PBMA/PMMA nanospheres had 27.4 ± 16.4 , 27.0 ± 12.5 , 17.5 ± 15.2 and $13.1 \pm 10.3\%$ of the total uptake distributed to the heart, central blood compartment, spleen and lungs, respectively. Despite both formulations being similar in size and morphology, they had largely different biodistribution profiles. The different surface chemistries likely result in the varied biodistribution profiles and lends potential to passive targeting of nanospheres from the oral route by altering the surface properties of nanospheres.

It is evident from the uptake data that the bioadhesive formulation (PBMA/PMMA nanospheres) resulted in a much higher amount of intestinal uptake. Although many research groups, including our own, have theorized the potential benefit of bioadhesive nanospheres in oral drug delivery, this is the first time that a direct, quantitative study has confirmed these theories. Comparison of the bioadhesion measurements to the total intestinal uptake

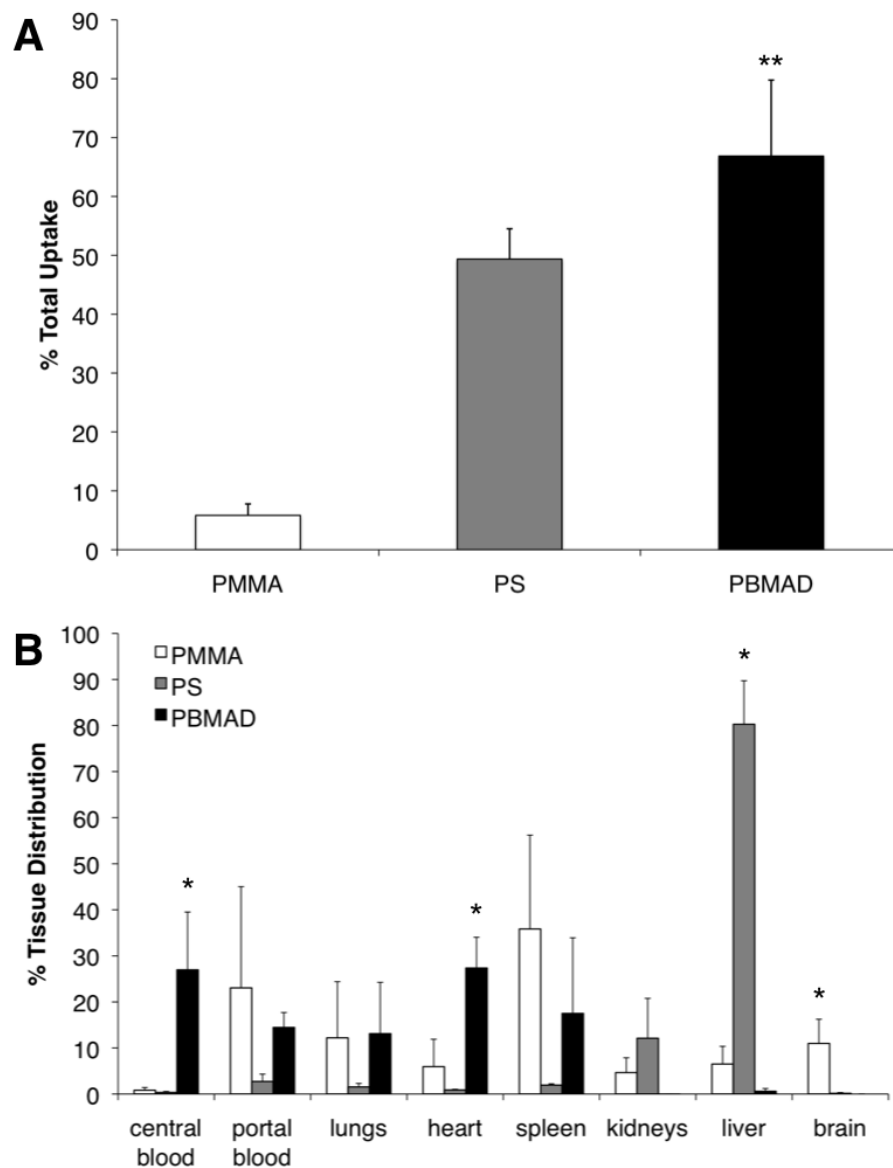


Figure 4.4: (A) In vivo uptake: total uptake of 500 nm nanospheres from isolated loop uptake studies in the jejunum (n = 6), ** p < 0.01; (B) biodistribution: % tissue distribution of translocated 500 nm nanospheres following administration to the jejunum via isolated loop uptake study (n=6), * p < 0.05.

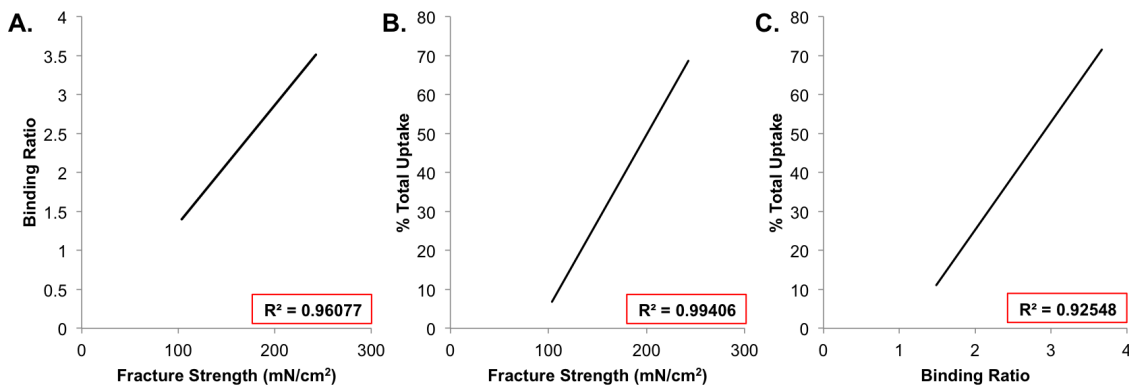


Figure 4.5: Correlation between texture analyzer (in vitro), everted sac bioadhesion (ex vivo) and isolated loop uptake studies (in vivo); (A) fracture strength vs. binding ratio, (B) fracture strength vs. % total uptake, (C) binding ratio vs. % total uptake.

showed a strong linear correlation regardless of whether the nanosphere formulations were measured ($R^2 = 0.9255$) or the bioadhesion of the bulk materials were measured ($R^2 = 0.9941$) (Figure 4.5B and C).

4.5 Conclusions

In summary, this work demonstrates the high potential of bioadhesive nanospheres for applications in the delivery of therapeutics via oral administration as evidenced by the strong correlation of intestinal uptake to the bioadhesive property of the bulk material as well as the bioadhesive property of the nanosphere formulation. Furthermore, the strong correlation observed between the bioadhesion testing of the bulk polymer and the nanospheres suggests that effective methods of testing bioadhesive phenomena can provide insight into both the macro- and nanoscale behavior of bioadhesive polymers. Lastly, nanospheres of different surface chemistries (PMMA, PS and PMAD) were observed to distribute differently to organs following internalization and may allow for the passive targeting of oral nanosphere drug delivery systems. This finding is currently under further investigation by the authors.

4.6 References

- [1] K. Park, J.R. Robinson, Bioadhesive polymers as platforms for oral-controlled drug delivery: method to study bioadhesion, *International Journal of Pharmaceutics*. 19 (1984) 107–127.
- [2] E. Mathiowitz, D. Chickering, J.S. Jacob, C. Santos, Bioadhesive Drug Delivery Systems, in: E. Mathiowitz (Ed.), *Encyclopedia of Controlled Drug Delivery*, 1st ed. John Wiley & Sons, Inc., New York, NY, n.d.: pp. 9–945.
- [3] M. Szycher, *High performance biomaterials*, CRC, 1991.
- [4] Y. Huang, W. Leobandung, A. Foss, N.A. Peppas, Molecular aspects of muco- and bioadhesion: tethered structures and site-specific surfaces., *J Control Release*. 65 (2000) 63–71.
- [5] G. Ponchel, J.M. Irache, Specific and non-specific bioadhesive particulate systems for oral delivery to the gastrointestinal tract, *Advanced Drug Delivery Reviews*. (1998).
- [6] M.A. Longer, H.S. Ch'ng, J.R. Robinson, Bioadhesive polymers as platforms for oral controlled drug delivery III: oral delivery of chlorothiazide using a bioadhesive polymer., *J. Pharm. Sci.* 74 (1985) 406–411.
- [7] N.A. Peppas, J.J. Sahlin, Hydrogels as mucoadhesive and bioadhesive materials: a review., *Biomaterials*. 17 (1996) 1553–1561.
- [8] S. Tamburic, D.Q.M. Craig, A comparison of different in vitro methods for measuring mucoadhesive performance, *European Journal of Pharmaceutics and Biopharmaceutics*. 44 (1997) 159–167.
- [9] N.A. Peppas, P.A. Buri, Surface, interfacial and molecular aspects of polymer bioadhesion on soft tissues, *Journal of Controlled Release*. 2 (1985) 257–275.
- [10] F. Madsen, K. Eberth, J.D. Smart, A rheological examination of the mucoadhesive/mucus interaction: the effect of mucoadhesive type and concentration., *J Control Release*. 50 (1998) 167–178.
- [11] S.-H.S. Leung, J.R. Robinson, The contribution of anionic polymer structural features to mucoadhesion, *J Control Release*. 5 (1988) 223–231.
- [12] D. Chickering, J. Jacob, E. Mathiowitz, Poly(fumaric-co-sebacic) microspheres as oral drug delivery systems., *Biotechnol. Bioeng.* 52 (1996) 96–101.
- [13] C.M. Lehr, J.A. Bouwstra, W. Kok, A.B. Noach, A.G. de Boer, H.E. Junginger, Bioadhesion by means of specific binding of tomato lectin., *Pharm Res.* 9 (1992) 547–553.

- [14] D. Norris, N. Puri, P. Sinko, The effect of physical barriers and properties on the oral absorption of particulates., *Advanced Drug Delivery Reviews*. 34 (1998) 135–154.
- [15] L.A. Kotkoskie, M.T. Butt, E. Selinger, C. Freeman, M.L. Weiner, Qualitative investigation of uptake of fine particle size microcrystalline cellulose following oral administration in rats., *J. Anat.* 189 (Pt 3) (1996) 531–535.
- [16] G. Ponchel, F. Touchard, D. Duchêne, N.A. Peppas, Bioadhesive analysis of controlled-release systems. I. Fracture and interpenetration analysis in poly(acrylic acid)-containing systems, *Journal of Controlled Release*. 5 (1987) 129–141.
- [17] N.A. Peppas, *Hydrogels in Medicine and Pharmacy*, CRC Press, Boca Raton, FL, 1987.
- [18] J. Vasir, Bioadhesive microspheres as a controlled drug delivery system, *International Journal of Pharmaceutics*. 255 (2003) 13–32.
- [19] S. Sakuma, R. Sudo, N. Suzuki, H. Kikuchi, M. Akashi, Y. Ishida, et al., Behavior of mucoadhesive nanoparticles having hydrophilic polymeric chains in the intestine., *J Control Release*. 81 (2002) 281–290.
- [20] G.P. Carino, J.S. Jacob, E. Mathiowitz, Nanosphere based oral insulin delivery., *J Control Release*. 65 (2000) 261–269.
- [21] P. Arbós, M.A. Campanero, M.A. Arangoa, M.J. Renedo, J.M. Irache, Influence of the surface characteristics of PVM/MA nanoparticles on their bioadhesive properties., *J Control Release*. 89 (2003) 19–30.
- [22] B. Kriwet, E. Walter, T. Kissel, Synthesis of bioadhesive poly(acrylic acid) nano- and microparticles using an inverse emulsion polymerization method for the entrapment of hydrophilic drug candidates., *J Control Release*. 56 (1998) 149–158.
- [23] M.A. Jepson, N.L. Simmons, D.T. O'Hagan, B.H. Hirst, Comparison of poly(DL-lactide-co-glycolide) and polystyrene microsphere targeting to intestinal M cells., *Journal of Drug Targeting*. 1 (1993) 245–249.
- [24] C. Porta, P. James, A. Phillips, Confocal analysis of fluorescent bead uptake by mouse Peyer's patch follicle-associated M cells, *Experimental Physiology*. 77 (1992) 929–932.
- [25] N. Hussain, V. Jaitley, A.T. Florence, Recent advances in the understanding of uptake of microparticulates across the gastrointestinal lymphatics., *Advanced Drug Delivery Reviews*. 50 (2001) 107–142.
- [26] A. Florence, A. Hillery, N. Hussain, Nanoparticles as carriers for oral peptide absorption: Studies on particle uptake and fate., *Journal of Controlled Release*. 36 (1995) 39–46. [27] P. Jani, G.W. Halbert, J. Langridge, A.T. Florence, The uptake and

- translocation of latex nanospheres and microspheres after oral administration to rats., *J. Pharm. Pharmacol.* 41 (1989) 809–812.
- [28] A. des Rieux, V. Fievez, M. Garinot, Y.-J. Schneider, V. Préat, Nanoparticles as potential oral delivery systems of proteins and vaccines: a mechanistic approach., *J Control Release.* 116 (2006) 1–27.
- [29] E. Mathiowitz, J.S. Jacob, Y.S. Jong, G.P. Carino, D.E. Chickering, P. Chaturvedi, et al., Biologically erodable microspheres as potential oral drug delivery systems., *Nature.* 386 (1997) 410–414.
- [30] C.A. Santos, J.S. Jacob, B.A. Hertzog, B.D. Freedman, D.L. Press, P. Harnpicharnchai, et al., Correlation of two bioadhesion assays: the everted sac technique and the CAHN microbalance., *J Control Release.* 61 (1999) 113–122.
- [31] C. Santos, Evaluation of anhydride oligomers within polymer microsphere blends and their impact on bioadhesion and drug delivery in vitro, *Biomaterials.* 24 (2003) 3571–3583.
- [32] B. Laulicht, P. Cheifetz, A. Tripathi, E. Mathiowitz, Are in vivo gastric bioadhesive forces accurately reflected by in vitro experiments?, *J Control Release.* 134 (2009) 103–110.
- [33] D.E. Chickering, W.P. Harris, E. Mathiowitz, A microtensiometer for the analysis of bioadhesive microspheres., *Biomed Instrum Technol.* 29 (1995) 501–512.
- [34] C.G. Thanos, Z. Liu, M. Goddard, J. Reineke, N. Bailey, M. Cross, et al., Enhancing the oral bioavailability of the poorly soluble drug dicumarol with a bioadhesive polymer., *J. Pharm. Sci.* 92 (2003) 1677–1689.
- [35] J.J. S, M. Edith, A Novel Mechanism for Spontaneous Encapsulation of Active Agents: Phase Inversion Nanoencapsulation, in: *ACS Symposium Series CY -*, American Chemical Society, Washington, DC, 2011: pp. 214–223–223.

Chapter 5

Fabrication of Double-Walled Polymeric Nanospheres by Sequential Phase Inversion Nanoencapsulation

5.1 Abstract

A single step method for the preparation of double-walled polymeric nanospheres is presented. The process, based on phase inversion nanoencapsulation (PIN), depends on the sequential phase separation of two polymers. By choosing the correct solvent and non-solvent pairs for the two polymers and engineering the sequential phase inversion of the core polymer followed by the shell polymer, double-walled nanospheres can be prepared nearly instantaneously in a single step. A series of double-walled formulations were prepared with various core and shell polymers then characterized using laser diffraction particle sizing, scanning electron microscopy, Fourier transform infrared spectroscopy, differential scanning calorimetry and confocal microscopy. This method is particularly advantageous because it can be used to produce 500 nm nanoparticles with core-shell morphologies through a rapid spontaneous self-assembly process avoiding high shear rates and prolonged exposure to harsh solvents, which could result in damage to or the loss of bioactivity of the therapeutic cargo.

Daniel Y. Cho, Kenneth Estrellas, Alexis Mancini, Alexander Leydon, Cheng Tian, Taylor Peak, Joshua Reineke and Edith Mathiowitz

5.2 Introduction

Polymer microspheres have long been researched as a means for the controlled delivery of therapeutic agents. With the recent surge of research in nanotechnology, nanospheres have become an area of significant interest for the field of drug delivery. While there have been many advances in the development of more sophisticated methods for fabricating microspheres, translating these technologies to the nanoscale has proven to be quite challenging. One such example is double-walled microspheres, which have potential for use in drug delivery by enhancing controlled release, reducing the effect of the initial burst release of drug trapped on the surface and also allowing for the sequential release of multiple drugs localized to each layer [1]; however, to the best of our knowledge, methods to fabricate double-walled nanospheres for drug delivery applications have been elusive.

Traditionally, the preparation of large double-walled microspheres involved multiple steps in which single-walled microspheres were fabricated then coated with a second polymer by pan-coating, dip-coating, spray drying or other similar processes. However, these methods often result in the incomplete or uneven deposition of the coating polymer while the need for additional steps in the manufacturing process decreases yields and presents difficulties in quality control and scale up. As micro- and nanoencapsulation techniques have become more sophisticated and yield smaller diameter particles, the technical challenges of coating them have made methods for the single-step preparation of double-walled micro- and nanospheres essential.

First described by Mathiowitz et. al., a modified solvent evaporation method was developed using polymer-polymer phase separation phenomena to prepare double-walled microspheres in the range of 20 to 1000 μm in a single step [2]. Since then, many groups have evaluated microspheres fabricated by this method using a variety of different polymer and drug combinations to evaluate fabrication parameters, drug localization, in vitro and in vivo degradation as well as drug release kinetics [2-9]. More recently, smaller diameter microspheres were fabricated by precision particle fabrication (PPF), which relies on a series of annular nozzles to create a compound jet of core and shell polymer solutions broken into uniform droplets by acoustic excitation into an aqueous bath for solvent evaporation [10-12].

Most recently, triple-walled microspheres were fabricated with layer localization of multiple drugs based on their affinity to each polymer [13]. All these studies have shown that the addition of a polymer shell results in smaller bursts and more sustained release of drugs encapsulated in the core than their single-walled counterparts.

The small diameter of nanospheres offers many advantages for drug delivery but also presents a variety of challenges in successfully obtaining controlled release. The high surface area to volume ratio of nanospheres often results in significant drug release during the initial burst, especially for proteins and peptides. In this case, the use of double-walled nanospheres could play an important role in the use of nanospheres for controlled release applications. Furthermore, given their high surface area, double-walled nanospheres could be further advantageous with the use of bioactive polymers as the shell to enhance nanoparticle-tissue interactions – for example, the use of bioadhesive polymers to enhance the retention of the nanospheres on mucosal surfaces.

In this study, we describe a single step method for the fabrication and characterization of double-walled nanospheres; this process is based on phase inversion nanoencapsulation (PIN), a method developed in our lab for the encapsulation of small molecules, proteins and genes in small diameter micro- and nanospheres [14,15].

5.3 Materials and Methods

5.3.1 Materials

Poly(lactic-co-glycolic acid) (PLGA, Resomer RG 502, 50:50; i.v. range 0.16 – 0.24 dL/g in CHCl_3), was purchased from Boehringer-Ingelheim, Inc. (Ridgefield, CT), PLGA (75:25; i.v. 0.67 dL/g in CHCl_3) and D,L-poly(lactic acid) (D,L-PLA; i.v. 0.21 dL/g in CHCl_3) were purchased from Birmingham Polymers, Inc. (Pelham, AL); these polymers were stored at -15°C until use. Poly(methyl methacrylate) (PMMA, atactic; MW: 100 kDa) was obtained from Polysciences, Inc. (Warrington, PA) and stored at room temperature.

All novel bioadhesive poly(butadiene-maleic anhydride) (PBMA; MW=10 - 15 kDa)-derivative and poly(ethylene-maleic anhydride) (PEMA; MW= 400 kDa)-derivative polymers were synthesized via a ring-opening, side-chain conjugation reaction in dimethyl sul-

foxide (DMSO); the pBMA and pEMA were purchased from Polysciences, Inc. (Warrington, PA) while the amino acid conjugates were obtained from Sigma-Aldrich Co. (St. Louis, MO). These polymers include poly(butadiene-maleic anhydride-co-L-phenylalanine) (PBMAP), poly(butadiene-maleic anhydride-co-L-tyrosine) (PBMAT), poly(butadiene-maleic anhydride-co-L-DOPA) (PBMAD), poly(ethylene-maleic anhydride-co-L-phenylalanine) (PEMAP), poly(ethylene-maleic anhydride-co-L-tyrosine) (PEMAT) and poly(ethylene-maleic anhydride-co-L-DOPA) (PEMAD); these polymers were stored at room temperature. All solvents were of the highest commercial grade available.

5.3.2 Cloud Point Testing

The cloud point of a polymer solution corresponds to the transition from a translucent to a metastable cloudy turbid state due to the separation of the solution into two distinct phases, one with a high relative polymer concentration and another with a low relative polymer concentration.

For the each formulation, THF was selected as the solvent for the core polymer and ethanol was selected as the solvent for the shell polymer; other polymer/solvent combinations have also been successfully evaluated for use with sPIN. To determine the cloud point of the each core polymer solution, ethanol was added in 100 μ L increments to a 2% w/v polymer solution until the solution became turbid and resulted in no increase in the turbidity of the solution with the further addition of ethanol but without precipitation of the polymer. Once the cloud point was determined, the cloud point testing was repeated using a 2% w/v solution of the shell polymer in ethanol to ensure the presence of the second polymer did not affect the cloud point. In each case evaluated, we have found that the polymer that was added to the ethanol did not change the cloud point. To prepare particles with PMMA as a core the cloud point of PMMA in THF was determined with PBMAP, PBMAT, PBMAD, PEMAP, PEMAT and PEMAD in ethanol, as they were used as the second shell-layers (Table 5.1). The following polymers were used as core materials: PMMA, PLGA (75:25), PLGA (50:50) and D,L-PLA and thus the cloud point was determined in THF using PBMAD in ethanol as a second layer (Table 5.1).

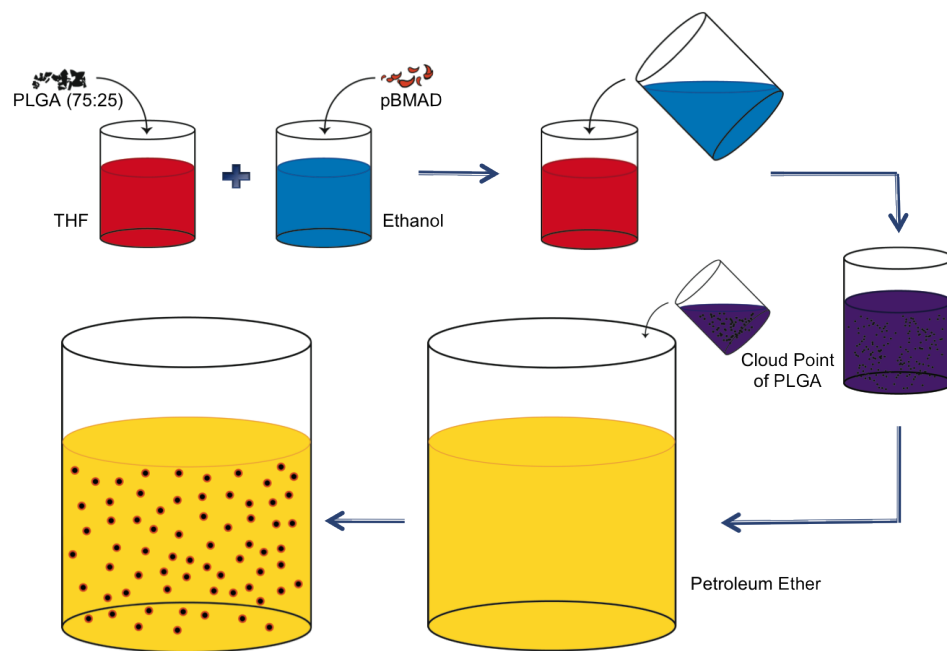


Figure 5.1: Schematic of the sequential phase inversion nanoencapsulation (sPIN) process for the PBMA/PLGA (75:25) double-walled formulation.

5.3.3 Sequential Phase Inversion Nanoencapsulation

To fabricate double-walled nanospheres consisting of PMMA-cores with various shell polymers, the appropriate volume of 2% w/v solution of each shell polymer in ethanol was added to 5 mL of a 2% w/v solution of PMMA in tetrahydrofuran (THF) until the cloud point for PMMA was reached. Similarly, to prepare nanospheres consisting of a PBMA-shell with various core polymers, the appropriate volume (as determined in section 5.3.2) of 2% w/v solution of PBMA in ethanol was added to 5 mL of a 2% w/v solution of each core polymer in THF until the cloud point for the core polymer was reached.

Each solution at the cloud point for the core polymer was then rapidly added to a non-solvent bath of petroleum ether with a total solvent to non-solvent ratio of 1:75 resulting in the spontaneous formation of double-walled nanospheres then stirred manually for 2 minutes to allow for the extraction of residual solvent and nanospheres curing (Figure 5.1). The resulting spheres were filtered using a 0.2 μm Fluoropore PTFE membrane filter in a positive pressure filtration column (Millipore, Inc.; Billerica, MA), flash frozen in liquid nitrogen and lyophilized for 24 hours.

To allow for the localization of the polymers using confocal microscopy, fluorescein isoth-

iocyanate (FITC)-labeled insulin was incorporated into PLGA using a cryogenic emulsion method. Briefly, 1 mg of FITC-insulin dissolved in 200 μ L of water was emulsified in 2 mL of a 10% w/v PLGA/methylene chloride solution by vortexing for 1 minute. The resulting w/o emulsion was flash frozen in liquid nitrogen and lyophilized for 24 hours. This method allows for the preferential association of the protein with the polymer in the form of a solid dispersion of nanonized FITC-insulin within the continuous phase of PLGA, which was reconstituted in 10 mL of THF for encapsulation with PBMA via sPIN as described above to produce spheres with a PBMA shell and a fluorescently-labeled PLGA core.

5.3.4 Characterization

5.3.4.1 Particle Size Analysis

All formulations were reconstituted from powder form into reconstitution media consisting of an aqueous 1% sodium lauryl sulfate (SLS) / 1% polyvinylpyrrolidone (PVP) solution in 0.5 – 2% w/v suspensions with bath sonication. A Beckman Coulter LS230 Laser Diffraction Particle Size Analyzer (Beckman Coulter, Inc.; Brea, CA) was used to evaluate the size distribution of nanoparticle populations.

5.3.4.2 Fourier Transform Infrared Spectroscopy

All formulations and bulk polymers were analyzed as dry powders using a Perkin Elmer Spectrum One FTIR with a Universal ATR Sampling Accessory and analyzed using built-in Spectrum software (Perkin-Elmer, Inc.; Waltham, MA).

5.3.4.3 Differential Scanning Calorimetry

3 – 7 mg of each formulation and bulk polymers were sealed into aluminum sample pans for DSC analysis. Thermal analyses of the nanospheres were performed using a model DSC 7 (Perkin-Elmer, Inc.; Waltham, MA) equipped with controller model TAC 7/DX (Perkin-Elmer, Inc.; Waltham, MA). After equilibration at -20°C, samples were heated from -20 to 200°C, cooled to -20°C then reheated to 200°C at a rate of 10°C/min. Thermograms were analyzed using Perkin-Elmer Thermal Analysis software for the calculation of glass

transition temperatures (T_g).

5.3.4.4 Scanning Electron Microscopy

The morphology and particle size of the nanosphere formulations were analyzed by scanning electron microscopy. Formulations were placed on a carbon-backed adhesive and sputter-coated with Au-Pd for 4 minutes at 20 mA and imaged on a Hitachi 2700 Scanning Electron Microscope (Hitachi High Technologies America, Inc.; Pleasanton, CA) with an accelerating voltage of 8 kV.

5.3.4.5 Confocal Microscopy

The localization of the polymers within the nanospheres was evaluated using confocal microscopy using the 1% FITC-insulin PBMA/PLGA formulation. These spheres were suspended in 1% SLS/1% PVP and mounted onto glass slides with coverslips. A Zeiss LSM 510 Meta Confocal Laser Scanning Microscope was used to visualize the spheres using a 100x oil objective using DIC as well as fluorescence at excitation and emission wavelengths of 495 and 521 nm, respectively. Z-stack images were taken at 30 nm increments.

5.4 Results and Discussion

5.4.1 Sequential Phase Inversion Nanoencapsulation (sPIN) Rationale

Phase inversion nanoencapsulation (PIN) is a method developed by Mathiowitz et al. that can be used to spontaneously encapsulate therapeutic agents in microspheres and nanospheres in the range of 200 nm and 30 μ m [14]. This method is a relatively simple process in which a dilute polymer solution containing the target drug is rapidly added to an excess of non-solvent. The polymer in the solution undergoes a transition from a homogenous solution to an unstable two-phase mixture in which spherical droplets of non-solvent or drug in the polymer rich phase serve as nucleation sites for the precipitation of the polymer. In contrast to many of the traditional microencapsulation techniques that often require multiple steps and often expose therapeutic agents to denaturing conditions, PIN is advantageous in that it is a one-step encapsulation process that is nearly instantaneous and relatively gentle on

the target drug. The only requirement for the successful fabrication of microspheres by PIN is the appropriate selection of solvent and non-solvent: the solvent and non-solvent must be miscible for particle formation with a minimal solvent:non-solvent ratio of 1:40, though a ratio of 1:100 is optimal. Furthermore, the diameter of the particles produced by PIN is dependent on the viscosity of the initial polymer solution rather than the mixing rate of the initial emulsion as in traditional techniques. Using initial polymer concentrations in the range of 1 – 2 % (w/v) corresponding to solution viscosities of 1 – 2 cps generates particles of 100 – 500 nm while polymer concentrations of 2 – 5 % (w/v) with viscosities of 2–3 cps result in particles between 500 – 3000 nm [14].

The preparation of double-walled nanoparticles by sequential phase inversion nanoencapsulation (sPIN), is a single-step process that relies on phase separation and phase inversion phenomena to achieve the core-shell arrangement of the two polymers in the system. By modifying this method to allow for the controlled sequential phase inversion of two polymers, double-walled nanospheres can be prepared in a single step. In contrast to the methods for the preparation of double-walled microspheres described above which rely on polymer-polymer interactions, this method relies on the phase separation of polymers in solution upon addition of a non-solvent, a much more rapid process. Therefore, the formation of nanospheres is instantaneous and requires no shear forces, which is further beneficial for the preservation of bioactivity when encapsulating sensitive therapeutic agents, such as proteins or genetic material.

The careful selection of polymers and solvents is required to engineer conditions for the successful preparation of double-walled nanoparticles:

1. The core polymer solvent must be a solvent for the core polymer and shell polymer.
2. The shell polymer solvent must be a solvent for the shell polymer and a non-solvent for the core polymer; thus, upon addition of the shell polymer solution to the core polymer solution, the solubility of the core polymer will be decreased to induce phase separation while the shell polymer remains dissolved in the continuous phase.
3. A strong non-solvent for both polymers must be selected for the final phase inversion to produce double-walled nanospheres; all three solvents must be miscible and there

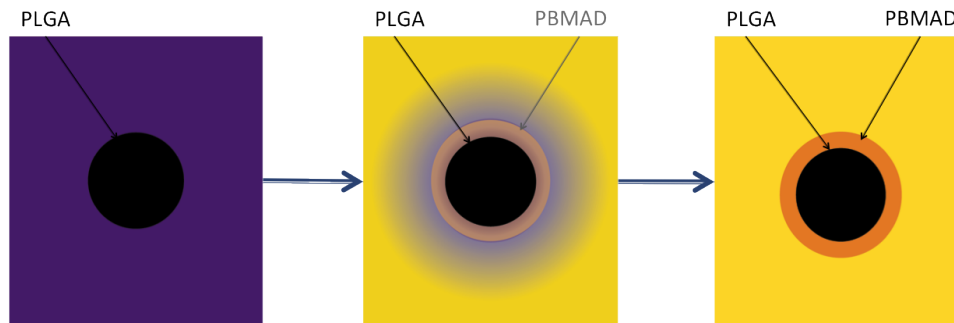


Figure 5.2: Hypothesized mechanism of particle formation via the sequential phase inversion nanoencapsulation (sPIN) process; (a) cloud point of PLGA in THF-ethanol mixed solvent solution, the PBMAD is fully soluble; (b) phase inversion of the PBMAD shell around the PLGA core in the environment of excess non-solvent; (c) double-walled PBMAD-shell/PLGA-core nanosphere.

must an excess of non-solvent with a solvent to non-solvent ratio of at least 1:40 but ideally 1:100.

Thus, to preferentially create the core of the nanoparticles, the two dilute polymer solutions are mixed together at the appropriate experimentally determined ratio where the non-solvent behavior of the shell polymer solution induces the cloud point of the core polymer, thus forming a cloudy suspension of phase separated core polymer droplets (Figure 5.2). The rapid addition of this cloudy suspension to an excess of a non-solvent for both polymers results in the diffusion of the polymer solvents into the continuous phase of non-solvent causing the spontaneous, nearly instantaneous self-assembly of double-walled nanospheres (Figures 5.2b-c), since the phase separated core polymer droplets serve as nucleation sites for the phase inversion of the shell polymer.

To illustrate the versatility this process, double-walled nanospheres with different core- and shell-polymer combinations have been prepared by sequential phase inversion nanoencapsulation (sPIN), as listed in tables 5.2 and 5.3. The first step was the evaluation the cloud point of each polymer solution.

5.4.2 Cloud Point Testing

The cloud points of each 2% w/v core polymer solution in THF was evaluated using ethanol, as it is a non-solvent for the core polymer but a solvent for the bioadhesive shell polymers.

Polymer	Solvent 1 (S1)	Solvent 2 (S2)	Cloud Point (mL _{S2} /mL _{S1})
PMMA	THF	Ethanol	2.801
PLGA (50:50)	THF	Ethanol	1.560
PLGA (75:25)	THF	Ethanol	2.519
D,L-PLA	THF	Ethanol	1.280

Table 5.1: Cloud point testing of 2% w/v core polymer solutions in THF with shell polymer solvent, ethanol; the cloud point results are presented as the ratio of the volume of shell polymer solvent to volume of core polymer solution at stable cloudy turbidity.

As indicated in table 5.1, the cloud point of each core polymer solution was successfully induced with the addition of a sufficient volume of ethanol; the cloud points of PMMA, PLGA(50:50), PLGA(75:25) and D,L-PLA were achieved with ethanol:THF ratios of 2.801, 1.560, 2.519 and 1.280, respectively. This difference in the required volume of ethanol to induce cloud point formation is likely the result of the solubility parameter of each polymer in THF.

Additionally, to confirm that the presence of the secondary shell polymer does not affect the cloud point of the core polymer, these tests were performed using 2% solutions of the shell polymer in ethanol. The results indicated that the presence of the shell polymer in the system gave the same cloud point for the core polymer, suggesting that the phase separation in this system is the consequence of the reduction of polymer solubility in the presence of a non-solvent rather than the polymer/polymer interactions previously used in the preparation of double-walled microspheres.

5.4.3 Nanosphere Preparation

Using the experimentally determined cloud point for each core polymer solution, a series of double-walled nanospheres with various core and shell polymer combinations were prepared, as listed in tables 5.2 and 5.3.

For example, a 2% solution of PLGA(50:50) in THF reaches its cloud point with the addition of 1.560 mL of ethanol per mL of core polymer solution (Table 5.1). Thus, for the preparation of the PBMA/PLGA(50:50) formulation, 7.8 mL of a 2% solution of PBMA in ethanol was added to 5 mL of the PLGA solution. The rapid addition of the shell polymer solution results in the phase separation of the PLGA causing the solution to take

on a turbid, cloudy appearance while the amber colored PBMA_D remains in solution. This cloudy solution was immediately added to 1000 mL of petroleum ether, for a solvent to non-solvent ratio of approximately 1:75, resulting in the phase inversion of the PBMA_D polymer around the already phase separated PLGA droplets (Figure 5.1). The final nanospheres took on the form of a fine amber-colored powder, which suggests that the colored PBMA_D polymer is indeed coating the white colored PLGA. All other formulations were prepared in a similar manner.

To illustrate the versatility of this process, a series of nanospheres with different shell polymers were fabricated using PBMA_P, PBMA_T, PBMA_D, PEMA_P, PEMA_T and PEMA_D with PMMA as a core polymer. Likewise, using a PBMA_D shell, formulations with different cores of PMMA, PLGA(50:50), PLGA(75:25) and D,L-PLA were also prepared and characterized.

5.4.4 Nanosphere Characterization

Double-walled nanospheres were prepared using the sPIN method to form particles with various shell polymers and core polymers; some representative formulations have been presented in tables 5.2 and 5.3. For the shell polymers, we have evaluated a series of novel bioadhesive polymers that we have developed in our lab for use in oral drug delivery applications; for the core polymers, we have evaluated PMMA as a model polymer as well as a variety of biodegradable polymers commonly used in drug delivery applications. While the results of the characterization of multiple formulations will be presented, the PBMA_D-shell/PLGA (75:25)-core formulation will be the focus of the discussion.

5.4.4.1 Particle Size Analysis

As can be seen in figure 5.3, laser diffraction particle size analysis revealed that the sPIN process using 2% w/v polymer solutions results in two populations of particles, one in the range of 500 nm and another in the range of 2 μ m. The production of particles in the 500 nm range is consistent with previous work in our lab preparing single-walled nanoparticles with PIN, which revealed that particle size is directly related to the polymer solution viscosity, a function of polymer concentration [14]. Like PIN, this method requires the use

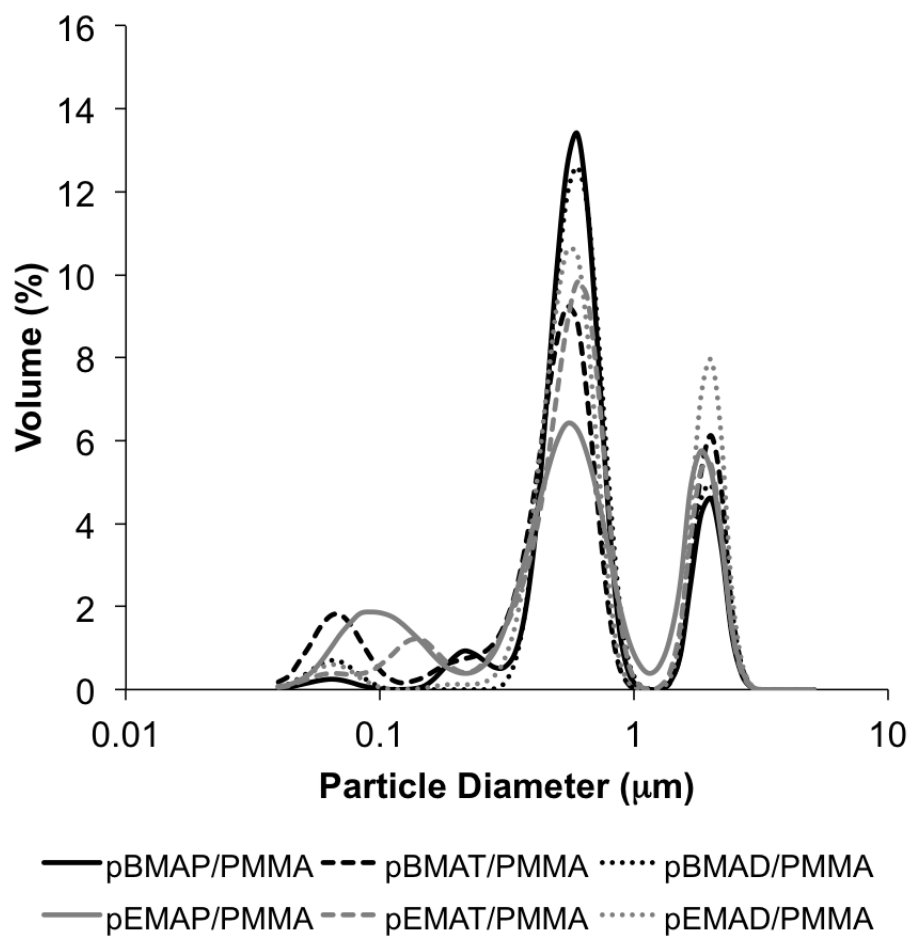


Figure 5.3: Coulter laser particle size analysis of prepared by sPIN with different shell polymers and PMMA cores.

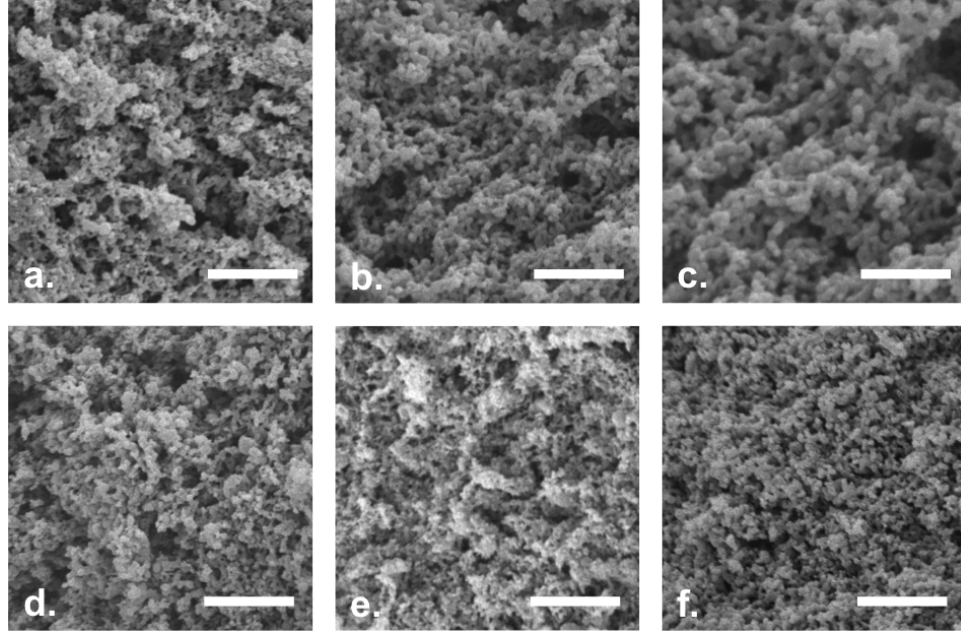


Figure 5.4: Scanning electron micrographs of double-walled nanosphere formulations prepared by sPIN with PBMA-shells and different core polymers (a.) PBMA/PLGA (75:25), 3000x, scale bar: 5 μm , (b.) PBMA/PLGA (75:25), 6000x, scale bar: 2.5 μm , (c.) PBMA/PLGA (75:25), 10000x, scale bar: 1.5 μm , (d.) PBMA/PLGA (75:25), 3000x, scale bar: 5 μm , (e.) PBMA/PLGA (50:50), 3000x, scale bar: 5 μm , (f.) PBMA/D,L-PLA, 3000x, scale bar: 5 μm .

of dilute polymer solutions to produce discrete spheres, typically less than 5% w/v, with higher concentrations resulting in the formation of polymeric membranes and networks. The secondary peak in the 2 μm range could either reflect the production of a polydisperse population of spheres or more likely, the presence of aggregates, which was further explored by SEM analysis.

5.4.4.2 Scanning Electron Microscopy

As seen in the scanning electron micrographs of the PBMA/PLGA nanospheres (Figure 5.4a-c), the sequential phase inversion process results in the formation of discrete monodisperse nanospheres with an average particle diameter of ~ 500 nm (Figure 5.4c). SEM analysis revealed the presence of nanospheres in aggregates (Figure 5.4a), which explains the additional peak at ~ 2 μm seen in the laser diffraction particle size analysis (Figure 5.3); this may be the result of the adhesive nature of the PBMA and other bioadhesive shell polymers, processing of the spheres in hydrophobic conditions as well as the effect of lyophilization as

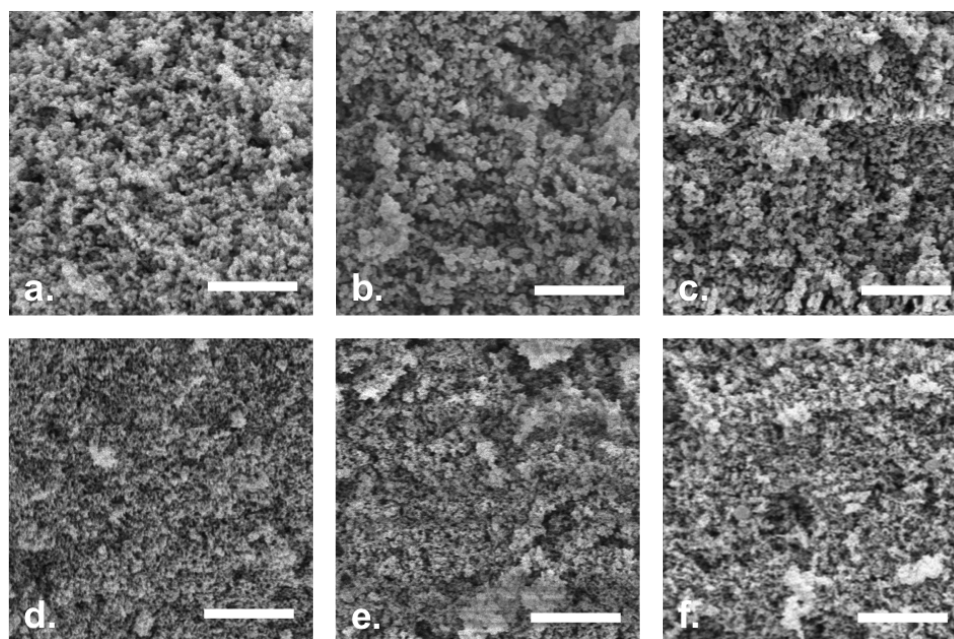


Figure 5.5: Scanning Electron Micrographs of double-walled nanosphere formulations prepared by sPIN with different shell polymers and PMMA cores. (a.) PBMAP/PMMA 75:25, 3000x, scale bar: 5 μm , (b.) PBMAT/PMMA 75:25, 3000x, scale bar: 5 μm , (c.) PB-MAD/PMMA 75:25, 3000x, scale bar: 5 μm , (d.) PBMAP/PMMA 75:25, 3000x, scale bar: 5 μm , (e.) PBMAT/PMMA 75:25, 3000x, scale bar: 5 μm , (f.) PBMAP/PMMA 75:25, 3000x, scale bar: 5 μm .

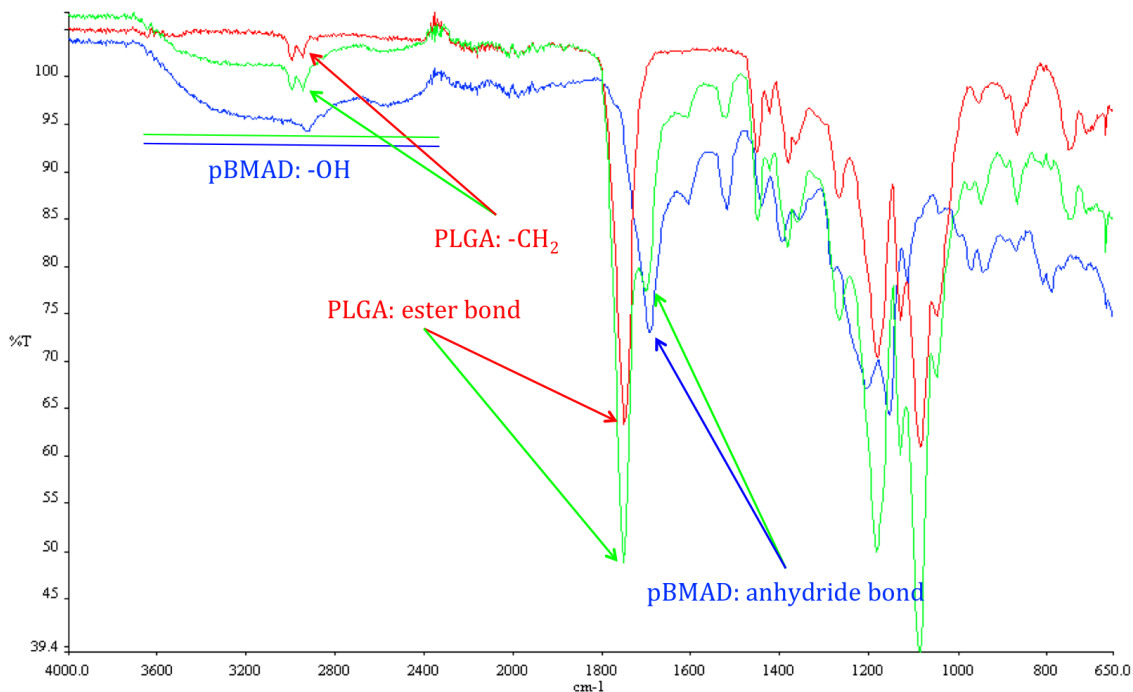


Figure 5.6: Fourier transform infrared spectroscopy (FTIR) analysis of PLGA (red), PB-MAD (blue) and PB-MAD-shell/PLGA (75:25)-core (green) nanospheres; characteristic peaks have been identified.

the final step in preparation all of which likely promote the aggregation of the nanospheres [8]. Thus, the results of the particle size analysis are due to the tendency of the spheres to aggregate and probably not because of nanosphere polydispersity. The resolution of the images was not sufficient to identify any distinct characteristics in the surface morphology of the nanospheres. Similar results were seen for SEM analysis of all formulations (Figures 5.4 and 5.5).

5.4.4.3 FTIR Analysis

Fourier transform infrared spectroscopy (FTIR) of the PB-MAD/PLGA double-walled nanospheres identified peaks characteristic of the core polymer (PLGA) and the shell polymer (PB-MAD) as well as their presence in the PB-MAD-shell/PLGA-core double-walled formulation (Figure 5.6). Characteristic peaks include the ester bond of PLGA (1750 cm^{-1}) and the anhydride bond of pBMAD (1700 cm^{-1}), which are seen together in the PB-MAD/PLGA spheres. Additionally, the $-\text{CH}_2$ stretch of the PLGA (3000 cm^{-1}) and the broad hydroxyl peak of the

Formulation		DSC	
Shell	Core	T _{g1} (°C)	T _{g2} (°C)
PBMAP	PMMA	56.82	148.58
PBMAT	PMMA	53.44	161.97
PBMAD	PMMA	54.30	159.99
PEMAP	PMMA	59.91	167.51
PEMAT	PMMA	56.27	179.46
PEMAD	PMMA	55.23	174.96

Table 5.2: Thermal analysis of double-walled nanosphere formulations prepared by sPIN with different shell polymers and PMMA cores; glass transition temperatures (T_g) were determined from the first heating run on DSC.

Formulation		DSC	
Shell	Core	T _{g1} (°C)	T _{g2} (°C)
PBMAD	PMMA	54.30	159.99
PBMAD	PLGA (50:50)	42.27	132.59
PBMAD	PLGA (75:25)	49.39	159.21
PBMAD	D,L-PLA	46.15	147.69

Table 5.3: Thermal analysis of double-walled nanosphere formulations prepared by sPIN with PBMAD-shells and different core polymers; glass transition temperatures (T_g) were determined from the first heating run on DSC.

PBMAD (2400 – 3600 cm⁻¹) are additive in the spectrum of the PBMAD/PLGA spheres. While these results confirm the presence of both polymers within double-walled formulation, FTIR does not provide any information regarding the interaction between the two polymers. Similar results were observed for the evaluation of the spectra of the core polymers, shell polymers and double-walled formulations described above (data not shown).

5.4.4.4 Thermal Analysis

Thermal analysis of the bulk polymers and double-walled nanospheres was performed using differential scanning calorimetry to probe the interactions between to the polymers within each formulation (Tables 5.2 and 5.3). PLGA exhibited a glass transition at 42.69°C with no T_m due to the amorphous nature of the polymer. In contrast, the PBMAD exhibited a high glass transition temperature at 174.29°C; no melting temperature was detected in the temperature ranges analyzed. The PBMAD/PLGA formulation exhibited two glass transition temperatures: 49.39°C and 159.21°C, the first corresponding to the PLGA (50:50) and the second to PBMAD (Table 5.2). As seen in tables 5.2 and 5.3, two glass transitions

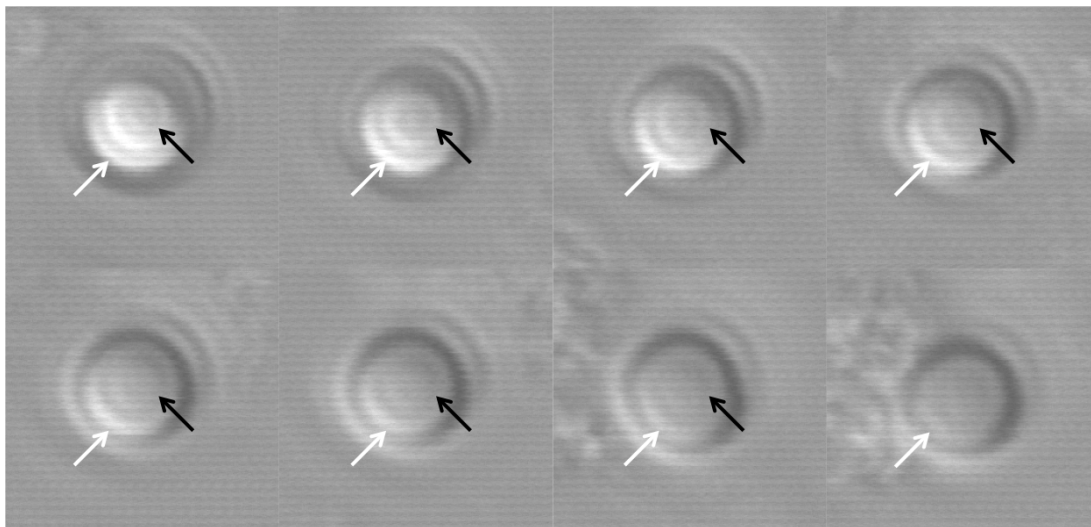


Figure 5.7: High magnification z-stack images of PBMA/PLGA-FITC insulin nanospheres. Arrows indicate the two layers of the polymer, core (black) and shell (white). 500x magnification.

were detected in each formulation – one corresponding to the core polymer and another to the shell polymer. The detection of two distinct glass transition temperatures may only indicate the phase separation of the two polymers within the formulation [3,6]. This phase separation could be in the form of double-walled core-shell nanospheres or be the result of the two polymers phase inverting separately. DSC analysis does not provide information about the localization of the two polymers in the system; however, given that this method relies on the preferential sequential phase separation process of the first polymer at its cloud point to form the core particle prior its engulfment upon the phase inversion of the second polymer, the formation of double-walled nanospheres is expected.

Interestingly, evaluation of the thermograms for the second heat, reveal the loss of the glass transition of the shell polymer, revealing a single second-order transition corresponding to the T_g of each core polymer (data not shown). The loss of this glass transition may be due to the formation of a polymeric solid solution of the two polymers after heating above the two glass transitions upon the cooling of the polymers after the first heat during the DSC run.

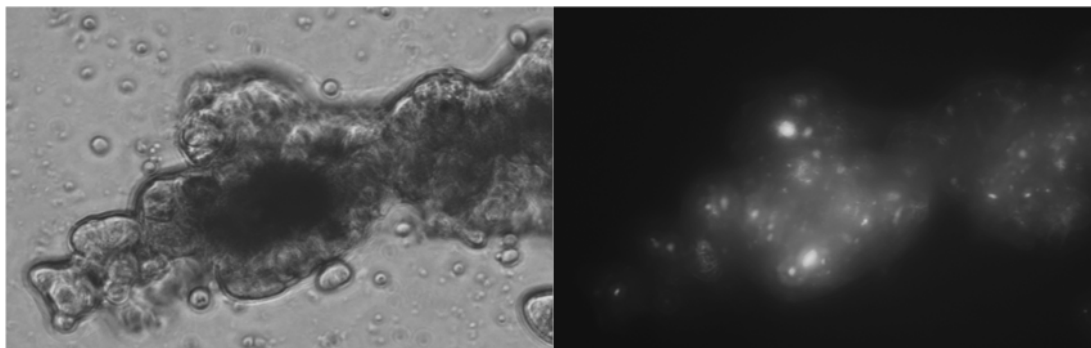


Figure 5.8: Light (left) and fluorescence (right) microscopy of PBMA/PLGA-FITC insulin nanospheres. 40x magnification.

5.4.4.5 Polymer Localization

Light microscopy was used to confirm the double-walled structure of the sPIN nanospheres (Figure 5.7). As can be seen in the serial z-stack images of a single PBMA/PLGA-FITC insulin nanosphere, two distinct polymeric phases can be seen within the nanosphere due to the different refractive indices of the polymer. As the z-stacks progress through different focal planes within the nanosphere, the diffraction pattern of the core disappears indicating the complete engulfment within the other polymer.

Light and fluorescence microscopy of the PBMA/PLGA-FITC insulin formulation confirmed the localization of the two polymers within the nanospheres. As can be seen in figure 5.8, within an aggregate of the nanospheres under light microscopy, discrete droplets of one polymer can be seen within the other polymer due to the differences in the refractive indices of the two polymers. Due to the adhesive nature of the PBMA, the nanospheres have a tendency to aggregate in solution. Using fluorescence microscopy, the FITC-insulin which is located within the PLGA can be found as discrete regions within the aggregate of PBMA, confirming the localization of the PLGA to the core and the bioadhesive PBMA to the shell of the nanospheres.

5.5 Conclusions

Sequential phase inversion nanoencapsulation (sPIN) requires the selection of two polymer/solvent pairs that allow for the controlled sequential phase inversion of each polymer.

When preparing double-walled nanospheres, solubility tests must be performed to ensure the proper localization of each polymer to find a solvent system that meets the following criteria. For the core polymer, a solvent must be selected that is also a solvent for the shell polymer while the solvent for the shell polymer must be a non-solvent for the core polymer; all solvents must be miscible with the non-solvent for both polymers. When the shell polymer solution is added to the core polymer solution, the resulting decrease in the core polymer solubility at its cloud point results in its preferential phase separation to produce the core of the nanosphere and if desired, encapsulation of the target drug. When this unstable solution is rapidly added to the non-solvent, the shell polymer engulfs the core polymer as the phase inversion is completed to form double-walled nanospheres.

This method is particularly advantageous because it can be used to produce nanoparticles with core-shell morphologies in a single step through a rapid spontaneous self-assembly process avoiding high shear rates and prolonged exposure to harsh solvents, which could result in damage to or the loss of bioactivity of the therapeutic cargo. These double-walled particles can modulate drug release kinetics for controlled release as well as alter the surface properties of nanoparticles for better biomaterials-tissue interactions, both of which could be advantageous for drug delivery applications.

5.6 References

- [1] S. Freiberg, X. Zhu, Polymer microspheres for controlled drug release., *International Journal of Pharmaceutics*. 282 (2004) 1–18.
- [2] K.J. Pekarek, J.S. Jacob, E. Mathiowitz, Double-walled polymer microspheres for controlled drug release., *Nature*. 367 (1994) 258–260.
- [3] E. Tan, R. Lin, Fabrication of double-walled microspheres for the sustained release of doxorubicin, *Journal of Colloid and Interface Science*. (2005).
- [4] Y. Yang, T. Chung, X. Bai, W. Chan, Effect of preparation conditions on morphology and release profiles of biodegradable polymeric microspheres containing protein fabricated by double-emulsion method, *Chemical Engineering Science*. 55 (2000) 2223–2236.
- [5] W. Zheng, A water-in-oil-in-oil-in-water (W/O/O/W) method for producing drug-releasing, double-walled microspheres, *International Journal of Pharmaceutics*. (2009).

- [6] K. Leach, K. Noh, E. Mathiowitz, Effect of manufacturing conditions on the formation of double-walled polymer microspheres., *Journal of Microencapsulation*. 16 (1999) 153–167.
- [7] K.J. Leach, E. Mathiowitz, Degradation of double-walled polymer microspheres of PLLA and P(CPP:SA)20:80. I. In vitro degradation., *Biomaterials*. 19 (1998) 1973–1980.
- [8] K.J. Leach, S. Takahashi, E. Mathiowitz, Degradation of double-walled polymer microspheres of PLLA and P(CPP:SA)20:80. II. In vivo degradation., *Biomaterials*. 19 (1998) 1981–1988.
- [9] N.A. Rahman, E. Mathiowitz, Localization of bovine serum albumin in double-walled microspheres., *J Control Release*. 94 (2004) 163–175.
- [10] C. Berkland, E. Pollauf, N. Varde, D.W. Pack, K.K. Kim, Monodisperse liquid-filled biodegradable microcapsules., *Pharm Res*. 24 (2007) 1007–1013.
- [11] C. Berkland, A. Cox, K.K. Kim, D.W. Pack, Three-month, zero-order piroxicam release from monodispersed double-walled microspheres of controlled shell thickness, *J. Biomed. Mater. Res*. 70A (2004) 576–584.
- [12] C. Berkland, E. Pollauf, D.W. Pack, K. Kim, Uniform double-walled polymer microspheres of controllable shell thickness., *J Control Release*. 96 (2004) 101–111.
- [13] W.L. Lee, E. Widjaja, S.C.J. Loo, One-step fabrication of triple-layered polymeric microparticles with layer localization of drugs as a novel drug-delivery system., *Small*. 6 (2010) 1003–1011.
- [14] J.J. S, M. Edith, A Novel Mechanism for Spontaneous Encapsulation of Active Agents: Phase Inversion Nanoencapsulation, in: *ACS Symposium Series CY -*, American Chemical Society, Washington, DC, 2011: pp. 214–223–223.
- [15] E. Mathiowitz, J.S. Jacob, Y.S. Jong, G.P. Carino, D.E. Chickering, P. Chaturvedi, et al., Biologically erodable microspheres as potential oral drug delivery systems., *Nature*. 386 (1997) 410–414.

Chapter 6

Comparative Analysis of Insulin Nanoencapsulation by Phase Inversion

6.1 Abstract

The phase inversion nanoencapsulation (PIN) method was used to prepare poly(lactic-co-glycolic acid) nanospheres loaded with bovine zinc insulin (BZI) using three micronization techniques: water-in-oil (w/o) emulsion, w/o cryogenic emulsion and nanonized-BZI solid-in-oil (s/o) dispersion. The effect of protein loading and salt addition on the encapsulation and release kinetics was studied for each case. Nanosphere formulations were characterized by laser diffraction particle size analysis, scanning electron microscopy, Fourier transform infrared spectroscopy and differential scanning calorimetry. Formulations had high loadings and varied encapsulation efficiencies depending on the method used. An inverse relationship between encapsulation efficiency and total protein loading was observed for the cryogenic emulsion and s/o methods. While protein loading and salt addition did not have a significant effect on release kinetics, the nanonized-BZI formulations exhibited reductions in initial release as well as sustained release for a period of 42 days from 500 nm PLGA nanospheres. Transmission electron microscopy depicted a reservoir type delivery systems consisting of a protein core and polymer shell structure, which was achieved with loadings up to 20%.

Daniel Y. Cho, Cheng Tian, Lakir Patel, Marissa Anto, Taylor Peak, Shara Azad, Paula Weston and Edith Mathiowitz

6.2 Introduction

With the advent of modern biotechnology, a significant amount of research has focused on the development of protein and peptide therapeutics for the management of a variety of disease processes, many of which have been approved or are in advanced clinical trials [1,2]. However, these drugs typically need to be administered parentally due to their physical and chemical instability, short half-lives as well as low oral bioavailability [2], necessitating the use of engineered delivery systems. The use of polymeric matrices for the controlled delivery of therapeutics has been of significant interest to allow for the temporal and spatial control of drug release. With the recent surge in nanotechnology research, the use of polymeric nanospheres in particular has been evaluated as an approach to overcome the issues in protein delivery [1]. Unfortunately, with the increase in the surface area to volume ratio that results from a reduction in particle size, the use of nanoparticles often requires compromising control of over release kinetics for the potential advantages presented by nanoscale geometries. Notwithstanding the many advances in microencapsulation techniques [1,3], an effective method for controlling the release of therapeutic proteins from polymeric nanospheres has been difficult to achieve and many research groups are working on strategies to optimize these delivery systems [1,2,4].

The most common method described in the literature for the preparation of polymeric nanospheres is solvent evaporation. This method relies on the emulsification of a polymer solution in an aqueous bath followed by the evaporation of the polymer solvent resulting in the formation of microspheres. By using high shear rates, particle size can be reduced to form spheres with nanoscale dimensions. Unfortunately, these high shear forces along with the prolonged exposure to organic solvents often result in a loss in the bioactivity of the protein. First described by Mathiowitz et al., phase inversion nanoencapsulation (PIN) is a method that is used to encapsulate therapeutic agents via the spontaneous self-assembly of polymeric nano- and microspheres [5,6]. When a dilute polymer solution is rapidly added to the environment of an excess of non-solvent, the polymer in the solution undergoes a transition from a homogenous solution to an unstable two-phase mixture in which spherical droplets of non-solvent or drug in the polymer rich phase serve as nucleation

sites for the precipitation of the polymer to form spheres [5]. The only requirement for the successful fabrication of microspheres by PIN is the appropriate selection of solvent and non-solvent: the solvent and non-solvent must be miscible for particle formation with a minimal solvent:non-solvent ratio of 1:40, though a ratio of 1:100 is optimal. The preparation of small diameter nanospheres using traditional methods such as solvent evaporation often require multiple steps and expose therapeutic agents to harsh denaturing conditions for prolonged periods of time, including high shear rates in order to reduce particle size into the nanoscale. In contrast, PIN is advantageous in that it is a one-step encapsulation process that is nearly instantaneous and relatively gentle; the short time of exposure to solvents and the absence of high shear forces make this an attractive method for the encapsulation of sensitive therapeutics, particularly proteins [7-13].

A variety of different strategies have been employed to achieve better release profiles for proteins from polymeric matrices. Particularly in the case of polymeric nanospheres, the high surface area of the particles results in a large initial burst in which the protein trapped on the surface is very rapidly released, reducing the potential for controlled or sustained release properties. The polymer selection and fabrication parameters affect the properties of the final formulation and can alter release kinetics; these include: polymer molecular weight and concentration, polymer crystallinity, chemical characteristics of the protein, physical state of the protein during encapsulation, selection of organic solvents and surfactants, temperature, etc [14-16]. Additionally, the use of excipients has been a common approach to stabilize the proteins during encapsulation and to control release. Commonly studied excipients include salts, sugars, polyols, amino acids, polymers (including PEG, PVP, dextrans) as well as surfactants [3,17]. Salts have been shown to act as osmotic agents, which can help to draw water into the polymeric matrix and allow for more rapid protein release; however, studies with sparingly soluble metal salts (i.e. $\text{Mg}(\text{OH})_2$, MgCO_3 , ZnCO_3 , CaCO_3) have shown that despite increased water uptake, the rate of polymer degradation is not affected while the rate of protein release is reduced [18], potentially due to a reduction in the solubility of the protein upon complexation with the metal salt [19]. Other approaches have looked at the effect of surfactant to reduce the initial burst, including ion pairing of the protein with charged surfactants [20] as well as using surfactants to alter the affinity of the protein for

the polymer [21]. Most recently, chemical modifications have been used to further modulate release kinetics, including the use of PEGylated proteins and covalent bonding of the protein to the polymer [3].

While the use of excipients as a strategy to overcome the challenges of achieving controlled release from nanospheres has great potential, control of the physical state in which the protein is incorporated and its physical interactions with the polymer during the encapsulation process would allow for an additional level of control over release kinetics. Thus, the aim of this work was to comparatively investigate the effect of three different methods (water-in-oil (w/o) emulsion, w/o cryogenic emulsion and solid-in-oil (s/o) dispersion) for the incorporation of a model protein, bovine zinc insulin (BZI), in poly(lactic-co-glycolic acid) (PLGA) nanospheres prepared using phase inversion encapsulation techniques on the efficiency of protein encapsulation, loading and release kinetics.

6.3 Materials and Methods

6.3.1 Materials

Poly(lactic-co-glycolic acid) (PLGA; Resomer RG 502, 50:50; i.v. range 0.16 – 0.24 dL/g in CHCl₃), was purchased from Boehringer-Ingelheim, Inc. (Germany) and stored at -15°C until use. Bovine zinc insulin (BZI) was obtained from Gibco Life Technologies (Rockville, MD). All solvents were of the highest commercial grade available.

6.3.2 Insulin Encapsulation

Bovine zinc insulin (BZI) was prepared for encapsulation using three methods: solid-in-oil (s/o) nanonized dispersion, water in oil (w/o) emulsion and a w/o cryogenic emulsion method (Figure 6.1). All nanospheres were prepared using the phase inversion nanoencapsulation method. For the s/o method, BZI was nanonized using a tert-butyl alcohol precipitation method resulting in insulin particles with an average diameter of 100 – 200 nm. Briefly, 100 mg stock BZI was dissolved in 10 mL of 0.01N HCl then precipitated with 1 mL of 10% ZnSO₄ then re-solubilized with 25 mL of 0.01N HCl. An excess of tert-butyl alcohol (~450 mL) was added to the solution to nanoprecipitate the BZI, which was collected by

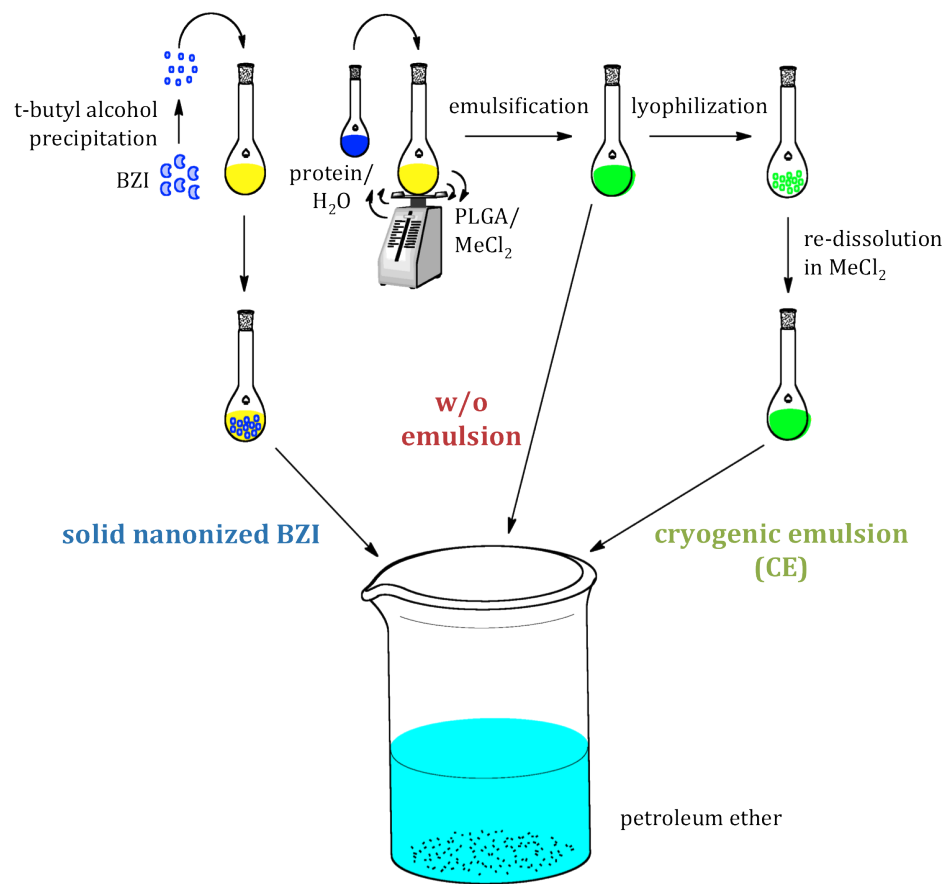


Figure 6.1: Schematic of the nanoencapsulation methods used for the preparation of insulin-loaded PLGA nanospheres.

centrifugation and lyophilization. The appropriate loading (4 mg, 20 mg, 40 mg, 80 mg and 160 mg) of the solid nanonized BZI was suspended in 20 mL of a 2% solution of PLGA in methylene chloride prior to phase inversion to prepare nanospheres with 1%, 5%, 10%, 20% and 40% w/w BZI/PLGA loading.

For the emulsification methods, 4 mg, 20 mg and 40 mg of BZI was dissolved in 200 μ L of 0.01 N HCl to form the aqueous phase prior to emulsification. The w/o emulsion was formed by adding the aqueous BZI solution to 20 mL of a 2% w/v solution of PLGA in methylene chloride then vortexing for 1 minute prior to phase inversion. In contrast, the cryogenic emulsion method involved the emulsification of 200 μ L of each aqueous BZI solution in 4 mL of a 10% w/v PLGA/methylene chloride solution by vortexing for 1 minute. The resulting w/o emulsion was flash frozen in liquid nitrogen and lyophilized for 24 hours. This method allows for the preferential association of the protein with the polymer in the form of a solid dispersion of nanonized BZI within the continuous phase of PLGA. The polymeric matrix was reconstituted in 20 mL of methylene chloride to form a 2% w/v solution containing suspended BZI particles prior to phase inversion. For both emulsification methods, the protein loading was altered by increasing the concentration of the BZI solution prior to the emulsification step. To evaluate the effect of salt on release kinetics, NaCl was added to the aqueous phase in a 1% w/w salt to polymer ratio prior to emulsification. The following formulations were prepared: 1% w/w BZI loading with and without 1% w/w salt using the w/o emulsion method; 1% w/w-loading with and without 1% w/w salt, 5% and 10% w/w BZI loading using the w/o cryogenic emulsion method.

To prepare the nanospheres, 20 mL of the appropriate protein/polymer dispersion was rapidly added to 2 L of non-solvent, petroleum ether, for a solvent to non-solvent ratio of 1:75 resulting in the spontaneous formation of nanospheres. The suspension was stirred manually for 1 minute to allow for complete solvent extraction and polymer phase inversion. The nanospheres were collected using a 0.2 μ m PTFE filter paper in a positive pressure filtration column (Millipore; Billerica, MA), flash frozen and lyophilized overnight. The yields for all formulations were between 50 – 80%.

6.3.3 Particle Size Analysis

All formulations were reconstituted from powder form into reconstitution media consisting of an aqueous 1% sodium lauryl sulfate (SLS) / 1% polyvinylpyrrolidone (PVP) solution in 0.5 – 2% w/v suspensions with bath sonication. A Beckman Coulter LS230 Laser Diffraction Particle Size Analyzer (Beckman Coulter, Inc.; Brea, CA) was used to evaluate the size distribution of nanoparticle populations.

6.3.4 Differential Scanning Calorimetry

3 – 7 mg of each formulation were sealed into aluminum sample pans for DSC analysis. Thermal analyses of the nanospheres were performed using a model DSC 7 (Perkin-Elmer, Inc.; Waltham, MA) equipped with controller model TAC 7/DX (Perkin-Elmer, Inc.; Waltham, MA). After equilibration at -20°C, samples were heated from -20 to 100°C, cooled to -20°C then reheated to 100°C at a rate of 10°C/min. Thermograms were analyzed using Perkin-Elmer Thermal Analysis software for the calculation of glass transition temperatures (T_g).

6.3.5 Scanning Electron Microscopy

The morphology and particle size of the nanosphere formulations were analyzed by scanning electron microscopy. Formulations were placed on a carbon-backed adhesive and sputter-coated with Au-Pd for 4 minutes at 20 mA and imaged on a Hitachi 2700 Scanning Electron Microscope (Hitachi High Technologies America, Inc.; Pleasanton, CA) with an accelerating voltage of 8 kV.

6.3.6 Encapsulation Efficiency

To determine the insulin-loading of the nanospheres, 10 mg of each formulation was incubated in 2 mL of 0.1 N NaOH on a end-over-end rotating rack for 24 hours to allow for the complete degradation of the PLGA ($n = 3$). The resulting solutions were assayed in triplicate using the micro-bicinchoninic (micro-BCA) assay as per manufacturers instructions. The encapsulation efficiency was calculated as the ratio between actual loading and theoretical loading multiplied by 100; the results are presented as the mean encapsulation

efficiency $\pm$ standard deviation (Table 6.1).

6.3.7 Release Study

Release kinetics were determined by incubating 10 mg of each formulation in 1.5 mL of phosphate buffered saline (PBS, pH = 7.4) at 37°C (n = 4). At each time point (1, 2, 4 and 8 hours, 1, 2, 3, 5, 7, 14, 21, 28, 35 and 42 days), the nanospheres suspensions were centrifuged and 1 mL of the release buffer was removed then replaced with fresh PBS and vortexed briefly to re-suspend the spheres. The protein concentration of the release samples was determined using the micro-BCA assay as per manufacturers instructions; all samples were analyzed in triplicate. The results are presented as mean release $\pm$ standard deviation. For the cumulative percent release curves, the release data was normalized to total protein release at the end of the 42-day study.

6.3.8 Transmission Electron Microscopy

Samples were fixed by placing them in a desiccator with an opened ampule of osmium tetroxide for 7 days. Samples were then embedded directly in Epofix cold-setting embedding resin (Electron Microscopy Sciences, Hatfield, PA) and allowed to polymerize overnight. Blocks were sectioned at a thickness of 85 nm on a Reichert Ultramicrotome with a diamond knife. Sections were placed on copper grids and viewed on a Phillips 410 transmission electron microscope equipped with an Advantage HR CCD camera. Images were acquired with Advanced Microscopy Techniques imaging software.

6.4 Results and Discussion

6.4.1 Nanosphere Preparation

PLGA (50:50) nanospheres were prepared using three different loading methods by phase inversion (Figure 6.1). The versatility of PIN allows for the encapsulation of proteins directly as micronized solids and aqueous solutions via emulsification as well as proteins that have been micronized into the polymer by cryogenic emulsion. When the protein is encapsulated as a micronized solid or by cryogenic emulsion, the protein acts as a core nucleus on which

Loading Method	Insulin Loading		EE (%)	Salt (%w/w)	Particle Size		T _g (°C)	
	Theoret. (%w/w)	Actual (%w/w)			Num. Avg. (nm)	Vol. Avg. (nm)	1st Heat	2nd Heat
E0	0	-	-	0	323 ± 115	718 ± 592	44.78	42.15
E1	1	0.48	47.79	0	174 ± 132	765 ± 654	42.77	41.83
ES0	0	-	-	1	330 ± 128	770 ± 629	44.07	42.24
ES1	1	1.48	148.6	1	144 ± 115	722 ± 629	37.56	43.15
CE0	0	-	-	0	273 ± 118	726 ± 630	43.79	42.91
CE1	1	1.49	148.83	0	330 ± 126	748 ± 578	41.92	42.49
CE2	5	6.44	128.83	0	344 ± 106	725 ± 618	43.63	42.94
CE3	10	9.58	95.78	0	335 ± 111	702 ± 577	44.58	43.38
CES0	0	-	-	1	329 ± 100	695 ± 618	41.37	41.26
CES1	1	1.53	153.43	1	329 ± 130	783 ± 618	44.13	42.42
N0	0	-	-	0	282 ± 118	720 ± 624	45.08	43.57
N1	1	1.13	112.78	0	269 ± 119	716 ± 625	42.05	41.51
N2	5	5.19	103.89	0	316 ± 109	671 ± 591	40.19	42.79
N3	10	9.29	92.98	0	359 ± 112	771 ± 595	41.90	44.33
N4	20	13.44	67.22	0	293 ± 126	719 ± 555	44.97	43.54
N5	40	19.20	47.99	0	286 ± 125	734 ± 579	43.98	42.79

Table 6.1: BZI-loaded PLGA PIN formulations, encapsulation efficiencies, theoretical and actual loadings, particle size and thermal properties.

the polymer in solution can precipitate to form a coating around the particle [5]. Likewise, in the use of an aqueous solution for the encapsulation of the protein, the formation of an emulsion of the protein solution in hydrophobic solvent for the polymer results in the emulsion droplets serving as the nucleation sites [5].

Formulations with 1% BZI with and without 1% NaCl were prepared using a water-in-oil emulsion method in which an aqueous phase containing the protein and salt was emulsified into the polymer solution prior to phase inversion (Formulations E1 & ES1). For the cryogenic emulsion formulations, the water-in-oil emulsion was flash frozen and lyophilized to preserve the architecture of the droplets of protein distributed in a continuous phase of polymer; when reconstituted in methylene chloride, the protein is encapsulated as a solid. Cryogenic emulsion formulations of 1%, 5%, and 10% w/w protein/PLGA BZI as well as 1% BZI with 1% NaCl were prepared (Formulations CE1, CE2, CE3 and CES1 respectively). For the nanonized formulations, a tert-butyl alcohol nanonization method was used to re-complex the bulk BZI with ZnSO_4 to reduce particle size from 25 – 40 μm to 100 – 200 nm (Figure 2). 1%, 5%, 10%, 20% and 40% w/w protein/PLGA-loaded formulations were prepared by dispersing the nanonized BZI in the PLGA solution prior to phase inversion (Formulations N1, N2, N3, N4 and N5). A summary of the formulations can be found in table 6.1.

6.4.2 Nanosphere Characterization

Physical characterization revealed that regardless of the protein loading method, incorporation of salt and total protein loading, the phase inversion process resulted in formulations with similar particle size distributions, with mean number average diameters of 295 ± 59 nm and volume average diameters of 730 ± 30 across all formulations (Table 6.1). The discrepancy between the number and volume average particle sizes suggests that the PIN method generates a population of polydisperse nanospheres, which is likely the result of the spontaneous and instantaneous polymeric self-assembly of the nanospheres during the phase inversion process. SEM analysis of the nanospheres revealed a population of spheres with a size range consistent with the results from laser diffraction particle analysis (Figure 6.2). There were no traces of unencapsulated BZI crystals visible on the scanning electron

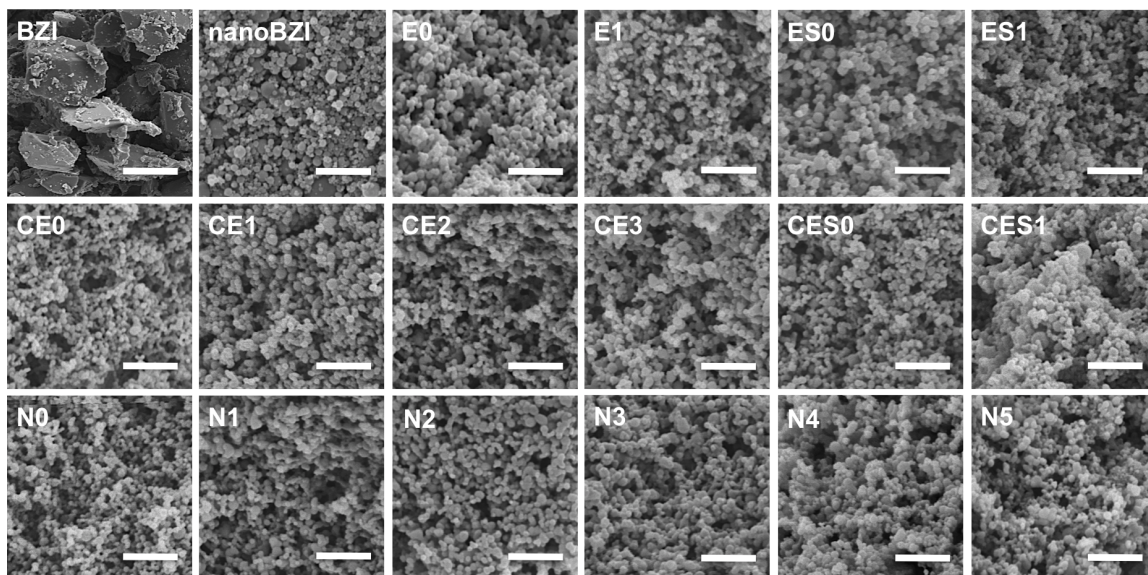


Figure 6.2: Scanning electron micrographs of the stock BZI (BZI; scale bar: 25 μm), nanonized BZI (nanoBZI; scale bar: 2.5 μm) and all formulations (scale bars: 2.5 μm).

micrographs; this may be the result of the nanosphere collection method which relies of filtration causing the loss of the small particles of unencapsulated insulin. DSC analysis revealed slight increases in the glass transition temperature (T_g) of the polymer in the first heat relative to the second heat (Table 6.1). No melting temperature (T_m) was observed due the amorphous nature of the PLGA (50:50). FTIR analysis revealed no changes in the spectra of the polymer, suggesting that no chemical changes occurred during the fabrication process (data not shown).

6.4.3 Encapsulation Efficiencies

The protein content for each formulation was determined and revealed high encapsulation efficiencies and actual loadings regardless of protein loading method, with the exception of the highest loaded nanonized formulations (Table 6.1). In some cases, the formulations revealed entrapment efficiencies of greater than 100%; in particular, the 1% BZI-loaded cryogenic emulsion formulations resulted in apparent encapsulation efficiencies of 148.84% (CE1) and 153.43% (CES1), with and without salt (Table 1). This is probably an indication of losses of the polymer relative to the protein, which was also observed in the E, ES and formulations. Phase inversion is a commonly used technique for the purification of polymers;

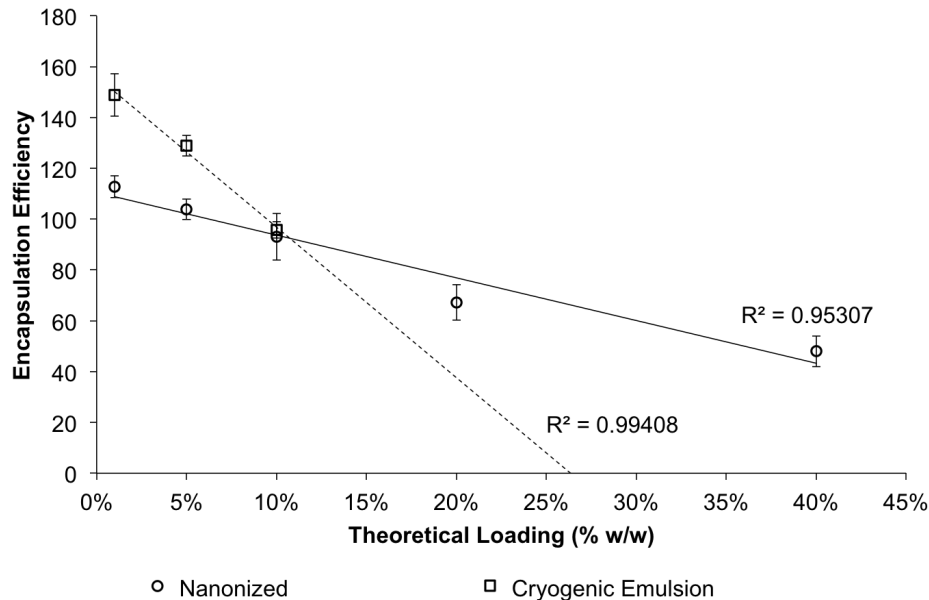


Figure 6.3: prepared using the cryogenic emulsion (○) and nanonized-BZI (□) loading methods.

during this process, higher molecular weight polymers precipitate while low molecular weight components remain soluble in the solvents and are eliminated from the system. The high entrapment efficiencies could be attributed to the preferential losses of low molecular weight components of the polymer.

Additionally, when the higher protein loaded formulations were evaluated for both the solid nanonized and the cryogenic emulsification methods, an inverse relationship between total protein loading and encapsulation efficiency were observed (Figure 6.3). For the cryogenic emulsion formulations with 1% (CE1), 5% (CE2) and 10% (CE3) BZI-loadings, encapsulation efficiencies of 148.83%, 128.83% and 95.78% were achieved, which could again indicate the preferential losses of the polymer (Table 6.1). Using the nanonized-BZI method, encapsulation efficiencies ranged from 112.78% for the 1% loading to 47.99% for the 40% theoretical loading, which correlate to actual loadings of 1.13%, 5.19%, 9.29%, 13.44% and 19.20% for the 1% (N1), 5% (N2), 10% (N3), 20% (N4) and 40% (N5) theoretical loadings (Table 6.1). With an increase in the amount of insulin to be encapsulated, it is expected that there may be higher losses of the drug causing a reduction of encapsulation efficiencies. This could be the result of the filtration method use to collect the nanospheres; at higher ratios

of protein to polymer, a larger proportion of the insulin can remain unencapsulated and the smaller size of these particles can result in their loss during nanosphere collection. As can be seen in figure 6.3, an inverse relationship between theoretical loading and encapsulation efficiency was observed.

For the purposes of the discussion of insulin release from the formulations, all formulations will be referred to by their approximate actual loadings. Thus, the E1, ES1, CE1 and CES1 formulations will be referred to as the 1% loaded formulations. Given the actual loadings of the cryogenic emulsion formulations, these will be referred to as 1%, 5% and 10% cryogenic emulsion formulations. Given the reduction of encapsulation efficiency observed with increased theoretical loadings in the nanonized formulations, N1, N2, N3, N4 and N5 will be referred to as the 1%, 5%, 9%, 13% and 19% formulations, respectively.

6.4.4 Effect of Loading Method on Insulin Release from 1% Loaded Formulations

The 28-day cumulative percent release of insulin from the 1% BZI-loaded formulations prepared by the emulsion, cryogenic emulsion and nanonized-BZI methods are graphed in figure 6.4A. Regardless of the loading methods, all the formulations exhibited a large initial burst release of protein over the first 2 hours of the release study, which accounted for $79.23 \pm 4.01\%$, $88.12 \pm 5.26\%$ and $82.16 \pm 6.29\%$ of the total release from formulations E1, CE1 and N1 during the study period. The initial burst is a commonly observed phenomenon from polymeric microspheres and is typically the result of the diffusion of drug trapped close to the surface of the formulations; this is particularly apparent for these formulations because their small size corresponds to a high surface area exposed to the aqueous environment. This initial release was followed by a short lag phase over the next 48 hours followed by a secondary burst between days 2 and 3, during which time, most of the remaining BZI was released as the polymer began to hydrolyze. These results suggest that the majority of the polymer in these 1% BZI-loaded formulations is trapped at or near the surface of the nanospheres in easily accessible pores, which results in a limited ability to control the release of insulin from this system.

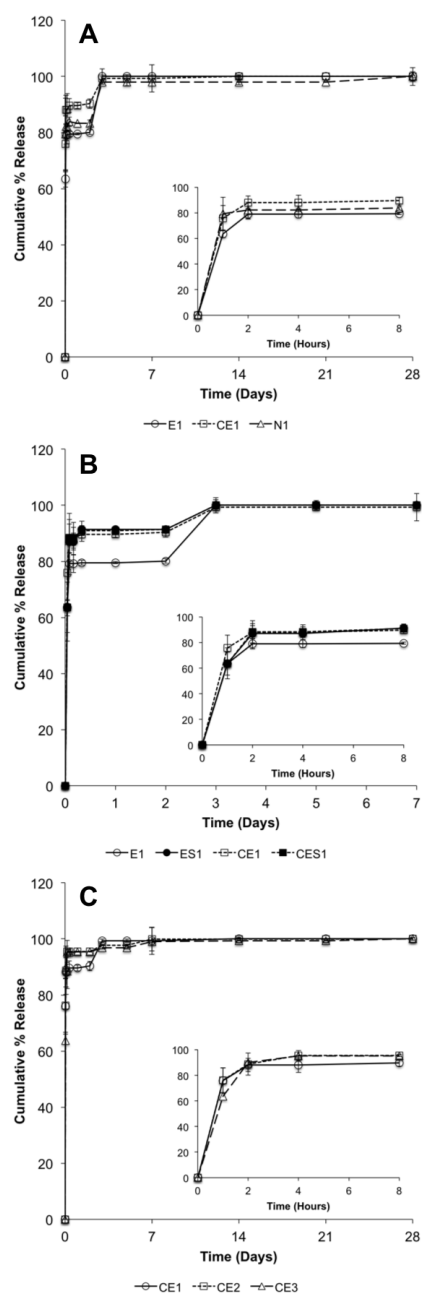


Figure 6.4: (A) 28-day cumulative percent release of insulin from 1% BZI-loaded formulations prepared using the emulsion (E1, ○), cryogenic emulsion (CE1, □) and nanonized-BZI methods (N1, △) (B) 7-day cumulative percent release of insulin from 1% BZI-loaded formulations prepared using the emulsion (○, ●) and cryogenic emulsion (□, ■) methods with (black) and without (white) 1% salt. (C) 28-day cumulative percent release of insulin from 1% (○), 5% (□) and 10% (△) BZI-loaded formulations prepared using the cryogenic emulsion method. Inserts: cumulative percent release over the first 8 hours. Fractional release is normalized to the total mass of protein released during the 42-day study period.

6.4.5 Effect of Salt Excipient on Insulin Release from 1% Loaded Formulations

Salts have been studied as excipients in the encapsulation of therapeutic agents in polymers as both stabilizers and as a release-modifying agents. In particular, insoluble salts have been used to modulate the swelling of polyesters and result in the improved release of high molecular weight proteins from PLGA films [18]. Previous work from our group has shown that soluble salts can act as osmotic agents, resulting in the more rapid swelling and uptake of release buffer by PLGA films [22]. This swelling allows for the generation of pores that allow for the modulation of drug release as well as the elimination of free monomers from the system to prevent autocatalysis of polymer degradation [22] and in the case of protein delivery, reduce the acidity of the local microenvironment, which has been implicated in protein aggregation and denaturation.

The cumulative release of insulin from 1% BZI-loaded formulations prepared using the emulsion and cryogenic emulsion methods with and without 1% NaCl is presented in figure 6.4B. A similar pattern of release is observed for these formulations with a rapid initial release of a large amount of insulin followed by a short lag over the next 48 hours then a secondary release between day 2 and 3. For all formulations, nearly all of the encapsulated insulin is released by day 3, regardless of loading method and salt-loading. In the case of the cryogenic emulsion formulations, no difference was observed in the initial release pattern; without salt, $88.12 \pm 5.26\%$ of the protein was released by 2 hours while $88.42 \pm 8.70\%$ was released with salt (Figure 6.4B). In contrast, the emulsion formulations revealed a slight difference in the intensity of the initial burst. The salt formulation exhibited $87.05 \pm 7.84\%$ release at 2 hours while the salt-free formulation had an initial release of $79.23 \pm 4.01\%$ (Figure 6.4B).

The immediate release found in all these formulations may be the result of the localization of the protein near the surface of the nanospheres. If the protein is entrapped at the surface of the particles, the generation of pores or the penetration of the release medium into the polymeric matrix will cause rapid release from such small particles. The difference between the cryogenic emulsion and emulsion formulations may be the result of the localization of the

salt. In the emulsion formulations, the salt will co-localize with the protein in the aqueous phase so the increased penetration of water into these pores could enhance the osmotic pressure and account for enhanced swelling and the larger initial release. However, in the cryogenic emulsion step, the salt could redistribute throughout the polymeric matrix upon the reconstitution of the polymer while the protein localizes to the surface and thus, no effect is seen with the addition of salt to these formulations.

6.4.6 Effect of Total Protein Loading on Insulin Release

Both the cryogenic emulsion and nanonized-BZI methods were evaluated to determine the effect of total protein loading on insulin release; the 28-day release graphs of the 1%, 5% and 10% loaded cryogenic emulsion formulations are presented in figure 6.4C. Regardless of total protein loading, the cryogenic emulsion formulations followed the same pattern observed for the other formulations. $88.12 \pm 5.26\%$, $88.82 \pm 8.66\%$ and $89.91 \pm 7.12\%$ of the total protein release for the 1%, 5% and 10% cryogenic emulsion formulations occurred in the first two hours. However, while the rest of the release occurred between days 2 and 3 in a secondary burst for the 1% formulation, the 5% and 10% loaded formulations exhibited continuous release over the first week.

In contrast, a strikingly different release pattern was observed for the nanonized formulations. Once again, given the losses of protein during the fabrication process with increased theoretical loadings in the nanonized formulations, N1, N2, N3, N4 and N5 will be referred to as the 1%, 5%, 9%, 13% and 19% formulations, which reflect the actual loadings, respectively. Figure 6.5A shows the release of insulin from these formulations over the first 8 hours. As the loading increased from 1%, 5%, 9%, 13% to 19%, the initial release over the first hour decreased from $79.48 \pm 12.81\%$, $31.02 \pm 10.08\%$, $11.88 \pm 3.42\%$, $3.85 \pm 0.54\%$ and $1.25 \pm 0.75\%$, indicating a significant reduction in the initial burst based on actual protein loading. Over the course of a week, all the formulations continued to release insulin over the first 3 to 4 days to varying degrees with the exception of the 1% formulation, which had a lag in release between 8 hours and 2 days (Figure 6.5B). The trend of decreased release from higher protein loadings was preserved over the first week for the 1%, 5%, 9% and 13% formulations but a unique release pattern was observed for the 19% formulation.

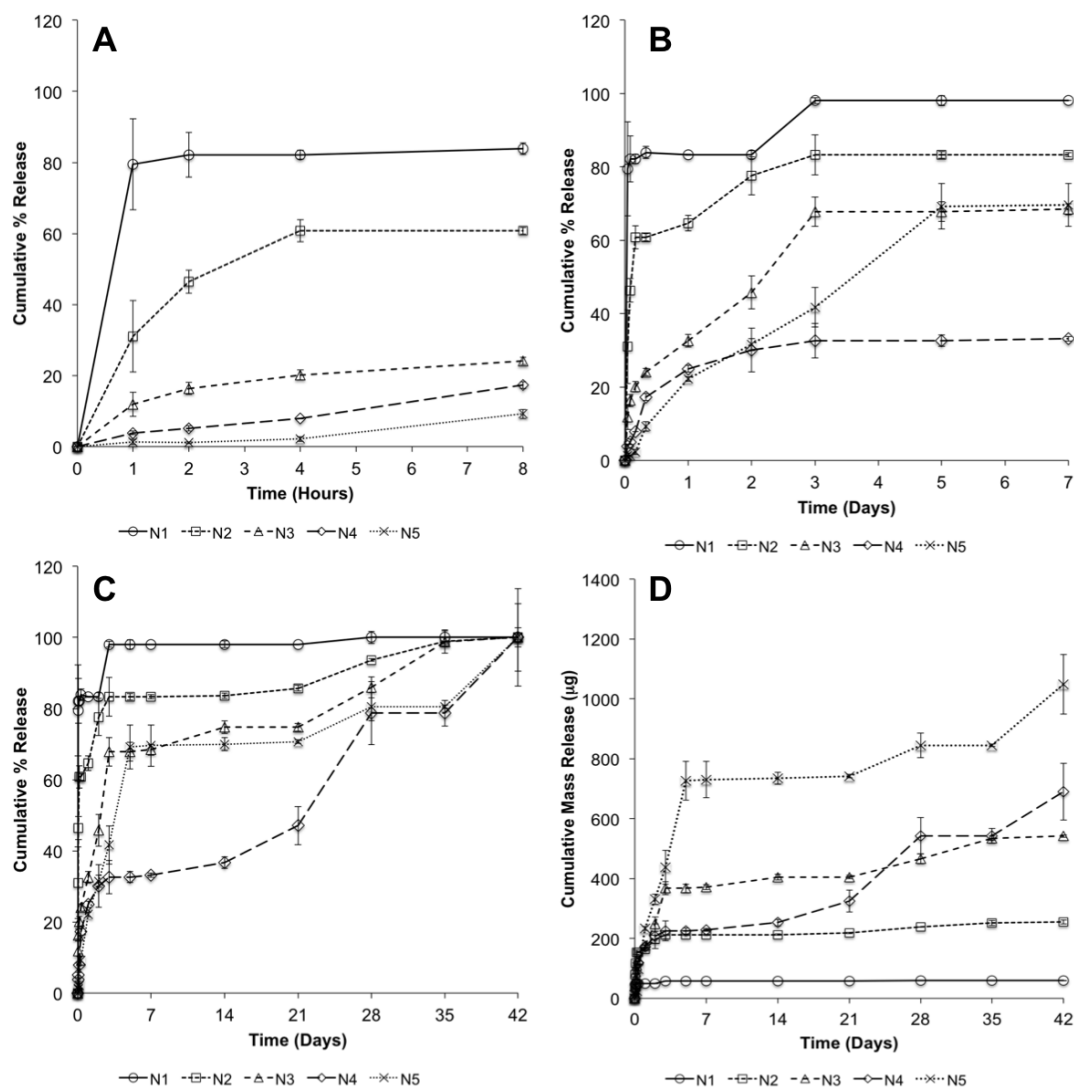


Figure 6.5: (A) Initial 8-hour, (B) 7-day and (C) 42-day cumulative percent release of insulin from 1% (N1, ○), 5% (N2, □), 9% (N3, △), 13% (N4, ◇) and 19% (N5, ×) BZI-loaded formulations prepared using the nanonized-BZI method. Fractional release is normalized to the total mass of protein released during the 42-day study period. (D) 42-day cumulative mass release of insulin 1% (N1, ○), 5% (N2, □), 9% (N3, △), 13% (N4, ◇) and 19% (N5, ×) BZI-loaded formulations prepared using the nanonized-BZI method.

Interestingly, this formulation had a near linear release of $69.26 \pm 6.20\%$ over the first 5 days before entering the lag phase observed with the other formulations; while the release of insulin from the 19% formulation was the slowest during the first 24 hours, the release was more rapid than the 13% formulation following day 2 (Figure 6.5B).

All formulations exhibited a period of slower release between the middle of the first week through 21 days (Figure 6.5C); this is a behavior typical to PLGA and is often referred to as the lag phase. An interesting observation during this period was that as the actual protein loading increased from 1 to 19%, there was a shorter lag phase for some of the formulations with release starting at 21 to 7 days. While the 5% and 19% formulations started the secondary release of insulin at day 21 following the lag, the other formulations continued to release insulin during this time. The 9% loaded nanospheres exhibited some release during the second week followed by a lag between days 14 and 21 until the release of insulin continued to the end of the study – an almost tri-phasic release profile. In contrast, the 13% formulation had a short lag period between days 5 and 7 before continuously increasing insulin release throughout the duration of the study.

In terms of total insulin release, the 1%, 5% and 10% cryogenic emulsion formulations released $99.75 \pm 0.42 \mu\text{g}$, $475.75 \pm 2.77 \mu\text{g}$ and $699.37 \pm 4.00 \mu\text{g}$ of insulin over the course of a week corresponding to $2.19 \pm 0.01 \text{ IU}$, $10.46 \pm 0.06 \text{ IU}$ and $15.37 \pm 0.09 \text{ IU}$ of insulin from a 10 mg dose (Figure 6.5D). The 1%, 5%, 9%, 13% and 19% nanonized formulations released $56.25 \pm 0.73 \mu\text{g}$, $254.80 \pm 6.82 \mu\text{g}$, $542.04 \pm 4.01 \mu\text{g}$, $690.02 \pm 94.46 \mu\text{g}$ and $1049.18 \pm 99.72 \mu\text{g}$ of insulin corresponding to $1.24 \pm 0.02 \text{ IU}$, $5.60 \pm 0.15 \text{ IU}$, $11.91 \pm 0.09 \text{ IU}$, $15.17 \pm 2.08 \text{ IU}$ and $23.06 \pm 2.19 \text{ IU}$ of insulin (Figure 5D). All of these values are well within the therapeutic range of insulin for the maintenance of euglycemia in diabetic human patients.

This release data demonstrates the ability of PIN nanospheres loaded using the nanonized-BZI method to effectively control and sustain the release of insulin over a 42-day period. The prolonged release observed with higher loaded formulations may be a result of the localization of the protein within the nanospheres. While the 1% loaded formulation with a large initial burst and rapid release kinetics suggests a localization of the polymer to the surface, the reduction in initial burst seen with the higher loaded formulations indicates that a smaller portion of the protein resides at or near the surface of the spheres. Furthermore, the

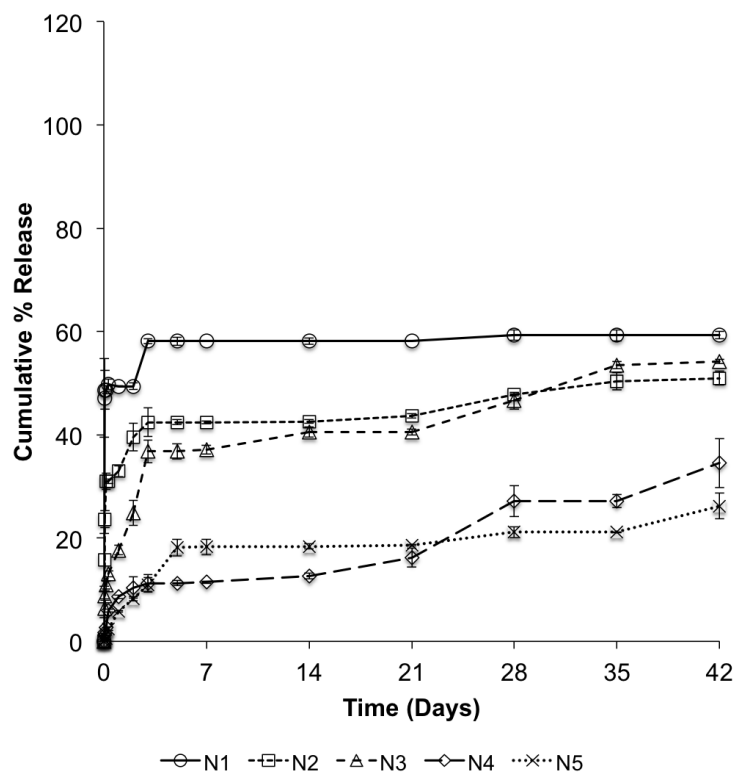


Figure 6.6: 42-day cumulative percent release of insulin normalized to theoretical loading from 1% (N1, ○), 5% (N2, □), 9% (N3, △), 13% (N4, ◇) and 19% (N5, ×) BZI-loaded formulations prepared using the nanonized-BZI method.

sustained release kinetics confirms that the protein is well encapsulated within the polymer. It is possible that during the encapsulation process, the protein has a higher affinity to itself than the polymer and serves as nucleation sites for the polymer, thus creating a polymeric shell around a core of insulin, which would serve as a rate-controlling barrier to release. The highest loaded 19% formulation exhibits different release kinetics but with a pattern characteristic of PLGA, suggesting that the higher ratio of protein relative to polymer may not provide a sufficient barrier to achieve more sustained release kinetics. Additionally, the tert-butyl alcohol nanoprecipitation method involves the dissolution and re-complexing of insulin with zinc ions, which creates another level of control over release not present with the other methods in which the insulin is completely solubilized in the aqueous phase prior to encapsulation. In order for the insulin to be released, it must first solubilize and dissociate from the zinc then diffuse through channels and pores in the polymeric matrix, which may account for the slow sustained release over the 42 days.

6.4.7 Transmission Electron Microscopy

To gain a better understanding of the morphology of the drug inside the nanoparticles, cross-sections of the formulations were evaluated by transmission electron microscopy; the protein was stained using osmium tetroxide and appears dark in the micrographs. As can be seen in figure 6.7, the majority of the insulin in the 9% nanonized formulation (N3) is distributed in the center of the polymeric matrix and surrounded by a layer of a continuous polymer phase. The micrographs in figure 6.8 are representative images of the 19% nanonized formulations (N5). It is apparent from these images that a different configuration was achieved with these nanospheres in which a core of protein is surrounded by a polymer coating, as can be seen in figure 6.8A and 6.8D. In contrast, figures 6.8B and 6.8C demonstrate a thicker shell of polymer; the irregular shape of the particle in figure 6.8C may be the result of the spontaneity and rapidity of the phase inversion process which can result in the formation of some irregularly shaped and polydispersed particles. In some cases, particles are observed as dark spheres with no coating, indicating that almost no encapsulation occurred in those particles. This variation in morphology and protein distribution among the nanospheres may be an explanation for the different release pattern as well as the lower encapsulation

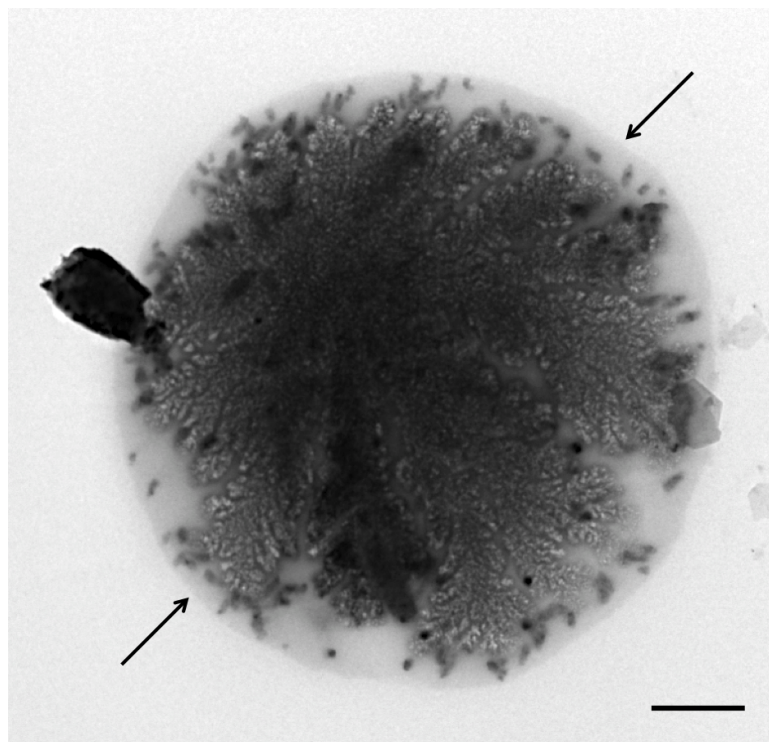


Figure 6.7: Transmission electron micrograph of a 9% nanonized-BZI-loaded nanosphere (N3) cross-section stained with osmium tetroxide; arrows indicate polymeric matrix; scale bar: 500 nm.

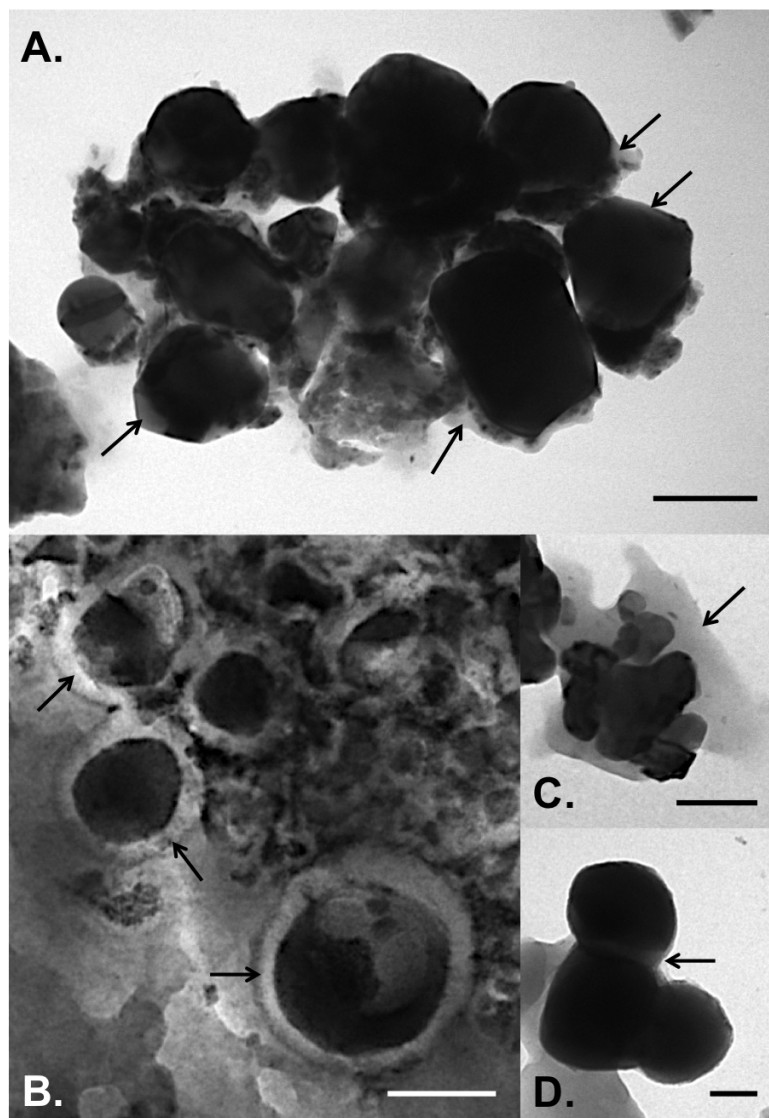


Figure 6.8: Transmission electron micrographs of 19% nanonized-BZI-loaded nanospheres (N5) cross-sections fixed with osmium tetroxide; arrows indicate polymeric matrix; scale bars: (A, C) 250 nm, (B,D) 100 nm.

efficiency observed with the 19% formulation. Some unencapsulated insulin particles may have been lost through the filter membrane during the nanosphere collection process due to their small size.

6.5 Conclusions

The phase inversion nanoencapsulation process was used to prepared PLGA nanospheres loaded with BZI using a w/o emulsion, w/o cryogenic emulsion and nanonized-BZI methods to determine the effect of protein loading method, salt and total protein loading on encapsulation and release. Nanosphere characterization revealed a polydispersed population of nanospheres with similar physical and chemical properties. Studies of encapsulation efficiencies revealed high encapsulation efficiencies with most formulations, with the exception of the 13% and 19% nanonized formulations, and showed the preferential loss of polymer over protein during the encapsulation process. For the higher loaded formulations, an inverse relationship was observed between total protein loading and encapsulation efficiency. Release studies revealed no difference in the release kinetics between the loading methods for the 1% BZI-loaded formulations and a slight difference in the emulsion formulations with 1% salt, due to its potential osmotic behavior and localization. Studies of the 1%, 5% and 10% loaded cryogenic emulsion nanospheres revealed similar rapid release kinetics regardless of loading. The higher loaded nanonized formulations revealed a reduction in initial burst and more sustained release kinetics with an increase in protein loading from 1% to 19%. Further morphological analysis of the nanospheres confirmed than the nanospheres took on a core-shell type arrangement with a core of protein surrounded by a shell of polymer, which may serve as an explanation of the more controlled release properties achieved using this loading method.

6.6 References

- [1] S.C. Yadav, A. Kumari, R. Yadav, Development of peptide and protein nanotherapeutics by nanoencapsulation and nanobioconjugation., *Peptides*. 32 (2011) 173–187.

- [2] S.D. Putney, P.A. Burke, Improving protein therapeutics with sustained-release formulations., *Nat Biotechnol.* 16 (1998) 153–157.
- [3] A. Taluja, Y.S. Youn, Y.H. Bae, Novel approaches in microparticulate PLGA delivery systems encapsulating proteins, *J. Mater. Chem.* 17 (2007) 4002.
- [4] A. Giteau, M.C. Venier-Julienne, A. Aubert-Pouëssel, J.P. Benoit, How to achieve sustained and complete protein release from PLGA-based microparticles?, *International Journal of Pharmaceutics.* 350 (2008) 14–26.
- [5] J.J. S, M. Edith, A Novel Mechanism for Spontaneous Encapsulation of Active Agents: Phase Inversion Nanoencapsulation, in: *ACS Symposium Series CY -*, American Chemical Society, Washington, DC, 2011: pp. 214–223–223.
- [6] E. Mathiowitz, J.S. Jacob, Y.S. Jong, G.P. Carino, D.E. Chickering, P. Chaturvedi, et al., Biologically erodable microspheres as potential oral drug delivery systems., *Nature.* 386 (1997) 410–414.
- [7] N.K. Egilmez, Y.S. Jong, E. Mathiowitz, R.B. Bankert, Tumor vaccination with cytokine-encapsulated microspheres., *Methods Mol. Med.* 75 (2003) 687–696.
- [8] A. Sharma, C.M. Harper, L. Hammer, R.E. Nair, E. Mathiowitz, N.K. Egilmez, Characterization of cytokine-encapsulated controlled-release microsphere adjuvants., *Cancer Biother. Radiopharm.* 19 (2004) 764–769.
- [9] A. Arora, G. Su, E. Mathiowitz, J. Reineke, A.E. Chang, M.S. Sabel, Neoadjuvant intratumoral cytokine-loaded microspheres are superior to postoperative autologous cellular vaccines in generating systemic anti-tumor immunity., *J Surg Oncol.* 94 (2006) 403–412.
- [10] S. Furtado, D. Abramson, R. Burrill, G. Olivier, C. Gourd, E. Bubbers, et al., Oral delivery of insulin loaded poly(fumaric-co-sebacic) anhydride microspheres., *International Journal of Pharmaceutics.* 347 (2008) 149–155.
- [11] S. Furtado, D. Abramson, L. SIMHKAY, D. WOBBEKIND, E. Mathiowitz, Subcutaneous delivery of insulin loaded poly(fumaric-co-sebacic anhydride) microspheres to type 1 diabetic rats, *European Journal of Pharmaceutics and Biopharmaceutics.* 63 (2006) 229–236.
- [12] M. Sandor, S. Mehta, J. Harris, C. Thanos, P. Weston, J. Marshall, et al., Transfection of HEK cells via DNA-loaded PLGA and P(FASA) nanospheres., *Journal of Drug Targeting.* 10 (2002) 497–506.
- [13] G.P. Carino, J.S. Jacob, E. Mathiowitz, Nanosphere based oral insulin delivery., *J Control Release.* 65 (2000) 261–269.
- [14] E. Mathiowitz, M.R. Kretiz, L. Brannon-Peppas, *Microencapsulation*, 1st ed. John Wiley & Sons, Inc., New York ,NY, 1999.
- [15] S. Freitas, H.P. Merkle, B. Gander, Microencapsulation by solvent extraction/evaporation: reviewing the state of the art of microsphere preparation process technology, *Journal of Controlled Release.* 102 (2005) 313–332.

- [16] M. Li, O. Rouaud, D. Poncelet, Microencapsulation by solvent evaporation: state of the art for process engineering approaches., *International Journal of Pharmaceutics*. 363 (2008) 26–39.
- [17] L. Jorgensen, S. Hostrup, E.H. Moeller, H. Grohgan, Recent trends in stabilising peptides and proteins in pharmaceutical formulation - considerations in the choice of excipients., *Expert Opin Drug Deliv.* 6 (2009) 1219–1230.
- [18] Y. Zhang, S. Zale, L. Sawyer, H. Bernstein, Effects of metal salts on poly(DL-lactide-co-glycolide) polymer hydrolysis., *J. Biomed. Mater. Res.* 34 (1997) 531–538.
- [19] M. Tracy, Development and Scale-up of a Microsphere Protein Delivery System, *Biotechnology Progress*. (1998).
- [20] K. Fu, R. Harrell, K. Zinski, C. Um, A. Jaklenec, J. Frazier, et al., A potential approach for decreasing the burst effect of protein from PLGA microspheres., *J. Pharm. Sci.* 92 (2003) 1582–1591.
- [21] D. Blanco, Protein encapsulation and release from poly(lactide-co-glycolide) microspheres: effect of the protein and polymer properties and of the co-encapsulation of surfactants, *European Journal of Pharmaceutics and* (1998).
- [22] W.L. Webber, F. Lago, C. Thanos, E. Mathiowitz, Characterization of soluble, salt-loaded, degradable PLGA films and their release of tetracycline., *J. Biomed. Mater. Res.* 41 (1998) 18–29.

Chapter 7

Conclusions

The ultimate goal of this work is to provide a foundation for the development of more effective oral drug delivery platforms based on the knowledge obtained from systematic evaluations of particle uptake and tissue distribution from the small intestine, bioadhesion, nanosphere preparation and protein encapsulation. The conclusions of these studies and their potential applications in oral drug delivery will be discussed in this chapter.

7.1 Particle Uptake from the Small Intestine

There have been many reports of particle uptake from the gastrointestinal tract in scientific literature but there have been few quantitative studies to provide a fundamental understanding of particle uptake for oral drug delivery applications [1-7]. Thus, our evaluations begun qualitatively on the cellular level using transmission electron microscopy, which revealed that particle uptake can occur through intestinal enterocytes, potentially through an endocytotic mechanism. Evaluating particle uptake on the tissue level with confocal microscopy revealed that these nanospheres are capable of translocating through intestinal enterocytes and can enter the core of intestinal villi, which would allow for their distribution throughout the body by way of the circulatory system. These results motivated us to systematically evaluate particle uptake from the small intestine utilizing a quantitative in vivo isolated loop technique.

Quantitative uptake, biodistribution and uptake kinetics of microspheres from the small intestine are dependent on microsphere size and intestinal location. An inverse relationship between microsphere uptake and microsphere size exists in both the jejunum and ileum as

well as when spheres are administered orally. The highest level of uptake was observed when 500 nm polystyrene nanospheres were administered to the jejunum with $45.8 \pm 8.6\%$ of the total dose detected in the in the target tissues, demonstrating the great potential of polymeric micro- and nanospheres in oral drug delivery applications and provides the first evidence of high uptake levels by enterocytes in Peyer’s patch-free regions of the small intestine, which were believed to play an insignificant role in this process. Furthermore, particle size and uptake location plays a role in the tissue distribution of these nanospheres, which had a significant degree of localization in the liver but different biodistribution profiles based on these parameters.

Given the lack of studies systematically evaluating uptake mechanisms in the small intestinal tract and our data suggesting that particle translocation may occur via mechanisms typically thought to be uninvolved, we utilized the isolated loop technique with specific chemical uptake inhibitors to evaluate the pathways involved. The results were in direct contradiction to the understanding in the field, which believed that particles in this size range are taken up by M-cells in the Peyer’s patches as part of a GI immune surveillance function. The uptake and tissue distribution data indicate that the predominant mechanism in the translocation of polymeric microspheres is endocytotic, likely a synergistic effort between clathrin- and caveolae-mediated endocytosis.

This is significant because previous attempts at optimizing oral drug delivery systems for uptake by M-cell phagocytosis were unsuccessful due to low levels of uptake since these cells account for less than 1% of the surface area. By utilizing endocytotic uptake mechanisms by enterocytes, which comprise the vast majority of the surface area of the small intestine, combined with optimized particle sizes, more effective oral drug delivery may be achieved.

7.2 Bioadhesion

Many approaches have been evaluated to enhance the translocation of micro- and nanospheres from the small intestine [6,8,9]. In particular, our lab has focused on bioadhesion as a means of increasing gastrointestinal transit time and promoting interactions with the absorptive epithelium to achieve higher levels of particle uptake [10-14]. By combining the polyanhydride

backbone chemistry, which is capable of generating carboxylic acids upon hydrolysis, with the bioadhesive DOPA functionality, our group has developed novel bioadhesive polymers in which two maleic anhydride co-polymer backbones, a low molecular weight poly(butadiene-maleic anhydride) (PBMA) and a high molecular weight poly(ethylene-maleic anhydride) (PEMA), were conjugated to catechol-precursor amino acids. Using a texture analyzer apparatus, the bioadhesive properties of these polymers as a function of molecular weight, hydroxyl content and duration of interaction with small intestinal tissue was systematically evaluated.

The results suggest that an increase in the hydroxyl content of these polymers results in the generation of higher bioadhesive forces with intestinal mucosa; however, this comes at the cost of more rapid dissolution due to the increased hydrophilicity of the polymer. By increasing the molecular weight of the polymer backbone, the dissolution of the polymer can be controlled to allow for a longer duration of bioadhesion. The novel bioadhesive polymers developed in our lab are capable of achieving higher fracture strengths and tensile work values than poly(acrylic acid), a bioadhesive polymer currently used in pharmaceutical applications, with more desirable kinetics of bioadhesion. The highest levels of bioadhesion were observed for the PBMA and PEMA polymers, which had average tensile work values 1.51 and 1.88 times the average work of PAA.

Additionally, these bioadhesives exhibited pH-dependent solubilities which are advantageous for oral drug delivery applications due to their ability to remain in their solid form in the more acidic environment of the stomach to protect any encapsulated therapeutic cargo followed by the “activation” of the bioadhesive behavior of the polymer in the more basic small intestine to allow for increased GI transit time, enhanced interactions with the absorptive mucosal epithelium of the small intestine as well as pH-dependent controlled release characteristics for more effective drug delivery.

7.3 Uptake of Bioadhesive Nanospheres

Utilizing the knowledge gained from the uptake and bioadhesion studies, the effect of bioadhesion on the uptake and translocation of 500 nm nanospheres from the jejunum were evalu-

ated. Three nanosphere formulations were developed and characterized – a non-bioadhesive poly(methyl methacrylate) (PMMA), a moderately adhesive polystyrene and a bioadhesive PBMA-shell/PMMA-core formulation. The bioadhesiveness of these formulations were determined both using the bulk polymer in vitro with the texture analyzer apparatus and ex vivo using an everted sac bioadhesion assay. Bioadhesion testing revealed that the PMMA was least bioadhesive and the PBMA was most adhesive. Furthermore, a strong correlation was observed for the two testing methods indicating that bioadhesion testing with the bulk polymer can be used to predict bioadhesive behavior in the nanoscale.

The non-adhesive PMMA formulation had $5.8 \pm 1.9\%$ while the PBMA/PMMA formulation achieved $66.9 \pm 12.9\%$ uptake to the specific organs that we have evaluated, indicating that the addition of a bioadhesive PBMA coating on the non-adhesive PMMA spheres was able to enhance particle uptake 11.5-fold. Comparison of the bioadhesion testing results with uptake results confirmed a strong, direct correlation between bioadhesion and uptake. These results provide strong evidence for the use of bioadhesion as a strategy for enhancing particle uptake from the small intestine for more effective drug delivery. Furthermore, nanospheres of different surface chemistries (PMMA, PS and PBMA) were observed to distribute differently to organs following internalization and may allow for the passive targeting of oral nanosphere drug delivery systems based on polymer selection.

7.4 Sequential Phase Inversion Nanoencapsulation (sPIN)

As a method to minimize the burst effect commonly seen with micro- and nanospheres as well as to modify surface chemistry for the enhancement of particle uptake from the gastrointestinal tract, the use of double-walled nanospheres was proposed. Previous methods for the preparation of double-walled microspheres are not suitable for the efficient preparation of sub-micron sized nanospheres so we turned to the phase inversion nanoencapsulation (PIN) method [15], which relies on phase separation and phase inversion phenomena to achieve the core-shell arrangement of the two polymers in the system. By modifying this method to allow for the controlled sequential phase inversion of two polymers, double-walled nanospheres can be prepared in a single step via the sPIN process. In contrast to the methods for the

preparation of double-walled microspheres based on solvent evaporation and removal, which rely on polymer-polymer interactions, this method relies on the phase separation of polymers in solution upon addition of a non-solvent, a much more rapid process. Therefore, the formation of nanospheres is instantaneous and requires no shear forces, which is further beneficial for the preservation of bioactivity when encapsulating sensitive therapeutic agents, such as proteins or genetic material.

When preparing double-walled nanospheres using the sPIN method, care must be taken to engineer the conditions for the proper localization of the two polymers within the nanospheres. For the core polymer, a solvent must be selected that is also a solvent for the shell polymer while the solvent for the shell polymer must be a non-solvent for the core polymer; all solvents must be miscible with the non-solvent for both polymers. When the shell polymer solution is added to the core polymer solution, the resulting decrease in the core polymer solubility at its cloud point results in its preferential phase separation to produce the core of the nanosphere and if desired, encapsulation of the target drug. When this unstable solution is rapidly added to the non-solvent, the shell polymer engulfs the core polymer as the phase inversion is completed to form double-walled nanospheres.

Using this method, double-walled nanospheres with different core- and shell-polymer combinations were prepared and characterized. Physical characterization revealed particles in the 500 nm size range and confirmed the phase separation of the two polymers in the formulations. Furthermore, insulin was encapsulated within a PBMA/PLGA formulation, confirming that bioadhesive and biodegradable double-walled nanospheres for controlled oral drug delivery could be engineered using the sPIN method.

7.5 Protein Nanoencapsulation

The use of polymeric nanospheres has been evaluated as an approach to overcome the challenges of protein delivery [9,16-20]. However, the high surface area to volume ratio that results from a reduction in particle size with the use of nanoparticles often requires compromising control of over release kinetics for the potential advantages presented by nanoscale geometries. Despite the many advances in microencapsulation techniques, an effective method

for controlling the release of therapeutic proteins from polymeric nanospheres has been difficult to achieve.

The phase inversion nanoencapsulation process (PIN) was used to prepare PLGA nanospheres loaded with BZI using a w/o emulsion, w/o cryogenic emulsion and s/o nanonized-BZI dispersion methods to determine the effect of protein loading method, salt and total protein loading on encapsulation and release. Nanosphere characterization revealed a polydispersed population of nanospheres with similar physical and chemical properties. Studies of encapsulation efficiencies revealed high encapsulation efficiencies for most formulations and showed the preferential loss of polymer over protein during the encapsulation process. For the higher loaded formulations, an inverse relationship was observed between total protein loading and encapsulation efficiency.

Release studies revealed no difference in the release kinetics between the loading methods for the 1% BZI-loaded formulations and a slight difference in the emulsion formulations with 1% salt, due to its potential osmotic behavior and localization. Studies of the 1%, 5% and 10% loaded cryogenic emulsion nanospheres revealed similar rapid release kinetics regardless of loading. The higher loaded nanonized formulations revealed a reduction in initial burst and more sustained release kinetics over 42 days with an increase in protein loading from 1% to 19%. TEM analysis of the nanospheres indicated that the nanospheres consisted of a core of protein surrounded by a shell of polymer, which may serve as an explanation of the more controlled release properties achieved using this loading method. These results indicate the potential of the PIN method to create nanospheres that are capable of delivering therapeutically relevant doses of insulin (up to 23.06 ± 2.19 IU or 1049 ± 99.72 μ g from a 10 mg dose) with sustained release over the course of 42 days.

7.6 Potential Oral Drug Delivery System

The culmination of this work is a set of design criteria and methods for the development of a potentially effective oral drug delivery system. While the findings of each individual study provides essential information for the development of all oral drug delivery systems, the combined results motivate the development of a oral delivery platform based on bioadhesive

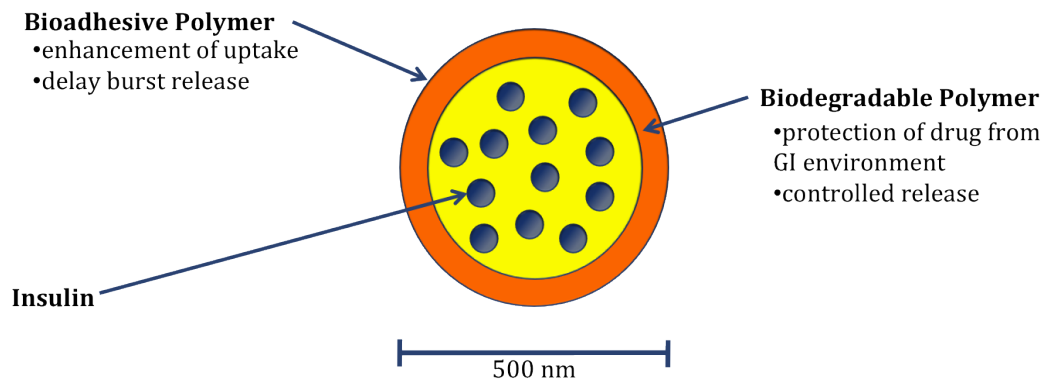


Figure 7.1: Schematic of potential oral drug delivery system based on bioadhesive and biodegradable double-walled nanospheres.

double-walled nanospheres.

The uptake studies revealed that particle translocation can be achieved by the intestinal enterocytes, which account for >98% of the surface area of the small intestine, and so oral drug delivery systems need not be optimized for M-cell phagocytosis as the primary means of translocation. Additionally, given the inverse relationship between particle size and uptake, the development of smaller nanospheres for oral delivery would allow for higher levels of uptake but potentially at the cost of total drug loading. The protein encapsulation studies have shown that with 500 nm nanospheres, high loadings can be achieved and that using the nanonized-BZI method, the total protein loading can be used to modulate release kinetics. This is further advantageous in that the 500 nm spheres achieved the highest levels of uptake from the jejunum. Using the novel bioadhesive polymers combined with the sequential phase inversion nanoencapsulation (sPIN) method developed in this work, bioadhesive and biodegradable protein-loaded nanospheres can be prepared in a single step. Furthermore, uptake studies using bioadhesive nanospheres showed a strong correlation between bioadhesion and uptake; most importantly, the addition of a bioadhesive PBMAAD coating to a non-adhesive polymer using the sPIN method was able to significantly enhance particle uptake. Thus, we propose an oral drug delivery platform based on 500 nm bioadhesive shell, biodegradable core nanospheres prepared via sPIN (Figure 7.1). Protein loading can be achieved using the cryogenic emulsion method at high loadings for rapid release kinetics or by the nanonized-BZI method for sustained release kinetics.

Using the data obtained in this work, we can evaluate the potential of our new oral delivery system. By fabricating particles of 500 nm with the extremely bioadhesive PBMA^D-shell, we can achieve protein loadings of up to 19% and our *in vivo* uptake data predicts the uptake of 66.86% of the administered dose. Furthermore, the unique bioadhesive properties of PBMA^D allow for it to function as a protective coating on each nanosphere to prevent the premature release of insulin in the harsh environment of the stomach followed by activation into a bioadhesive matrix in the small intestine to enhance the translocation of the insulin-containing core into the circulation to minimize insulin loss. The result is a theoretical bioavailability of 66.9% with an insulin delivery of 70.55 IU (3210.24 μ g) from a 25 mg dose, well within the therapeutically relevant range required for the management of diabetes. If a PBMA^D/PLGA formulation with 1%, 5% or 9% insulin loading is administered, the theoretical insulin delivery is 4.15 IU (188.94 μ g), 19.07 IU (867.77 μ g) or 33.55 IU (1526.54 μ g), respectively, indicating the ability to tailor the amount of insulin delivered. Furthermore, our release data demonstrates the ability to achieve near complete protein release from our formulations over the course of a few hours using the cryogenic emulsion method or over the course of many weeks using the nanonized method, which allows for the selection of the appropriate loading method to obtain the optimal release kinetics for the target therapeutic agent and application.

7.7 Future Directions

Despite the promising results of our potential oral protein delivery system, a significant amount of work would be required to bring this product fruition. In the case of diabetes, the formulation would need to be engineered to meet the dosage and release kinetics required for either the prevention of glucose excursions following meals or the maintenance of basal levels of insulin. Once this is determined, the formulations would need to be prepared and characterized chemically and physically using techniques such as laser diffraction particle size analysis, dynamic light scattering (DLS) for particle size, electrophoretic mobility for surface energy, FTIR for chemical composition, DSC for thermal properties and electron microscopy for morphology. Additionally, the encapsulation efficiencies, release studies and

insulin bioactivity would need to be determined in vitro prior to animal studies to determine their behavior in vivo. The therapeutic effect of the NS formulations can be evaluated in vivo in the diabetogenic BioBreeder Diabetes Prone (BBDP) rat model. These rats are genetically predisposed to spontaneously develop type I diabetes mellitus due to autoimmune destruction of the pancreatic β -cells. These rats provide an excellent model for the evaluation of this therapeutic system because they do not produce endogenous insulin following the onset of diabetes [13]; therefore, any insulin detected in the plasma is the result of the experimental manipulations. The efficacy of the orally administered encapsulated insulin should be evaluated compared to injectable insulin and a control group following the administration of the appropriate dose of nanospheres to the rats by oral gavage. Blood samples should be collected at hourly intervals by tail vein bleeds into heparinized tubes and centrifuged to recover the plasma for analysis. A glucose trinder assay can be used to determine plasma glucose levels while insulin levels can be detected using an enzyme-linked immunosorbent assay (ELISA).

The relative oral bioavailability of the insulin is calculated by comparing the plasma concentrations from oral administration of the nanospheres to those from the rats receiving subcutaneous insulin according to the following equation:

$$relative\ bioavailability = \frac{\frac{[AUC]_{PO}}{dose_{PO}}}{\frac{[AUC]_{SQ}}{dose_{SQ}}}$$

where the AUC is the area under the curve using the trapezoidal rule by plotting average plasma insulin level over time, PO indicates the oral nanosphere administration and SQ indicates the subcutaneous injection of unencapsulated insulin. These results would ideally indicate that in vivo bioavailabilities are equivalent to those of subcutaneous insulin but given the complexities of oral drug delivery, high bioavailabilities combined with evidence of therapeutic efficacy would provide compelling evidence for the further development and optimization of this system.

7.8 Conclusions

Through the systematic evaluation of particle uptake and tissue distribution, the synthesis and characterization of novel bioadhesive polymers, the development of novel nanoencapsulation methods and systematic evaluation of protein nanoencapsulation by phase inversion, we have provided a framework upon which to develop more effective oral drug delivery platforms.

7.9 References

- [1] F. Delie, Evaluation of nano- and microparticle uptake by the gastrointestinal tract., *Advanced Drug Delivery Reviews*. 34 (1998) 221–233.
- [2] M. Desai, V. Labhasetwar, G. Amidon, Gastrointestinal uptake of biodegradable microparticles: effect of particle size, *Pharm Res.* (1996).
- [3] G. Hodges, E. Carr, R. Hazzard, Uptake and Translocation of Microparticles in the Small Intestine, *Digestive Diseases and . . .* (1995).
- [4] P. Jani, G.W. Halbert, J. Langridge, A.T. Florence, The uptake and translocation of latex nanospheres and microspheres after oral administration to rats., *J. Pharm. Pharmacol.* 41 (1989) 809–812.
- [5] N. Hussain, V. Jaitley, A.T. Florence, Recent advances in the understanding of uptake of microparticulates across the gastrointestinal lymphatics., *Advanced Drug Delivery Reviews*. 50 (2001) 107–142.
- [6] A.T. Florence, Issues in Oral Nanoparticle Drug Carrier Uptake and Targeting, *Journal of Drug Targeting*. 12 (2004) 65–70.
- [7] F. Delie, M.J. Blanco-Príeto, Polymeric particulates to improve oral bioavailability of peptide drugs., *Molecules*. 10 (2005) 65–80.
- [8] V.F. Patel, F. Liu, M.B. Brown, Advances in oral transmucosal drug delivery., *J Control Release*. 153 (2011) 106–116.
- [9] A. Taluja, Y.S. Youn, Y.H. Bae, Novel approaches in microparticulate PLGA delivery systems encapsulating proteins, *J. Mater. Chem.* 17 (2007) 4002.
- [10] C.G. Thanos, Z. Liu, M. Goddard, J. Reineke, N. Bailey, M. Cross, et al., Enhancing the oral bioavailability of the poorly soluble drug dicumarol with a bioadhesive polymer., *J. Pharm. Sci.* 92 (2003) 1677–1689.
- [11] J. Jacob, E. Mathiowitz, Poly(fumaric-co-sebacic) microspheres as oral drug delivery systems, *Biotechnology and . . .* (1996).

- [12] C.A. Santos, J.S. Jacob, B.A. Hertzog, B.D. Freedman, D.L. Press, P. Harnpicharnchai, et al., Correlation of two bioadhesion assays: the everted sac technique and the CAHN microbalance., *J Control Release*. 61 (1999) 113–122.
- [13] S. Furtado, D. Abramson, R. Burrill, G. Olivier, C. Gourd, E. Bubbers, et al., Oral delivery of insulin loaded poly(fumaric-co-sebacic) anhydride microspheres., *International Journal of Pharmaceutics*. 347 (2008) 149–155.
- [14] C. Santos, Evaluation of anhydride oligomers within polymer microsphere blends and their impact on bioadhesion and drug delivery in vitro, *Biomaterials*. 24 (2003) 3571–3583.
- [15] J.J. S, M. Edith, A Novel Mechanism for Spontaneous Encapsulation of Active Agents: Phase Inversion Nanoencapsulation, in: *ACS Symposium Series CY -*, American Chemical Society, Washington, DC, 2011: pp. 214–223–223.
- [16] S. Cohen, T. Yoshioka, M. Lucarelli, L.H. Hwang, R. Langer, Controlled delivery systems for proteins based on poly(lactic/glycolic acid) microspheres., *Pharm Res*. 8 (1991) 713–720.
- [17] S.D. Putney, P.A. Burke, Improving protein therapeutics with sustained-release formulations., *Nat Biotechnol*. 16 (1998) 153–157.
- [18] A. Vila, A. Sanchez, M. Tobio, P. Calvo, Design of biodegradable particles for protein delivery, *Journal of Controlled . . .* (2002).
- [19] S.C. Yadav, A. Kumari, R. Yadav, Development of peptide and protein nanotherapeutics by nanoencapsulation and nanobioconjugation., *Peptides*. 32 (2011) 173–187.
- [20] S. Freiberg, X. Zhu, Polymer microspheres for controlled drug release., *International Journal of Pharmaceutics*. 282 (2004) 1–18.

Appendix A

Development of an Artificial Melanosome by Engineering a Lipid-Polymer Hybrid Microparticle

A.1 Introduction

Pigmentation is a crucial protection mechanism against UV-induced cellular damage [1]. Specialized cells called melanocytes are responsible for the production, storage and transfer of pigment present in the skin, hair and eyes of mammals and some vertebrates [2]. Under normally functioning conditions, biopolymeric pigments called melanins are synthesized through subsequent oxidation reactions of an amino acid, tyrosine, into one of two forms: eumelanin (black) or pheomelanin (red) [1-3]. The synthesis machinery for these melanin pigments is found within organelles, called melanosomes, which are specific to melanocytes and retinal pigment epithelium in mammals [2]. Structurally, melanosomes are liposome-like organelles ranging from 0.94 – 1.44 μm in diameter and containing homogenous dark pigment, as observed by light microscopy [4].

Melanocytes transport their products through filapodia processes to keratinocytes located in the external skin layer [5]. Despite extensive research, the exact method of the pigment translocation and uptake by skin cells remains elusive. However, with this work,

Daniel Y. Cho^{*}, Lauren Quattrochi^{*}, Stacia Furtado, Kenneth Estrellas, Natalie Chavez, Nadine Wicks, Edith Mathiowitz and Elena Oancea

^{*}both authors contributed equally to this work

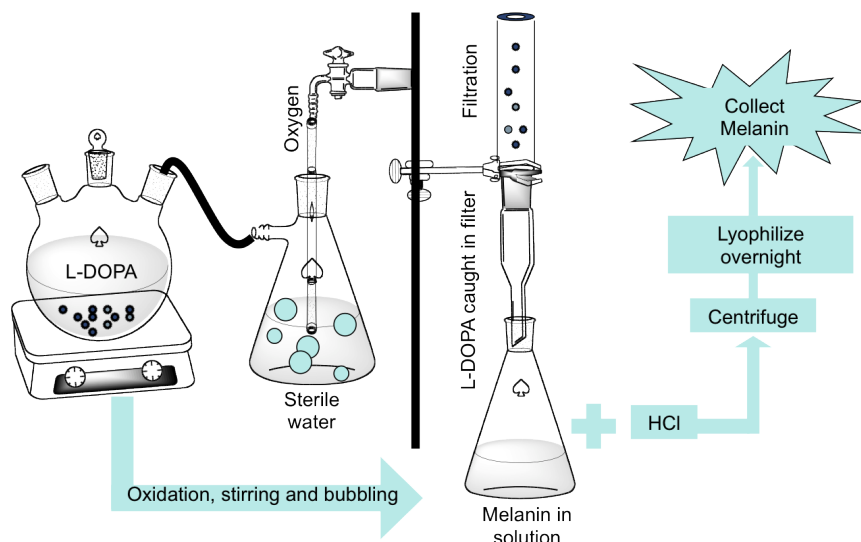


Figure A.1: Schematic of melanin synthesis.

we hope to provide a novel tool to enable the transport of an artificial melanosome in an in vitro setting, in order to elucidate this pathway. Through development of synthetic melanosomes, we provide a biodegradable, cytocompatible, purified and readily modifiable organelle to characterize uptake mechanisms in epithelial cells, as well as, possibly develop a novel melanin delivery approach in order to minimize the damaging effects of UV on human skin cells.

A.2 Materials and Methods

A.2.1 Materials

L-DOPA was purchased from Sigma Aldrich (St. Louis, MO). Poly(L-lactic acid) (i.v. 0.90 - 1.20) was purchased from Durect, Inc. Lecithin was obtained from Spectrum Chemical Mfg. Corp. (Gardena, CA).

A.2.2 Melanin Synthesis

Melanin was synthesized from a natural precursor, 3,4-dihydroxy-L-phenylalanine (L-DOPA), using a method adapted from the auto-oxidation protocol described by Arnow (Figure A.1) [3]. Briefly, air was bubbled through a saturated solution of L-DOPA in 0.01 N NaOH to

allow for the oxidation of L-DOPA, with a concurrent color change from pink to the jet black of melanin. The excess insoluble L-DOPA sediment was filtered using 0.2 μm filter and the resulting melanin solution was acidified using 6M HCl to precipitate the melanin. Thereafter, the melanin precipitate was centrifuged and lyophilized. Melanin synthesis was confirmed by comparison to commercially available melanin using UV-VIS, X-Ray Diffraction and Fourier Transform Infrared Spectroscopy (FTIR).

A.2.3 Melanin Micronization by Phase Inversion

The dried melanin particles were in the $\sim 200\ \mu\text{m}$ range so the pigment was micronized using a novel phase inversion method. Lab synthesized melanin was dissolved in 1N NaOH to a concentration of 60mg/mL. 1mL of the concentrated melanin solution was then rapidly added to a beaker containing 1 L of isopropyl alcohol (IPA) for a solvent to non-solvent ratio of 1:1000 then stirred at 500 rpm with an overhead stirrer resulting in the spontaneous formation of melanin microparticles. Melanin particles were then centrifuged and lyophilized to remove residual solvent (Figure A.2).

A.2.4 Microsphere Preparation

Melanin was encapsulated in poly-L-lactic acid (PLLA), using a S/O/W solvent evaporation method (Figure A.3). 20 mg of the micronized melanin was added to a 10 mL of a 2% solution of PLLA in methylene chloride with bath sonication to create the solid-in-oil (s/o) suspension. A 250 mL aqueous phase of 1 % w/v polyvinyl alcohol (25 kDa, 88 % hydrolyzed) was prepared and mixed under a VirTis Cyclone IQ² SENTRY microprocessor at 25,000 rpm with a straight open blade and VirTis baffled homogenizer flask; 200 μL of 1-octanol was added to the solution minimize bubble formation. After 5 minutes of shearing, the s/o dispersion was added to the PVA solution to form the solid-in-oil-in-water (s/o/w) emulsion, which was further emulsified for 15 minutes. The resulting emulsion was poured into 100 mL of 1 % w/v PVA and stirred at 13,000 rpm with an overhead mixer for 5 hours to allow for solvent evaporation and polymer precipitation. The resulting microspheres suspension was centrifuged at 6000 rpm for 20 minutes and the supernatant poured off. The pellet was resuspended and washed in deionized water three times then the formulation was lyophilized

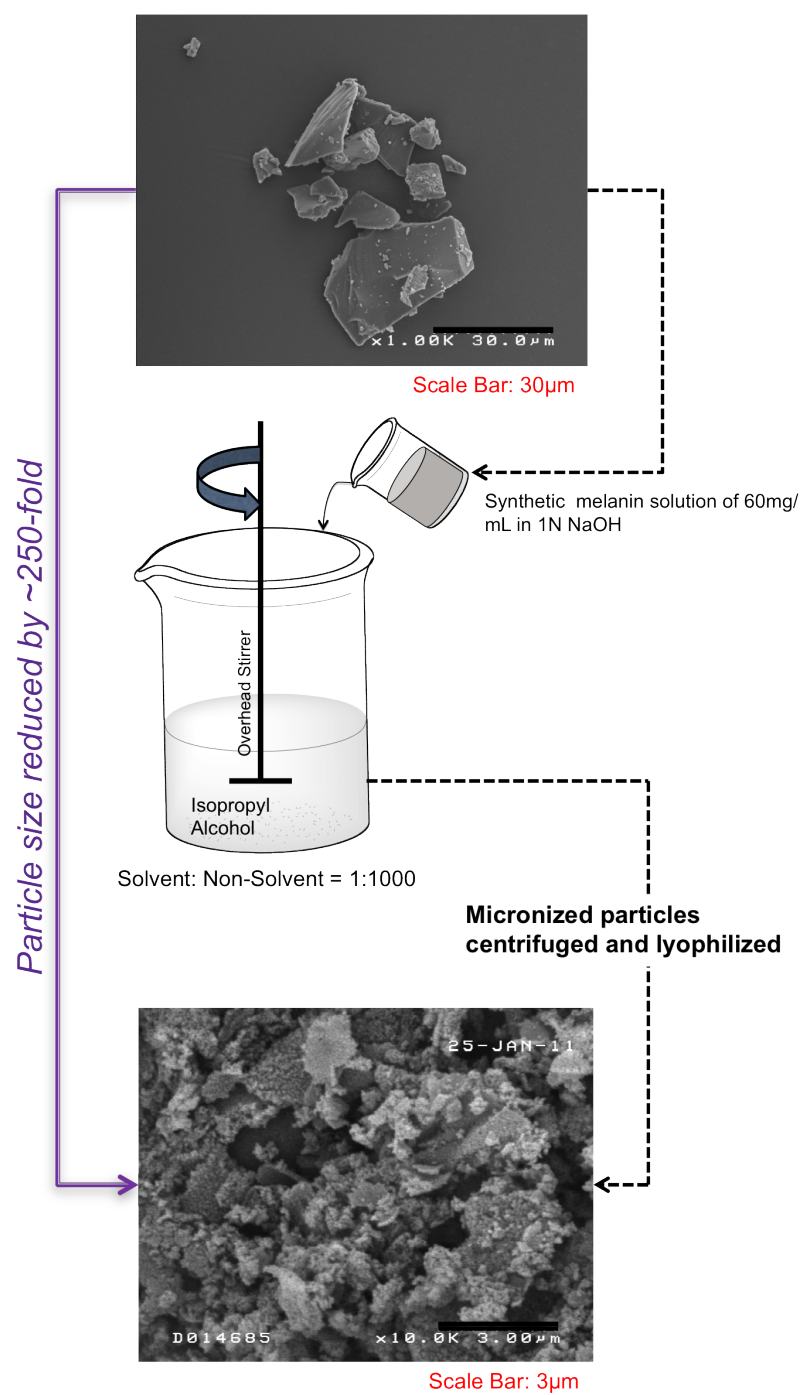


Figure A.2: Schematic of melanin phase inversion micronization.

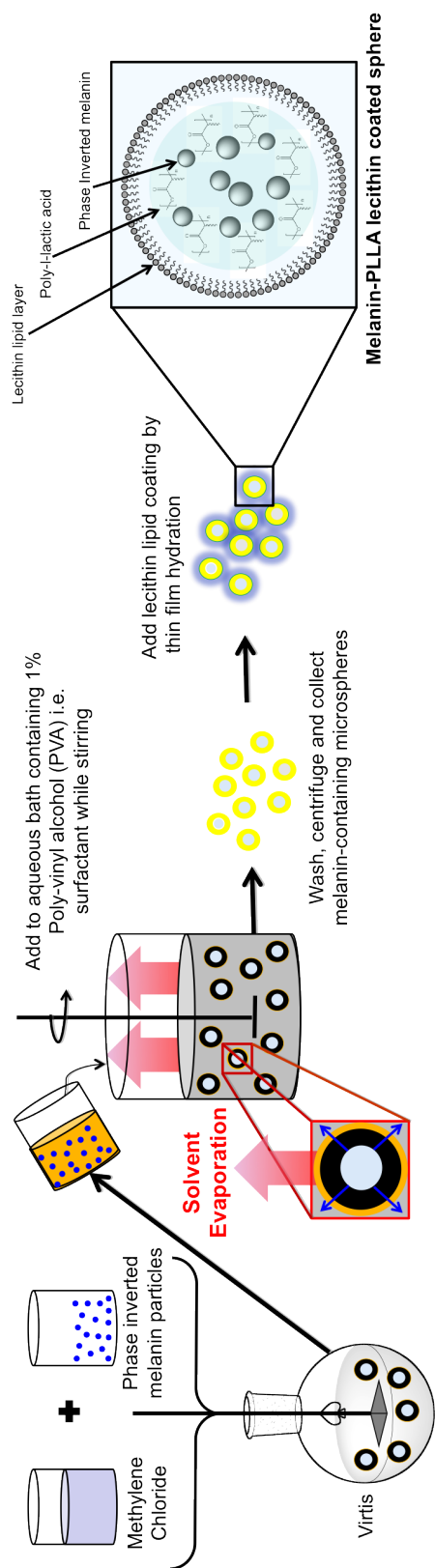


Figure A.3: Schematic of artificial melanosome preparation.

for 48 hours and stored at -4°C until use.

A.2.5 Dynamic Light Scattering

Samples suspended in 1 % PVP and 1 % SLS were also analyzed for polydispersity using dynamic light scattering (DLS) using a Malvern Zetasizer Nano (Worcestershire, United Kingdom). Studies were also performed with lecithin coated samples suspended in cell culture.

A.2.6 Fourier Transform Infrared Spectroscopy

Fourier-transform infrared spectroscopy (FTIR) was used to study the chemical interactions between DOX and the polymer. FTIR analysis was performed on each formulation using a Perkin-Elmer Spectrum One FTIR (Waltham, MA) and analyzed using Spectrum software (Perkin-Elmer, Waltham, MA). Samples were scanned in a range between 4000 and 650 cm^{-1} . All measurements were taken at room temperature.

A.2.7 Differential Scanning Calorimetry

3 – 7 mg of each formulation and bulk polymers were sealed into aluminum sample pans for DSC analysis. Thermal analyses of the nanospheres were performed using a model DSC 7 (Perkin-Elmer, Inc.; Waltham, MA) equipped with controller model TAC 7/DX (Perkin-Elmer, Inc.; Waltham, MA). After equilibration at -20°C, samples were heated from -20 to 200°C, cooled to -20°C then reheated to 200°C at a rate of 10°C/min. Thermograms were analyzed using Perkin-Elmer Thermal Analysis software for the calculation of glass transition temperatures (T_g), melting temperatures (T_m) and change in enthalpy of the melt. The percent crystallinity (X_c) was calculated using:

$$X_c = \frac{\Delta H_m}{\Delta H_{PLLA}} \times 100$$

where ΔH_m is the enthalpy of melting of the sample and ΔH_{PLLA} (93.6 $\text{J}\cdot\text{g}^{-1}$) is the specific heat of melting of a 100% crystalline PLLA as reported in the literature [6].

A.2.8 Scanning Electron Microscopy

The morphology and particle size of the nanospheres formulations were analyzed by scanning electron microscopy. Formulations were placed on a carbon-backed adhesive and sputter-coated with Au-Pd for 4 minutes at 20 mA and imaged on a Hitachi 2700 Scanning Electron Microscope (Hitachi High Technologies America, Inc.; Pleasanton, CA) with an accelerating voltage of 8 kV.

A.2.9 Lecithin Coating & In Vitro Studies

To create a lipid layer around the spheres, as to mimic liposome-like melanosomes, lecithin micelles were formed around the PLLA microspheres using a thin layer hydration method just prior to use for in vitro assays (Figure A.3). 30 mg of lecithin was dissolved in 1 mL of chloroform and the solvent was evaporated overnight to form a thin film of lecithin on a glass beaker bottom. Approximately 1 hr prior to in vitro studies, a known concentration of melanin-containing polymer-based spheres were added to 5mL of phosphate buffer (PBS). This suspension was added to the dried lecithin film and bath sonicated for 10 minutes (to ensure sufficient interaction had occurred). The suspension was centrifuged and washed with PBS 3x prior to the last centrifugation, forming a pellet of spheres. Residual PBS was decanted and cell media was added to resuspend the pellet; this mixture was introduced to live cells. A second batch of microspheres was created using a fluorescent hydrophobic dye, tetramethyl rhodamine isothiocyanate (TRITC), in place of melanin, to facilitate visualization. These spheres were incubated with melanocytes for 24 hrs and observed using confocal microscopy. Cultures with spheres were analyzed using differential interference contrast (DIC) and confocal microscopy.

A.3 Results and Discussion

A.3.1 Preparation of Melanin for Microencapsulation

Using an auto-oxidation method as described by Arnow et al, melanin was synthesized from L-DOPA, the precursor in the melanin biosynthetic pathway. During the course of

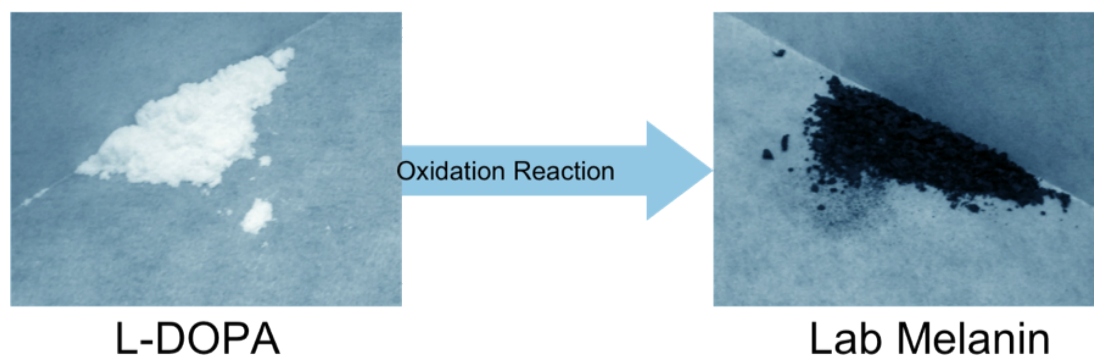


Figure A.4: Visual observation of the starting material, a melanin precursor called L-DOPA, to synthetic melanin after 2 days of auto-oxidizing.

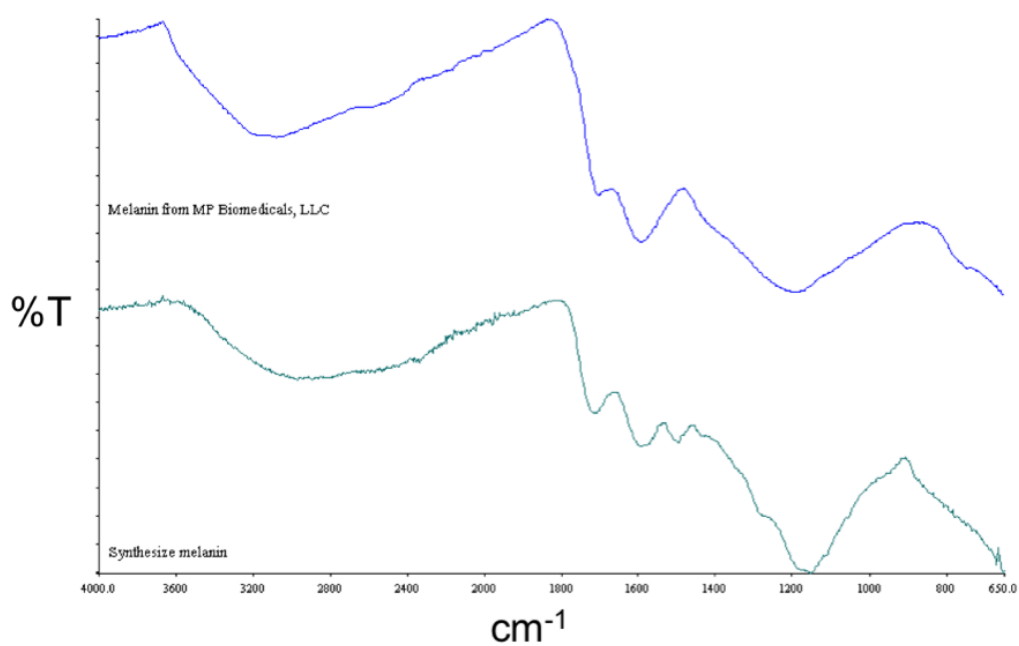


Figure A.5: FTIR spectral comparison of commercial melanin (top) to the lab-created melanin (bottom).

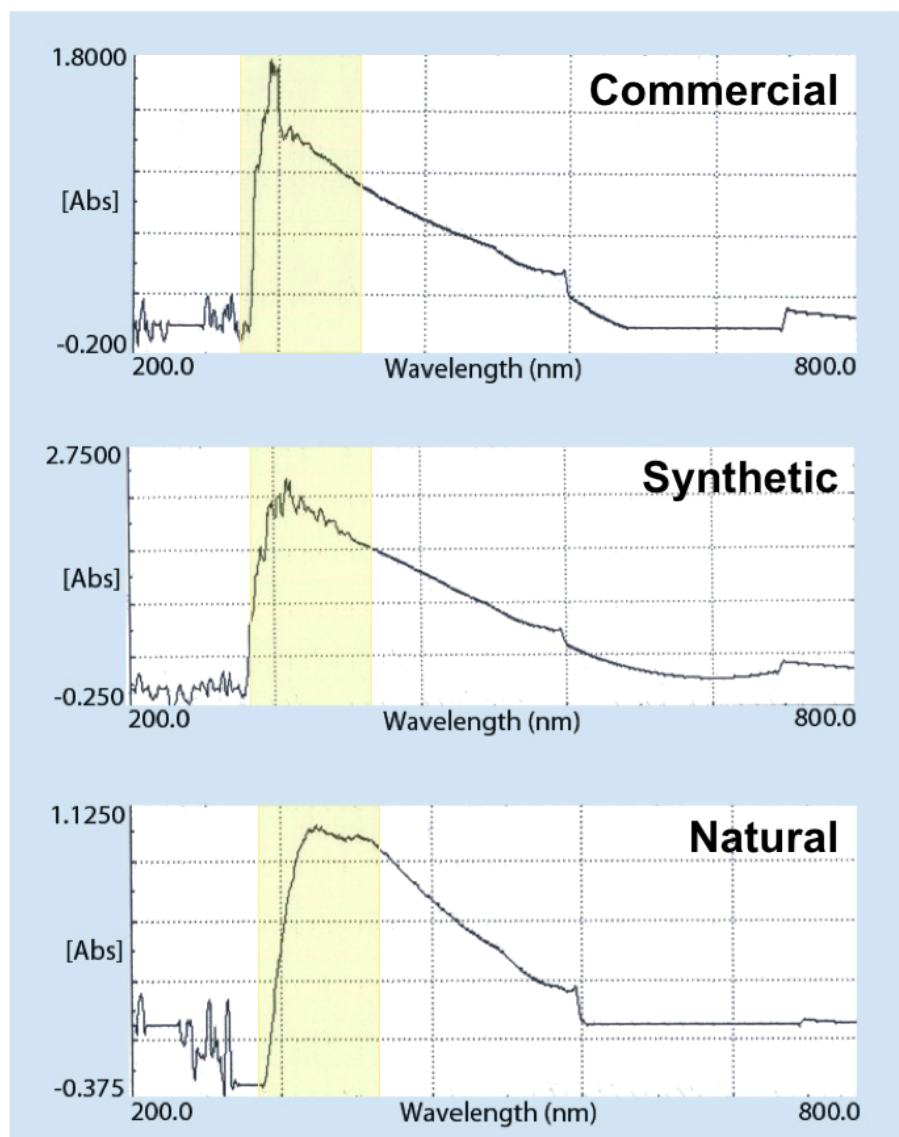


Figure A.6: UV-VIS spectral analysis of melanin from three sources: (top) Commercial from MP Biomedicals, LCC; (middle) Lab synthesized melanin; (bottom) Natural melanin extracted from human melanocytes in vitro. The highlighted area of each spectrum indicates the expected maximal wavelength absorbance range for melanin (i.e. 280-400 nm), where the peak absorbance of human melanin pigment occurs at 335 nm.

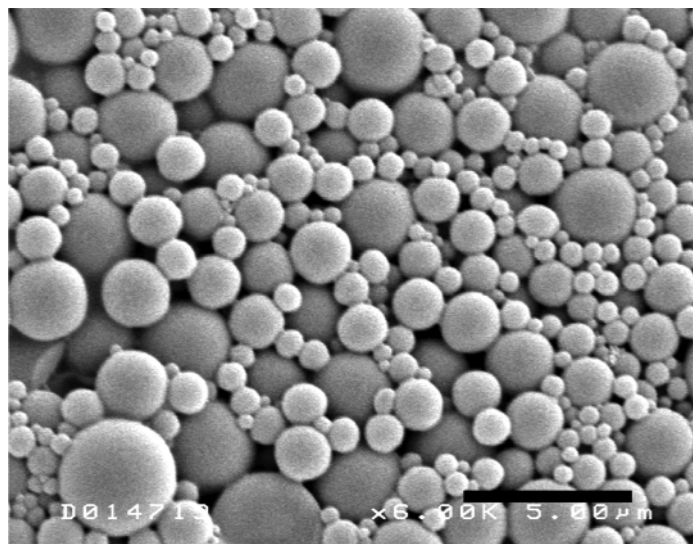


Figure A.7: Scanning electron micrograph of melanin-PLLA spheres; scale bar: 5 μm .

the reaction, the pink DOPA solution oxidized to form a black solution of melanin, which was precipitated by acidification of the solution. The resulting synthetic melanin was a fine black powder while the reactant was a fine white powder (Figure A.4). FTIR analysis of the synthetic melanin revealed similar absorption spectrum as commercially available melanin (Figure A.5). Furthermore, UV-VIS spectra analysis revealed that the light absorption characteristics of the synthetic melanin matched those of both naturally occurring and commercially available melanin confirming the successful preparation of melanin for encapsulation in a synthetic melanosome (Figure 1A).

Analysis of the synthetic analysis by SEM revealed particles in the range of 20 microns, dimensions too large for encapsulation in 500 nm microspheres (Figure A.2). Thus, a phase inversion micronization method was developed for the micronization of melanin (Figure A.2). A dilute aqueous solution of melanin was rapidly added to a large excess of isopropanol. Since isopropanol is miscible with water and a non-solvent for the melanin, the introduction of the melanin solution to the isopropanol bath results in the diffusion of the water into the continuous phase of the non-solvent to form sub-micron sized melanin particles (Figure A.2) in a size range suitable for encapsulation.

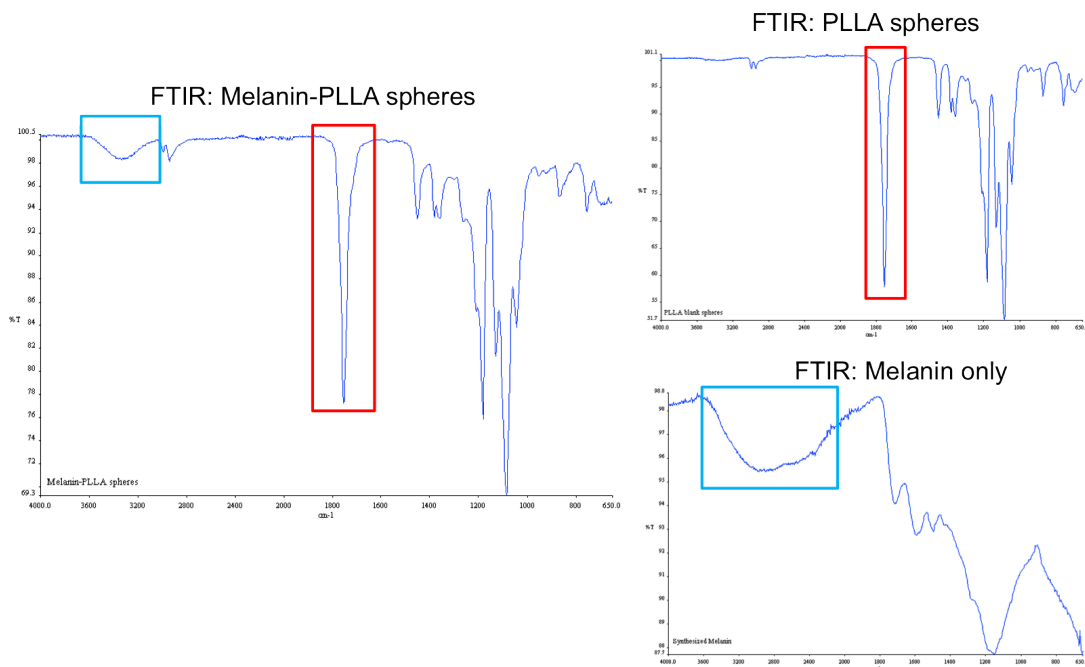


Figure A.8: FTIR spectra of melanin-PLLA microspheres (left), blank spheres (top right) and synthetic melanin (bottom right), red box indicates a carbonyl peak and the blue box shows a hydroxyl stretch.

A.3.1.1 Encapsulation of Melanin by Solvent Evaporation

Melanin-loaded PLLA microspheres were prepared using an oil-in-water (o/w) solvent evaporation method (Figure A.3) resulting in the formation of a fine light brown powder. These microspheres were characterized using dynamic light scattering (DLS), scanning electron microscopy (SEM), Fourier transform infrared spectroscopy (FTIR) and differential scanning calorimetry (DSC). Particle size analysis by DLS revealed a mean particle diameter of 687 and 821 nm for the control and melanin loaded formulations (Table A.1), indicating that the encapsulation of melanin resulted in an increase in particle size perhaps due to the loading of the melanin particles. Visualization of the microspheres by SEM revealed discrete spherical particles in a size range consistent with the DLS results; the spheres exhibit some polydispersity in particle size but within a range that approximates naturally occurring melanosomes (Figure A.7).

FTIR of the melanin, control formulation and the melanin-loaded spheres confirmed the encapsulation of melanin within the PLLA (Figure A.8). The spectra obtained for the

melanin shows a broad absorption characteristic of the -OH bond in the 3000 cm^{-1} range, which is not present in the analysis of PLLA, due to the absence of this chemical group in the polymer. A characteristic carbonyl peak ($\sim 1750\text{ cm}^{-1}$) is observed in the PLLA spectra, characteristic of the ester bond. The melanin-loaded microspheres exhibit both the carbonyl peak and the hydroxyl stretch, each unique to the PLLA and melanin, respectively, providing evidence for the successful encapsulation of the melanin within the polymer. Thermal analysis of the control PLLA microspheres revealed glass transition at 48.92°C and a melting temperature of 169.76°C while the melanin formulation had a T_g of 57.67°C and a melting temperature of 171.70°C (Table A.1). Determination of the percent crystallinity showed that the encapsulation of melanin resulted in a reduction in crystallinity from 28.46% to 19.63% while the bulk polymer from the manufacturer had a crystallinity of 23.92% (Table A.1). This suggests that the incorporation of the melanin within the polymeric matrix interferes with the crystallization during the solvent evaporation and microsphere hardening process. However, melanin encapsulation results in an increase in the glass transition of the polymer, indicating a relative reduction in the amorphous regions of the PLLA.

A.3.2 Lecithin Coating to form Artificial Melanosomes

Using the thin layer hydration method, lecithin films were allowed to self-assemble around the microspheres to produce a phospholipid layer that mimics the natural structure of melanosomes, a cellular organelle (Figure A.3). Dynamic light scattering of the microparticles following lipid coating revealed an increase in the hydrodynamic radius of the synthetic melanosomes to 903 nm and 1010 nm for the control and melanin-loaded formulations, respectively (Table A.1). The increase in the diameter of the microspheres confirms the formation of a lecithin layer around the PLLA microspheres. However, the results indicate particles much larger than would be expected given the relatively small size of a phospholipid monolayer or bilayer, this may be the result of the DLS technique, which relies on fluctuations in scattered light intensity to determine particle size. The increase in hydrophilicity of the particles following the lecithin coating and the polydispersity of the PLLA microspheres could explain the higher than expected perceived hydrodynamic radius.

	Particle Size (nm)	T _g (°C)	T _m (°C)	% Crystallinity
PLLA MS	687	48.92	169.76	28.46
PLLA MS + Lecithin	903	-	-	-
Melanin-PLLA MS	821	57.67	171.70	19.63
Melanin-PLLA MS + Lecithin	1010	-	-	-

Table A.1: Particle size and thermal properties of melanin lipid-polymer hybrid microparticles.

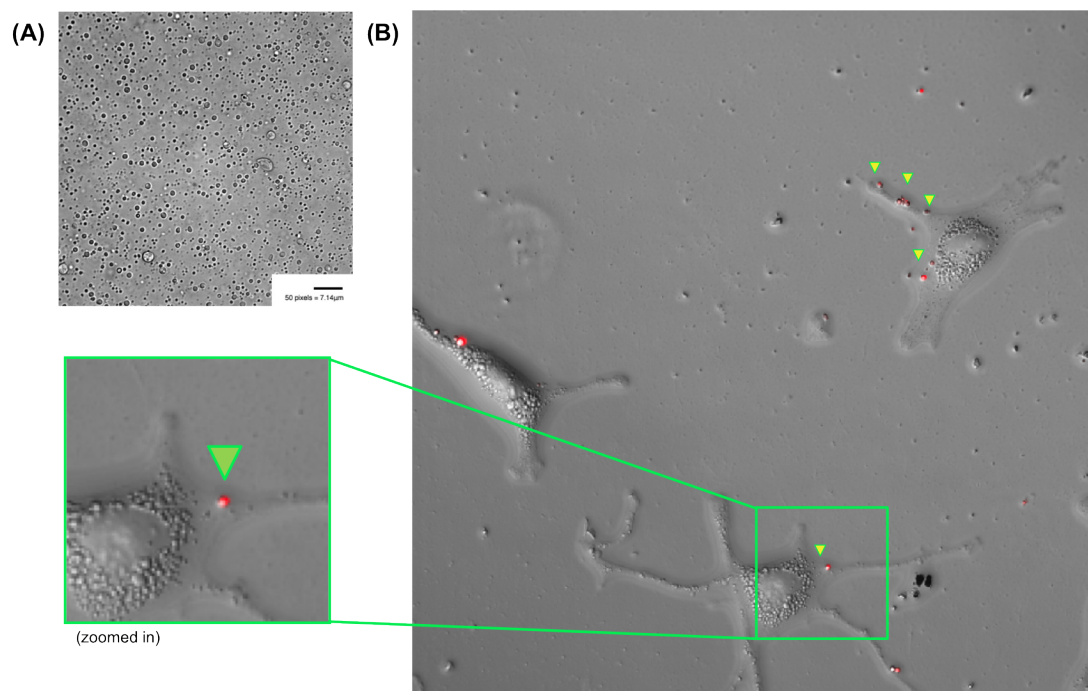


Figure A.9: (A) DIC microscopy image of melanin-containing polymer spheres in melanocyte culture media (scale bar = 7.14 μm). (B) Confocal microscopy of fixed human neonatal melanocytes co-incubated for 24 hrs with 100 $\mu\text{g}/\text{mL}$ of TRITC-hybrid microspheres; green arrows indicate presence of TRITC-incorporated polymeric spheres interacting with human melanocytes.

A.3.3 In Vitro Cytocompatibility

Using lipid-polymer microparticles containing TRITC or melanin, the cytocompatibility of these synthetic organelles was evaluated in culture using human neonatal melanocytes in culture. At concentrations up to 100 $\mu\text{g}/\text{mL}$, the lipid-polymer hybrid spheres were cytocompatible and did not indicate any adverse effects on cell survival or morphology (Figure A.9). Furthermore, the synthetic particles were closely associated with cells but their uptake into the melanocytes has yet to be confirmed.

A.4 Conclusions

Melanin was synthesized from the auto-oxidation of DOPA and was confirmed to be chemically and physically equivalent to naturally occurring and commercially available melanin. A phase inversion micronization method was developed for the particle reduction of melanin

for encapsulation. Melanin-loaded PLLA microspheres were prepared using the solvent evaporation method and coated with a phospholipid layer using the thin layer hydration method to produce a synthetic melanosome that mimics these naturally occurring organelles. Preliminary in vitro studies confirmed that these artificial melanosomes are cytocompatible and could be a tool used to study melanosome trafficking in cell culture.

A.5 References

- [1] H.Y. Park, M. Kosmadaki, M. Yaar, B.A. Gilchrest. *Cell. Mol. Life Sci.* 66 (2009) 1493-1506.
- [2] M. Schiaffino. *Inter. Jour. of Biochem. & Cell Bio.* 42 (2010) 1094-1104.
- [3] E. Arnow. *The Preparation of DOPA-Melanin.* *Science.* 87 (1938) 308.
- [4] H.Y. Thong, Jee SH, Sun CC, Boissy RE. *British Jour. of Derm.* 149 (2003) 498-505.
- [5] S. Singh, R. Kurfurst, C. Nizard, S. Schnebert, E. Perrier, D. Tobin. *FASEB Journal.* 24 (2010) 3756-3769.
- [6] Fischer EW, Sterzel HJ, Wegner G. *Kolloid Z Z Polym* 1973;251:980–90.

Appendix B

PEGylated Superparamagnetic Double-Walled Nanospheres for Micromagnetic-Microfluidic Dialysis as a Novel Contrast Imaging Modality

B.1 Introduction

Current injectable radiocontrast agents can lead to irreversible renal failure or death in healthy and at risk patients. The highest risk is in patients with compromised renal function and diabetes – thus, the use of contrast agents is contraindicated in this patient population. This is further complicated as many patients with chronic renal failure, often on dialysis, require contrast imaging for diagnosis and treatment of other conditions and diseases. For example, patients with compromised renal function are considerably more likely to require a coronary intervention compared to an otherwise healthy patient. Cardiac angiograms or coronary intervention assisted by radiocontrast imaging exposes the kidney to high level of contrast agent resulting in complications from acute toxicity to total renal failure. Renal failure requiring dialysis after a coronary intervention is associated with poor outcomes, including 40% in-hospital mortality and 19% two-year survival [1, 2].

The Ingber Group has developed a blood-cleansing device that uses micromagnetic and

Daniel Y. Cho, Benjamin Lux, Lakir Patel and Edith Mathiowitz

microfluidic forces to remove pathogens from the body [3-5]. Briefly, magnetic antibodies are combined with the blood where they rapidly seek out and attach to pathogens. The blood then travels through microchannels where magnets remove the antibodies (and attached pathogens) from the blood into a dialysis fluid. Early in vivo models have demonstrated the system to be over 80% effective in rapidly clearing blood of specific pathogens in a single pass through the machine. While the micromagnetic-microfluidic device was originally developed as a means to rapidly remove fungal and bacterial pathogens from septic patients, our group views its existence as a platform for the use of temporarily implanted non-degradable biomaterials. Prior to the device, non-degradable materials could not be implanted unless the body could naturally clear them, they were removed surgically or permanent placement was desirable. The blood-cleansing device allows for magnetic nanospheres to be introduced into the body and cleared. Radiocontrast agents are a logical application to initially investigate, as there is a significant need for non-nephrotoxic contrast agents for patients with end stage renal failure. These patients are the same patients in need of contrast angiography, though are limited in their current options. As many of these patients already have artificial fistulas for dialysis, they are ideal candidates for this procedure.

Superparamagnetic iron oxide (SPIO) contrast agents have been investigated extensively as contrast agents due to their radio-opacity, low radio-artifact, and low renal toxicity [6, 7]. However, they encountered limited success due to slow systemic clearance (>90 days) and rapid settling of particles in the liver and spleen [6, 7]. Though systemic toxicity did not present itself as an issue, the slow excretion and biodistribution of the particles prevented widespread acceptance [8]. The purpose of this work is to develop PEGylated superparamagnetic double-walled nanospheres using the solvent evaporation method for use in conjunction with the micromagnetic-microfluidic device to provide a means to leverage the desirable radio and toxicological properties of iron oxide while allowing for rapid systemic clearance. The PEGylation of the iron oxide enhances the circulation of these particles and reduces the settling of the particles in the liver and spleen, allowing for sufficiently high availability in the blood stream for the dialysis to be effective.

B.2 Materials and Methods

B.2.1 Nanosphere Preparation

10% w/v solutions of PLLA in methylene chloride (MeCl_2) and PEG-PLGA in MeCl_2 were prepared then mixed together in a 1:1 ratio by vortexing. The mixed polymer solution was emulsified into a 1% PVA solution using a VirTis Cyclone IQ homogenizer at 25,000 rpm for 20 minutes. This O/W emulsion was stirred in a second PVA bath at 1000 rpm for 3 hours to allow for solvent evaporation, phase separation and microsphere curing. The resulting microspheres were washed and collected by centrifugation, flash frozen in liquid nitrogen and lyophilized for 24 hours.

In order to fabricate SPIO-loaded double-walled nanospheres as a potential contrast agent for biomedical imaging applications, NANOCAT MB120 Magnetic Iron Oxide (Fe_3O_4) Powder was suspended in the mixed polymer solution prior to the emulsification process described above. The suspension was bath sonicated to break up the aggregates of iron oxide, which has an average particle size of 70-100 nm, for the nanoencapsulation procedure.

B.2.2 Particle Size Analysis

All formulations were reconstituted from powder form into reconstitution media consisting of an aqueous 1% sodium lauryl sulfate (SLS) / 1% polyvinylpyrrolidone (PVP) solution in 0.5 – 2% w/v suspensions with bath sonication. A Beckman Coulter LS230 Laser Diffraction Particle Size Analyzer (Beckman Coulter, Inc.; Brea, CA) was used to evaluate the size distribution of nanoparticle populations.

B.2.3 Fourier Transform Infrared Spectroscopy

All formulations and bulk polymers were analyzed as dry powders using a Perkin Elmer Spectrum One FTIR with a Universal ATR Sampling Accessory and analyzed using built-in Spectrum software (Perkin-Elmer, Inc.; Waltham, MA).

B.2.4 Differential Scanning Calorimetry

3 – 7 mg of each formulation and bulk polymers were sealed into aluminum sample pans for DSC analysis. Thermal analyses of the nanospheres were performed using a model DSC 7 (Perkin-Elmer, Inc.; Waltham, MA) equipped with controller model TAC 7/DX (Perkin-Elmer, Inc.; Waltham, MA). After equilibration at -20°C, samples were heated from -20 to 200°C, cooled to -20°C then reheated to 200°C at a rate of 10°C/min. Thermograms were analyzed using Perkin-Elmer Thermal Analysis software for the calculation of glass transition (T_g) and melting (T_m) temperatures.

B.2.5 Scanning Electron Microscopy

The morphology and particle size of the nanospheres formulations were analyzed by scanning electron microscopy. Formulations were placed on a carbon-backed adhesive and sputter-coated with Au-Pd for 4 minutes at 20 mA and imaged on a Hitachi 2700 Scanning Electron Microscope (Hitachi High Technologies America, Inc.; Pleasanton, CA) with an accelerating voltage of 8 kV.

B.3 Results and Discussion

B.3.1 Modified Solvent Evaporation Method

PEGylated double-walled nanospheres consisting of a poly-L-lactic acid (PLLA) core and a polyethylene glycol-poly(lactic-co-glycolic acid) (PEG-PLGA) shell were fabricated using a modified solvent evaporation method. While previous methods have utilized phase separation phenomena to prepare double-walled microspheres of PLGA and PLLA in the 300 – 500 μm range [9-11], increasing the shear rate allows for the reduction of particle size to the nanoscale. However, the increased surface area to volume ratio of the nanoparticles results in more rapid solvent evaporation and consequently less time for phase separation. Thus, PEG-PLGA was selected as the shell polymer since the hydrophilicity of the PEG would help to rapidly localize the copolymer to the surface and result in the formation of ‘core-shell-corona’ type nanospheres suitable for bioimaging applications.

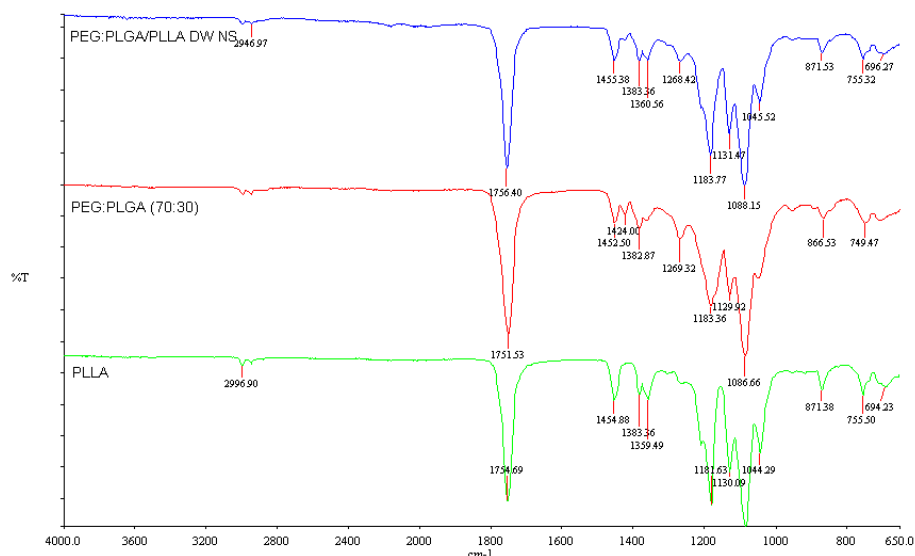


Figure B.1: FTIR spectra of the PEG:PLGA/PLLA double-walled nanospheres formulation, bulk PEG:PLGA and bulk PLLA.

B.3.2 Control Nanosphere Characterization

The nanospheres were characterized using a variety of polymer characterization techniques including Coulter laser particle sizing, Fourier Transform Infrared Spectroscopy (FTIR), Differential Scanning Calorimetry (DSC) and Scanning Electron Microscopy (SEM). Laser particle size analysis of the nanoparticles showed an average particle size of 640 ± 295 nm – large enough to minimize uptake by cells but small enough to prevent vascular complications. Furthermore, the extensions of the PEG chain into the aqueous environment to form a corona-structure during the particle size analysis may artificially increase the perceived particle size; however, the PEGylation can serve as an impediment to cellular uptake and result in prolonged circulation times in vivo.

Comparison of the FTIR spectra for PEG-PLGA and PLLA with the double-walled formulation revealed the characteristic peaks of both polymers in the double-walled formulation (Figure B.3). This indicates that both polymers are present within the nanospheres; however, the FTIR does not provide any significant information about the interaction or localization of the two polymers.

DSC was used to evaluate the thermal characteristics of the bulk polymers and the double-walled formulations (data not shown). Analysis of the bulk PEG-PLGA and PLLA

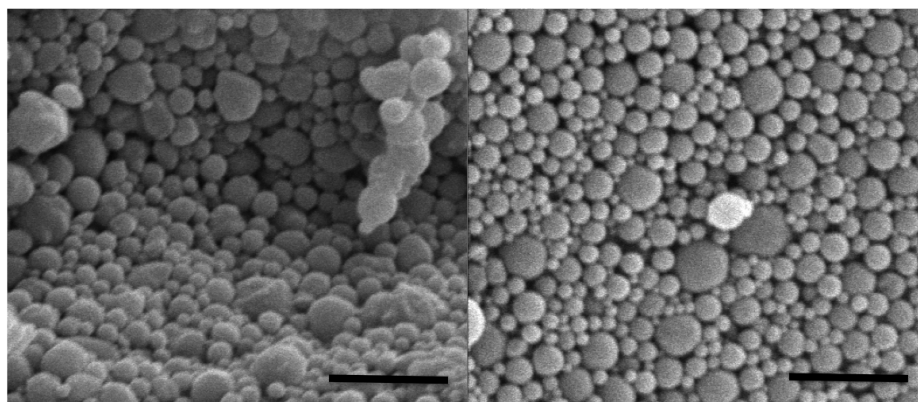


Figure B.2: SEM images of the PEG:PLGA/PLLA nanospheres (left) and PLLA nanospheres (right), scale bars: 2.5 μm .

showed glass transitions at 40.4°C and 57.2°C respectively; the PLLA also showed a melting temperature of 172.2°C. DSC analysis of the double-walled nanospheres showed two glass transitions at 41.7°C and 56.2°C as well as a T_m at 175.0°C. The slight shift of the glass transitions of the two polymers in the double-walled formulation towards the T_g of the other suggests a small degree of incomplete phase separation at the interface. The high surface area of the nanoparticles in the initial emulsion allows for rapid solvent evaporation, which may not leave enough time for the two polymers to completely phase separate. While the DSC results confirm the phase separation of the two polymers, the localization of the two polymers within the nanospheres cannot be determined.

Various microscopy techniques were used to characterize the nanospheres. While PLGA is a fully amorphous polymer, PLLA has a crystalline structure and will exhibit birefringence under polarized light. During the fabrication process, cross-polarized light microscopy was used to look for birefringence in the nanospheres to determine the localization of PLLA. Starting at 1.5 hours into the solvent evaporation process, the core of the nanospheres began to exhibit birefringence which was observed through the rest of the processing and in the final formulation. Scanning electron micrographs of the double-walled nanospheres confirm the results of laser particle size analysis (Figure B.2). Furthermore, the morphology of the double-walled nanospheres compared to PLLA nanospheres fabricated by a similar solvent evaporation method provide further insight into the localization of the two polymers within the nanospheres. While the PLLA nanospheres are very smooth, the PEG:PLGA/PLLA

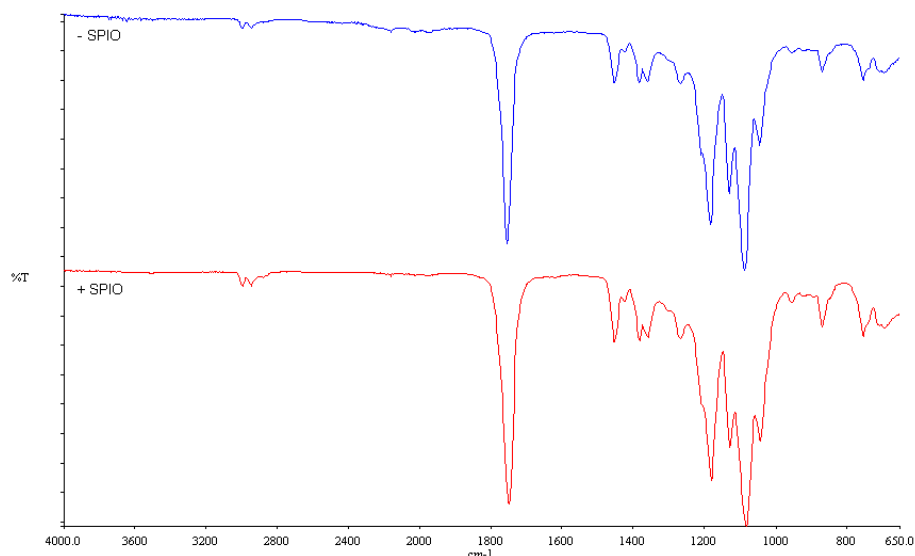


Figure B.3: FTIR spectra of the unloaded and SPIO-loaded PEG:PLGA/PLLA formulations.

nanospheres have a uneven appearance (Figure B.2). This is the result of the PEG:PLGA localizing to the surface during the solvent evaporation process, which takes place in an aqueous environment, allowing for the hydrophilic PEG chains to extend into the surroundings. When these nanospheres are flash frozen and lyophilized, the PEG chains collapse down on the surface of the spheres resulting in their crumpled appearance. Therefore, using microscopy, we were able to conclude that the PEG:PLGA localized to the surface while the PLLA phase separated to form the core of these nanospheres.

B.3.3 PEGylated Superparamagnetic Double-Walled Nanospheres

In contrast to the fine white powder of the blank nanospheres, the iron-loaded nanospheres had a light brown color, indicative of the encapsulated iron oxide.

Laser particle size analysis of the SPIO-loaded nanospheres revealed a slighter larger particle size and distribution with an average particle size of 704 ± 459 nm. FTIR analysis showed no differences between the unloaded and SPIO-loaded formulations (Figure B.3).

Thermal characterization of the SPIO-loaded nanospheres by DSC demonstrate glass transitions at 52.8 °C and 56.5 °C as well as a melting temperature at 173.7 °C. This data indicates that the incorporation of the iron into the structure of the nanospheres causes the

PLLA chains to better orient themselves resulting in an increase in the T_g and T_m seen in the DSC thermograms. Similar to the unloaded nanospheres, there seems to be incomplete phase separation of the two polymers.

B.4 Conclusions

Using the solvent evaporation method, PEGylated superparamagnetic core-shell double-walled nanospheres have been prepared as a proof of concept for evaluation as a potential radiocontrast agent in patients with renal failure to be used in combination with the micromagnetic-microfluidic dialysis device developed by the Ingber lab. As this work is preliminary, further studies for these particles include the optimization of formulations as well as investigation of their systemic effects, toxicity, natural clearance and clearance via magnetic dialysis with the micromagnetic-microfluidic device; and evaluate their potential for different types of radiocontrast imaging.

B.5 References

- [1] Chertow, G.M. Christiansen, C.L. and Cleary, D.P. Prognostic stratification in critically ill patients with acute renal failure requiring dialysis. *Arch Intern Med* 155 (1995), pp. 1505–1511.
- [2] McCullough, P.A. Wolyn, R. Rocher, L.L. Levine, R.N. and O'Neill, W.W. Acute renal failure after coronary intervention: incidence, risk factors, and relationship to mortality. *Am J Med* 103 (1997), pp. 368–375.
- [3] Polte, T. et al. Nanostructured magnetizable materials that switch cells between life and death. *Biomaterials* 28, 2783-2790.
- [4] Mannix, R. et al. Nanomagnetic actuation of receptor-mediated signal transduction. (2008).
- [5] Yung, C., Flering, J., Mueller, A. & Ingber, D. Micromagnetic–microfluidic blood cleansing device. *Lab on a Chip* 9, 1171-1177 (2009).
- [6] Weissleder, R. et al. Ultrasmall superparamagnetic iron oxide: characterization of a new class of contrast agents for MR imaging. *Radiology* 175, 489-493 (1990).
- [7] Weissleder, R. et al. Superparamagnetic iron oxide: pharmacokinetics and toxicity. *Am. J. Roentgenol.* 152, 167-173 (1989).

- [8] Stark, D. et al. Superparamagnetic iron oxide: clinical application as a contrast agent for MR imaging of the liver. *Radiology* 168, 297-301 N291 - (1988).
- [9] Pekarek, J.J., Jacob, J.S. and E. Mathiowitz, Double-walled polymer microspheres for controlled drug release. *Nature*, 1994. 367: p. 258-260.
- [10] Pekarek, K.J. , Jacob J.S. and E. Mathiowitz, One-step preparation of DW microspheres. *Advanced Materials*, 1994. 6: p. 684-687.
- [11] Leach, K., Noh K. and E. Mathiowitz, Effect of manufacturing conditions on the formation of double-walled polymer microspheres. *J. Microencapsulation*, 1999. 16: 153-167.

Appendix C

Abstracts and Conference Proceedings

1. J. Reineke, **D.Y. Cho**, Y. Liu, E. Mathiowitz. Quantitative and Mechanistic Analysis of Bioadhesive Microparticle Uptake for Oral Drug Delivery. 2009 Annual MD/PhD Program Retreat. Brown University. Providence, RI. September 2009.
2. **D.Y. Cho**, J. Reineke, E. Mathiowitz. Quantitative Analysis of Bioadhesive Nanoparticle Uptake From the Small Intestine for Oral Drug Delivery Applications. The Joint Meeting of the American Society for Clinical Investigation and the Association of American Physicians. Chicago, IL. April 2010.
3. R. Patel, **D.Y. Cho**, C. Tian, A. Chang, D. Decoteau, J. Morgan, E. Mathiowitz. A Novel W/O/O Double Emulsion Solvent Removal Method for the Nanoencapsulation of Doxycycline. 37th Annual Meeting and Exposition of the Controlled Release Society. Portland, Or. July 2010.
4. **D.Y. Cho**, C. Tian, K.M. Estrellas, E. Mathiowitz. Effect of Encapsulation Method on Protein Loaded Nanospheres Fabricated by Phase Inversion Nanoencapsulation. 37th Annual Meeting and Exposition of the Controlled Release Society. Portland, OR. July 2010.
5. **D.Y. Cho**, K.M. Estrellas, C. Tian, J. Reineke, E. Mathiowitz. A Novel Method for the Fabrication of Double-Walled Nanospheres by Sequential Phase Inversion Nanoencapsulation. 37th Annual Meeting and Exposition of the Controlled Release Society. Portland, OR. July 2010.
6. C. Tian, S. Azad, **D.Y. Cho**, B.D. Lux, T. Peak, S. Furtado, E. Mathiowitz. Comparing the Effects of Fabrication Parameters on Micro- and Nanospheres for Drug Delivery Applications. 2010 Brown University Summer Research Symposium for the Humanities, Sciences and Social Sciences. Providence, RI. August 2010.
7. **D.Y. Cho**, C. Tian, K.M. Estrellas, J. Reineke, E. Mathiowitz. Development of a Rapid Release Oral Insulin Formulation for Diabetes Mellitus. 2010 Annual MD/PhD Program Retreat. Brown University. Providence, RI. September 2010.

8. **D.Y. Cho**, L. Patel, E. Mathiowitz. Fabrication of PEG-ylated Double-Walled Nanospheres by Solvent Evaporation. 2010 Annual Meeting of the Biomedical Engineering Society. Austin, TX. November 2010.
9. **D.Y. Cho**, C. Tian, J.J. Reineke, E. Mathiowitz. Progress in the development of an oral insulin formulation for diabetes mellitus. The Joint Meeting of the American Society for Clinical Investigation and the Association of American Physicians. Chicago, IL. April 2011.
10. D. Decoteau, **D.Y. Cho**, L. Zhang, R. Stefani, R. Patel, E. Mathiowitz. Single Step Fabrication of Wet Spun Binary Phase Composite Microfibers. 38th Annual Meeting and Exposition of the Controlled Release Society. Baltimore, MD. August 2011.
11. L. Quattrochi, **D.Y. Cho**, K.M. Estrellas, N.L. Wicks, E. Oancea, E. Mathiowitz. Development of an Artificial Melanosome Utilizing a Lipid-Polymer Hybrid Microparticle. 38th Annual Meeting and Exposition of the Controlled Release Society. Baltimore, MD. August 2011.
12. **D.Y. Cho**, C. Tian, K.M. Estrellas, Y.T. Liu, J.J. Reineke, S. Furtado, E. Mathiowitz. Bioadhesive Double-Walled Nanospheres as an Oral Insulin Delivery Platform. 38th Annual Meeting and Exposition of the Controlled Release Society. Baltimore, MD. August 2011.
13. L. Quattrochi, **D.Y. Cho**, N. Chavez, Z. Pfeiffer, E. Oancea, E. Mathiowitz. In Vitro Assessment of Lipid-Polymer Hybrid Melanosomes in Human Neonatal Epidermal Melanocytes. 2011 Annual Meeting of the Biomedical Engineering Society. Hartford, CT. October 2011

Quantitative and Mechanistic Analysis of Bioadhesive Microparticle Uptake for Oral Drug Delivery

Joshua Reineke, Daniel Y. Cho, Yu-Ting Liu, Edith Mathiowitz
 Dept. of Molecular Pharmacology, Physiology and Biotechnology
 Artificial Organs, Biomaterials and Cellular Technology
 Brown University, Providence, RI 02912

Introduction

Although the oral administration of therapeutic proteins is the preferred method to maximize patient comfort and compliance, the challenges presented by the gastrointestinal (GI) system have prevented the development of an effective oral delivery system. Polymeric, microsphere-based systems demonstrate great potential for oral drug delivery due to their ability to protect the encapsulated drug from degradation in the GI tract while allowing for controlled drug release kinetics. However, the primary obstacle in developing an efficient oral drug delivery system has proven to be microparticle uptake.

Previous work in our lab has shown that microspheres formulated with bioadhesive polymers enhanced the oral bioavailability of therapeutic molecules *in vivo*. Behrens, et al. have shown a correlation between bioadhesion and nanoparticle uptake both in cell culture and qualitatively with imaging of intestinal tissue *in vivo* [1]. This previous work provides evidence that uptake can be enhanced using bioadhesive microspheres.

The purpose of this work was to determine the percent uptake, cellular internalization mechanisms and bioadhesion studies that may serve as important quantitative guidelines for selecting the optimal particle sizes for oral drug delivery.

Methods

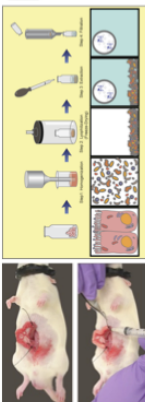


Figure 1. Isolated loop surgery for local administration of MS.

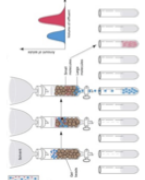


Figure 2. Schematic of tissue processing.

To study the effect of bioadhesion on microparticle uptake, MS were fabricated using polymers with varying degrees of bioadhesiveness. Non-bioadhesive (NA) 0.5 μ m poly(methylmethacrylate) (PMMA) MS were fabricated by the solvent evaporation method. Bioadhesive (BA) MS were fabricated by Phase Inversion Nanocapsulation (PIN) [3], a novel technique developed in our lab. A mixed solution consisting of 2% w/v PMMA in dichloromethane and 2% w/v poly(diphenylmaleic anhydride-co-L-DOPA) (pDMAD) in ethanol was rapidly added to a non-solvent bath of petroleum ether, resulting in the spontaneous formation of MS consisting of a pDMAD shell encapsulating a PMMA core. The pDMAD-PMMA MS were confirmed to be 0.5 μ m in diameter.

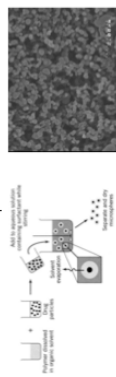


Figure 3. Schematic of gel permeation chromatography.

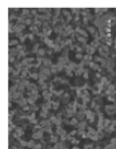


Figure 4. The solvent evaporation method of microparticle fabrication.

To quantify the uptake of microparticles from specific regions of the GI tract, an isolated loop procedure was performed on 6 cm intestinal sections as previously described [2] in male Sprague-Dawley rats weighing 200-250 g with a 1 mL (2.5% w/v) dosage of polystyrene (PS) microspheres (MS). Following a 5 hour incubation period, tissue samples were collected and processed to extract the polymer into chloroform for quantification by gel permeation chromatography. For fed studies, MS were administered directly to the stomach by oral gavage. Mechanistic studies were performed with an initial dose of inhibitor administered by direct injection into the isolated loop 30 minutes prior to injection of the 0.5 μ m PS MS and at appropriate intervals during the incubation period. The percentage of uptake was calculated by taking the sum of all amounts detected in tissues divided by the total dose administered and multiplied by 100. Mass balance calculations were performed by taking the sum of all amounts detected in every tissue sample by the total administered dose; for all study groups, mass balance results were within 11.3% of the total dose.

Results

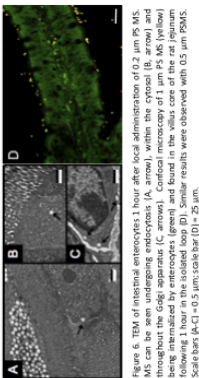
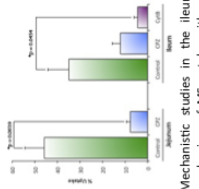


Figure 6. TEM of intestinal enterocytes 1 hour after local administration of 0.2 μ m PS MS. MS can be seen undergoing endocytosis (A, arrow), within the cytosol (B, arrow) and throughout the Golgi apparatus (C, arrows). Confocal microscopy of 1 μ m PS MS (yellow) following 1 hour in the isolated loop (D). Similar results were observed with 0.5 μ m PSMS. Scale bar (A-C) = 0.5 μ m; scale bar (D) = 20 μ m.



Studies in the jejunum using chlorpromazine (CPZ) showed a reduction in uptake from $45.86 \pm 8.6\%$ to $3.6 \pm 2.1\%$ indicating that clathrin-mediated endocytosis is the primary mechanism by which the MS are internalized. TEM imaging of 0.2 and 0.5 μ m MS as well as confocal microscopy of 1 μ m MS show endocytic internalization by enterocytes (Fig. 6). The clathrin-mediated uptake of particles has previously only been described in cell culture studies and most *in vivo* studies have attributed the uptake of microparticles to an unknown non-phagocytic mechanism. It is likely that this unknown mechanism of microparticle uptake is actually clathrin-dependent endocytosis.

Mechanistic studies in the ileum also indicated that clathrin-mediated endocytosis was the primary mechanism of MS uptake with a reduction from $32.2 \pm 11.5\%$ to $12.1 \pm 3.6\%$ in CPZ treated ileal loops. However, the uptake was not completely inhibited with administration of CPZ suggesting that another mechanism was involved. The presence of Peyer's patches (PP) in the ileum led us to hypothesize this other mechanism to be phagocytosis by M-cells, which function to sample antigens and deliver them to antigen presenting cells and lymphocytes in the PP. Administration of cyclophosphamide B (CyB) to inhibit both endocytosis and phagocytosis further decreased uptake to $4.5 \pm 1.6\%$ indicating that both phagocytosis and clathrin-mediated endocytosis play significant roles in the internalization of MS the ileum.

The bioadhesive pDMAD coating enhanced the uptake of non-bioadhesive PMMA MS from $5.8 \pm 1.9\%$ to $66.9 \pm 12.9\%$. The total uptake for the bioadhesive pDMAD-PMMA spheres was also significantly higher than for PS MS. Linear regression of total uptake with the fracture strength of these polymers, a measure of the bioadhesion to the GI mucosa, resulted in an R^2 value of 0.99142, providing strong evidence for the correlation between bioadhesion and MS uptake from the GI tract.

Conclusions

Our results show that the uptake of microspheres from the GI tract is strongly size dependent. Using inhibitors of specific uptake pathways, we determined that clathrin-mediated endocytosis by enterocytes dominates microparticle uptake in Peyer's patch free jejunum, which reflects the fact that enterocytes comprise over 98% of the surface area of the small intestine. In the Peyer's patch dense ileum, the uptake of microparticles is dependent on both phagocytosis by the M-cells in the Peyer's patches as well as endocytosis by enterocytes. The extensive clathrin-mediated endocytosis of microspheres throughout the GI tract provides a new paradigm for oral drug delivery.

The elucidation of non-competing transcellular pathways that are size-selective holds great promise for designing oral protein delivery systems with sufficient uptake for use by human patients. In addition, the correlation between bioadhesion and uptake provides evidence that the use of bioadhesive polymers is another method to enhance the uptake of microparticles for oral drug delivery.

Acknowledgements

- Roanne Burrill for her assistance with the animal work
- Arthur Peter Morello for the TEM and confocal images
- Peter Chellett for bioadhesion testing.
- Spherics, Inc. for pDMAD.

Literature Cited

- [1] Behrens L, Pena AI, Alonso MJ, Kissel T. Comparative uptake studies of bioadhesive and non-bioadhesive nanoparticles in human intestinal cell lines and rats: the effect of mucous on particle adsorption and transport. *Pharm Res*. 2002; 19(8):1185-93.
- [2] Florence AT, Hillery AM, Hussain N, Jani PU. Factors affecting the oral uptake and translocation of polystyrene nanoparticles: histological and analytical evidence. *J Drug Target*. 1995; 3(1): p. 65-70.
- [3] Mathiowitz E, Jacob JS, Jong YS, Carino GP, Chikering DE, Chaturvedi P, Santos CA, Vijayaraghavan K, Montgomeri S, Bassett M, Morrell C. Biologically erodable microspheres as potential oral drug delivery systems. *Nature*. 1997; 386(6623): p. 410-4.

Quantitative analysis of bioadhesive nanoparticle uptake from the small intestine for oral drug delivery applications

DY Cho[†], JJ Reineke[‡], E Mathiowitz[†].

[†] Dept. of Molecular Pharmacology, Physiology and Biotechnology, Brown University. Providence, RI;

[‡] College of Pharmacy and Health Sciences, Wayne State University. Detroit, MI.

Although the oral administration of therapeutic proteins is the preferred method to maximize patient comfort and compliance, the challenges presented by the gastrointestinal (GI) system have prevented the development of an effective oral delivery system. Polymeric micro- and nanosphere-based systems demonstrate great potential for oral drug delivery due to their ability to protect the encapsulated drug from degradation in the GI tract while allowing for controlled drug release kinetics. To evaluate the potential for the use of bioadhesive polymers for oral drug delivery applications, the relationship between the bioadhesion and uptake of 500 nm nanospheres (NS) from the small intestines was evaluated. NS fabrications included moderately bioadhesive polystyrene (PS) NS, non-bioadhesive poly(methyl methacrylate) (PMMA) NS fabricated by solvent evaporation and highly bioadhesive poly(butadiene-maleic anhydride-co-L-DOPA) (pBMAD) shell/PMMA core NS fabricated by a modified phase inversion nanoencapsulation method. Bioadhesion testing of the bulk polymers was performed using a texture analyzer while the in vitro bioadhesion of the NS formulations was evaluated using a modified everted sac assay. This bioadhesion data was correlated with the results from NS uptake studies using an isolated loop protocol in the jejunum of rats and showed a direct correlation between bioadhesion and NS uptake. Delivery of 500 nm PMMA, PS and pBMAD/PMMA microspheres locally to the rat jejunum for a 5-hour period resulted in uptake of $5.8 \pm 1.9\%$, $45.8 \pm 8.6\%$, and $66.9 \pm 12.9\%$ of the administered dose, respectively. Comparison of bioadhesion to intestinal uptake revealed a direct correlation with a 2.5-fold increase in bioadhesion resulting in a >10-fold increase in uptake. Furthermore, the differential distribution of the NS to various tissues suggests that surface chemistry also plays a significant role in the localization of these NS within the body following uptake. This is the first quantitative study to confirm the benefit of using bioadhesive polymers to enhance nanoparticle uptake from the small intestine. Furthermore, the results provide evidence for the possibility of passive targeting of NS to specific tissues for oral drug delivery applications.

Quantitative Analysis of Bioadhesive Nanoparticle Uptake From the Small Intestine For Oral Drug Delivery Applications

Daniel Y. Cho, Joshua Reineke, Edith Mathiowitz

Dept. of Molecular Pharmacology, Physiology and Biotechnology
Program in Artificial Organs, Biomaterials and Cellular Technology
Brown University, Providence, RI 02912

Introduction

Although the oral administration of therapeutic agents is the preferred method to maximize patient comfort and compliance, the challenges presented by the gastrointestinal (GI) system have prevented the development of effective oral delivery systems for many classes of drugs, including proteins and DNA. Polymeric micro- and nanosphere-based systems demonstrate great potential for oral drug delivery due to their ability to protect the encapsulated drug from degradation in the GI tract while allowing for controlled drug release kinetics. However, the primary obstacle in developing an efficient oral drug delivery system has proven to be nanosphere (NS) uptake from the small intestine.

Previous work in our lab has evaluated the uptake of NS from the small intestine using an isolated loop assay that has resulted in both qualitative and quantitative evidence of nanoparticle uptake via endocytosis by the enterocytes (figure 1). The use of bioadhesive polymers for oral drug delivery allows for longer GI transit time and closer interaction of the NS with the absorptive epithelium, which could play a role in enhancing particle uptake. Previous work in our lab has also shown that NS formulated with bioadhesive polymers enhanced the oral bioavailability of therapeutic molecules including insulin and DNA *in vivo*. Behrens, et al. have shown a correlation between bioadhesion and nanoparticle uptake both in cell culture and qualitatively with imaging of intestinal tissue *in vivo* [1]. This previous work provides evidence that uptake can be enhanced using bioadhesive NS. However, as of yet, no studies have quantitatively evaluated bioadhesive nanoparticle uptake *in vivo*.

The purpose of this work was to evaluate the effect of bioadhesion on the uptake and systemic biodistribution of 500 nm NS from the jejunum for oral drug delivery applications.

Acknowledgements

The authors would like to acknowledge Roxanne Burrill for her assistance with the animal studies, Peter Cheliez for the texture analyzer bioadhesion testing data, A. Peter Morello for the TEM images, Yu-Ting Liu for figures 5 & 6 and Spherics, Inc. for providing the pBMAD.

Methods

To study the effect of bioadhesion on nanoparticle uptake, 500 nm NS were fabricated using polymers with varying degrees of bioadhesiveness. Non-bioadhesive polymethylmethacrylate (PMMA) NS were fabricated by the solvent evaporation method. Moderately bioadhesive polystyrene (PS) NS were obtained from Polysciences, Inc. Bioadhesive NS were fabricated by Sequential Phase Inversion Nanoemulsification (SPIN), a novel technique developed in our lab. A mixed solution consisting of 2% w/v PMMA in chloroform and 2% w/v poly(butadiene-maleic anhydride-co-L-DOPA) (pBMAD) in ethanol was rapidly added to a non-solvent bath of petroleum ether resulting in the spontaneous formation of NS consisting of a pBMAD shell encapsulating a PMMA core. All NS were confirmed to be 500 nm in diameter.

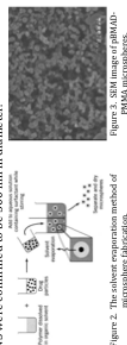


Figure 2. SEM image of pBMAD-coated PMMA nanoparticles.

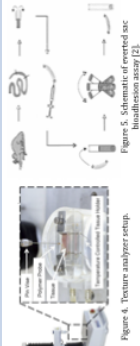


Figure 3. Isolated loop assay setup.

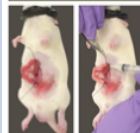


Figure 4. Schematic of tissue processing.

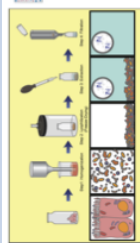


Figure 5. Schematic of gel permeation chromatography.

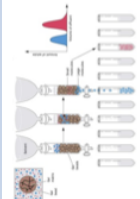


Figure 6. Schematic of drug permeation assay.

To quantify the uptake of NS from specific regions of the GI tract, an isolated loop procedure was performed on 6 cm intestinal sections as previously described [3] in male Sprague-Dawley rats weighing 200-250 g with a 1 mL (2.5% w/v) dosage of NS in solution (figure 6). Following a 5 hour incubation period, tissue samples were excised as shown in figure 6, and the uptake of the polymer into the intestinal wall was quantified by GPC (figure 8). The amount of drug detected in each tissue was the sum of all amounts detected in tissues divided by the total dose administered and multiplied by 100. The percent bioadhesion was calculated by dividing the amount of polymer detected in each tissue by the total amount of polymer detected in all tissues; all data was normalized to total uptake. Mass balance calculations were performed by taking the sum of all amounts detected in all samples by the total administered dose.

Results

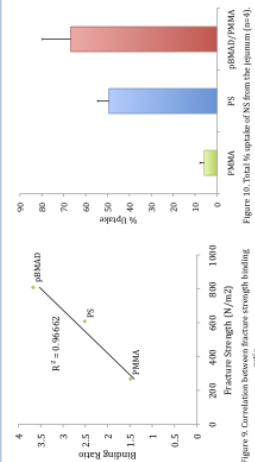


Figure 9. Total % uptake of NS from the jejunum (n=4).

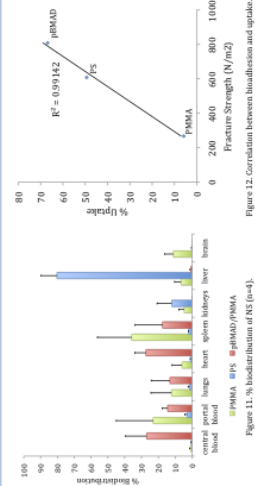


Figure 10. % bioadhesion of NS (n=4).

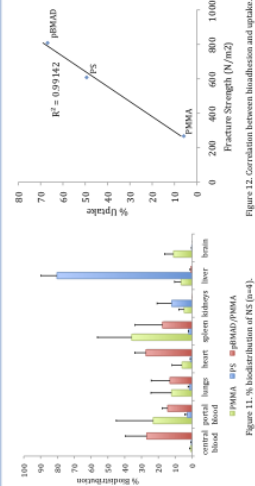


Figure 11. Correlation between bioadhesion and uptake.

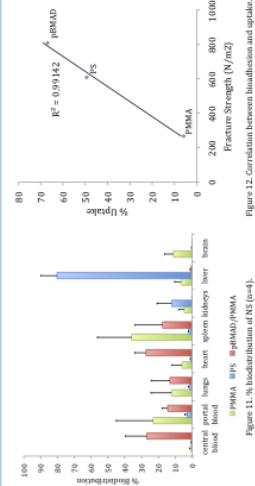


Figure 12. Correlation between fracture strength and uptake.

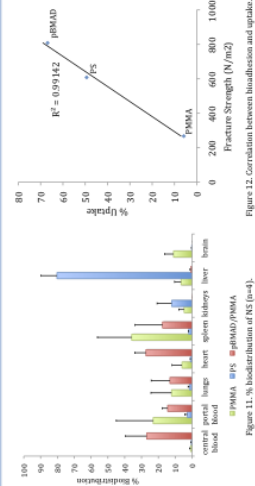


Figure 13. Schematic of drug-loaded bioadhesive NS for oral drug delivery.

Conclusions

Our results show that the use of bioadhesive polymers can significantly enhance the uptake of 500 nm NS from the jejunum. Furthermore, the use of our novel bioadhesive polymer, pBMAD, resulted in higher levels of particle uptake from the small intestine than previously described in literature.

These findings provide strong support for the use of bioadhesive polymers as a strategy to enhance particle uptake in the development of more sophisticated and efficient drug delivery systems for the oral administration of a variety of therapeutic agents.

A potential NS-based delivery system consisting of a bioadhesive polymer and a biodegradable polymer core loaded with a therapeutic agent may prove to be the optimal formulation for high levels of particle uptake with a controllable drug release kinetics (figure 13).

Comparison of fracture strength to intestinal uptake revealed a direct correlation with a 2.5-fold increase in bioadhesion resulting in a >10-fold increase in uptake. Similar results were obtained when the bridging ratio was correlated with specific drug delivery.

Literature Cited

- [1] Behrens T, Pena AI, Alonso MJ, Kissel T. Pharm Res. 2002; 19:1185-1193.
- [2] Santos CA, Jacob JS, Hertzog BA, Freedman BD, Press DL, Hampichamchal P, Mathiowitz E. J Controlled Release. 1999; 6:113-122.
- [3] Florence AT, Hillery AM, Hussain N, Jani PU. J Drug Target. 1995; 3:65-70.

A Novel W/O/O Double Emulsion Solvent Removal Method for the Nanoencapsulation of Doxycycline

R. Patel, D. Cho, C. Tian, A. Chang, D. Decoteau, J. Morgan, and E. Mathiowitz

Center for Biomedical Engineering, Brown University, Providence, RI, 02912, USA
roshni_patel@brown.edu

ABSTRACT SUMMARY

A novel method has been developed for the nanoencapsulation of doxycycline that does not include the use of an aqueous non-solvent phase and as a result, enhances encapsulation efficiency and minimizes drug loss. Based on the solvent removal method with the incorporation of a water/oil/oil (W/O/O) double emulsion, PLGA nanospheres containing doxycycline nanospheres (PLGA-DOX NS) can be fabricated with sustained release kinetics. The versatility of this method allows for its use in the nanoencapsulation of small hydrophilic therapeutic molecules for drug delivery applications.

INTRODUCTION

Traditional encapsulation techniques, such as solvent evaporation, have typically yielded poor encapsulation efficiencies for the microencapsulation of hydrophilic drugs due to the affinity of the drug to the aqueous non-solvent phase during the process of spherification. We are propose a modified encapsulation technique utilizing solvent removal, a method originally developed for the microencapsulation of drugs in polyanhydride microspheres due to the rapid hydrolysis of the anhydride bond in the presence of water, and later used for the encapsulation of proteins in biodegradable polyesters [1]. Using a model hydrophilic drug, doxycycline, we report here on a technique that is easily adapted for the encapsulation of hydrophilic drugs in biodegradable polyesters, such as PLGA, by the incorporation of a W/O/O double emulsion.

Previous encapsulation of doxycycline (DOX, a hydrophilic tetracycline derivative) within PLGA microspheres using solvent evaporation has resulted in poor encapsulation efficiencies, requiring high theoretical drug loadings to achieve release kinetics compatible with therapeutic activity [2,3]. Such methods yield significant drug loss and large particle diameters due to the higher drug loading. Other methods for encapsulating doxycycline within PLGA using ultrasonic atomization or spray drying are not easily accessible, making these techniques difficult to translate into therapeutics for widespread clinical use [4]. The modified solvent removal method reported here is a relatively simple procedure that results in high encapsulation efficiencies and small particle diameters. Furthermore, this technique can be adapted for use with a wide range of hydrophilic drugs for a variety of applications.

EXPERIMENTAL METHODS

A W/O/O double emulsion solvent removal process was modified for the fabrication of PLGA nanospheres

encapsulating DOX. In this method, DOX equivalent to 20% w/w of the dry weight of the polymer was dissolved in distilled water to form the aqueous phase. RG502 (PLGA 50:50) in methylene chloride formed the first oil phase. The DOX solution was added to the polymer solution and probe sonicated using a homogenizer (40% amplitude) to create the first W/O emulsion. The primary emulsion was then poured into a 20% v/v methylene chloride/silicon oil bath and probe sonicated to create the W/O/O double emulsion. For the final curing of the nanospheres, the secondary emulsion was poured into petroleum ether and stirred (2000 rpm) before filtration and washing with petroleum oil. The resulting nanospheres (PLGA-DOX NS) were collected and lyophilized for 2 days and stored at -20°C for further assaying. Unloaded nanospheres (PLGA NS) were also fabricated using the above method and served as a negative control for further characterization.

To determine the encapsulation efficiency, 10 mg of the PLGA-DOX NS were dissolved in 1 mL 0.1 N NaOH for 24 hours in the dark with constant agitation. The resulting solution was filtered using a 0.2 µm syringe filter and analyzed by UV spectrophotometry at an absorbance of 255 nm.

Particle diameters were quantified using a Beckham Coulter LS230 Diffraction Particle Size Analyzer. Surface morphology was examined using a Hitachi 2700 Scanning Electron Microscope. The thermal properties of bulk PLGA, bulk DOX, unloaded PLGA NS, and PLGA-DOX NS, as well as the interaction and incorporation of DOX within the PLGA, were evaluated using differential scanning calorimetry (DSC) using a Perkin Elmer DSC calorimeter.

The release kinetics of the nanospheres were determined by adding 10 mg of PLGA-DOX NS to 1.25 mL of phosphate buffered saline (PBS, pH = 7.4) and incubating at 37 °C. 1 mL of the supernatant was removed for UV spectrophotometric analysis and replaced with fresh PBS at the following time points: 1 hour, 2 hours, 3 hours, 4 hours, 8 hours, 1 day, 2 days, 3 days, 5 days, 1 week, 2 weeks, 3 weeks and 4 weeks. In addition, at 1 day, 1-, a 2-, 3-, and 4-week time point, the supernatant was removed completely and the microspheres were lyophilized for 24 hours.

RESULTS AND DISCUSSION

A 20% w/w loading of DOX to dry PLGA yielded PLGA-DOX NS with an encapsulation efficiency of 87.2%. Particle sizing indicated two populations for the PLGA-DOX NS in the micro- and nano-scale with diameters of approximately 576 nm and 1801 µm.

Similarly, for unloaded PLGA NS, two populations were sized at the 567 nm and 1812 μm .

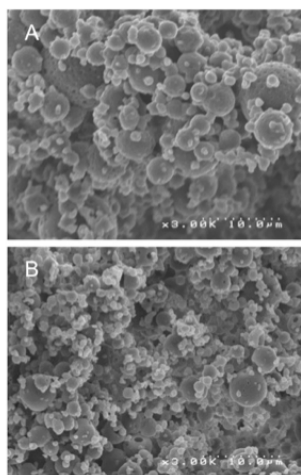


Figure 1 – SEM micrographs of (A) PLGA-DOX NS and (B) unloaded PLGA NS

Figure 1 illustrates SEM micrographs of (A) PLGA-DOX NS and (B) unloaded PLGA NS. Generally spherical particles were observed for both loaded and unloaded formulations, each with the majority of the particles distributed between two size populations as confirmed with Coulter particle sizing. There were some larger particles in both formulations, in the 5-10 μm range. These larger particles are likely due to inconsistency in the W/O/O emulsification process.

Preliminary *in vitro* release studies of DOX from PLGA-DOX NS indicated that within the first hour, approximately 63% of encapsulated DOX was released from the spheres. This burst was followed by a sustained release of the next 28 days with a cumulative release of 66%. An increase in release occurred at day 21 and continued for the duration of study. This increase is likely attributed to the degradation of the polymeric matrix core of each sphere and studies are still ongoing to determine release post-28 days. The high initial burst is most likely due to the high drug loading in this formulation and hydrophilic nature of drug within the spheres. If loading is decreased, it is believed that a more controlled release will be achieved. Particle porosity, a parameter that has been demonstrated to modulate protein release in microspheres fabricated with this method [1], may also be adjusted to control DOX release from the nanospheres.

DSC analysis was used to understand the physical state of the DOX within the nanospheres. The endotherm peaks corresponding to the glass transition of PLGA were observed at 42.530°C and 42.253°C for the control and DOX-loaded spheres, respectively. The T_m of the DOX was observed at 168.033°C. However, the DOX-loaded spheres had no observable thermal activity, indicating a molecular dispersion within the PLGA and which may

explain the sustained release of the DOX from the nanospheres as seen in the preliminary release studies.

CONCLUSION

We report here on a novel method for the nanoencapsulation of hydrophilic drugs without the use of an aqueous non-solvent phase, a phase which is present in most solvent evaporation methods and which can result in considerable loss of drug during the encapsulation process. Using a solvent removal method coupled with a water/oil/oil double emulsion, PLGA nanospheres containing DOX nanospheres can be fabricated with enhanced encapsulation efficiency of 87.2%. Preliminary release studies indicate sustained release of DOX over the course of 28 days *in vitro*. Further characterization using DSC suggests molecular dispersion of DOX within the PLGA and which may explain the sustained release of the DOX. Together, these preliminary results illustrate potential use of the solvent removal method incorporating a water/oil/oil double emulsion for the nanoencapsulation of hydrophilic drugs for drug delivery.

REFERENCES

1. D.M. Ciombor, A. Jaklenec, A. Liu, C. Thanos, N. Rahman, P. Weston, R. Aaron, E. Mathiowitz. J. Microencapsulation. 2006; 23 (2), 183-194.
2. R.C. Mundargi, S. Srirangarajan, S.A. Agnihotri, S.A. Patil, S. Ravindra, S.B. Setty, T.M. Aminabhavi. J. Controlled Release. 2007; 199, 59-68.
3. D.Z. Liu, W.P. Chen, C.P. Lee, S.L. Wu, Y.C. Wang, T.W. Chung. J. Microencapsulation. 2004; 21 (6), 643-652.
4. P. Patil, R.C. Mundargi, V.R. Babu, D. Jain, V. Rangaswamy, T.M. Aminabhavi. J. Applied Polymer Science. 2008; 108, 4038-4046.



A Novel W/O/O Double Emulsion Solvent Removal Method For the Nanoencapsulation of Doxycycline

R. Patel, D. Cho, C. Tian, A. Chang, D. Decoteau, J. Morgan, and E. Mathiowitz
Center for Biomedical Engineering, Brown University, Providence RI
rosnhi_patel@brown.edu

ABSTRACT

A novel method has been developed for the nanoencapsulation of doxycycline that does not utilize an aqueous non-solvent phase. Drug loss during fabrication is minimized and encapsulation efficiency is enhanced. Based on a solvent removal method with the incorporation of water/oil (W/O/O) double emulsion, PLGA nanospheres containing doxycycline (PLGA-DOX NS) can be fabricated with sustained release kinetics for up to 85 days *in vitro*. Higher loading formulations (10% and 5%) yielded classic biphasic PLGA release profiles, while lower loading formulations gave rise to a more constitutive, linear release. The addition of 1% NaCl modulated release from most formulations by increasing the initial burst. The versatility of this method allows for its use in the nanoencapsulation of other small, hydrophilic therapeutic molecules for drug delivery applications.

INTRODUCTION

Previous encapsulation of DOX within PLGA using solvent evaporation has yielded poor encapsulation efficiencies, requiring higher theoretical drug loading to achieve therapeutically relevant release kinetics [1, 2]. Significant drug loss during fabrication and large particle sizes due to higher drug loading are also common to these methods.

We present a modified encapsulation technique utilizing solvent removal, a method originally developed for the microencapsulation of drugs within poly(hydroxyacids) due to the rapid hydrolysis of the anhydride bond in the presence of water, and later used for the encapsulation of proteins in biodegradable polyesters [3]. Based on our work with the encapsulation of DOX, we propose that this technique can be adapted for a wide range of hydrophilic drugs for a variety of applications.

METHODS

Nanosphere Fabrication

As shown in Figure 1, the first W/O emulsion was created by adding DOX to the polymer solution and probe-sonicated (40% amplitude for 1 min). This emulsion was then added to 20% v/v methylene chloride/silicon oil solution and probe-sonicated to create the W/O/O double emulsion. Loading of DOX varied from 0.1%-10% (w/w drug/polymer). For formulations containing 1% NaCl, NaCl was dissolved in the DOX solution prior to creating the first emulsion. Unloaded nanospheres served as a negative control.

Characterization

- Coulter particle sizing for particle diameters
- SEM for surface morphology
- DSC for thermal analysis and assess interaction of DOX within the PLGA
- In vitro* release studies using UV spec (255 nm)

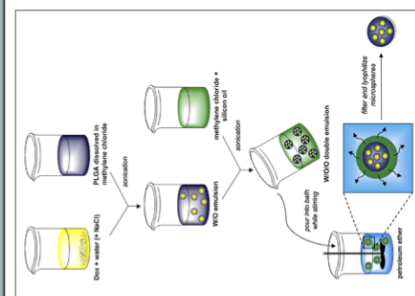


Figure 1 – W/O/O Double Emulsion Solvent Removal

RESULTS

Formulation	% Doxycycline Loading	% NaCl Loading	% Encapsulation Efficiency	Mean Particle Size (µm)	T _g (°C)	T _m (°C)
A	10	---	51.1 ± 5.0	617 ± 247	35.514	---
B	10	1	38.9 ± 5.4	608 ± 247	35.301	65.909
C	5	---	70.1 ± 3.3	605 ± 220	36.455	---
D	5	1	73.9 ± 3.7	609 ± 239	36.973	68.452
E	1	---	69.6 ± 1.1	603 ± 223	36.566	62.843
F	1	1	73.9 ± 9.2	598 ± 218	34.762	64.482
G	0.1	---	67.4 ± 2.6	603 ± 234	37.844	65.870
H	0.1	1	56.4 ± 2.6	598 ± 216	38.476	64.694

DOX T_g = 168.53°C PLGA T_g = 40.85°C PLGA T_m = 46.09°C

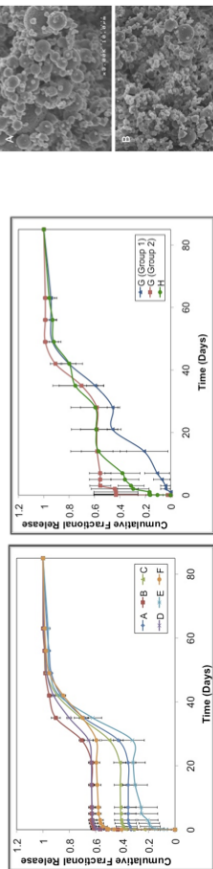


Figure 2 – Cumulative fractional release of DOX from 0.1% loading without (A) and with salt (B). 5% loading without (C) and with salt (D), (E) and (F) loading without (G) and with salt (H). n=6 for all formulations.

DISCUSSION/CONCLUSIONS

- High encapsulation efficiencies for DOX in PLGA as compared to previously reported methods
- Particle size in nanoscale
- Sustained release for hydrophilic drug over course of 85 days
 - High loading formulations (10%, 5%) followed the biphasic classic PLGA release profiles (initial burst → lag phase → second burst due to degradation of matrix core)
 - Salt addition yielded higher initial burst
 - Two release profiles observed for low loading formulations (0.1%)
 - Classic biphasic
 - Continuous release
 - Salt addition yielded more continuous release
- No thermal activity was observed for any formulation with respect to DOX (T_m = 168.533)
 - Molecular dispersion of DOX within the PLGA
 - May explain the sustained release of the drug
- May be an attractive approach for encapsulation of a variety of small, hydrophilic therapeutics for sustained delivery using PLGA nanospheres.

ACKNOWLEDGEMENTS

This work was partially supported by the Office of Research and Development Rehabilitation R&D Service, Department of Veterans Affairs.

REFERENCES

- R.C. Mundargi, S. Srikantharaj, S.A. Agnihotri, S.A. Palli, S. Ravindra, S.B. Sanyal, T.M. Annabhavi, J. Controlled Release, 2007; 199, 59-68.
- D.Z. Lu, W.P. Chen, C.P. Lee, S.L. Wu, Y.C. Wang, T.W. Chung, J. Microencapsulation, 2004; 21 (6), 643-652.
- D.M. Combar, A. Jakelenc, A. Liu, C. Tharos, N. Rahman, R. Weston, E. Mathiowitz, J. Microencapsulation, 2006; 23 (2), 183-194.

Effect of Encapsulation Method on Protein Loaded Nanospheres Fabricated by Phase Inversion Nanoencapsulation

D.Y. Cho, C. Tian, K.M. Estrellas, E. Mathiowitz

Brown University, Providence, RI, 02912, USA
daniel_cho@brown.edu

ABSTRACT SUMMARY

In the fabrication of nanospheres by phase inversion nanoencapsulation, proteins can be encapsulated by the formation of a water-in-oil emulsion, micronization into the polymer by frozen emulsion or as a micronized solid. Each of these methods can influence the dispersion of the protein within the polymer and ultimately affect the loading and release kinetics of the proteins from the nanospheres. The effect of the encapsulation method on PLGA nanospheres loaded with bovine serum albumin and bovine zinc insulin has been investigated.

INTRODUCTION

Phase inversion nanoencapsulation (PIN) is a method of encapsulating small molecules, proteins and genes in small diameter micro- and nanospheres [1]. The formation of nanospheres is dependent on the phase inversion of a dilute polymer solution when it is rapidly added to a non-solvent bath. The versatility of PIN allows for the encapsulation of proteins directly as micronized solids and aqueous solutions via emulsification as well as proteins that have been micronized into the polymer by frozen emulsion.

When the protein is encapsulated as a micronized solid or by frozen emulsion, the protein acts as a core nucleus on which the polymer in solution can precipitate to form a coating around the particle [2]. Likewise, in the use of an aqueous solution for the encapsulation of the protein, the formation of an emulsion of the protein solution in hydrophobic solvent for the polymer results in the emulsion droplets serving as the nucleation sites [2].

The differences in the interaction of the polymer and protein resulting from each of the encapsulation methods are likely to influence the dispersion of the protein in the nanospheres. This can have an effect on particle size, protein loading and release kinetics. This study compares the method of protein micronization for nanospheres fabrication by PIN. Thus bovine serum albumin (BSA) and bovine zinc insulin (BZI) have been encapsulated in PLGA nanospheres by PIN to determine the effect of each method on the resulting formulations.

EXPERIMENTAL METHODS

Nanospheres of PLGA (RG502) were fabricated by the rapid addition of 2% w/v solutions of PLGA in methylene chloride into petroleum ether with a solvent to non-solvent ratio of 1:75.

For the solid micronized protein loadings, BZI was micronized by a precipitation method resulting in the formation of particles with an average diameter of 300

nm. The BZI was suspended into the PLGA solution before addition into the non-solvent bath. For the emulsion formulations, the appropriate weight of protein was dissolved in distilled water and emulsified into the polymer solution by vortexing with an aqueous to organic ratio of 1:50 before phase inversion. For the frozen emulsion nanospheres, the previously described emulsion was flash-frozen and lyophilized for 24 hours. The polymer/protein mixture was then re-dissolved in methylene chloride before phase inversion. The nanospheres were collected using a 0.2 μ m PTFE filter in a positive pressure filtration column and lyophilized for 24 hours.

The nanospheres were characterized using a Beckman Coulter LS230 Laser Diffraction Particle Size Analyzer, a Perkin Elmer DSC calorimeter, and a Hitachi 2700 Scanning Electron Microscope.

To determine the protein loading, 10 mg of each formulation was dissolved in 2 mL of 0.1N NaOH with constant agitation for 24 hours. The resulting solution was filtered using a 0.2 μ m PTFE syringe filter tip and the protein concentration was analyzed using a bicinchoninic acid (BCA) assay.

RESULTS AND DISCUSSION

Laser diffraction particle size analysis revealed that regardless of encapsulation method, the nanospheres were very similar in size with diameters ranging from 556.6 nm and 573.6 nm (Table 1). These results are consistent with previous research published by the Mathiowitz lab showing that particle size is dependent on polymer viscosity rather than the mixing rate of the initial emulsion, as seen with traditional microencapsulation techniques [2]. SEM analysis of the nanospheres revealed a monodisperse population of spheres with a size range consistent with the results from laser diffraction particle analysis (Figure 1). There were no traces of unencapsulated BZI crystals visible on the scanning electron micrographs.

Table 1
Particle Size (nm)

	Control	BSA	BZI
Emulsion	559.3	556.6	562.6
Frozen Emulsion	573.6	568.0	567.4
Micronized	565.7	—	558.4

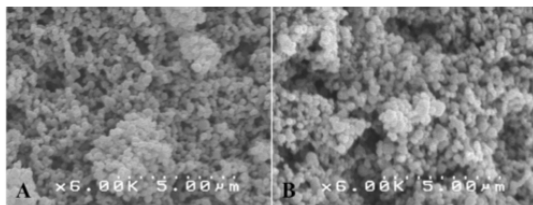


Figure 1. Scanning electron microscopy of frozen emulsion control nanospheres (A) and frozen emulsion BZI nanospheres (B)

The only thermal activity observed by DSC for the both the control and protein-loaded nanospheres was the glass transition of the PLGA (Table 2). The protein-loaded nanospheres showed a slight increase in the T_g of the PLGA, with the highest increases in the glass transition seen in the formulations in which the protein was loaded by emulsification. These variances in the T_g of the PLGA could be indicative of different arrangements of the protein within the nanospheres, which can result in distinctive release kinetics for each formulation.

Table 2
Glass Transition (T_g)

	Control	BSA	BZI
Emulsion	41.941	43.256	44.588
Frozen Emulsion	41.390	42.480	41.754
Micronized	40.613	—	41.428

Analysis of the protein content of the nanospheres revealed differences in the % loading for each encapsulation method and protein (Table 3). BZI had higher rates of encapsulation than BSA regardless of encapsulation method. When compared to the encapsulation of micronized solid proteins, the use of emulsions resulted in higher protein loadings for BZI, 1.385% and 1.449% compared to 0.943%. The micronization of protein into the polymer by frozen emulsion resulted in the highest rates of protein encapsulation for both BSA and BZI. This could be due to the close interaction of the protein and polymer during the frozen emulsion process or the increased loss of low molecular weight components of the polymer during the phase inversion process.

Table 3
Protein Loading (% w/w protein to polymer)

	BSA	BZI
Emulsion	0.895	1.385
Frozen Emulsion	1.190	1.449
Micronized	—	0.943

Further studies with the formulations described above will include the effect of the encapsulation method on the release kinetics of the proteins from the nanospheres. The

changes in the morphology and FTIR spectra of the nanospheres as they degrade will also be evaluated.

CONCLUSION

PIN is a rapid and versatile method for the fabrication of drug-loaded nanospheres. Since this method is nearly instantaneous, it is especially useful for the encapsulation of sensitive bioactive molecules such as proteins. The encapsulation of proteins into the nanospheres can be achieved directly as micronized solids and aqueous solutions via emulsification as well as proteins that have been micronized into the polymer by frozen emulsion. Preliminary results have shown that although the method of encapsulation does not affect the size of the nanospheres, it does play a role in protein loading. It is expected that the release kinetics of the protein from the nanospheres will also be affected by the method of protein encapsulation. These results will prove to be useful in the preparation of protein-loaded nanospheres by PIN for drug delivery applications.

REFERENCES

1. E. Mathiowitz, J.S. Jacob, Y.S. Jong, G.P. Carino, D.E. Chickering, P. Chartuvedi, C.A. Santos, L. Vijayaraghavan, S. Montgomery, M. Bassett, C. Morrell. *Nature*. 1997, 386 (6623): 410-414.
2. J.S. Jacob, E. Mathiowitz, *Carrier-Based Drug Delivery*, Vol. 879, American Chemical Society, 2004.

ACKNOWLEDGMENTS

The authors would like to acknowledge Shara Azad for her help with the protein assays and Stacia Furtado for her assistance with all aspects of this project.



Effect of Encapsulation Method on Protein-Loaded Nanospheres Fabricated by Phase Inversion Nanoencapsulation

Daniel Y. Cho, Cheng Tian, Kenneth M. Estrellas, Edith Mathiowitz

Program in Artificial Organs, Biomaterials and Cellular Technology, Department of Molecular Pharmacology, Physiology and Biotechnology, Brown University, Providence, RI 02912



Introduction

Phase inversion nanoencapsulation (PIN) is a method of encapsulating small molecules, proteins and genes in small diameter micro- and nanospheres [1]. The formation of nanospheres is dependent on the phase inversion of a dilute polymer solution when it is rapidly added to a non-solvent bath. The versatility of PIN allows for the encapsulation of proteins directly as nanonized solids and aqueous solutions via emulsification as well as proteins that have been nanonized into the polymer by frozen emulsion.

When the protein is encapsulated as a nanonized solid or by frozen emulsion, the protein acts as a core nucleus on which the polymer in solution can precipitate to form a coating around the particle [2]. Likewise, in the use of an aqueous solution for the encapsulation of the protein, the formation of an emulsion of the protein solution in hydrophobic solvent for the polymer results in the aqueous droplets serving as the nucleation sites [2].

The differences in the interaction of the polymer and protein resulting from each of the encapsulation methods are likely to influence the dispersion of the protein in the nanospheres. This can have an effect on particle size, protein loading and release kinetics. This study compares the method of protein nanonization for nanospheres fabrication by PIN. Thus bovine serum albumin (BSA) and bovine zinc insulin (BZI) have been encapsulated in PLGA nanospheres by PIN to determine the effect of each method on the resulting formulations

Methods

Nanospheres were fabricated by the rapid addition of 2% w/v solutions of PLGA in methylene chloride into petroleum ether with a solvent to non-solvent ratio of 1:7.5. For the solid nanonized protein loadings, BZI was micronized by a *tert*-butyl alcohol precipitation method. The nanonized BZI was suspended into the PLGA solution before phase inversion. For the emulsion formulations, the appropriate weight of protein was dissolved in distilled water and emulsified into the polymer solution by vortexing with an aqueous to organic ratio of 1:50 before phase inversion. For the frozen emulsion nanospheres, the previously described emulsion was flash-frozen and lyophilized for 24 hours. The polymer/protein mixture was then re-dissolved in methylene chloride before phase inversion. The nanospheres were collected using a 0.2 μ m PTFE filter in a positive pressure filtration column and lyophilized for 24 hours.

The formulations were characterized using a Beckman Coulter LS230 Laser Diffraction Particle Size Analyzer, a Perkin Elmer DSC calorimeter, and a Hitachi 2700 Scanning Electron Microscope. A micro-bicinchoninic acid (micro BCA) assay was used to determine protein loading and release kinetics.

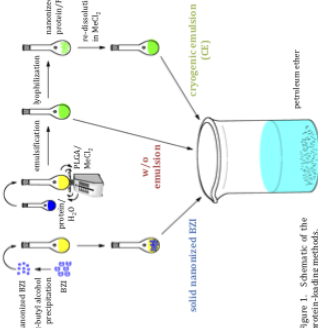


Figure 1. Schematic of the protein-loading methods.

Results

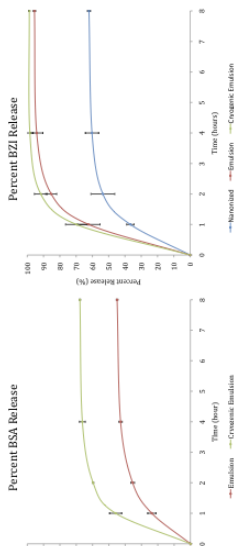
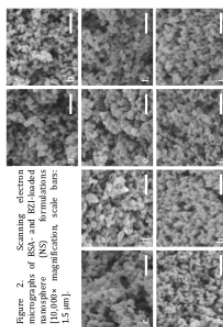


Figure 3. Percent Release of BSA and BZI from nanosphere formulations. Release data calculated as a percent of the total protein loading (n=6).

Formulation	Particle Size (nm)	T _g (°C)		Loading (% w/w)		T _g (°C)		Loading (% w/w)	
		1st Heat	2nd Heat	Actual	Theoretical	1st Heat	2nd Heat	Actual	Theoretical
Emulsion	541 ± 210	41.85	39.65	0.609	1.000	39.79	39.30	0.641	1.000
Emulsion Control	550 ± 221	40.95	38.65	-	-	41.16	39.53	-	-
Cryogenic Emulsion	530 ± 159	40.36	39.55	0.906	1.000	41.35	39.30	0.75	1.000
Cryogenic Emulsion Control	540 ± 203	39.81	39.73	-	-	40.03	39.63	1.099	1.000
						40.16	39.63	0.994	1.000

Table 1. Table of formulations with particle size, DSC data and protein loading (n = 3).

Literature Cited

1. E. Mathiowitz, J.S. Jacob, Y.S. Jong, G.P. Carino, D.E. Chickering, P. Chaturvedi, C.A. Santos, K. Vijayaraghavan, S. Montgomery, M. Bassett, C. Morrell. Biologically erodable microspheres as potential oral drug delivery systems. *Nature*. 1997; 386 (6623): 410-414.

2. J.S. Jacob, E. Mathiowitz. A Novel Mechanism for Spontaneous Encapsulation of Active Agents: Phase Inversion Nanoencapsulation. *Carrier-Based Drug Delivery*, Vol. 879. assistance with this project. American Chemical Society, 2004.

Acknowledgements

The authors would like to acknowledge Shara Azad Taylor Peak, Benjamin Lux and Stacia Furrado for their assistance with this project.

Conclusions

- The protein loading method did not affect the size of the nanospheres fabricated by PIN. As previously described, particle size was determined by polymer solution viscosity.
- The encapsulation of proteins and the protein-loading method did not significantly affect the thermal properties of the nanospheres. The DSC data suggests that the protein and the polymer are completely phase separated.
- For both BSA and BZI, the cryogenic emulsification method resulted in the highest protein loading. For the BZI, the emulsification method resulted in higher protein encapsulation than that of the protein as a nanonized solid.
- All formulations resulted in the burst release of protein, which is characteristic of the release kinetics from polyester depot drug delivery systems. The results suggest that the release of protein from PLGA nanospheres depends more on the characteristics of the protein rather than the polymer.
- In the case of BZI, both the cryogenic emulsification and the w/o emulsion resulted in near complete release of the encapsulated protein by 4 hours. In contrast, the release of the nanonized BZI exhibited a smaller burst, possibly suggesting better entrapment of the protein.

The results of this study show that while the encapsulation method does not significantly affect the physical properties of the nanospheres, it can play a role in determining the release kinetics of the protein. However, it seems that the properties of the protein play a more significant role than the modulating the intensity of the initial burst, which warrants further evaluation.

A Novel Method for the Fabrication of Double-Walled Nanospheres by Sequential Phase Inversion Nanoencapsulation

D.Y. Cho¹, K.M. Estrellas¹, C. Tian¹, J.J. Reineke^{1,2} and E. Mathiowitz¹

¹Brown University, Providence, RI, 02912, USA; ²Wayne State University, Detroit, MI, 48201, USA
daniel_cho@brown.edu

ABSTRACT SUMMARY

A novel technique for the preparation of double-walled nanospheres was developed based on the phase inversion nanoencapsulation method and phase separation phenomena encountered in polymer/solvent/non-solvent systems. Nanospheres consisting of a biodegradable polyester core with an encapsulated drug and a bioadhesive polymer shell can be fabricated in a single step for oral drug delivery applications.

INTRODUCTION

Phase inversion nanoencapsulation (PIN) was originally developed as a simple and rapid method for encapsulating small molecules, proteins and genes in small diameter polymer micro- and nanospheres [1]. This method relies on the spontaneous formation of nanospheres when a dilute polymer solution is added to the environment of a non-solvent [2]. While the fabrication of nanospheres by this method is a versatile and powerful technique, the small diameters of particles formed by PIN make traditional coating processes to prepare double-walled microspheres a challenge to adapt for nanoparticles.

As a method to minimize the burst effect commonly seen with micro- and nanospheres as well as to modify surface chemistry for the enhancement of particle uptake from the gastrointestinal system, the PIN method was modified to fabricate double-walled nanospheres in a single step. Sequential phase inversion nanoencapsulation (sPIN) requires the selection of two polymer/solvent pairs that allow for the sequential phase inversion of each polymer. For the core polymer, a solvent must be selected that is also a solvent for the shell polymer while the solvent for the shell polymer must be a non-solvent for the core polymer; all solvents must be miscible with the non-solvent for both polymers. Thus, when the shell polymer solution is added to the core polymer solution, the resulting decrease in the core polymer solubility at its cloud point results in its preferential phase separation and if desired, encapsulation of the target drug. When this unstable solution is rapidly added to the non-solvent, the shell polymer engulfs the core polymer as phase inversion is completed to form double-walled nanospheres.

Prior studies of particulate uptake from the gastrointestinal tract have shown higher levels of uptake with smaller diameter particles [3]. The use of bioadhesive polymers to increase gastrointestinal transit time as method of uptake enhancement has been also been identified as an approach for effective oral drug delivery [1]. Thus, bioadhesive poly(butadiene-maleic anhydride-co-L-DOPA) shell, poly(lactic-co-glycolic acid) (75:25)

core (pBMAD-PLGA) nanospheres were fabricated by sPIN and characterized by scanning electron microscopy (SEM), laser particle size analysis, fourier transform infrared spectroscopy (FTIR), differential scanning calorimetry (DSC) and transmission electron microscopy (TEM).

EXPERIMENTAL METHODS

Solubility testing identified polymer/solvent pairs that fit the parameters described above for sPIN. In the case of pBMAD and PLGA, the solvents were ethanol and tetrahydrofuran (THF), respectively. While both pBMAD and PLGA are soluble in THF, only the pBMAD is soluble in ethanol; petroleum ether was selected as the non-solvent for both polymers. In order to determine the ratio of solvents required to induce the phase separation of PLGA, the cloud point of a 2% w/v PLGA/THF solution with ethanol was determined. The addition of ethanol to the PLGA solution causes phase separation at 1.25% w/v polymer to total solvent. Since PIN requires dilute polymer solutions between 1 – 5% w/v, this concentration of polymer fits within the parameters required to form discrete nanospheres [2].

To fabricate double-walled nanospheres from pBMAD and PLGA, a 2% w/v solution of pBMAD in ethanol was added to a 2% w/v solution of PLGA until the cloud point for PLGA was reached. This solution was then rapidly added to a non-solvent bath with a solvent to non-solvent ratio of 1:75 and stirred for 1 minute to cure the nanospheres. The resulting nanospheres were filtered using a 0.2 μ m PTFE filter in a positive pressure filtration column and lyophilized for 24 hours.

The nanospheres were characterized using a Beckman Coulter LS 230 Laser Diffraction Particle Size Analyzer, a Perkin Elmer Spectrum One FTIR and a Perkin Elmer DSC7 calorimeter. Microscopy was performed using a Hitachi 2700 SEM equipped with a lanthanum hexaboride gun and a Philips 410 TEM.

RESULTS AND DISCUSSION

Laser particle size analysis of the nanospheres revealed a biphasic distribution of particle sizes with peaks at 545 nm and 1668 nm. To confirm particle size and evaluate morphology, SEM was used to visualize the nanospheres. As can be seen in Figure 1, the majority of the nanospheres are in the 500 nm range. In contrast to the results from laser particle size analysis, the spheres seem to be fairly monodisperse but with a tendency to aggregate due to the bioadhesiveness of the pBMAD. The larger peak detected by particle size analysis most likely reflects the aggregation of the 500 nm particles.

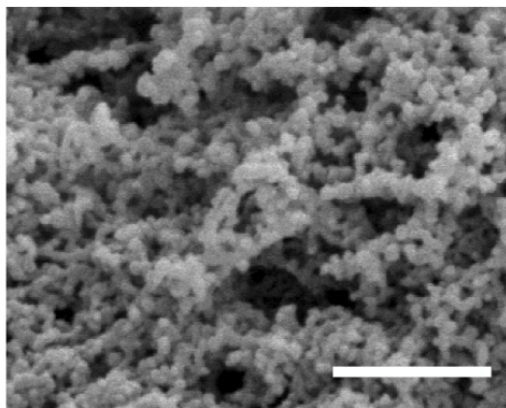


Figure 1. Scanning Electron microscopy of pBMAD/PLGA nanospheres, scale bar = 2.5 μm .

To determine the interaction of the two polymers within the nanospheres, FTIR analysis and DSC were used. FTIR analysis of the bulk pBMAD and PLGA as well as the double-walled formulation revealed the presence of both polymers within the nanospheres. The FTIR spectra of the double-walled formulation contains peaks that are characteristic of both the core and shell polymers, such as the ester bond of PLGA (1750 cm^{-1}) and the anhydride bond of pBMAD (1700 cm^{-1}) (Figure 2). DSC analysis of the thermal properties of the bulk polymers revealed a T_g for PLGA at 42.7°C and a T_g for pBMAD at 174°C . In contrast, the pBMAD/PLGA formulation showed two glass transitions at 47.1°C and 173°C , corresponding to the glass transition temperatures for PLGA and pBMAD, respectively. The shift in the glass transitions of each polymer towards the T_g of the other could be indicative of incomplete phase separation of the two polymers within the nanospheres.

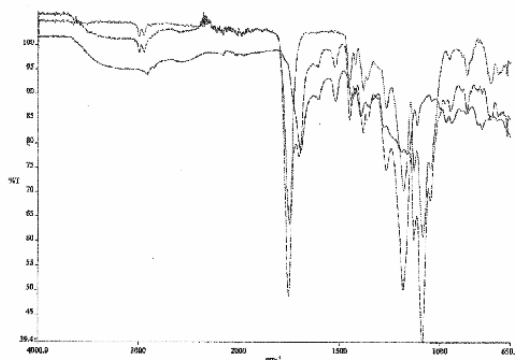


Figure 2. FTIR spectra of bulk pBMAD (straight line), bulk PLGA (dotted line) and pBMAD-PLGA double-walled nanospheres (dashed line).

Further studies will include the evaluation of the morphology of the core and shell polymers in cross-sections of the nanospheres by TEM.

Double-walled nanospheres of other polymer combinations have also been fabricated and characterized, indicating the potential for the use of this technique for the fabrication of more sophisticated oral drug delivery systems. Methods of nanoencapsulating proteins within these nanospheres has also been evaluated but a discussion of these methods is not within the scope of this abstract.

CONCLUSION

While the coating of small diameter micro- and nanoparticles has been a technical challenge, sPIN is a quick and easy method for the fabrication of double-walled nanospheres in a single step. By utilizing the phenomenon of polymer phase separation in the environment of a non-solvent, nanospheres with a polyester core for controlled drug release and a bioadhesive polymer shell for uptake enhancement can be prepared by the controlled sequential phase inversion of the two polymers. The small size of these particles combined with the bioadhesive polymer shell makes these particles especially suitable for oral drug delivery applications.

REFERENCES

1. E. Mathiowitz, J.S. Jacob, Y.S. Jong, G.P. Carino, D.E. Chickering, P. Chaturvedi, C.A. Santos, K. Vijayaraghavan, S. Montgomery, M. Bassett, C. Morrell. *Nature*. 1997, 386 (6623): 410-414.
2. J.S. Jacob, E. Mathiowitz, *Carrier-Based Drug Delivery*, Vol. 879, American Chemical Society, 2004.
3. N. Hussain, V. Jaitley, A.T. Florence. *Adv Drug Deliv Rev*. 2001, 50(1-2):107-142.

ACKNOWLEDGMENTS

The pBMAD used in these studies was obtained from Spherics, Inc. The authors would also like to acknowledge Geoff Williams for his assistance with the microscopy and Stacia Furtado for all her help with this project.



A Novel Method for the Fabrication of Double-Walled Nanospheres by Sequential Phase Inversion Nanoencapsulation

Daniel Y. Cho¹, Kenneth M. Estrellas¹, Cheng Tian¹, Joshua J. Reineke², Edith Mathiowitz¹

¹Program in Artificial Organs, Biomaterials and Cellular Technology, Department of Molecular Pharmacology, Physiology and Biotechnology, Brown University, Providence, RI 02912.

²College of Pharmacy and Health Sciences, Wayne State University, Detroit, MI 48201.



Introduction

Phase inversion nanoencapsulation (PIN) was originally developed as a simple and rapid method for encapsulating small molecules, proteins and genes in small diameter polymer micro- and nanospheres [1]. This method relies on the spontaneous formation of nanospheres when a dilute polymer solution is added to the environment of a non-solvent [2]. While the fabrication of nanospheres by this method is a versatile and powerful technique, the small diameters of particles formed by PIN make traditional coating processes to prepare double-walled microspheres a challenge to adapt for nanoparticles.

As a method to minimize the burst effect commonly seen with micro- and nanospheres as well as to modify surface chemistry for the enhancement of particle uptake from the gastrointestinal system, the PIN method was modified to fabricate double-walled nanospheres in a single step.

Methods

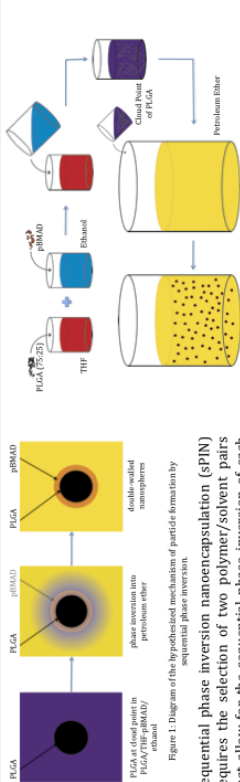


Figure 1. Diagram of the hypothesized mechanism of particle formation by sequential phase inversion.

Sequential phase inversion nanoencapsulation (SPIN) requires the selection of two polymer/solvent pairs that allow for the sequential phase inversion of each polymer. For the core polymer, a solvent must be selected that is also a solvent for the shell polymer while the solvent for the shell polymer must be a non-solvent for the core polymer; all solvents must be miscible with the non-solvent for both polymers.

To fabricate double-walled nanospheres from pBMAD and PLGA, a 2% w/v solution of pBMAD in ethanol was added to a 2% w/v solution of PLGA until the cloud point for PLGA was reached. This solution was then rapidly added to a non-solvent bath with a solvent to non-solvent ratio of 1:75 and stirred for 1 minute to cure the nanospheres. The resulting nanospheres were filtered using a 0.2 µm PTFE filter in a positive pressure filtration column and lyophilized for 24 hours.

The dry nanospheres were characterized using a Perkin Elmer Spectrum One FTIR and a Perkin Elmer DSC7 calorimeter. The nanospheres were suspended in an aqueous 1% SLS/PVP solution for determination of particle size using a Beckman Coulter LS 230 Laser Diffraction Particle Size Analyzer. Microscopy was performed using a Hitachi 2700 SEM equipped with a lanthanum hexaboride gun.

Results

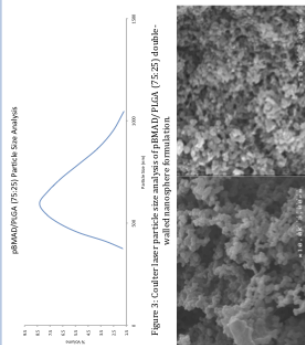


Figure 3. Goulet laser particle size analysis of pBMAD/PLGA (75:25) double-walled nanosphere formation. Inset: SEM image of the nanospheres.

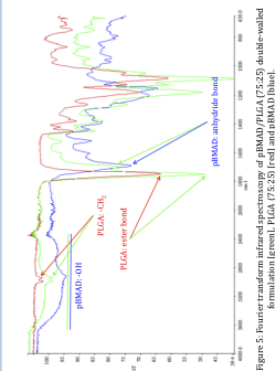


Figure 5. Fourier transform infrared spectra of pBMAD/PLGA (75:25) double-walled nanosphere formation. Inset: SEM image of the nanospheres.

Fourier transform infrared spectra of the double-walled nanospheres showed peaks characteristic of both the core polymer (PLGA) and the shell polymer (pBMAD). Characteristic peaks include the ester bond of PLGA (1750 cm⁻¹) and the anhydride bond of pBMAD (1700 cm⁻¹). However, the FTIR does not provide any information regarding the interaction between the two polymers.

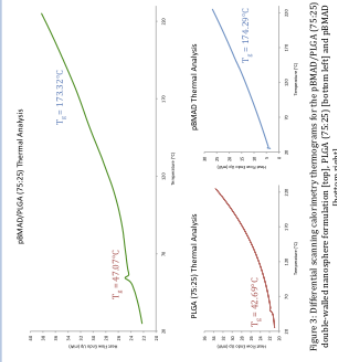


Figure 3. Differential scanning calorimetry thermograms for the pBMAD/PLGA (75:25) double-walled nanosphere formation. Inset: SEM image of the nanospheres.

Differential scanning calorimetry of the double-walled nanosphere formation revealed glass transitions characteristic of the PLGA and pBMAD, indicating phase separation between the two polymers.

Conclusions

While the coating of small diameter micro- and nanoparticles has been a technical challenge, SPIN is a quick and easy method for the fabrication of double-walled nanospheres in a single step. By utilizing the phenomenon of polymer phase separation in the environment of a non-solvent, nanospheres with a polyester core for controlled drug release and a bioadhesive polymer shell for uptake enhancement can be prepared by the controlled sequential phase inversion of the two polymers. The small size of these particles combined with the bioadhesive polymer shell makes these particles especially suitable for oral drug delivery applications.

Further characterization of the nanospheres will include advanced microscopy techniques to allow for the conclusive localization of both polymers. Multiple other formulations have been fabricated using different bioadhesive polymer shells and biodegradable polymer cores. SPIN has been used to encapsulate various therapeutic agents in double-walled nanospheres and they have been evaluated for potential use in oral drug delivery.

Literature Cited

1. E. Mathiowitz, J.S. Jacob, Y.S. Jong, G.P. Carino, D.E. Chickering, P. Chaturvedi, C.A. Santos, K. Vijayaraghavan, S. Montgomery, M. Bassett, C. Morrell. Biologically erodable microspheres as potential oral drug delivery systems. *Nature*. 1997; 386 (6623): 410-414.
2. J.S. Jacob, E. Mathiowitz. A Novel Mechanism for Spontaneous Encapsulation of Active Agents: Phase Inversion Nanoencapsulation. *Carrier-Based Drug Delivery*. Vol. 879. Alexis Mancini and Stacia Furtado for their assistance with this project.

Acknowledgements

The pBMAD used in these studies was obtained from Spherics, Inc. The authors would like to acknowledge Alexis Mancini and Stacia Furtado for their assistance with this project.

Comparing the Effect of Fabrication Parameters on Polymeric Micro- and Nanospheres for Drug Delivery Applications

Cheng Tian*, Shara Azad*, Daniel Y. Cho, Benjamin D. Lux, Taylor C. Peak, Stacia Furtado, Edith Mathiowitz
Department of Molecular Pharmacology, Physiology, and Biotechnology; Program in Artificial Organs, Biomaterials, and Cellular Technology.

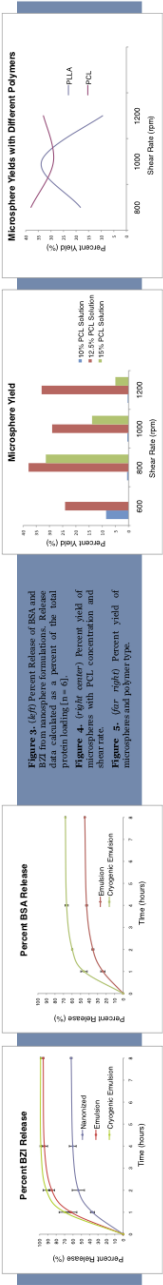
Introduction

Protein-loaded microspheres were fabricated by phase inversion nanosuspension (PIN), a novel method of encapsulating small molecules, proteins and genes in small diameter polymeric micro- and nanospheres that is dependent on the phase inversion of a dilute polymer-solvent solution added rapidly to a non-solvent bath. PIN spontaneously forms discrete uniform sized particles independent on polymer viscosity. Utilizing three different protein loading methods, the interactions between poly(lactide-co-glycolide) (PLGA) and encapsulated proteins were studied. The effect of protein type, concentration, and molecular weight as well as shear rate were evaluated to fabricate biodegradable microspheres for drug-delivery applications.

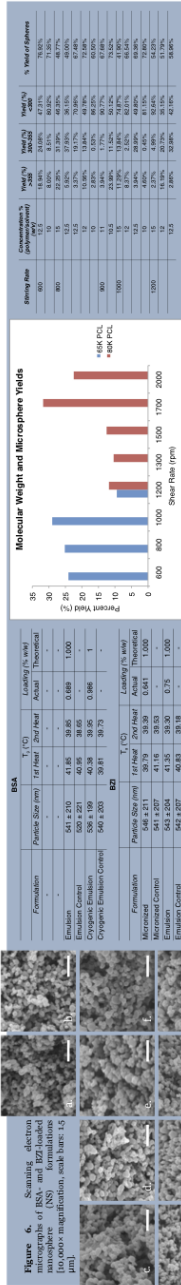
Materials & Methods

Protein-loaded microspheres were fabricated by PIN - 2% w/v polymer solutions of PLGA in methylene chloride were rapidly added to a non-solvent bath of petroleum ether in a 1:75 ratio to obtain discrete uniform microspheres. The effect of protein type, concentration, and molecular weight as well as shear rate were evaluated to fabricate biodegradable microspheres for drug-delivery applications. The microspheres were collected following PIN using a 0.2 µm PTFE filter. The microspheres were characterized using a Beckman Coulter Laser DLS, Particle Size Analyzer, Perkin Elmer DSC, and a Perkin Elmer Spectrum One Fourier Transform Infrared spectrometer. The effect of protein type, concentration, and molecular weight as well as shear rate were evaluated to fabricate biodegradable microspheres for drug-delivery applications. The microspheres were characterized using a Beckman Coulter Laser DLS, Particle Size Analyzer, Perkin Elmer DSC, and a Perkin Elmer Spectrum One Fourier Transform Infrared spectrometer. The effect of protein type, concentration, and molecular weight as well as shear rate were evaluated to fabricate biodegradable microspheres for drug-delivery applications.

Results



Microsphere yields in the target size range of 300 – 855 µm were found to be affected by both the concentration of the polymer solution and the shear rate at which the polymer solution was added to the aqueous phase. The 12.5% PCL solution resulted in consistently high yields at all shear rates. The 10% PCL solution resulted in lower yields at lower shear rates. The 15% PCL solution resulted in higher yields at higher shear rates. The polymer type was also found to affect yield at the target size range of microspheres at various shear rates. For PLGA, there was a higher percent yield of spheres at a shear rate of 1000 rpm, with a 12.5% concentration whereas for PCL, the percent yield remained fairly high (around 30%) for all shear rates at a 12.5% concentration.



Microsphere yields in the target size range of 300 – 855 µm were found to be affected by both the concentration of the polymer solution and the shear rate at which the polymer solution was added to the aqueous phase. The 12.5% PCL solution resulted in consistently high yields at all shear rates. The 10% PCL solution resulted in lower yields at lower shear rates. The 15% PCL solution resulted in higher yields at higher shear rates. The polymer type was also found to affect yield at the target size range of microspheres at various shear rates. For PLGA, there was a higher percent yield of spheres at a shear rate of 1000 rpm, with a 12.5% concentration whereas for PCL, the percent yield remained fairly high (around 30%) for all shear rates at a 12.5% concentration.

Discussion

Three methods of protein encapsulation by PIN were examined in this study. As expected, polymer solution concentration, a direct determinant of nanoparticle size regardless of protein encapsulation method. The results of the release study for BSA show that the nanosuspension method resulted in a less intense burst release, which is suggestive of better encapsulation of the BSA within the nanosphere matrix. Most importantly, the results indicate that protein characteristics play a more important role in affecting release than polymer type. Polymer concentration, molecular weight of the polymer, and shear rate were found to affect the size of microspheres fabricated by solvent evaporation. When targeting the 300 – 355 µm size range for the purpose of drug loading, a 12.5% solution of both PCL and PLGA in methylene chloride at a shear rate of 1000 rpm was found consistently to produce the greatest yield of spheres. This was most likely because of the aforementioned factors play a role in determining the viscosity of the polymer solution. Viscosity directly affects the size of the polymer droplets in the emulsion, which are the precursors to the microspheres, because from the polymer droplets the solvent merely needs to evaporate to form the final microsphere product. Therefore, viscosity remains a critical factor in microsphere sizing when using solvent evaporation as well as PIN.

Daniel Y. Cho, Cheng Tian, Kenneth M. Estrellas, Joshua Reineke, Edith Mathiowitz
 BROWN
 Dept. of Molecular Pharmacology, Physiology and Biotechnology
 Program in Artificial Organs, Biomaterials and Cellular Technology
 Brown University, Providence, RI 02912

Experimental Methods & Results

The use of polymeric nanospheres shows significant promise for the oral delivery of insulin due to their ability to protect the encapsulated protein from the harsh GI environment while allowing for controlled drug release kinetics. By combining data from systematic studies of particle translocation from the small intestinal lumen with the development of novel nanoencapsulation technologies, we hope to create more sophisticated and effective bioadhesive polymeric nanosphere-based drug delivery systems.

bioabsorbable polymer bead from GI environment
 controlled release
 insulin
 bioabsorbable polymer bead

The authors would like to thank A. Peter Morello, Yu-Ting Liu, Shara Azad, Taylor Peak, Stacia Furtado, Roxanne Burrill and Spherics, Inc. for their assistance with this research.

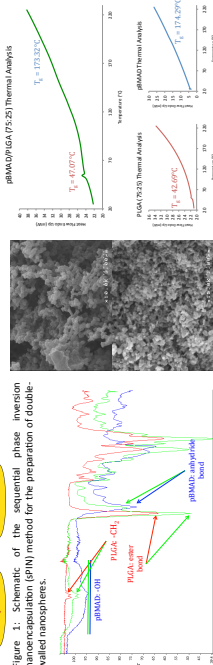


Figure 3: Fourier transform infrared spectroscopy of pBMAO/PLGA (75:25) double-walled formulation [green], PLGA (75:25) double-walled formulation [red] and pBMAO [blue].

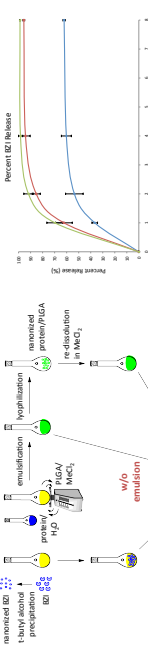
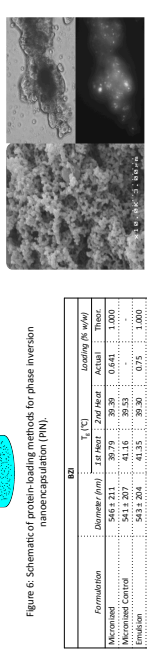


Figure 7. Percent cumulative release of B21 from PLGA 50/50 nanoparticles fabricated by PN. Both emulsification techniques exhibit very rapid and near complete release of encapsulated insulin within 4 hours. These release kinetics are suitable for a rapid release oral insulin dosage form administered prior to meals in postprandial glucose control.



	542 ± 203	40.83	39.18
Emulsion Control	542 ± 203	40.83	-
Cryogenic Emulsion	541 ± 207	40.16	1.099
Cryogenic Emulsion Control	543 ± 205	40.61	39.94

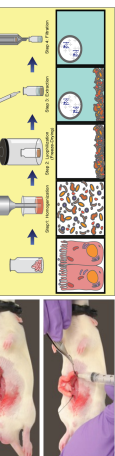
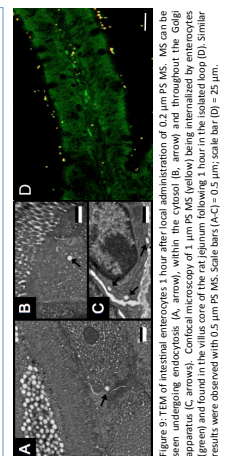


Figure 10: Protocol for isolated top studies. 2.5% w/v nanosphere suspensions were injected into isolated loops of jejunum in male Sprague-Dawley rats (top left). Following a 5 hour incubation to allow for particle uptake and penetration, the loops were removed and processed to extract the polymer (top right) for quantification by gel permeation chromatography (GPC) analyses (left). The elution volume of the polymer was determined by calculating the AUC of the standard vs. time curve and comparing to a standard concentration curve for the polymer (right).

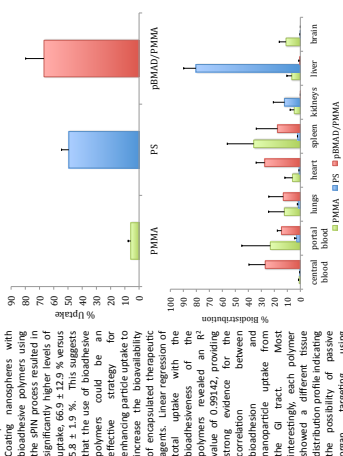


Figure 11: Results of *in vivo* uptake studies of nanosphere formulations.

Fabrication of PEGylated Double-Walled Nanospheres By Solvent Evaporation

Daniel Cho, Lakir Patel and Edith Mathiowitz
Brown University. Providence, RI 02912.

Double-walled microspheres can minimize the initial “burst” effect caused by the release of drug trapped on the surface and also allow for the sequential release of multiple therapeutic agents. Unfortunately, traditional coating methods cannot be adapted to nanospheres (NS) due to their small particle sizes. To overcome this problem, PEGylated double-walled NS consisting of a poly-L-lactic acid (PLLA) core and a polyethylene glycol-poly(lactic-co-glycolic acid) (PEG-PLGA) shell were fabricated using a modified solvent evaporation method. While previous methods have utilized phase separation phenomena to prepare double-walled microspheres in the 300 – 500 μm range, increasing the shear rate allowed for the reduction of particle size to the nanoscale. However, the increased surface area to volume ratio of the nanoparticles results in more rapid solvent evaporation and consequently less time for phase separation. Thus, PEG-PLGA was selected as the shell polymer since the hydrophilicity of the PEG would help to rapidly localize the copolymer to the surface and result in the formation of ‘core-shell-corona’ type NS suitable for drug delivery and bioimaging applications. The resulting NS were determined to be $640\text{ nm} \pm 295\text{ nm}$ in diameter as determined by laser particle sizing and were characterized by Fourier transform infrared spectroscopy (FTIR), differential scanning calorimetry (DSC) and scanning electron microscopy. Work with these NS has included the encapsulation of therapeutic agents and contrast agents for a variety of biomedical applications.

Progress in the development of an oral insulin formulation for diabetes mellitus.

DY Cho[†], C Tian[†], JJ Reineke[‡], E Mathiowitz[†].

[†] Dept. of Molecular Pharmacology, Physiology and Biotechnology, Brown University. Providence, RI;

[‡] College of Pharmacy and Health Sciences, Wayne State University. Detroit, MI.

Since its discovery in 1922, insulin has revolutionized the treatment of diabetes and many advances have been made in the preparation of insulin and its analogues. However, insulin is still predominantly delivered via subcutaneous injection and with the recent failure of inhaled insulin, there exists a clinical need for the development of technologies to allow for the non-invasive administration of insulin – ideally, per os (PO). Unfortunately, the gastrointestinal (GI) tract presents a variety of chemical, physical and enzymatic barriers that prevent the effective oral delivery of insulin for therapeutic purposes.

Our group has approached the challenge of oral drug delivery through the encapsulation of therapeutic proteins within biodegradable polymeric micro- and nanospheres, which serve to protect the cargo from the harsh GI environment and can be designed to enhance translocation from the intestinal lumen into systemic circulation. Using a quantitative *in vivo* assay to study the effects of particle size, surface chemistry and bioadhesion on translocation and biodistribution of polymeric micro- and nanospheres from the small intestines of rats, we have identified specific design criteria for an optimal oral insulin delivery system. Furthermore, we have developed sophisticated novel nanoencapsulation techniques for the preparation of insulin-loaded bioadhesive and biodegradable double-walled nanoparticles with encapsulation efficiencies > 90% capable of delivering up to 8 IU of insulin from a 10 mg dose.

Combining the results of our previous work with insulin encapsulation and delivery, we have fabricated 500 nm poly(butadiene-maleic anhydride-co-DOPA) (pBMAD)-shell, poly(lactic-co-glycolic acid) (PLGA)-core nanoparticles loaded with 10% w/w bovine zinc insulin (BZI). These particles are designed to remain intact in the low pH environment of the stomach followed by the dissolution of the pH-sensitive pBMAD polymer shell into an extremely mucoadhesive matrix in the small intestine. This allows for the enhanced translocation of the insulin-containing PLGA core into the bloodstream for controlled insulin release. Using predictive modeling methods, we have determined that a 25 mg dose of this oral insulin formulation can theoretically deliver 33.6 IU of insulin to the systemic circulation, a 38-fold increase over our previous attempts at insulin delivery and well within the range of therapeutically relevant levels.

Single Step Fabrication of Wet Spun Binary Phase Composite Microfibers

D. Decoteau¹, D. Cho¹, L. Zhang¹, R. Stefani¹, R. Patel¹, and E. Mathiowitz¹

¹Brown University, Providence, RI, 02906, USA
Danya_Decoteau@Brown.edu

ABSTRACT SUMMARY

A single step method was developed to fabricate a multi-layer polymeric microfiber delivery system. Phase separated binary blends of poly(L-lactide) (PLLA) and poly(lactide-co-glycolide) (75:25) (PLGA) were wet spun by phase inversion. Polarized light microscopy, differential scanning calorimetry (DSC), scanning electron microscopy (SEM), and fluorescence microscopy (FM) were used to assess the formation of composite microfibers. The technique developed in this study may be used to fabricate small diameter drug-eluting monofilaments with minimal initial burst and controlled delivery of therapeutics.

INTRODUCTION

The delivery of hydrophilic therapeutics from small diameter wet spun microfibers is often characterized by an initial burst due to drug trapped on the surface during encapsulation [1,2]. This can be detrimental for the delivery of drugs with narrow therapeutic ranges, as local drug concentrations can quickly become toxic. To date, little research has been done to slow the release of therapeutics from wet spun microfiber-based delivery systems [3]. One potential solution to control release kinetics from fibers could be to localize drug to the inner core. Previous research in our lab has shown the ability to create double-walled microspheres with drug localized to inner or outer core for controlled release kinetics [4,5]. Using our knowledge of microencapsulation and phase separation, we have developed a technique for the fabrication of a microfiber-based drug delivery system.

EXPERIMENTAL METHODS

To determine the conditions for phase separation of PLLA and PLGA solutions, 20% w/v 1:1, 1:2, and 1:3 w/w ratios of each of the polymers were made in dichloromethane (DCM). Solutions were mixed for 1 h and left to equilibrate for 24 h. The appearance of two distinct phases was observed by a difference in color between the polymer solutions. Films were then cast from the liquid-liquid phase separated solutions. Once liquid-liquid phase separated solutions were verified using polarized light microscopy, PLLA/PLGA microfibers were wet spun by phase inversion. Polymers were dissolved in DCM at appropriate w/w ratios. The phase separated polymer solution was loaded into a glass syringe fitted with a 22-gauge spinneret and placed in a syringe pump. Since DCM is miscible with petroleum ether, the immersion of the spinneret into the coagulation bath resulted in the continuous precipitation of monofilament microfibers.

DSC was used to determine the existence of two glass transition temperatures to verify phase separation. Microfibers were analyzed for thermal transitions using a Perkin-Elmer model DSC-7 and Perkin-Elmer Intracooler 2 intercooling system. Glass transition temperature, melting temperature, and change in enthalpy were measured from the resulting thermograms.

The cross sectional (C-S) morphology of monofilament microfibers was observed by SEM. Lyophilized microfibers were cross sectioned, mounted on adhesive metal stubs and sputter coated with a layer of gold-palladium using a Emitech K550 sputter coater set at a deposition current of 20-30 A for 4 minutes. Samples were viewed and digital images were taken with a Hitachi S-2700 scanning electron microscope.

A model hydrophilic agent, dextran labeled with fluorescein isothiocyanate (FITC-dextran) was used to visualize polysaccharide localization within microfibers. FITC-dextran was dissolved in Milli Q water, added to 1 g PLLA dissolved in 10 mL DCM and vortexed for 60 s. The primary emulsion was flash frozen in liquid nitrogen and lyophilized for 24 h. Following the cryogenic emulsion process, a 1:1, FITC-dextran/PLLA:PLGA solution was made by dissolving 0.2 ± 0.005 g of each polymer in 2 mL of DCM. The spin dope solution was added to a spinneret and wet spun as previously described.

RESULTS AND DISCUSSION

Figure 1 shows a typical micrograph of a phase separated PLLA:PLGA solution. PLLA spherulites are visible within the continuous PLGA phase.

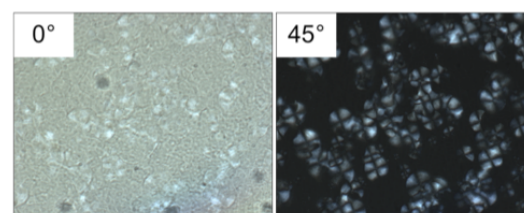


Figure 1. Cross-polarized optical micrographs of a PLLA:PLGA (1:1) phase separated film (magnification, 20x).

While the phase separation of 1:1, 1:2, and 1:3 PLLA:PLGA solutions was observed under cross-polarized optical microscopy, only the 1:1 spin dope solution was suitable for wet spinning. The inherent viscosity and molecular weight of 1:2 and 1:3 phase separated solutions was found to be too low to fabricate continuous monofilaments within the spin bath.

DSC thermograms exhibited the presence of two glass transitions in the first heating scan of PLLA:PLGA (1:1) microfibers (Figure 2). The presence of two glass transitions, each representative of PLLA and PLGA controls, is evidence of a phase separated blend. If the solution were a miscible blend of PLLA and PLGA, the thermal properties would be between those of the two unblended polymers.

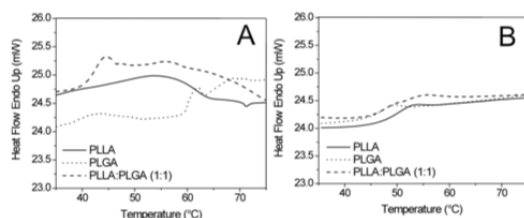


Figure 2. First (A) and second (B) heat DSC thermograms of PLLA, PLGA, and PLLA:PLGA (1:1) microfibers.

No apparent differences in the C-S morphology of binary phase microfibers were observed in comparison to PLLA and PLGA microfiber controls (Figure 3). However, since PLLA and PLGA microfibers display similar C-S morphologies, it is hard to distinguish variations in phase separated blends.

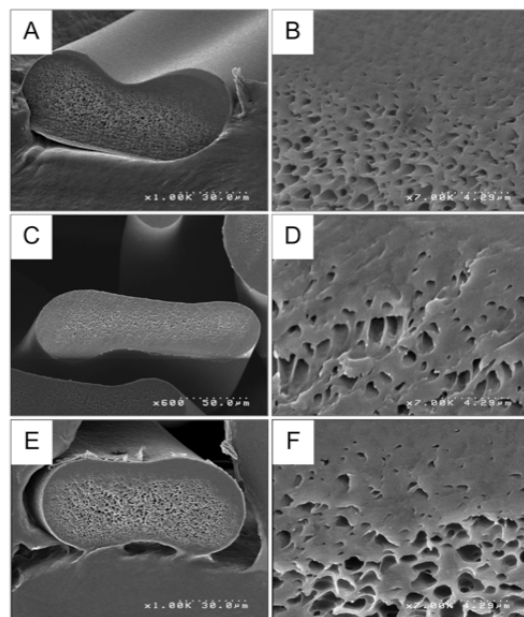


Figure 3. Scanning electron micrographs of PLLA (A, B), PLGA (C, D), and 1:1, PLLA:PLGA (E, F) microfibers. Scale bars, 30 µm (A, E), 50 µm (C), and 4.29 µm (B, D, F), respectively.

FITC-dextran was encapsulated to demonstrate the ability to localize potential therapeutics within binary phase composite microfibers. Localization of FITC-

dextran within the PLLA phase of the composite microfiber was observed with FM (Figure 4). The ability to localize drug to the inner core of composite microfibers could be used to control the spatiotemporal release of therapeutics from small diameter monofilament delivery systems. *In vitro* release studies of potential therapeutics are currently ongoing.

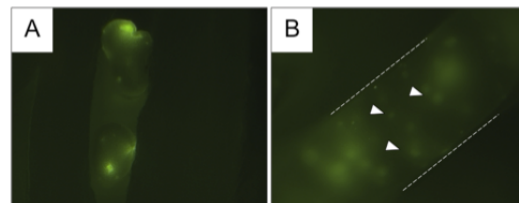


Figure 4. Top (A) and side (B) view fluorescent images of 1:1, 0.3 % w/w FITC-dextran/PLLA:PLGA binary phase composite microfibers (magnification, 20x).

Unlike the previous work done by our lab on the fabrication of double-walled microspheres, the composite microfibers fabricated in this study do not exhibit a distinct core-shell morphology. Partial separation is likely due to differences in fabrication technique. Double-walled microspheres are made by solvent removal and solvent evaporation, processes that can take several hours before microspheres are fully precipitated. Wet spinning by phase inversion is nearly an instantaneous process governed by solvent and nonsolvent miscibility. Hence, phase separated spin dope solutions do not fully separate during microfiber precipitation and PLLA regions are solidified within the continuous PLGA phase. Our wet spinning technique could be further optimized to achieve microfiber composites with core-shell morphology.

CONCLUSION

A single step wet spinning technique was used to fabricate binary phase composite microfibers. Microfibers fabricated from the phase separation of binary blends have the potential for therapeutic use where a high burst may be detrimental. Microfibers from this study may also be braided, woven or knitted into scaffolds for tissue engineering and regenerative medicine applications.

REFERENCES

1. Williamson, M.R., et al. *Biomaterials*. **2004** (25), 5053-5060.
2. Chang, H-I., et al. *Journal of Biomedical Materials Research Part A*. **2007** (84A) 1: 230-237.
3. Schakenraad, J.M. et al. *Biomaterials*. **1988** (9), 116-120.
4. Rahman, N.A., et al. *Journal of Controlled Release*. **2003** (94) 163-175.
5. Pekarek, K.J., et al. *Nature*. **1994** (367), 258-260.

ACKNOWLEDGMENTS

The authors of this study would like to thank the American Heart Association for predoctoral fellowship funding (I.D. 0910113G).



Single Step Fabrication of Wet Spun Binary Phase Composite Microfibers



D. Lavin (Decoteau), D. Cho, L. Zhang, R. Stefani, R. Patel, and E. Mathiowitz

Department of Molecular Pharmacology, Physiology, and Biotechnology, Center for Biomedical Engineering, Brown University, Providence, RI 02912
Email: Danya_Lavin@Brown.edu

Introduction

The delivery of hydrophilic therapeutics from small diameter wet spun microfibers is often characterized by an initial burst due to drug trapped on the surface during encapsulation [1,2]. This can be detrimental for the delivery of drugs with narrow therapeutic ranges, as local drug concentrations can quickly become toxic. To date, little research has been done to slow the release of therapeutics from wet spun microfiber-based delivery systems [3]. One potential solution to control release kinetics from fibers could be to localize drug to the inner core. Previous research in our lab has shown the ability to create double-walled microspheres with drug localized to inner or outer core for controlled release kinetics [4,5]. Using our knowledge of microencapsulation and phase separation, we have developed a technique for the fabrication of a microfiber-based drug delivery system.

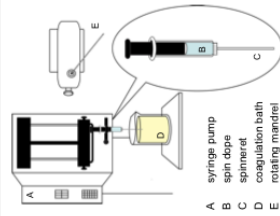
Methods

Spin Dope Preparation

FTC-dextran and bovine serum albumin (BSA) served as model therapeutics for this study. Cryogenic emulsions (CEs) containing protein and polymer were made one day prior to wet spinning. A micro-bicinchoninic acid (micro BCA) assay was used to determine protein loading and release kinetics.

Wet Spinning by Phase-Inversion

Following the CE process, micro/nano-sized droplets are distributed in a continuous phase of polymer. Once the cryogenic emulsion is lyophilized, the spinning solution is reconstituted, the spinning solution is loaded into a syringe and placed in a syringe pump. The introduction of the spinneret into the non-solvent coagulation bath leads to the precipitation of the polymer and subsequent encapsulation of the protein by phase-inversion.



Polymer	η (dL/g)	M _w (g/mol)	Spin Dope Concentration (%)
PLLA	1.4	120,000	20
PLGA (75:25)	0.55-0.75	90,000	20

Intrinsic Viscosity
M_w: Molecular Weight

Results and Discussion

FIGURE 1. Cross-polarized optical micrographs of a PLLA:PLGA (1:1) phase separated film (magnification, 20x).

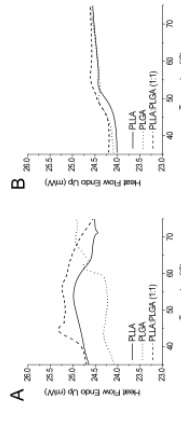
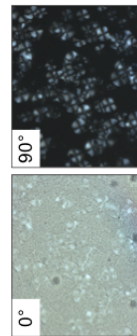


FIGURE 2. First (A) and second (B) heat DSC thermograms of PLLA, PLGA, and PLLA:PLGA (1:1) microfibers. The presence of two glass transitions, each representative of PLLA and PLGA controls, is evidence of a phase separated blend. If the solution were a miscible blend of PLLA and PLGA, the thermal properties would be between those of the two unblended polymers.

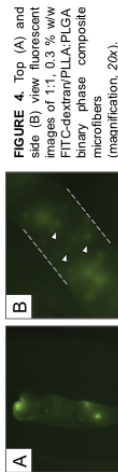


FIGURE 4. Top (A) and side (B) view fluorescent images of 1:1:1:1 PLLA:PLGA:BSA:PLGA (1:1) microfibers. The core-shell morphology is evident in both views.

Conclusions

A single step wet spinning technique was used to fabricate binary phase composite microfibers. Microfibers fabricated from the phase separation of binary blends have the potential for therapeutic use where a high burst may be detrimental.

Unlike the previous work done by our lab on the fabrication of double-walled microspheres, the composite microfibers fabricated in this study do not exhibit a distinct core-shell morphology. Partial separation is likely due to differences in fabrication technique. Double-walled microspheres are made by solvent removal and solvent evaporation, processes that can take several hours before microspheres are fully precipitated. Wet spinning by phase inversion is nearly an instantaneous process governed by solvent and nonsolvent miscibility. Hence, phase separated spin dope solutions do not fully separate during microfiber precipitation and PLLA regions are solidified within the continuous PLGA phase. Our wet spinning technique could be further optimized to achieve microfiber composites with core-shell morphology.

Acknowledgements

The authors of this study would like to thank the American Heart Association for predoctoral fellowship funding (D. Cho, 0910130), as well as Brown University for research support (D. Lavin, 0910130). We also thank the National Science Foundation's Materials Research Science & Engineering Program (DMR-0528551) summer fellowships.

Literature Cited

1. Williamson, M.R., et al. *Biomaterials*, **2004** (25), 5053-5060.
2. Cheng, H.-L., et al. *Journal of Biomedical Research Part A*, **2007** (84A) 1: 230-237.
3. Schmitt, J.M., et al. *Journal of Biomedical Research Part A*, **1998** (63), 165-170.
4. Rahman, N.A., et al. *Journal of Controlled Release*, **2003** (94) 163-175.
5. Pekarek, K.J., et al. *Nature*, **1994** (367), 259-260.

Development of an Artificial Melanosome Utilizing a Lipid-Polymer Hybrid Microparticle

L. Quattrocchi¹, D.Y. Cho¹, K.M. Estrellas¹, N. L. Wicks¹, E. Oancea¹ and E. Mathiowitz¹

¹Brown University, Providence, RI, 02903, USA

Lauren_Quattrocchi@brown.edu

ABSTRACT SUMMARY

Using an auto-oxidation and novel phase inversion micronization method, 3,4-dihydroxy-L-phenylalanine (L-DOPA) was converted into micronized synthetic melanin for encapsulation in a lipid-polymer hybrid microparticle to serve as an artificial organelle for the purpose of *in vitro* keratinocyte uptake and cytocompatibility studies.

INTRODUCTION

Pigmentation is crucial for protection against UV-induced cellular damage¹. Specialized cells called melanocytes are responsible for the production, storage and transfer of pigment present in the skin, hair and eyes of mammals and other vertebrates². Biopolymeric pigments called melanins are synthesized through subsequent oxidation reactions of an amino acid, tyrosine, into one of two forms: eumelanin (black) or pheomelanin (red)^{1,2,3}. Melanin synthesis machinery is found within organelles termed melanosomes, which are specific to skin cells called melanocytes and retinal pigment epithelium in mammals². Structurally, melanosomes are liposomal-like organelles ranging from 0.94 – 1.44 μm in diameter and contain homogenous dark pigment, as observed by light microscopy⁴.

Melanocytes transport melanin through filopodia processes to keratinocytes located in the upper epidermal skin layer⁵. Despite extensive research, the exact method of the pigment translocation and uptake by keratinocytes remains elusive. However, with this work, we hope to provide a novel tool to enable the transport of an artificial melanosomes *in vitro*, in order to elucidate this mechanism. Through development of synthetic melanosomes, we provide a purified, readily modifiable organelle to characterize uptake machinery using cultured keratinocytes.

Modifications of these artificial organelles could include, but are not limited to, fluorescent labeling, incorporating adhesive receptors, or tailoring of melanin loading and particle size. Further refinement can occur in the external coating region of the synthetic melanosome, where we have surrounded the melanin-containing microspheres with lecithin phospholipid, which provides a lipid bilayer-like coating, as proposed in Zhang et al⁶. The polymer-based melanosomes with lecithin shells constitute the basic melanosome structure. This design provides a biocompatible and bioerodible platform for application as a tool in molecular biology, as well as a potential therapeutic agent for the prevention of UV-induced pathologies.

EXPERIMENTAL METHODS

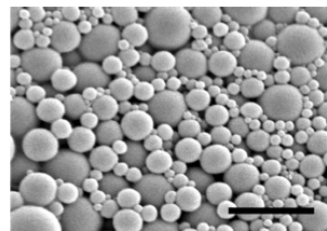
Melanin was synthesized from L-DOPA using a method adapted from the auto-oxidation protocol described by Arnow, E³. Briefly, air was bubbled through a saturated solution of L-DOPA in 0.01 N NaOH to allow for the oxidation of L-DOPA, with a concurrent color change from pink to the jet black of melanin. The excess insoluble L-DOPA sediment was filtered using 0.2 μm and the resulting melanin solution was acidified using HCl to precipitate the melanin. Thereafter, the melanin precipitate was centrifuged and lyophilized. The dried melanin particles were in the ~200 μm range so the pigment was micronized using a novel phase inversion method.

Melanin was encapsulated in PLLA using a S/O/W solvent evaporation method. 20 mg of the micronized melanin was added to a 10 mL of a 2% solution of PLLA in methylene chloride with bath sonication to create the S/O suspension. This mixture was emulsified in 400 mL of a 1% Poly-vinyl alcohol (PVA) solution at 5500rpm for 4.5 hrs with a Silverson homogenizer to allow for solvent evaporation. The resulting microspheres washed and collected by centrifugation before lyophilization. The microspheres were then analyzed using Fourier transform infrared spectroscopy (FTIR), Coulter laser diffraction particle sizing and scanning electron microscopy (SEM). In order to produce the final lipid-polymer hybrid microparticle, lecithin micelles were formed around the PLLA microspheres using a thin layer hydration method prior to use for *in vitro* assays.

RESULTS AND DISCUSSION

The auto-oxidation reaction resulted in the conversion of L-DOPA, a fine white powder, to a black powder with chemical and physical properties similar to commercially available melanin. However, SEM analysis of the melanin revealed particles in the 200 μm range, which far exceeds the desired dimensions of the artificial melanosome. Thus, a phase inversion micronization method was used to reduce particle size so that 90% of the particles were less than 0.904 μm in diameter for encapsulation.

Figure 1: SEM of melanin-PLLA microspheres (scale bar is 5 μm)



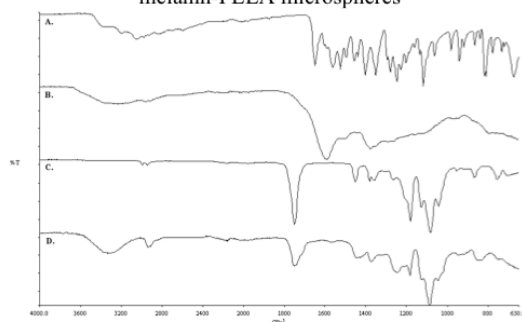
Upon visual inspection, melanin-containing microspheres were a darker color than the control PLLA formulation, indicating the presence of the pigment within the synthetic melanosomes. Laser diffraction particle size analysis revealed the average particle size for control and melanin-loaded PLLA microspheres to be $2.327 \pm 1.077 \mu\text{m}$ and $1.917 \pm 0.844 \mu\text{m}$, within the range of native melanosomes (Table 1); still, these microparticles are slightly larger than the average diameter of the naturally occurring melanosomes observed in dark skin, Asian skin and Caucasian skin⁶. The SEM of melanin-PLLA spheres confirmed the results observed by laser diffraction (Figure 1). Thus, further optimization of the solvent evaporation protocol will be performed to reduce particle size to $\sim 1 \mu\text{m}$.

Table 1: Particle Size Analysis

Formulation	Mean Diameter (μm)
PLLA MS	2.327 ± 1.077
Melanin-PLLA MS	1.917 ± 0.844

FTIR was used to analyze the spectra of L-DOPA, melanin, PLLA and melanin-loaded PLLA microspheres. As can be seen in Figure 1A and B, the auto-oxidation process results in a change in the FTIR spectra with the conversion of DOPA to melanin; comparison of the spectra of the synthetic melanin to commercially available eumelanin confirmed formation of the desired product (data not shown). FTIR comparison of control and melanin-loaded microspheres revealed the characteristic carbonyl peak of PLLA in both formulations; however, the spectrum of the melanin-loaded spheres reveals a broad alcohol stretch at $\sim 3200 \text{ cm}^{-1}$ that is also observed in the melanin spectra. In addition, there are changes in the peaks seen in the $1500 - 650 \text{ cm}^{-1}$ range, demonstrating incorporation of melanin into the polymeric microspheres (Figure 2).

Figure 2: FTIR spectra of (A.) L-DOPA, (B.) synthetic melanin, (C.) control PLLA microspheres and (D.) melanin-PLLA microspheres



Further analysis of these formulations is underway and includes the use of differential scanning calorimetry (DSC) to determine the thermal properties of the artificial

melanosomes, particularly the crystallinity of PLLA, as well as to gain insight into the distribution of the melanin within the polymeric matrix, which will be confirmed by transmission electron microscopy (TEM). *In vitro* cyto-compatibility and degradation studies of the synthetic melanosomes will be performed prior to their eventual use in the elucidation of melanosome uptake pathways and melanin signal transduction mechanisms. Furthermore, the use of modified phospholipids will allow for conjugation of ligands and other targeting molecules, which will be used to evaluate and enhance the effect of these particles *in vitro*.

CONCLUSION

An artificial organelle containing melanin was created using polymer-based encapsulation techniques, along with a lecithin coating to mimic native melanosomal architecture, in order to study pigment uptake by keratinocytes. Preliminary results have shown that this approach to engineer imitation cellular structures is feasible by hybridizing manmade polymers with natural elements (i.e. melanin). Through further optimization of the micronized melanin-loaded PLLA spheres, we hope to achieve a bioactive synthetic cellular organelle that could be applied in both laboratory and clinical setting.

Further investigation of these synthetic melanosome-like particles includes refinement of the artificial organelle to be more representative of naturally occurring melanosomes. The physiologically relevant composition of these melanin-encapsulating microspheres may provide some advantages over alternative uptake agents, such as fluorescent latex beads⁷. Ongoing *in vitro* keratinocyte uptake studies will assess the viability of this approach against current methods.

REFERENCES

1. H.Y. Park, M. Kosmadaki, M. Yaar, B.A. Gilchrist. *Cell. Mol. Life Sci.* 66 (2009) 1493-1506.
2. M. Schiaffino. *Inter. Jour. of Biochem. & Cell Bio.* 42 (2010) 1094-1104.
3. E. Arnow. *The Preparation of DOPA-Melanin.* *Science.* 87 (1938) 308.
4. H.Y. Thong, Jee SH, Sun CC, Boissy RE. *British Jour. of Derm.* 149 (2003) 498-505.
5. S. Singh, R. Kurfurst, C. Nizard, S. Schnebert, E. Perrier, D. Tobin. *FASEB Journal.* 24 (2010) 3756-3769.
6. L. Zhang, J. Chan, F. Gu, J. Rhee, A. Wang, A.Rodovic-Moreno, F. Alexis, R. Langer, O. Farokhzad. *ACS Nano.* 2 (2008) 1696-1702.
7. F. Belleudi, V. Purpura, C. Scrofani, F. Persechino, L. Leone, M. Torrisi. *FASEB Journal.* 25 (2011) 1-13.

ACKNOWLEDGMENTS

The authors would like to thank Stacia Furtado, Danya Decoteau and Linda Zhang for their support.



Development of an Artificial Melanosome by Engineering a Lipid-polymer Hybrid Microparticle

L. E. Quattrochi, D. Y. Cho, K. M. Estrellas, N. Wicks, E. Oancea, and E. Mathiowitz

Department of Molecular Pharmacology, Physiology and Biotechnology (MPPB), Brown University, Providence, RI, USA

Email: Lauren_Quattrochi@Brown.edu
Daniel_Cho@Brown.edu

Background

Pigmentation has proven to be crucial in protection against UV-induced cellular damage¹. Specialized cells, called melanocytes, are responsible for its production, storage and transfer of pigment present in the skin, hair and eyes of mammals and some vertebrates². Under normally occurring conditions, biopolymeric pigments called melanins are synthesized through sequential oxidation reactions of an amino acid, tyrosine, into one of two forms, eumelanin (black) or pheomelanin (red)^{3,4}. The synthesis machinery for these melanin pigments is found within organelles, called melanosomes, which are specific to melanocytes and certain pigment epithelial cells in mammals⁵. Structurally, melanosomes are liposomal-like, organelles ranging from 0.34 – 1.44 μm in diameter and containing homogeneous dark pigment, as observed by light microscopy⁶.

Melanocytes transport their products through filopodia processes to keratinocytes located in the external skin layer. Despite extensive research, the exact method of the pigment translation and uptake by skin cells remains elusive. However, with this work, we hope to provide a novel tool to enable the transport of an artificial melanosome in *in vitro* setting, in order to elucidate this mechanism. Additionally, if these spheres prove viable, steps could be taken to deliver melanin topically to UV-exposed human skin to provide another avenue to ameliorate the risk of skin cancer, especially in patients prone to melanin deficiency or who display improper melanin synthesis machinery.

Objective:

Through development of synthetic melanosomes, we provide a biodegradable, cytocompatible, purified and readily modifiable organelle to characterize uptake mechanisms in epithelial cells, as well as, possibly develop a novel melanin delivery approach in order to minimize the damaging effects of UV on human skin cells.

Methods

All experiments were performed in accordance with National Institutes of Health and institutional guidelines.

Melanin synthesis:

MeLanin was synthesized from a natural precursor, 5,6-dihydroxy-L-tryptophan (L-DOPA), using a method adapted from the size-oxidation protocol described by Arnow, P. Purdy, et al. (1969). Briefly, L-DOPA was dissolved in 100 mL of 0.1 M NaOH to allow for the oxidation of L-DOPA with a saturated solution of K₂S₂O₈ to form MeLanin. The excess oxidized L-DOPA sediment was filtered using 0.2 μ m filter and the resulting melanin solution was acidified using 6M HCl to precipitate the melanin. Thereafter, the melanin precipitate was centrifuged and washed with distilled water. The melanin was then lyophilized. Melanin synthesis was confirmed by comparison to commercially available melanin using UV-VIS x-ray diffraction and Fourier transform infrared spectroscopy (FTIR).

Melanin Micronization:

The dried melain particles were in the ~ 200 μm range so the pigment was micronized using a novel phase inversion method. Lab-created melain was dissolved in $\text{LiN}(\text{OH})_2$ to a concentration of $\sim 0.01\%$. Initially, deionized deionized water was then quickly added to a stirring beaker containing 1 liter of isopropyl alcohol (IPA) to yield a ratio of 1:1. The melain dispersed into microtonic-sized particles in the IPA nearly instantly. Melain particles were then centrifuged and dried in a vacuum oven at 50°C for 24 h. The dried melain particles were then dispersed into a non-polar solvent (hexane) and removed residual solvent and provided dried melain on the size range of naturally occurring melain pigments.

Solvent Evaporation

Solvent Evaporation. Melanin was encapsulated in a biodegradable, bio-compatible polymer, poly-l-lactic acid (PLLA), using a S/O/SO solvent evaporation method. 20 mg of the microzonized melanin was added to a 10 mL of a 2% solution of PLLA in methylene chloride with bath sonication to create the S/O suspension. This mixture was emulsified in 400 mL of a 1% poly-vinyl alcohol (PVA) solution at 22.5 K for 4.5 hrs with a Virtis mixer to allow for solvent evaporation. The resulting microcapsules were washed and centrifuged prior to overnight lyophilization. Thereafter, microcapsules were analyzed using FTIR, dynamic light scattering (DLS) and scanning electron microscopy (SEM).

Lecithin Coating

To create a lipid layer around the PLLA microsphere, as to mimic liposome-like microsphere, lecithin micelles were formed around the PLLA microsphere using a thin layer hydration method just prior to use for *in vitro* assays. 30 mg of lecithin was dissolved in 1 mL of chloroform and the solvent was evaporated overnight to form a thin film of lecithin on a glass dish bottom. Approximately 10 μ l of *in vitro* suspensions, a known concentration of melamine-containing polymerized spheres were added to 1 mL of phosphate buffer (pH 9.5). This suspension was added to the dried lecithin film and glass sonicated for 10 minutes (to ensure sufficient interaction had occurred). The suspension was centrifuged and the supernatant was removed. The pellet of spheres was resuspended in 100 μ l of phosphate buffer. The spheres were then added to centrifuge tubes and the tubes were sonicated for 10 minutes. The spheres were then removed to glass slides. Cultures with spheres were analyzed using differential interference contrast (DIC) and confocal microscopy.

Results

1 Synthetic Melanin is Comparable to Commercial Melanin

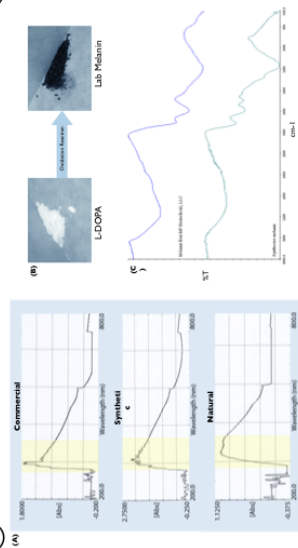


Figure 1. Lab-created melanin is comparable to commercially available melanin. (A) UV-Vis spectral analysis of melanin from three sources: (top) Commercial from NP Biomaterials, LLC; (middle) Lab synthesized melanin; (bottom) Lab synthesized melanin after 2 days of aging. (B) FTIR spectra of the same three melanin samples. The peak indicates the expected maximal wavelength absorbance range for melanin (i.e., 260–400 nm), where the peak absorbance of human melanin pigment occurs at 315 nm. (C) Visual observation of the starting material, a melanin precursor called L-DOPA, to synthetic melanin after 2 days of auto-oxidizing. (D) FTIR spectral comparison of commercial melanin (top) to the lab-created melanin (bottom). While FTIR is not an ideal technique to assess melanin, due to melanin's light absorbent profile, this analysis reveals that the two are not dissimilar, very similar.

2 Novel Phase Inversion Micronization Protocol of Melanin

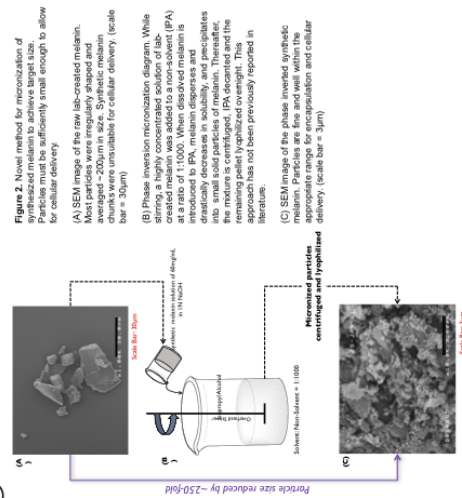


Figure 2. Novel method for micronization of synthesized melanin to achieve target size. Particles must be sufficiently small enough to allow for cellular delivery.

(A) SEM image of the raw lab-created melanin. Most particles were irregularly shaped and averaged ~200µm in size. Synthetic melanin chunks were unsuitable for cellular delivery. (scale bar = 30µm)

(B) Phase inversion micronization diagram. While stirring, a highly concentrated solution of lab-created melanin was added to a non-solvent (IPA) at a ratio of 1:1000. When dissolved melanin is introduced to IPA, melanin disperses and drastically decreases in solubility, and precipitates into small solid particles of melanin. Thereafter, the mixture is centrifuged, IPA decanted and the remaining pellet lyophilized overnight. This approach has not been previously reported in literature.

(C) SEM image of the phase inverted synthetic melanin. Particles are fine and well within the appropriate range for encapsulation and cellular delivery. (scale bar = 3 μ m)

Summary and Conclusions

A novel phase inversion protocol has been described. Polymer-lipid hybrid microspheres containing melanin mimetic natural melanosomes.

Artificial melanosomes applied to human melanocytes in culture are cytocompatible,

References

1. H-Y Park, M. Kosmadaki, M-Yan BA. *Glycine Toxicol Cell Mol Life Sci* 66 (2009) 1493-1506.
2. M. Schaffino *Inter Jour. of Biochem. & Cell Bio* 42 (2010) 1094-1104.
3. E. Annow. *The Preparation of DOPA-Melanin*. *Science* 87 (1938) 308.
4. H-Y Thong, Jee SH, Sun CC, Boissy RE. *British Jour of Derm.* 149 (2003) 498-505.
5. S. Singh, R. Kurlantz, C. Nizard, S. Schnebert. *E-Pernic D-Tohn J-FASEB Journal* 24 (2010) 3756-3769.

J. Quatrecchi et al., Poster to be presented at CIRS 2011 in Baltimore, Maryland

Acknowledgements: Eric Chan, Zachary Pfeiffer, Stacia Furtado, Anita Zimmerman, Geoff Williams

Bioadhesive Double-Walled Nanospheres as an Oral Insulin Delivery Platform

D.Y. Cho¹, C. Tian¹, K.M Estrellas¹, Y.T. Liu¹, J.J. Reineke², S. Furtado¹, and E. Mathiowitz¹

¹Brown University, Providence, RI, 02903, USA; ²Wayne State University, Detroit, MI, 48201, USA.
daniel_cho@brown.edu

ABSTRACT SUMMARY

We present the analysis of our quantitative uptake studies of particle translocation from the small intestine and studies of insulin nanoencapsulation that have guided the development of a potential oral drug delivery platform based on bioadhesive double-walled nanospheres (NS). Using predictive methods, we have determined the theoretical efficacy of our lead formulation, which has the potential to deliver up to 33.54 IU of insulin from a dose of 25 mg of NS.

INTRODUCTION

While the oral administration of therapeutic agents is desired due to increased patient compliance and comfort, the gastrointestinal (GI) tract presents a variety of physical, chemical and enzymatic barriers that prevent the oral delivery of proteins. Thus, a significant amount of research in the field of controlled drug delivery has been focused on the development of technologies that allow for protein administration per os (PO).

Prior work in our lab has focused on the use of bioadhesive polymeric micro- and nanoparticles as a carrier for therapeutics due to their ability to protect encapsulated proteins from the harsh GI environment while enhancing their translocation from the intestinal lumen to the bloodstream. By combining basic research into particle uptake from the small intestine with the development of novel encapsulation technologies, we have developed a more sophisticated and effective oral drug delivery platform using insulin as a model protein.

EXPERIMENTAL METHODS

To quantify the uptake of micro- and nanoparticles from the small intestine, an isolated loop procedure was performed on 6 cm intestinal sections of jejunum as previously described in [1] using male Sprague Dawley rats weighing 200-250 g with a 1 mL (2.5% w/v) dosage of particles in suspension. Following a 5-hour incubation period, tissue samples were collected and processed to extract the polymer for quantification by gel permeation chromatography (GPC). To assess the effect of particle size on uptake, polystyrene (PS) spheres of 500 nm, 1 μ m and 2 μ m were evaluated; to determine the effect of bioadhesion on uptake, 500 nm poly(methylmethacrylate) (PMMA), PS and poly(butadiene-maleic anhydride-co-DOPA)-shell/PMMA-core (pBMAD/PMMA) double-walled NS were evaluated *in vivo*. The bioadhesion of the bulk polymers was quantified *in vitro* using polymer-coated probes on a texture analyzer set up with fresh jejunal tissue to determine fracture strength and tensile work while the bioadhesion of each nanoparticle

formulation was evaluated using an everted sac bioadhesion assay.

Bovine zinc insulin (BZI), prepared using a nanoprecipitation method, was encapsulated as a nanonized solid in poly(lactide-co-glycolide) 50:50 (PLGA, RG502) by phase inversion nanoencapsulation (PIN). The nanonized BZI was suspended in a 2% w/v solution of RG502 in methylene chloride with the appropriate w/w ratio for desired loadings of 0%, 1%, 5% and 10%. The polymer suspension was then rapidly added to a non-solvent bath of petroleum ether with a solvent to non-solvent ratio of 1:75 resulting in the spontaneous formation of NS, which were collected using a 0.2 μ m PTFE filter and lyophilized.

The insulin-loaded NS were characterized using Coulter laser particle sizing, Fourier transform infrared spectroscopy (FTIR), differential scanning calorimetry (DSC), and scanning electron microscopy (SEM). Encapsulation efficiencies and release kinetics were quantified using a micro-bicinchoninic assay (μ BCA).

Insulin-loaded pBMAD-shell, PLGA-core NS were fabricated in a single step by sequential phase inversion nanoencapsulation (sPIN) using a pBMAD/ethanol and PLGA/THF mixed solvent system with petroleum ether as the non-solvent. The resulting NS were filtered, lyophilized and characterized as described above.

RESULTS AND DISCUSSION

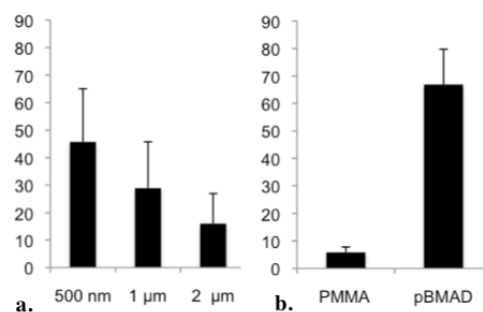


Figure 1 a. Total uptake of 500 nm, 1 μ m and 2 μ m nanospheres from the jejunum; b. total uptake of 500 nm NS from the jejunum (n = 4).

Studies of particle uptake from the small intestine provided insight for the development of design criteria for an effective oral protein delivery system based on bioadhesive and biodegradable polymeric micro- and nanoparticles. Although the final formulations will be based on bioerodible polymers to allow for the controlled release of therapeutics, non-erodible polymers were used in our uptake studies to allow for accurate quantification.

As can be seen in figure 1a, there is an inverse relationship between particle size and total uptake in the small intestine. The highest uptake was seen with 500 nm PS NS ($45.78 \pm 19.31\%$ of total dose) compared to the 1 μm ($28.90 \pm 16.89\%$) and 2 μm ($16.03 \pm 10.91\%$) PS MS. Linear regression of the uptake data revealed a strong inverse linear correlation between particle size and total uptake ($R^2 = 0.948$). Although we expect this relationship to hold true for particles smaller than 500 nm, this would come at the expense of total protein loading into each NS, which would ultimately compromise total drug delivery. Thus, a particle size of 500 nm was selected to allow for sufficient uptake and protein loading.

Tensile testing and everted sac analysis of PMMA, PS and pBMAD on small intestinal tissue revealed the PMMA to be least bioadhesive and the pBMAD to be most bioadhesive. Uptake studies of 500 nm particles of each polymer resulted in higher levels of uptake for pBMAD/PMMA spheres ($66.86 \pm 12.91\%$ of total dose) than PMMA ($5.84 \pm 1.94\%$) (figure 1b). Most interestingly, the addition of a bioadhesive pBMAD coating on PMMA particles using sPIN resulted in an enhancement of uptake of PMMA particles by 12.5-fold, providing higher levels of particle translocation than previously described in scientific literature and suggesting that bioadhesion is an effective strategy for enhancing particle uptake for oral drug delivery applications.

Using PIN, nanonized BZI was encapsulated in PLGA to evaluate protein loading and release from 500 nm spheres. NS with insulin loadings of up to 10% w/w were successfully prepared yielding encapsulation efficiencies greater than 91.33%. Release studies revealed that an increase in total protein loading modulates the release of insulin from the PLGA spheres and the encapsulation of BZI as a nanonized solid reduces the initial burst. Most notable was that the highest loaded 10% formulation resulted in a lower initial release of insulin than the 5% formulation as well as sustained release kinetics with nearly linear delivery of 8.14 IU ($370.23 \mu\text{g}$) of insulin over 4 days.

Combining the results of the studies described above with previous data from our lab described by Furtado et al (table 1), it is interesting to evaluate the performance of each delivery system. The system developed by Furtado et al, relies on bioadhesive microspheres of pFA:SA, a rapidly degrading polyanhydride, with a suboptimal size distribution with 90% of the spheres less than 5.9 μm with a 1% loading of insulin. When administered orally, assuming an average diameter of 2 μm , our data suggests that 16.03% of the dose is translocated into the circulation resulting in a delivery of 0.88 IU ($40.08 \mu\text{g}$). However, the actual bioavailability was only in the range of 5% due to the rapid degradation of the pFA:SA causing the release of insulin throughout the GI tract leading to loss of insulin to the stomach and intestine. In contrast, we have optimized our lead formulation, a 10% w/w BZI-loaded pBMAD/PLGA 500 nm double-walled NS fabricated by sPIN and characterized as described above (data not shown), to maximize insulin delivery. By fabricating

particles of 500 nm with the extremely bioadhesive pBMAD-shell, we can predict the uptake of 66.86% of the administered dose. Furthermore, the unique bioadhesive properties of pBMAD allow for it to function as a protective coating on each nanosphere to prevent the premature release of insulin in the harsh environment of the stomach followed by activation into a bioadhesive matrix in the small intestine to enhance the translocation of the insulin-containing core into the circulation while minimizing insulin loss. The result is a theoretical insulin delivery of 33.6 IU ($1537.9 \mu\text{g}$) from a 25 mg dose (table 1), a 38-fold improvement over our previous attempts and well within the therapeutically relevant range required for the management of diabetes. If a pBMAD/PMMA formulation with 1% or 5% insulin loading is administered, the theoretical insulin delivery is 4.78 IU ($217.36 \mu\text{g}$) or 18.68 IU ($850.05 \mu\text{g}$), respectively (data not shown), indicating the ability to tailor the amount of insulin delivered as well as release kinetics based on total protein loading. An additional benefit to our system is the potential to further increase drug loading to increase total insulin delivery per mass of polymer.

In vivo studies using diabetic rats are currently in progress.

	Furtado (2007)	Cho (2011)
Formulation	pFA:SA	pBMAD/PLGA
Theoretical Loading	2% w/w	10% w/w/
Actual Loading	1% w/w	9.13%
Particle Size	90% < 5.9 μm	557 nm
% Uptake	16.03%	66.86%
Total Uptake (mg)	4.01	16.72
Insulin Delivery (μg)	40.08	1526.08
Insulin Delivery (IU)	0.88	33.54

Table 1 Comparison of theoretical PO insulin delivery from 25 mg dose.

CONCLUSION

We have developed an oral drug delivery platform based on bioadhesive and biodegradable double-walled NS for the oral delivery of therapeutic peptides. By using data from studies of particle uptake and protein nanoencapsulation, we have been able to optimize our platform for the delivery of therapeutically relevant levels of insulin using small doses of NS.

REFERENCES

1. Florence AT, Hillery AM, Hussain N, Jani PU. J Drug Target, 3: 65-70.
2. Furtado S, Abramson, D, Burriel R, Olivier G, Gourd C, Bubbers E, Mathiowitz E. Int J Pharm, 347: 149-155.

ACKNOWLEDGMENTS

The authors would like to thank Lakir Patel, Shara Azad and Taylor Peak for their assistance.

***In Vitro* Assessment of Lipid-Polymer Hybrid Melanosomes in Human Neonatal Epidermal Melanocytes**

L. Quattrocchi, D.Y. Cho, N. Chavez, Z. Pfeiffer, E. Oancea and E. Mathiowitz

Department of Molecular Pharmacology, Physiology and Biotechnology.

Brown University. Providence, RI 02912. USA.

Introduction: Ultraviolet radiation (UV) is a leading cause of skin cancer in humans. One of the most prominent and notable mechanisms that the human body has developed to combat UV-induced DNA damage is pigmentation, commonly referred to as the “tanning response.” When UV light is sensed, skin cells upregulate their production of pigment to prevent DNA damage. Skin color is determined by specialized neural-crest derived cells, named melanocytes, which synthesize, store and transport biopolymeric pigment called melanin¹. Melanin is transferred in liposome-like organelles termed melanosomes, from melanocytes to keratinocytes, in order to play the crucial role of absorbing harmful UV light¹. In this research, a novel approach to protect cells against the adverse affects of UV has been explored. Melanin-containing lipid-polymer microspheres were engineered for use as artificial melanosomes to evaluate their potential to diminish UV-evoked damage on melanocytes.

Materials and Methods: L-DOPA was converted to melanin through auto-oxidation², and subsequently analyzed by Fourier-Transform Infrared (FTIR), X-ray crystallography and UV-VIS. It was then nanonized using a novel phase inversion method. The nanonized melanin particles were encapsulated in poly-L-lactic acid (PLLA) in a S/O/W solvent evaporation technique. The resulting microspheres were analyzed by FTIR, Dynamic Light Scattering (DLS), scanning electron microscopy (SEM) and transmission electron microscopy (TEM). The melanin-containing polymer spheres were coated with lecithin using thin layer hydration to approximate the phospholipid layer on natural melanosomes. In parallel, a second batch of microspheres was created using a fluorescent hydrophobic dye, tetramethyl rhodamine isothiocyanate (TRITC), in place of melanin, to facilitate visualization. The spheres were incubated with melanocytes in several time course experiments to assess cytocompatibility and uptake by melanocytes.

Results and Discussion: The synthesized melanin had chemical and physical properties consistent with commercially available melanin. SEM revealed that lab-made melanin particles were $\geq 200\mu\text{m}$ wide, too large for encapsulation into microspheres; thus, a novel phase inversion nanonization technique was used to reduce the particle size ~ 250 -fold. A S/O/W solvent evaporation method was employed to encapsulate the nano-melanin within PLLA, resulting in microspheres with an average particle size below $1\mu\text{m}$ (as determined by DLS). SEM and TEM analysis confirmed that the microsphere diameters were well within the size range of naturally occurring melanosomes (Fig. 1A). The efficiency of encapsulation was determined through UV-VIS analysis, confirming the presence of melanin within the polymer-based spheres. After characterization, melanin-containing or TRITC-encapsulated particles were evaluated with human embryonic melanocytes (HEMs). Sphere dosage was refined and cells incubated with $100\mu\text{g/mL}$ of lipid-polymer hybrid spheres had impressive cytocompatibility and survival, as visualized by differential interference contrast (DIC) microscopy. Preliminary data using fluorescent microscopy suggest that melanocytes may occasionally uptake microspheres within their environment (Fig. 1B), but further studies will be performed to determine if the HEMs could utilize these melanin-containing particles to reduce UV-damage and whether these artificial melanosomes can be delivered to melanocytes.

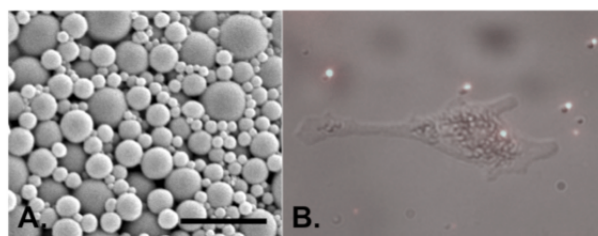


Figure 0 A. SEM micrograph of PLLA-Melanin microspheres, scale bar = $5\mu\text{m}$; B. HEM cells incubated with PLLA-TRITC artificial melanosomes for 48 hours, 40X magnification.

Conclusions: Future studies may include the addition of growth factors to the melanocyte media, which have previously shown to increase uptake³. If melanocytes are shown to engulf melanin-containing spheres in their environment, UV could be applied to test melanocyte durability to assess whether artificial organelles, like those described here, could be used to reduce the incidence of skin cancer.

- References:**
1. C. Wasmeier et al. *Jour. Cell Science*, 2008, 121, 3995-3999.
 2. E. Arnow et al. *Science*, 1938, 87, 308.
 3. F. Belleudi et al. *FASEB Journal*, 2011, 25, 1-13.



In Vitro Assessment of Lipid-Polymer Hybrid Melanosomes in Human Neonatal Epidermal Melanocytes

L.E. Quattrocchi, D.Y. Cho, N. Chavez, Z. Pfeiffer, E. Oancea & E. Mathiowitz

Department of Molecular Pharmacology, Physiology & Biotechnology, Brown University, Providence, RI, USA

Email: Lquattrocchi@brown.edu
Dycho@brown.edu

Background

Ultraviolet radiation (UV) is one of the leading causes of skin cancer in humans. When UV light is sensed by skin cells, the cells upregulate their production of a biopolymeric pigment to protect the potential for future DNA damage; this protection mechanism is commonly referred to as the "tanning response."

The cells responsible for pigmentation are specialized neural-crest derived skin cells called melanocytes. Melanocytes produce melanin, a natural skin pigment critical for tanning called melanin. Melanin is transferred in melanosomes, liposome-like organelles, from melanocytes to keratinocytes through an undefined pathway. Once inside the keratinocytes, the melanosomes predominantly place themselves at the perinuclear area (directly above the nucleus), thereby shielding the DNA from future UV-induced DNA damage. UV exposure, the production, packaging and transfer of melanin from melanocytes confers the skin's ability to absorb harmful UV light¹. This research attempts to imitate the most prominent and notable biological mechanism that the human body has developed to combat UV-induced DNA damage: pigmentation. **Objective:** A novel approach to mimic the beneficial effects of melanosomes was explored by designing cell-deliverable pigmented polymer-based microspheres. These microspheres were designed to mimic the morphology and cellular uptake capabilities of melanosomes on these engineered particles and 2. optimize the study design(s) that could confirm cellular uptake.

Methods

Melanin & Microspheres Synthesis:

L-DOPA was converted to melanin through auto-oxidation² and subsequently analyzed by Fourier-Transform Infrared (FTIR), X-ray crystallography and UV-VIS. The size of the resulting particles was reduced using a novel phase inversion method. The micronized melanin particles were encapsulated in poly-L-lactide (PLLA) microspheres. The microspheres were analyzed by Dynamic Light Scattering (DLS) and scanning electron microscopy (SEM). The melanin-containing polymer spheres were coated with lecithin using thin layer hydration to approximate the phospholipid layer on natural melanosomes.

In Vitro Experiments:

The spheres were incubated with human neonatal epidermal melanocytes to determine if the spheres were taken up by the cells. The uptake of the spheres was visualized by skin cells. Initial experiments involved incubating healthy melanocytes with the first generation of spheres at various concentrations. After the optimal sphere concentration was obtained, melanin-containing microspheres were assessed in vitro in various trials involving fluorescent markers, such as phalloidin, FITC, and DAPI. The spheres were analyzed by Atomic Force Microscopy (AFM), Interference Contrast (DIC), Total Internal Reflection Fluorescent (TIRF) or fluorescence microscopy.

I. Melanocytes were incubated with 100µg/mL of melanin-containing microspheres for 24hrs and thereafter, fixed in 4% paraformaldehyde (PFA). The cellular actin was stained using phalloidin (in order to fluorescently label the entire outline of the cell). Cells were sufficiently rinsed with PBS during the entire experiment, ensuring surplus spheres were removed before observation.

II. Melanocytes were incubated with 100µg/mL of fluorescently-labeled spheres over the course of 24hrs. Sphere membranes were marked for ease of visualization using DAPI. In short, DAPI was added and allowed to attach to the lipid coating of the spheres, illuminating the spheres in contrast to the cells. Spheres were rinsed with PBS to remove residual dye before being placed in culture. After 24hrs, the cells were fixed in 4% PFA and observed using confocal microscopy observations, those microspheres were rinsed away with new cell media.

III. pAEM marked melanocytes were incubated with 100µg/mL of fluorescent melanin-polymer spheres for 24 hrs. Using the same paradigm as in experiment (i) above, DAPI labeled microspheres were assessed for possible uptake. Cells were fixed in 4% PFA and observed using confocal microscopy (pAEM). Spheres were rinsed away after 24hrs and microspheres were used to observe the possibility of DAPI transfer from the spheres to the cells (perhaps providing false positives for uptake).

In parallel, a second batch of microspheres was created using a fluorescent hydrophobic dye, tetramethylrhodamine isothiocyanate (TRITC), in place of melanin, to facilitate visualization. These spheres were incubated with melanocytes for 24hrs and observed using confocal microscopy.

Lastly, cell co-culture optimization was initiated to determine whether spheres could be taken up by keratinocytes. The spheres were incubated with keratinocytes for 24hrs and observed using confocal microscopy. Parameters of cell culture tested were cell density, cell ratio (melanocyte/keratinocyte), co-culture incubation time, UV-exposure and the presence of the hormone, α-MSH (Melanocyte Stimulating Hormone).

Results

1. Melanin-Encapsulated Microspheres Characterization

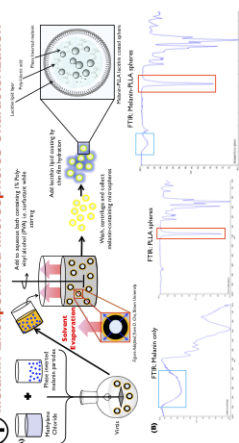


Figure 1. Artificial melanosomes, in the form of lipid-polymer hybrid naturally occurring human melanosomes. (A) Solvent evaporation of microspheres. (B) SEM image of microspheres. (C) FTIR spectra of microspheres. (D) DLS analysis of microspheres. (E) AFM image of microspheres. (F) TIRF image of microspheres. (G) AFM image of microspheres. (H) AFM image of microspheres. (I) AFM image of microspheres. (J) AFM image of microspheres. (K) AFM image of microspheres. (L) AFM image of microspheres. (M) AFM image of microspheres. (N) AFM image of microspheres. (O) AFM image of microspheres. (P) AFM image of microspheres. (Q) AFM image of microspheres. (R) AFM image of microspheres. (S) AFM image of microspheres. (T) AFM image of microspheres. (U) AFM image of microspheres. (V) AFM image of microspheres. (W) AFM image of microspheres. (X) AFM image of microspheres. (Y) AFM image of microspheres. (Z) AFM image of microspheres.

2. Molecular Approaches to Attempt Observation of Spheres in Human Melanocytes

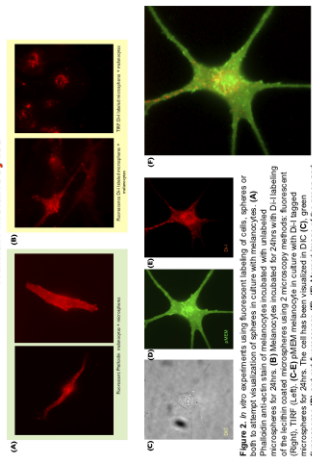


Figure 2. In vitro experiments using fluorescent labeling of cells, spheres or microspheres. (A) Fluorescently labeled microspheres. (B) Fluorescently labeled microspheres. (C) Fluorescently labeled microspheres. (D) Fluorescently labeled microspheres. (E) Fluorescently labeled microspheres. (F) Fluorescently labeled microspheres. (G) Fluorescently labeled microspheres. (H) Fluorescently labeled microspheres. (I) Fluorescently labeled microspheres. (J) Fluorescently labeled microspheres. (K) Fluorescently labeled microspheres. (L) Fluorescently labeled microspheres. (M) Fluorescently labeled microspheres. (N) Fluorescently labeled microspheres. (O) Fluorescently labeled microspheres. (P) Fluorescently labeled microspheres. (Q) Fluorescently labeled microspheres. (R) Fluorescently labeled microspheres. (S) Fluorescently labeled microspheres. (T) Fluorescently labeled microspheres. (U) Fluorescently labeled microspheres. (V) Fluorescently labeled microspheres. (W) Fluorescently labeled microspheres. (X) Fluorescently labeled microspheres. (Y) Fluorescently labeled microspheres. (Z) Fluorescently labeled microspheres.

Microscopy Technique	How technique may enable visualization?	Theoretical Hypothesis	Observations and Conclusions
Phalloidin (actin stain)	Spheres appear as dark spots against bright background (fluorescent actin)	No visible "dark spot" against bright background (fluorescent actin)	No visible "dark spot" against bright background (fluorescent actin)
DAPI (lipid dye)	DAPI labels lipid coat of spheres to reveal location of particles on the cells in real-time	DAPI labels lipid coat of spheres to reveal location of particles on the cells in real-time	DAPI labels lipid coat of spheres to reveal location of particles on the cells in real-time
DAPI & AEM	Test reduction of dye transfer from lecithin sphere coat to cell phospholipid bilayer on 24hrs	Test reduction of dye transfer from lecithin sphere coat to cell phospholipid bilayer on 24hrs	Test reduction of dye transfer from lecithin sphere coat to cell phospholipid bilayer on 24hrs
TRITC (lipid dye)	TRITC is directly incorporated into the lipid coat of spheres to use as a tool for visualization in live cells in real-time	TRITC is directly incorporated into the lipid coat of spheres to use as a tool for visualization in live cells in real-time	TRITC is directly incorporated into the lipid coat of spheres to use as a tool for visualization in live cells in real-time

Table 1. Summary of Fluorescent Techniques & Microscopy Observations of Cyto-compatible Spheres in Culture

3. TRITC-embedded Spheres Efficiently Track in Culture

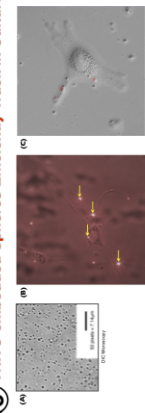


Figure 3. TRITC-embedded spheres. (A) TRITC-embedded spheres. (B) TRITC-embedded spheres. (C) TRITC-embedded spheres. (D) TRITC-embedded spheres. (E) TRITC-embedded spheres. (F) TRITC-embedded spheres. (G) TRITC-embedded spheres. (H) TRITC-embedded spheres. (I) TRITC-embedded spheres. (J) TRITC-embedded spheres. (K) TRITC-embedded spheres. (L) TRITC-embedded spheres. (M) TRITC-embedded spheres. (N) TRITC-embedded spheres. (O) TRITC-embedded spheres. (P) TRITC-embedded spheres. (Q) TRITC-embedded spheres. (R) TRITC-embedded spheres. (S) TRITC-embedded spheres. (T) TRITC-embedded spheres. (U) TRITC-embedded spheres. (V) TRITC-embedded spheres. (W) TRITC-embedded spheres. (X) TRITC-embedded spheres. (Y) TRITC-embedded spheres. (Z) TRITC-embedded spheres.

4. Cyto-compatible Spheres May Assist in Enlightening Natural Melanosome Pathway

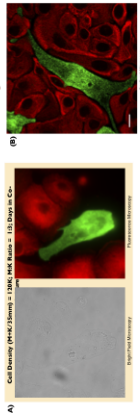


Figure 4. Optimization of co-cultures of Melanocytes (M) to Keratinocytes (K). (A) Optimization of co-cultures of Melanocytes (M) to Keratinocytes (K). (B) Optimization of co-cultures of Melanocytes (M) to Keratinocytes (K). (C) Optimization of co-cultures of Melanocytes (M) to Keratinocytes (K). (D) Optimization of co-cultures of Melanocytes (M) to Keratinocytes (K). (E) Optimization of co-cultures of Melanocytes (M) to Keratinocytes (K). (F) Optimization of co-cultures of Melanocytes (M) to Keratinocytes (K). (G) Optimization of co-cultures of Melanocytes (M) to Keratinocytes (K). (H) Optimization of co-cultures of Melanocytes (M) to Keratinocytes (K). (I) Optimization of co-cultures of Melanocytes (M) to Keratinocytes (K). (J) Optimization of co-cultures of Melanocytes (M) to Keratinocytes (K). (K) Optimization of co-cultures of Melanocytes (M) to Keratinocytes (K). (L) Optimization of co-cultures of Melanocytes (M) to Keratinocytes (K). (M) Optimization of co-cultures of Melanocytes (M) to Keratinocytes (K). (N) Optimization of co-cultures of Melanocytes (M) to Keratinocytes (K). (O) Optimization of co-cultures of Melanocytes (M) to Keratinocytes (K). (P) Optimization of co-cultures of Melanocytes (M) to Keratinocytes (K). (Q) Optimization of co-cultures of Melanocytes (M) to Keratinocytes (K). (R) Optimization of co-cultures of Melanocytes (M) to Keratinocytes (K). (S) Optimization of co-cultures of Melanocytes (M) to Keratinocytes (K). (T) Optimization of co-cultures of Melanocytes (M) to Keratinocytes (K). (U) Optimization of co-cultures of Melanocytes (M) to Keratinocytes (K). (V) Optimization of co-cultures of Melanocytes (M) to Keratinocytes (K). (W) Optimization of co-cultures of Melanocytes (M) to Keratinocytes (K). (X) Optimization of co-cultures of Melanocytes (M) to Keratinocytes (K). (Y) Optimization of co-cultures of Melanocytes (M) to Keratinocytes (K). (Z) Optimization of co-cultures of Melanocytes (M) to Keratinocytes (K).

Cell Density	Cell Ratio	Incubation Time	UV Exposure	Growth Factors
1000	1:1	24 hrs	2	2

Table 2. Optimized Parameters for Co-culture. (A) Optimization of co-cultures of Melanocytes (M) to Keratinocytes (K). (B) Optimization of co-cultures of Melanocytes (M) to Keratinocytes (K). (C) Optimization of co-cultures of Melanocytes (M) to Keratinocytes (K). (D) Optimization of co-cultures of Melanocytes (M) to Keratinocytes (K). (E) Optimization of co-cultures of Melanocytes (M) to Keratinocytes (K). (F) Optimization of co-cultures of Melanocytes (M) to Keratinocytes (K). (G) Optimization of co-cultures of Melanocytes (M) to Keratinocytes (K). (H) Optimization of co-cultures of Melanocytes (M) to Keratinocytes (K). (I) Optimization of co-cultures of Melanocytes (M) to Keratinocytes (K). (J) Optimization of co-cultures of Melanocytes (M) to Keratinocytes (K). (K) Optimization of co-cultures of Melanocytes (M) to Keratinocytes (K). (L) Optimization of co-cultures of Melanocytes (M) to Keratinocytes (K). (M) Optimization of co-cultures of Melanocytes (M) to Keratinocytes (K). (N) Optimization of co-cultures of Melanocytes (M) to Keratinocytes (K). (O) Optimization of co-cultures of Melanocytes (M) to Keratinocytes (K). (P) Optimization of co-cultures of Melanocytes (M) to Keratinocytes (K). (Q) Optimization of co-cultures of Melanocytes (M) to Keratinocytes (K). (R) Optimization of co-cultures of Melanocytes (M) to Keratinocytes (K). (S) Optimization of co-cultures of Melanocytes (M) to Keratinocytes (K). (T) Optimization of co-cultures of Melanocytes (M) to Keratinocytes (K). (U) Optimization of co-cultures of Melanocytes (M) to Keratinocytes (K). (V) Optimization of co-cultures of Melanocytes (M) to Keratinocytes (K). (W) Optimization of co-cultures of Melanocytes (M) to Keratinocytes (K). (X) Optimization of co-cultures of Melanocytes (M) to Keratinocytes (K). (Y) Optimization of co-cultures of Melanocytes (M) to Keratinocytes (K). (Z) Optimization of co-cultures of Melanocytes (M) to Keratinocytes (K).

Summary and Conclusions

- I. Artificial melanosomes are cyto-compatible in melanocyte cultures
- II. TRITC-incorporated spheres are necessary to track sphere movement in vitro
- III. There is no definitive evidence that melanocytes will independently uptake microspheres in culture
- IV. Optimized co-cultures of melanocytes and keratinocytes may provide an ideal in vitro environment to facilitate microsphere uptake and possibly transfer between cells

Future Directions:

- I. Test TRITC-embedded spheres in optimized co-culture to attempt sphere tracking. Is uptake feasible?
- II. Synthesize biocompatible microspheres that contain both TRITC and melanin
- III. Characterize TRITC-melanin loaded spheres into cells to ensure internalized microspheres are taken up by cells
- IV. Evaluate protection capabilities provided by polymer-melanin spheres

References

1. C. W. Oancea et al. *J. Invest. Dermatol.* 121, 3395-3399.
2. E. A. Oancea et al. *J. Invest. Dermatol.* 121, 3395-3399.
3. E. A. Oancea et al. *J. Invest. Dermatol.* 121, 3395-3399.

Special Thanks For: Chao-Kuo Erwin, Nishita Wada, Sara Frenkel and Jonathan Crooks

