

Analysis of Naïve vs Lipopolysaccharide (LPS) Activated Neutrophil Chemotaxis in 3D Collagen Matrices Using Traction Force Microscopy

by

David A. Stout

B. Sc, California State University, Long Beach, 2010

A Dissertation submitted in partial fulfillment of the
requirements for the Degree of Doctor of Philosophy
in the School of Engineering at Brown University

Providence, Rhode Island

May 2014

© Copyright 2014 by David A. Stout

This dissertation by David A. Stout is accepted in its present form
by the School of Engineering as satisfying the
dissertation requirement for the degree of Doctor of Philosophy.

Date _____
Christian Franck, Ph.D, Advisor

Recommended to the Graduate Council

Date _____
Jonathan Reichner, Ph.D, Reader

Date _____
Anubhav Tripathi, Ph.D, Reader

Date _____
Eric M. Darling, Ph.D, Reader

Date _____
Daniel A. Hammer, Ph.D, Reader

Approved by the Graduate Council

Date _____
Peter Weber, Ph.D, Dean of the Graduate School

Vitæ

David A. Stout was born in Long Beach, California, on July 15, 1983 to Dawn Elizabeth Stout. David attended many primary schools as a child. In 2001, David graduated from Fred C. Beyer High School in Modesto, California, in the top 2% of his class. In the fall of 2001, David attend flight school in Lincoln, California and earned his Private Pilots License.

For two years David served his church as a full-time missionary on the Native American Reservations of the American Southwest. In 2006, David graduated from Modesto Junior Collage with an A.A. in Transferable Studies and moved to Long Beach, California, (CSULB) to pursue his Bachelor's degree. During his tenure at CSULB, he moved to the East Coast twice to conduct research at different institutions. He moved to Virginia and Maryland for a NASA research position in the R&D division of the Advanced Special Projects Office. There he led a successful effort to design and build a satellite for NASA and HawkSpace which was launched by a Minotaur-1 rocket at NASA's Wallops Flight Facility on May 19, 2009. Then, he researched and designed an avionics systems for a two-stage vehicle that is being designed to have the capability to launch nanosat-class (up to 10 kg) payloads to low Earth orbit. In 2010, David, graduated from California State University, Long Beach with a B.Sc. in Aerospace Engineering and a minor in Chemistry. His senior project, under the direction of Eric Besnard, was titled, "Mini UAV for Troop Application Using Winglets."

After his undergraduate career, David moved to Providence, Rhode Island, to pursue his Ph.D in Biomedical Engineering under the guidance of Dr. Thomas J. Webster. At Brown, David used his aerospace and medical background on a multidisciplinary approach to investigate new nano-biomaterials for the cardiovascular

system. David transferred to Dr. Christian Franck's cell-mechanics lab in the summer of 2012, where he investigated the migration forces of human neutrophils in a 3D environment.

During his tenure at Brown University, David received numerous funding awards and fellowships, namely; The National Science Foundation (NSF) Graduate Student Fellowship, Indo-US Science and Technology Forum (IUSSTF) Research Fellowship, The Brown Simon Ostrach '50 Graduate Fellowship, Brown University Open Graduate Programs Scholarship, and many others. David has published over ten peer-reviewed journal articles, seven proceeding publication, over fifteen oral presentation, over ten poster presentation, and numerous invited presentations.

David A. Stout's Abbreviated CV

Refereed Journal Publications

- (1) **Stout, D.A.**, and H.H. Chen. "Development and Operations of CubeSat-Class Satellite Hawksat-1: Integrating New and Different Commercial-Off-The-Shelf (COTS) Products." *McNair Scholars Research Journal*, 13(1): 101–120, 2009.
- (2) Yang, Y., V. Chinthapenta, Q. Li, **D.A. Stout**, A. Liang, B. Sheldon, and T.J. Webster. "Understanding Osteoblast Responses on Stiff Nanotopographies through Experiments and Computational Simulation." *Journal of Biomedical Materials Research Part A*. 97A, 2011.
- (3) **Stout, D.A.**, B. Basu, and T.J. Webster. "Poly Lactic-Co-Glycolic Acid: Carbon Nanofiber Composites for Myocardial Tissue Engineering Applications." *Acta Biomaterialia*, 7(1): 3101–3112, 2011.
- (4) **Stout, D.A.**, and T.J. Webster. "Novel Nano Drug Systems for Cardiovascular Applications." *Recent Patents on Biomedical Engineering*, 5(1): 3–13, 2012.
- (5) **Stout, D.A.**, and T.J. Webster. "Carbon Nanotubes for Stem Cell Control." *Materials Today*, 15(7-8): 312–318, 2012.
- (6) Meng X., **D.A. Stout**, L. Sun, H. Fenniri, and T.J. Webster. "Novel injectable biomimetic hydrogels with carbon nanofibers and self-assembled rosette nanotubes for myocardial applications." *Journal of Biomedical Materials Research Part A*, 101(4):1095–102, 2012.
- (7) **Stout, D.A.**, J. Yoo, A.N. Santiago-Miranda, and T.J. Webster. "Mechanisms of Greater Cardiomyocyte Functions on Conductive Nanoengineered Composites for Cardiovascular Applications." *International Journal of Nanomedicine*, 7(1): 5653–5669, 2012.
- (8) **Stout, D.A.**, and T.J. Webster. "The Effect of Carbon Nanofiber Density in Poly(lactic-co-glycolic acid) Composites for Myocardial Tissue Engineering Applications." *Nano Bulletin*, 1(1): 120102–1:120102-6, 2012.

- (9) L. Sun, **D.A. Stout**, and T.J. Webster. “The Nano-Effect: Improving the Long-Term Prognosis for Musculoskeletal Implants.” *Journal of Long Term Effects of Medical Implants*, 22(3): 195-209, 2012.
- (10) Aninwene II G.E., **D.A. Stout**, Z. Yan, and T.J. Webster. “Nano-BaSO₄: A Novel Antimicrobial Additive to Pellethane.” *International Journal of Nanomedicine*, 8(1): 1197–1205, 2013.
- (11) Webster, T.J., **D.A. Stout**, S. Kalmodia, and B. Basu. “Experimental Methods and In Vitro Cytotoxicity and Genotoxicity of Nanomaterials.” *Nano LIFE*, 3(1): 1340-1361, 2013.

Refereed Proceeding Publications

- (1) **Stout, D.A.**, and H.H. Chen. “Hawksat-1: Introducing ‘COTS’ CubeSat Satellite System Variable.” *Proceedings of the National Conference on Undergraduate Research*. Asheville: University of North Carolina at Asheville, 9(1): 246–253, 2009.
- (2) Yang, L., **D.A. Stout**, A. Liang, and T.J. Webster. “The Impact of Substrate Topography on Cell Filopodia Extension and Cell Spreading.” in Business and Safety Issues in the Commercialization of Nanotechnology, edited by S.S. Mao, L. Merhari, J. van Schijndel, L. Tsakalakos, R. Hurt, H. Liu, T.J. Webster. *Mater. Res. Soc. Symp. Proc. Volume 1209*, Paper #1209-YY02-03, 2010.
- (3) **Stout, D.A.**, J. Yoo, and T.J. Webster. “Poly Lactic-Co-Glycolic Acid Carbon Nanofiber Composite for Enhancing Cardiomyocyte Function.” in Nanofunctional Materials, Nanostructures, and Nanodevices for Biomedical Applications II, *Mater. Res. Soc. Symp Proc. Volume 1316* Paper #913663-QQ06-33, 2011.
- (4) **Stout, D.A.**, B. Basu, and T.J. Webster. “Cytocompatible Poly-Lactic-co-Glycolic Acid: Carbon Nanofiber Composite Analysis for Cardiovascular Applications.” *Northeast Bioengineering Conference, IEEE 2011*, 978-1-61284-826-6, 2011.

- (5) **Stout, D.A.**, J. Yoo, and T.J. Webster. “Conductivity and Cardiomyocyte compatibility of Poly Lactic-co-Glycolic Acid Carbon Nanofiber Composites.” *8th International Conference on Composite Materials Proceedings*, Paper #F-26, 2011.
- (6) **Stout, D.A.**, E. Raimondo, and T.J. Webster. “Improved Cardiomyocyte Functions of Carbon Nanofiber Cardiac Patches,” in Biomaterials for Tissue Regeneration, *Mater. Res. Soc. Symp Proc. Volume 1417E*, Paper #KK036, 2012.
- (7) Meng, X., **D.A. Stout**, L. Sun, H. Fenniri, and T.J. Webster. “Injectable Composites with Carbon Nanofibers and Helical Rosette Nanotubes for Myocardial Tissue Engineering.” in Biomaterials for Tissue Regeneration, *Mater. Res. Soc. Symp Proc. Volume 1417E*, Paper #KK03.22, 2012.
- (8) L. Weng, **D.A. Stout**, and T.J. Webster. “Nanophased Magnesium for Orthopedic Applications.” *Northeast Bioengineering Conference, IEEE 2012*, 2012.

Book Chapters

- (1) Taylor, E., **D.A. Stout**, G. Aninwene II, and T.J. Webster. “In Vivo assessment of Nanotechnology in Biomaterials.” in Nanomedicine: Principles and Perspectives, Volume 1 & 2 ed Yi Ge (New York, NY): Springer, 2012.
- (2) Meng, X., **Stout, D.A.**, Sun, L., Fenniri, H. and Webster, T. “Injectable Biomimetic Hydrogels with Carbon Nanofibers and Novel Self Assembled Chemistries for Myocardial Applications.” in Biomaterials Science: Processing, Properties and Applications II: Ceramic Transactions, Volume 237, ed by R. Narayan, S. Bose and A. Bandyopadhyay, (Hoboken, NJ): John Wiley & Sons, Inc., 2012.
- (3) **Stout, D.A.**, N.G. Drumus, and T.J. Webster. “Synthesis of Carbon based Nanomaterials for Tissue Engineering.” in Nanomaterials in Tissue Engineering: Characterization, Fabrication and Applications ed by Gaharwar, Sant, Hancock, and Hancking (Cambridge, UK): Woodhead, 2013.

Oral Presentations

- **Stout, D.A.**, and T.J. Webster. Poly-lactic-co-glycolic acid: Carbon Nanofiber Nanomaterial Composites for Enhancing Cardiomyocyte Growth. International Symposium of Materials on Regenerative Medicine. Taipei Medical University, Taiwan, 2012.
- **Stout, D.A.**, J. Yoo, and T.J. Webster. Carbon Nanofiber Composites for Cardiovascular Applications. 4A, Nanotechnology-enhanced Biomaterials and Biomedical Devices and the XXI International Materials Research Congress. Cancun, Mexico, 2012.
- **Stout, D.A.**, and T.J. Webster. Nanotechnology: for Enhancing Cardiomyocyte Growth. 6th International Meeting on Developments in Materials, Processes and Applications of Emerging Technologies. Alvor, Portugal, 2012.
- **Stout, D.A.**, L. Yang, L. Zhang, and T.J. Webster. An Investigation of the Role of Nanoroughness in a 3D Environment. Society for Biomaterials 2012 Fall Symposium. New Orleans, LA, 2012.
- **Stout, D.A.**, and T.J. Webster. Nanotechnology: Healing Hearts, Fighting Infections, and Developing In Situ Sensors. IEEE-NANOMED 2011. Jeju, Korea, 2011.
- **Stout, D.A.**, and T.J. Webster. Enhanced Cardiomyocyte Function On Poly-Lactic-Co-Glycolic Acid: Carbon Nanofiber Composites Under Electrical Stimulation. American Institute of Chemical Engineers Annual Meeting. Minneapolis, MN, 2011.
- **Stout, D.A.**, and T.J. Webster. Improved Cardiomyocyte Function on Carbon Nanofiber Composites. Tissue Engineering International & Regenerative Medical Society North America 2011 Annual Conference. Houston, TX, 2011.
- **Stout, D.A.**, and T.J. Webster. Conductivity and Cardiomyocyte Compatibility of Poly Lactic co Glycolic Acid Carbon Nanofiber Composites. The 18th International Conference on Composite Materials. Jeju, Korea, 2011.

- **Stout, D.A.**, J. Yoo, and T.J. Webster. Cytocompatibility and Optimization of Cardiomyocytes on Poly Lactic-Co-Glycolic Acid Carbon Nanofiber Materials. Society For Biomaterials 2011 Annual Meeting and Exposition: Animating Materials. Orlando, FL, 2011.
- **Stout, D.A.**, B. Basu, and T.J. Webster. Effects of Material Concentration and Topography on Cardiovascular and Orthopedic Biomaterials. International Symposium on the Safe Use of Nanomaterials. Lucknow, UP. India, 2011.
- **Stout, D.A.**, M. Pocock, E. Silva, C. Huang, and E. Besnard. Mini UAV for Troop Application Through Winglet Usage. Region 6 American Institute for Aeronautics and Astronautics Student Research Competition. Tucson, AZ, 2010.
- **Stout, D.A.**, L. Yang, and T.J. Webster. Influences of Substrate Topography on Cell Spreading through Filopodia. 2010 California State University, Long Beach Undergraduate Student Research Competition. Long Beach, CA, 2010.
- **Stout, D.A.**, L. Yang, and T.J. Webster. Topography on Cell Filopodia Extension and Cell Spreading. The Leadership Alliance National Symposium. Washington D.C., 2009.
- **Stout, D.A.**, L. Yang, and T.J. Webster. The Impact of Substrate Topography on Cell Filopodia Extension and Cell Spreading. 2009 Material Research Society Fall Meeting. Boston MA, 2009.
- **Stout, D.A.**, L. Yang, and T.J. Webster. Impacts of Ultra-stiff Substrate Topography on Osteoblast Spreading. Southern California Conference for Undergraduate Research. Carson, CA, 2009.
- **Stout, D.A.**, and H.H. Chen. Hawksat-1: Introducing COTS CubeSat Satellite System Variables. National Conference on Undergraduate Research (NCUR). La Crosse, WI, 2009.

- **Stout, D.A.**, and H.H. Chen. CubeSat Satellite System Compatibility of COTS Products. 22nd California State University, Long Beach Undergraduate and Graduate Research Competition. Long Beach, CA, 2009.
- **Stout, D.A.**, G. Pickett, and H.H. Chen. CubeSat Satellite System Compatibility of COTS Products. California State University System Undergraduate and Graduate Research Competition. Los Angeles, CA, 2009.
- **Stout, D.A.**, G. Pickett, and H.H. Chen. Developing Compatibility of COTS Systems for CubeSat Satellites. Region 6 American Institute for Aeronautics and Astronautics Student Research Competition. Long Beach, CA, 2009.
- **Stout, D.A.**, G. Pickett, and H.H. Chen. CubeSat Compatibility of COTS Systems and Future Implications. 2009 California State University, Long Beach Undergraduate Student Research Competition. Long Beach, CA, 2009.
- **Stout, D.A.**, and H.H. Chen. Hawsat-1: Systems Variations for CubeSat Satellites. 20th Annual Research Symposium and McNair Research Conference. Stillwater, OK, 2009.
- **Stout, D.A.**, G. Pickett, and H.H. Chen. Hawksat-1: Introducing CubeSat Satellite System Variables. California State University, Long Beach McNair Research Symposium, Long Beach, CA, 2008.
- **Stout, D.A.**, G. Pickett, S. Schiare, and H.H. Chen. Hawksat-1: Introducing CubeSat Satellite System Variables. 16th Annual California McNair Scholars Symposium, Berkeley, CA, 2008.
- **Stout, D.A.**, G. Pickett, S. Schiare, and H.H. Chen. Hawsat-1: Variables of CubeSats. Hilltop Symposium at Howard University, Washington DC, DC, 2008.
- **Stout, D.A.**, G. Pickett, S. Schiare, and H.H. Chen. CubeSat: Satellite Systems Variations with COTS Products. Southern California Conference for Undergraduate Research. Pomona, CA, 2008.

- **Stout, D.A.**, and H.H. Chen. Hawksat-1: Introducing CubeSat Satellite System Variables. California State University, Long Beach Fall McNair Research Symposium, Long Beach, CA, 2008.

Poster Presentations

- Aninwene II G.E., **D.A. Stout**, Z. Yan, and T. J. Webster. Nano-BaSO₄: A Novel Antimicrobial Additive to Pellethane. Biomedical Engineering Society. Seattle, WA, 2013.
- **Stout, D.A.**, X. Meng, and T.J. Webster. Rosette Nanotubes Injectable Scaffolds for Myocardial Application. AIChE 2012 Annual Meeting. Pittsburgh, PA, 2012.
- **Stout, D.A.**, J. Yoo, and T.J. Webster. Material and Cytocompatibility Characteristics of Carbon Nanofibers for Cardiovascular Applications. Biomedical Engineering Society. Atlanta, GA, 2012.
- **Stout, D.A.**, and T.J. Webster. Material Affects and Mechanics of Carbon Nanofibers for Cardiovascular Biomaterial Applications. Society for Biomaterials 2012 Fall Symposium. New Orleans, LA, 2012.
- **Stout, D.A.**, and T.J. Webster. Cardiomyocyte Function on Poly-Lactic-co-Glycolic Acid: Carbon Nanofiber Composites under Electrical Stimulation. NanoTech 2012. Santa Clara, CA, 2012.
- **Stout, D.A.**, A.N. Santiago-Miranda, and T.J. Webster. Carbon Nanofibers in Polymer Promote Cardiac Function. World Biomaterials Conference 2012. Chendgu, China, 2012.
- **Stout, D.A.**, A.N. Santiago-Miranda, and T.J. Webster. Poly-lactic-co-glycolic acid Density Affects in Carbon Nanofiber Composites for Myocardial Tissue Engineering Applications. Material Research Society Spring Meeting 2012, San Francisco, CA, 2012.
- **Stout, D.A.**, and T.J. Webster. Improved Cellular Functions on Carbon Nanofiber Cardiac Patches. 2011 Material Research Society Fall Meeting. Group KK3.6, Boston, MA, 2011.

- **Stout, D.A.**, B. Basu, and T.J. Webster. Carbon Nanofibers for Myocardial Tissue Engineering Applications. Biomedical Engineering Society Annual Conference. Hartford, CT, 2011.
- **Stout, D.A.**, B. Basu, and T.J. Webster. Cytocompatible Poly-Lactic-co-Glycolic Acid: Carbon Nanofiber Composite Analysis for Cardiovascular Applications. 2011 Northeast Bioengineering Conference. Troy, NY, 2011.
- **Stout, D.A.**, and T.J. Webster. Conductivity and Compatibility of Polylactic-co-glycolic Acid Carbon Nanofiber Material. 2010 Material Research Society Fall Meeting. Group: QQ6.33 Boston, MA, 2010.
- **Stout, D.A.**, and T.J. Webster. Compatibility of Poly Lactic-Co-Glycolic-Acid Carbon Nanofiber material Ratios for Cardiomyocytes. Biomedical Engineering Society 2010 Annual Scientific Meeting. Austin, TX, 2010.
- **Stout, D.A.**, L. Yang, and T.J. Webster. Effects of NCD Topography on Osteoblast Function. Brown University SR-EIP/REU Conference. Providence, RI, 2009.
- **Stout, D.A.**, L. Yang, and T.J. Webster. Topography Impact on Osteoblast Spreading and Filopodia Extension on Diamond Substrates. BioInterface 2009 Symposium. San Mateo, CA, 2009.
- **Stout, D.A.**, L. Yang, and T.J. Webster. Impact of Nanotopography on Osteoblast Spreading and Filopodia Extension on Ultrastiff Substrates. Biomedical Engineering Society 2009 Annual Fall Scientific Meeting. Pittsburgh, PA, 2009.

“You know, Hobbes, some days even my rocketship underpants don’t even help with
my experiments.”

adapted from Calvin and Hobbes

Dedicated to My Family
(*Mom, Fernando, Tony, Taelor, Lisa D, Wilson, Gammie & Papa*)

Acknowledgements

First and foremost, I would like to thank my family for their persistent support and love they have always shown me. To my mother who has always been the perfect example of endurance and faith. To my siblings (Taelor, Elizabeth, Wilson) who have always told me that I was the best big brother. To Fernando who always told me that I could do anything I wanted. To my Grandparents (Gammie and Papa) who were the best cheerleaders anyone could ask for and their enduring love. To Tony, who has always been on my side from the very beginning. To the rest of my whole family who told me that it was worth it and for their continual interest in my research.

Next, I would like to thank my advisor, Dr. Christian Franck, who decided to take in an orphan graduate student at the hardest time of the research calendar. To Dr. Jonathan Reichner for his time, lab, and support. To everyone in the Franck Lab (Eyal, Jennet, Jon) and Reichner Lab (Angel, Cortney, Eliza) who taught me how to be a great researcher. To all my undergraduates advisees (Emilia Raimondo, Jennie Yoo, Daniel Hoff, Adriana Noemi Santiago-Miranda, Eugenia Gurvich, Giuliano Marostica, Christopher Corbett) for their hard work. I wish the best for you all. To my committee and Dr. Thomas J. Webster for their time and outside guidance.

Lastly, I would like to thank the many friends that I have met at Brown University who have supported me through the years, namely, Nancy Picard, Brian Walton, Peter Weber, Keeley Smith, and the administrative staff at the Brown University Graduate School and at the School of Engineering.

Preface

This dissertation is ultimately based on a novel experimental apparatus and data of a collaboration between two labs at Brown University and the Rhode Island Hospital. None of the text of the dissertation is taken directly from previously published or collaborative articles.

The experimental setup and design in Chapters 2 & 3 were done primarily by Toyjanova J and myself. Cell extraction protocols were adapted from Reichner's Lab protocols with assistance from Byrd A. Diffusion experiments were performed by Gurvich E and myself. MATLAB codes in Chapter 2 were written by Toyjanova J and myself, and data analysis performed by me.

The DVC MATLAB codes were written by Bar-Kochba E, Toyjanova J and Franck C. Three-dimensional cell experiments were my own design and all were performed by myself. Experimental cell MATLAB codes in Chapter 3 were written by Bar-Kochba E and myself, and data analysis and implementation performed by me.

A version of Appendix B has been published in the International Journal of Nanomedicine [Stout DA, Yoo J, Santiago-Miranda AN, Webster TJ. Mechanisms of greater cardiomyocyte functions on conductive nanoengineered composites for cardiovascular application. *Int J Nanomedicine* doi: 10.2147/IJN.S34574]. I was the lead investigator, responsible for all major areas of concept formation, data collection and analysis, as well as the majority of manuscript composition. Yoo J and Santiago-Miranda AN were involved in the early stages of data collection. Webster TJ was the supervisory author on this project and was involved throughout the project in concept formation and manuscript edits.

A version of Appendix C has been published in Nano Bulletin or in the Material Research Society Symposium Proceedings Vol. 1417E [Stout, DA and TJ Webster. The

Effect of Carbon Nanofiber Density in Poly(lactic-co-glycolic acid) Composites for Myocardial Tissue Engineering Applications. Nano Bulletin, 1(1): 1201021:120102-6, 2012.] or [Stout, DA, E Raimondo, and TJ Webster. Improved Cardiomyocyte Functions of Carbon Nanofiber Cardiac Patches, in Biomaterials for Tissue Regeneration, Mater. Res. Soc. Symp Proc. Volume 1417E]. I was the lead investigator, responsible for all major areas of concept formation, data collection and analysis, as well as the majority of manuscript composition. Raimondo E was involved in the early stages of data collection. Webster TJ was the supervisory author on this project and was involved throughout the project in concept formation and manuscript edits.

David A. Stout

Providence, Rhode Island

Contents

Vitæ	iv
Dedication	xv
Acknowledgements	xvi
Preface	xvii
List of Tables	xxii
List of Figures	xxiii
Chapter 1. Introduction	1
1. Cell Migration	1
1.1. Migration Force	5
1.2. Two-Dimensions (2D) vs. Three-Dimensions (3D)	5
2. Neutrophils	7
2.1. Migration Process	8
2.2. Sepsis	10
3. Digital Volume Correlation	12
4. Thesis Overview	15
Chapter 2. Planar Gradient Diffusion System to Investigate Chemotaxis in a 3D Collagen Matrix	17
1. Introduction	17
2. Methods & Materials	20
2.1. 3D Mold	20
2.2. Coverslip Preparation	20
2.3. Mold Assembly	21

1.1. Neutrophil Chemotaxis of naïve vs LPS activated neutrophils	67
2. Future Extensions	67
2.1. Force Generation of Neutrophil Migration	67
2.2. Signaling Pathway Alterations Between fMLP and LPS During Sepsis	68
3. Last Words	68
Appendix A. MATLAB and Mathematica codes	70
1. MATLAB codes for diffusion solutions	70
Appendix B. Mechanisms of Greater Cardiomyocyte Functions on Conductive Nanoengineered Composites for Cardiovascular Applications	78
1. Introduction	78
2. Methods & Materials	80
3. Results	88
4. Discussion	99
5. Conclusion	103
Appendix C. Enhanced Cardiomyocyte Function On Poly-Lactic-Co-Glycolic Acid: Carbon Nanofiber Composites Under Electrical Stimulation	104
1. Introduction	104
2. Methods & Materials	106
3. Results & Discussion	109
4. Conclusion	114
Bibliography	115
Index	131

List of Tables

2.1	3D chemotaxis assay obstacles	20
2.2	Collagen recipe for different collagen densities	23
2.3	Coefficients of diffusion.....	33
2.4	Planar gradient diffusion system meeting chemotaxis obstacles.....	35
3.1	Cell preparation control experiments	42
3.2	Recent clinical trials in sepsis	63

List of Figures

1.1	Cell movement types	2
1.2	Cell migration overview	4
1.3	Cell behavior in 2D vs. 3D.....	7
1.4	Neutrophil migration overview	9
1.5	Sepsis steps overview	11
1.6	Digital volume correlation	13
1.7	Incremental digital volume correlation (IDVC)	14
1.8	Understanding cell migration through force generation	15
2.1	Planar gradient diffusion system mold assembly	21
2.2	Planar gradient diffusion system mold assembly with collagen	22
2.3	Planar gradient diffusion system mold assembly prepared for imaging.	24
2.4	Planar gradient diffusion system mold assembly with Chambridge chamber	25
2.5	Planar gradient diffusion system mold assembly with gel	26
2.6	Diffusion graphs for 2.5 mg/ml collagen concentration	30
2.7	Rhodamine microscopy images in collagen	31
2.8	Concentration profile along collagen matrix at steady-state	32
2.9	Diffusion profile through collagen matrix	34
3.1	Pathway of healthy neutrophil to generate migration	37
3.2	Summary of neutrophil migration of healthy or in sepsis.....	39
3.3	Data calculation flow diagram.....	47
3.4	DiI stained neutrophil embedded in collagen matrix.....	48
3.5	fMLP induced chemotaxis of naïve neutrophil in planar gradient system	49

3.6	Naïve neutrophil chemotaxis displacement in 3D collagen molds.....	50
3.7	Naïve neutrophil chemotaxis principal strains in 3D collagen molds.....	51
3.8	LPS activated neutrophil chemotaxis displacement in 3D collagen molds	52
3.9	fMLP induced chemotaxis of LPS activated neutrophil in planar gra- dient system	53
3.10	LPS activated neutrophil chemotaxis principal strains in 3D collagen molds	54
3.11	Cell concentration between pseudopod and uropod of neutrophil.....	55
3.12	Principal strain magnitudes of LPS activated vs naïve neutrophil chemotaxis.....	56
3.13	Speed of LPS activated vs naïve neutrophil chemotaxis.....	57
3.14	Chemotactic index of LPS activated vs naïve neutrophil chemotaxis ...	58
3.15	Signaling pathway comparison between LPS activation and fMLP	59
3.16	Pincipal Strain concentration comparison between naïve and LPS activated Neutrophils	61
B.1	Scanning electron micrographs of CNF's in a PLGA matrix	88
B.2	PLGA:CNF sample contact angle and conductivity measurements	89
B.3	PLGA:CNF sample degradation and pH measurements	91
B.4	PLGA:CNF sample tensile measurements.....	92
B.5	PLGA:CNF sample topography and protein adsoption corolation.....	94
B.6	Cardiomyocyte proliferation on PLGA:CNF samples.....	96
B.7	Cardiomyocyte Live/Dead® assay on PLGA:CNF samples	97
B.8	Immunofluorescence staining for cardiomyocyte biomarkers on PLGA:CNF samples	98
B.9	PLGA:CNF sample surface schematic.....	101
C.1	Chemical structure of cardio-patch matrix.....	106
C.2	Electrical stimulation (ES) apparatus	107

C.3	Troponin complex structure	108
C.4	X-ray diffraction and scanning electron micrograph of cardio-patch.....	110
C.5	Electrical stiulation cardiomyocyte proliferation	111
C.6	Electrical stiulation cardiomyocyte Troponin I	113

Abstract of “Analysis of Naïve vs Lipopolysaccharide (LPS) Activated Neutrophil
Chemotaxis in 3D Collagen Matrices Using Traction Force Microscopy”

by David A. Stout, Ph.D., Brown University, May 2014

Understanding the fundamental mechanisms, and forces, underlying cell migration holds the promise of effective approaches for treating diseases and promoting cellular transplantation. One such disease is sepsis. During sepsis—a complex clinical syndrome that results from a harmful or damaging host response to infection—directed impairment has been shown of neutrophils to infectious foci and inadequate antimicrobial responses. In order to understand how sepsis affects neutrophil migration, we investigated the force generating characteristics and comparison between normal and septic neutrophil chemotaxis in a physiological relevant 3D mechanistically tractable system with the use of 3D FIDVC techniques. The development of a well controlled diffusion direct-viewing system was completed for chemotaxis studies, which allows one to overcome modern chemotaxis chamber obstacles. More compelling, we were able to successfully calculate the coefficient of diffusion of Rhodamine through five different concentrations of collagen as well as solve the governing diffusion equations to find the specific concentration along the surface of the cell. With the use of time-lapsed confocal microscopy and FIDVC we were able to probe the force generating chemotactic characteristic differences of naïve and LPS activated neutrophils. We were able to show that in 3D, LPS activated neutrophils have greater deformation and displacement capabilities, while increasing their speed and decreasing their directness. Investigating the signaling pathways between fMLP and LPS our research suggests a molecule used in both GPCR and TLR4 pathways is being affected which may shed light in understanding sepsis. By introducing the study of mechanics in the investigation of sepsis, we have presented insight (force generation location and mobility) on how sepsis may alter neutrophil function that may not have been noticed by traditional biological assays.

CHAPTER 1

Introduction

1. Cell Migration

The importance of cell migration is demonstrated during the beginning of human conception and throughout human life. For example, cell migration orchestrates morphogenesis (a biological process that causes an organism to develop its shape) throughout embryonic development [1]. From a migratory point of view, all embryonic cells are divided into cells of the epithelial morphology that form interconnected sheets and mesenchymal cells that originate from different lineages. During early development, the interconnected epithelial sheets undergo short-range migration during gastrulation. The mesenchymal cells migrate throughout the embryo as independent units to contribute to tissues and cell types around the body. Interestingly, this migration is driven not by the movement of the sheet itself, but by the repositioning of mesenchymal cells that define the embryonic mesodermal layer.

Cells of the mesenchymal morphology are actually responsible for most migratory events that take place during development. These cells migrate from various epithelial layers to special locations, where they differentiate to form the specialized cells that compose different tissues and organs. This can be seen through the development and patterning of the nervous system. During pre-differentiation stages, neuronal precursors of the neural crest lineage follow the same migration as any other mesenchymal cell, but after differentiation the neuronal cell bodies eventually find stationary positions within the developing nervous system. Next, their neurites continue to migrate through tissues to reach their destinations and form connections with their effectors (the gland or muscle that causes the response, stimulated by the nervous system) as part of the mature nervous system. This process can occur over considerable distances with relatively high speed, and constitutes a crucial part of development. In humans,

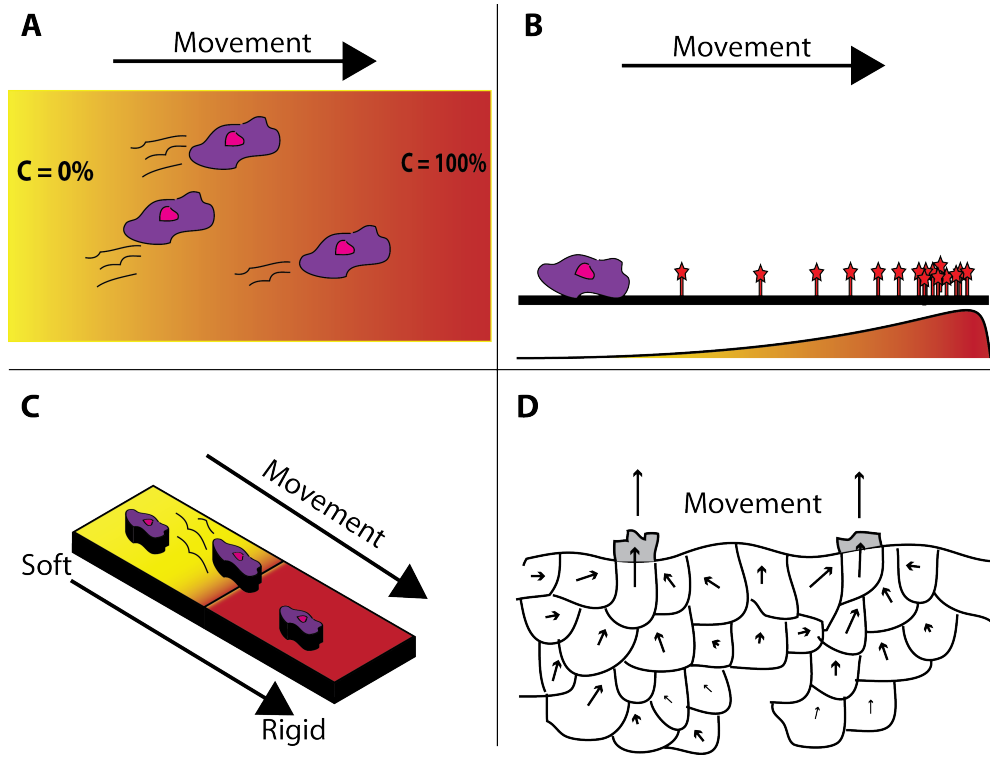


FIGURE 1.1. Taxis is the movement of cell in response to a stimulus that has motility and demonstrates guided movement towards or away from the stimulus source. Some of the most common taxes are: (A) chemotaxis where the cell moves along a chemical gradient; (B) haptotaxis, where the cell moves along a gradient of cell adhesion sites; (C) durotaxis, where the cell moves along a rigidity gradient; and (D) plithotaxis, where the cell moves along the local direction of the maximal principal stress or minimal intercellular shear stress in a monolayer.

brain development alone involves the laying down of approximately 10^{11} neurons, each forming around 1000 connections with other neurons and often traveling over distances of more than one meter, a 5^{th} order magnitude larger than the size of the neuronal cell body [2]. This migration, while highly specialized and morphologically distinct, is believed to occur by the same mechanisms as described with the epithelial sheet [3, 4, 5], and shows the importance of different types of cell migration during human development.

It is important to note that cell migration occurs as a response to an environmental stimulus to the cell (Figure 1.1). In chemotaxis, cells direct their movements according to certain soluble chemical cues in their environment [6]. This is illustrated

by movement of sperm toward an egg for fertilization, or movement of a neutrophil toward a bacteria in an epidermal cut (Figure 1.1A). In haptotaxis, a cell's motility is directed via a gradient of a chemoattractant expressed or bound on a surface, rather than in a soluble fluid (Figure 1.1B). During angiogenesis, a process through which new blood vessels form from pre-existing vessels, an extracellular matrix (ECM) directs the movement via haptotaxis [7]. In durotaxis—a subset of mechanotaxis, which refers to the directed movement of cell motility through mechanical cues—a cell is guided via gradients in substrate rigidity, usually in the direction of increasing substrate stiffness (Figure 1.1C) [8, 9]. In plithotaxis, a cell within a monolayer of cells migrates along the local direction of the maximal principal stress or minimal intercellular shear stress (Figure 1.1D) [10, 11].

Even after human development cell migration continues to demonstrate its importance. For instance, in tissue repair and immune surveillance, leukocytes from the circulation migrate into the surrounding tissue to destroy invading microorganisms and infected cells [12, 13]. During inflammatory response, neutrophils (a polymorphonuclear leukocyte and the most abundant type found in the body) move out of the bloodstream into an injured or infected tissue and migrate towards the site of infection. In response to gradients of chemokines released in the body, neutrophils sense these gradients and up the gradient towards the source. This begins with neutrophils attaching to the surface of the endothelial layer and beginning to roll along the surface of the vessel. As the attachment to the selectins strengthens, the increase in adhesion causes integrin activation which in turn causes the neutrophils to flatten out in preparation for the processes of diapedesis, during which they squeeze their way through the gaps that have appeared between the endothelial cell layer. Next, neutrophil chemotaxis (chemically prompted taxis in which the gradient develops in a soluble fluid) draws them toward the site of inflammation [14].

Unfortunately, excessive neutrophil activation does have its downside. Research has shown cell migration when unregulated, can bring an excessive number of neutrophils to an injured tissue thereby contributing to several pathological processes,

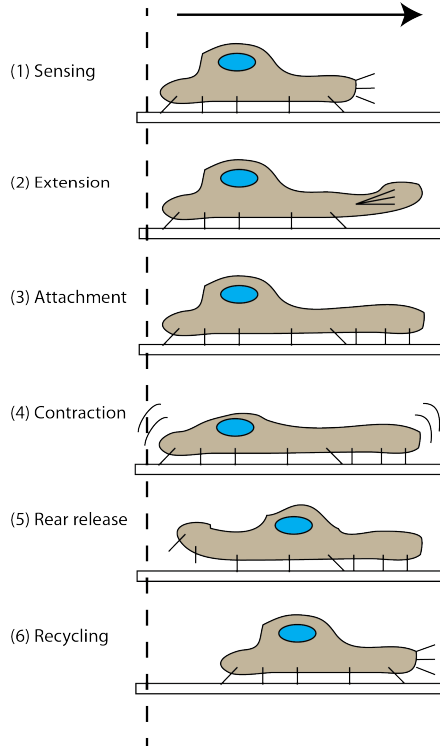


FIGURE 1.2. Major steps of mesenchymal cell migration. Cell migration can be divided in 6 sequential events: (1) Dependent sensing of the motile stimuli by filopodia; (2) cellular extension and formation of protruding lamellipodia; (3) attachment of the protrusions to the extracellular matrix at focal adhesions; (4) stress fiber-mediated contraction of the cell body allowing forward progression; (5) rear release by stress fiber-mediated traction forces; and (6) recycling of the adhesive and signaling components.

including vascular disease [15], osteoporosis [1], chronic inflammatory diseases [16] and cancer [17]. Understanding the fundamental mechanisms that underly cell migration, in general, holds the promise of effective approaches for treating disease, cellular transplantation and the preparation of artificial tissues.

Decoding cell migration has become a hot topic in research for the above reasons. Due to cell migration research groundwork and interest in understanding cell migration, progress has been made in comprehending this front. Such progress includes the establishment of polar structures, the regulation of the dynamic processes of actin and microtubule polymerization [18], and the regulation of spatial and temporal signal transduction [19].

Currently, our understanding of cell migration is derived from studies of different cell types and environments. Cell migration can be usefully conceptualized as a periodical process (Figure 1.2). The first response, usually of a cell to a migration promoting agent, is to polarize and extend protrusions in the direction of migration. These protrusions can be large broad lamellipodia (a cytoskeletal protein actin projection on the mobile or front edge of the cell), or sharp like filopodia (the skinny cytoplasmic projections that extend beyond the leading edge of lamellipodia). Both are driven by actin polymerization, and stabilized by adhering to the ECM or adjacent cells via transmembrane receptors linked to the actin cytoskeleton. These adhesions serve as traction sites for migration as the cell moves forward over them, then disassembles at the cell rear, allowing it to detach.

Many aspects of this process are shared among different cell types, but the details differ. For example, the above steps are observed most distinctly in slow moving cells, such as fibroblasts (which have been the classical model for studying cell migration in culture), but are not as obvious in fast-moving cells such as neutrophils, which seem to glide over the substratum [1].

1.1. Migration Force. Migration of all cell types requires force generation. When cells migrate, they exert traction forces on their surroundings [20]. This protrusion force at the leading edge of a cell is generated by the cytoskeleton, and controlled by multiple signaling cascades [21, 22]. The traction force generated by the cell on the extracellular matrix (ECM) regulates cell-ECM adhesions where the counter force exerted by the ECM on the cell drives the migration [23]. It has been established that a fundamental aspect of the motility mechanism is the transmission of these contractile forces to the surrounding matrix at the cell's leading and trailing edges [24, 25] (Figure 1.2). Understanding these forces can lead to a better understanding of cell motility.

1.2. Two-Dimensions (2D) vs. Three-Dimensions (3D). In addition, a cell's migratory behavior depends on its environment. This becomes very important

when investigating cell migration in vitro. Most of our current understanding of many biological processes is based largely on studies of homogeneous populations of cells on flat, 2D plastic or glass substrates. However, in vivo, cells primarily exist embedded within a complex and information-rich environment that contains multiple ECM components, a mixed cell population that interact hetero-typically, and a medley of cell-secreted soluble chemical factors.

These differences between 2D and in vivo has cast a “two sides to every coin” story; the simplicity of 2D culture and research has enabled minimalist approaches to comprehending individual cellular phenomena. Unfortunately, these findings have come with the caution that 2D systems might not completely capture the physiological behavior of cells in vivo.

Many cell types, when isolated from tissues and placed into 2D cell culture, become progressively flatter, divide and lose their differentiated phenotype [26, 27]. Some of these cell types can regain their physiological form and function when embedded in a 3D culture environment. For example, mammary epithelial cells embedded in a 3D environment halt uncontrolled division, assemble in acinar structures and establish a basement membrane [28, 29, 27].

Also, the process of spreading on a planar surface (2D) may be different from the processes that occur in a 3D matrix. For example, on 2D substrates integrin-mediated adhesion is followed by lamellipodial extension, myosin-mediated cytoskeletal tension and the reinforcement of focal adhesions [30, 31, 32]. This overall process takes a short period of time on these restraint-free substrates. In a 3D environment, cells must often negotiate or cleave the physical scaffold in order to extend [33]. Furthermore, the symphony of adhesive, mechanical and chemical cues that can regulate cell function, in a 3D context, can allow for higher-order phenomena to occur where the cell relocates.

Even simple processes can assume dramatically different forms in 2D and 3D settings. For example, migration on a 2D surface channels through a general step process (as stated above: extension of leading edge, adhesion formation, traction generation and final retraction of the trailing edge [21, 1] (Figure 1.2)). Cell migration in 3D

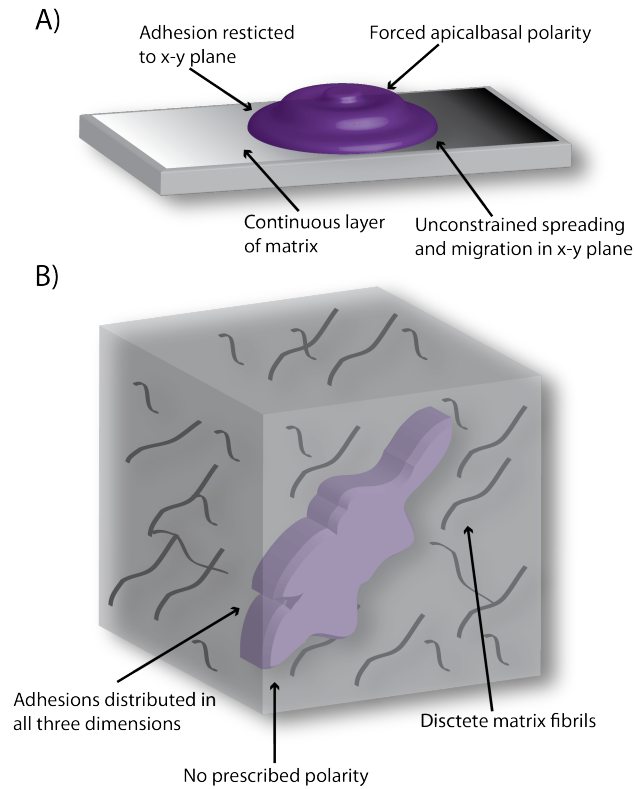


FIGURE 1.3. Cues and migration characteristics can be very different between an A) ECM coated glass or plastic surface (2D) and a B) 3D ECM, like collagen.

depends on the topography, steric hindrance and anisotropic mechanics specific to the ECM the cell is embedded in, as well as the mechanics the cells use to squeeze through potential pores [34, 35, 36] (Figure 1.3). It is therefore imperative to investigate cell migration in 3D environments to better understand in vivo force generating pathway characteristics of migrating cells.

2. Neutrophils

Understanding the fundamental mechanisms that make up cell migration holds the promise of producing effective approaches for treating disease and promoting cellular transplantation. One of the key components to this understanding is the neutrophil. Neutrophils are one of the major players during acute inflammation [37, 38, 39]. These cells are thought to be the first line of defense and the first leukocytes to be recruited to an inflammatory site [37]. Originated from the bone marrow as

progenitor stem cells and released as mature cells into the blood (process controlled by granulocyte colony stimulating factor(G-CSF) [40]), neutrophils circulate, waiting to be “activated” before being cleared by the liver, spleen or bone marrow [41].

Once activated, neutrophils migrate to their target and eliminate the pathogens by multiple avenues, both intra- and extracellular. When a neutrophil encounters microorganisms, they phagocytose the microorganisms. Intracellular microbial killing occurs by NADPH oxygenase-dependent mechanisms, reactive oxygen species (ROS) or antibacterial proteins [42, 43]. These antibacterial proteins can also be released from the neutrophil granules, either into phagosomes or into the extracellular milieu, thus acting on either intro- or extracellular pathogens. Also, highly activated neutrophils can eliminate extracellular microorganisms by releasing neutrophils extracellular traps (NETs). NETs are composed of a core DNA element to which histones, proteins and enzymes leased from neutrophil granules are attached [44], thus preventing the microorganism from spreading.

2.1. Migration Process. Before neutrophils can eliminate microorganisms, they must migrate from the blood stream to the source of infection. Research has shown there are five distinct phases of neutrophil migration to the focus site: (1) mobilization or capture, (2) rolling, (3) adhesion and spreading, (4) transmigration through the vessel wall, and (5) chemotaxis in response to chemotactic gradients (Figure 1.4). The initial step entails formation of a molecular interaction between the cell surface of the neutrophil and endothelial cell, which results in the neutrophil rolling along the luminal surface. This mobilization and rolling is dependent upon both physical and molecular forces and is mediated by selectins (a family of glycoprotein surface adhesion molecules) and ligands. This transient rolling assists in the high-affinity adhesive interaction between neutrophils and endothelial cells, which is mediated by a separate set of leukocyte adhesion molecules—namely integrins.

Following adhesion and spreading, neutrophils transmigrate through the vessel wall, then migrate to the site, but require a chemoattractant gradient to complete the process. Transmigration through the vessel wall occurs when the neutrophil

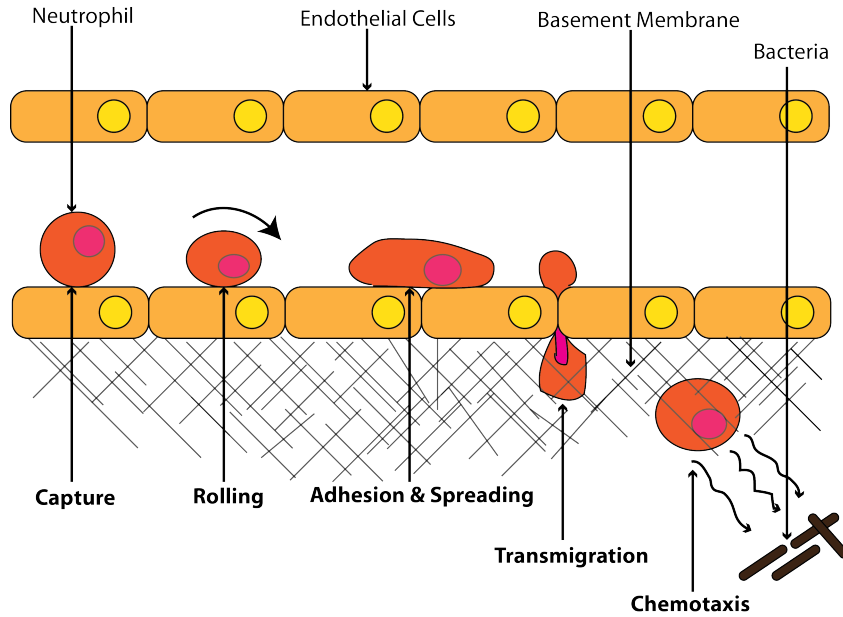


FIGURE 1.4. Neutrophils are the major leukocytes promptly recruited to the inflamed loci in response to infection or tissue injury. Neutrophil migration from the intravascular to the extravascular compartment predominantly occurs in the postcapillary venules, which is mediated by a combination of mechanical, chemical, and molecular processes. This process has distinct events that are linked in a temporal sequence: mobilization or capture, marination and rolling, adhesion and spreading, transmigration through the vessel wall, and chemotaxis in response to a chemotactic gradient.

senses the chemoattractant gradient and passes through the tight junctions of the endothelium. The neutrophil senses the chemoattractant gradient and migrates to the infection or injured tissue.

This last part, chemotaxis of the neutrophil within the tissue to the exact site of injury or infection, is the key, signature piece of the immune response. Since this response is important to human health, the complex mechanisms that allow a neutrophil to migrate so rapidly have been investigated over the past few years. For example, Sixt et al. [45] have shifted the paradigm by finding that integrins are desirable for motility in tissues and 3D collagen gels. Another finding by Oakes et al. [46] shows neutrophil morphology and migration are affected by substrate elasticity. They were able to show that neutrophils on stiffer gels moved with significantly smaller average

speed, indicating that the mechanical properties of substrates affect the principal cell functions of spreading, force generation and migration of neutrophils.

Even more interesting is the mechanism of the movement of the neutrophil itself. Oakes and Hammer [47] indicate neutrophils migrate in a sequence that is largely in the reverse of what has been seen in strongly adherent, slow moving cells, like fibroblasts. Neutrophil motility seems to be organized and initiated in the uropod (back of the cell), leading to forced lamellipodial polymerization and then the adhesion of the lamellipod. Taken together, work from these and other investigators emphasize that neutrophil response to physical as well as biochemical factors in the tissue microenvironment.

2.2. Sepsis. The mechanics and efficiency of neutrophil migration may be altered in the presence of disease. Sepsis describes a complex clinical syndrome that results from a harmful or damaging host response to infection by neutrophils [48]. Innate immune response to sepsis occur in two distinct and overlapping phases. The initial phase, referred to as the systemic inflammatory response syndrome (SIRS), is manifest by the release of a number of proinflammatory factors, such as tumor necrosis factor- α , IL-1, reactive oxygen species (ROS) and nitric oxide [49]. The second phase, compensatory anti-inflammatory response syndrome (CARS), is characterized by the expression and release of anti-inflammatory mediators and inhibitors of pathogen receptor signaling pathways [49]. This counter-regulatory phase is thought to limit the systemic inflammation induced during the SIRS response but also cause immunosuppression (Figure 3.2).

In spite of antibiotic therapy, fluid resuscitation and clinical trials looking at proinflammatory mediators, sepsis mortality is approximately 35%, causing 225,000 deaths in the US annually. This makes sepsis the leading cause of death in the intensive care unit in hospitals [51, 52], and the leading cause of death worldwide [53, 54]. In perspective, more people die every day from sepsis (589 deaths per day) than from an acute heart attack [51].

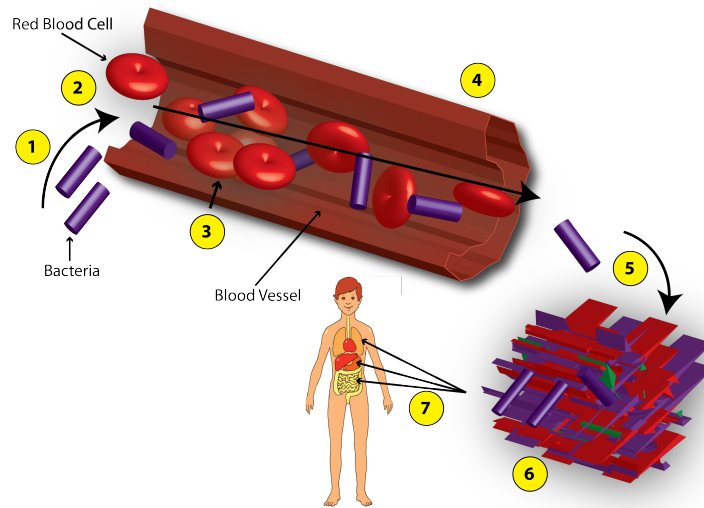


FIGURE 1.5. One avenue of sepsis starts with (1) bacteria from an abscess migrate into the bloodstream. Next, (2) bacteria is carried through the bloodstream to major organs, making them “sick.” (3) Blood clots form throughout the body which depletes available clotting factors. (4) This results in the body cells becoming “poisoned,” known as blood poisoning. This will cause a whole body inflammation state called (5) systemic inflammatory response syndrome (SIRS), which leads to (6) septic shock. After septic shock, the last results (7) are multiple organ failure and death.

Increasing evidence suggests sepsis impairs immune function by inducing defects to the innate immunity [48]. How the septic response impacts neutrophil function is incompletely understood. For example, there is evidence indicating that neutrophil function during severe sepsis is dysregulated, resulting in impaired directed migration of neutrophils to infectious foci and inadequate antimicrobial responses of PMNs at these sites [55]. Evidence also suggests there is deleterious accumulation of neutrophils within remote vital organs, leading to collateral tissue damage and ultimately multiple-organ failure [48]. Impaired neutrophil chemotactic responses and changes in neutrophil function have been directly correlated with increases in sepsis-mediated morbidity and mortality [55, 56], thus highlighting the clinical relevance of their altered responses.

Therefore, the understanding of how neutrophils cause tissue damage during sepsis is clearly not understood—a primary example being the neutralization of proinflammatory mediators in prevention of neutrophil hyper-activation (through blocking these molecules) which have failed to impair the clinical course of the disease. In the response of neutrophils to bacterially-derived activating molecules, improvement in clinical course of the disease has not affected morbidity or mortality by blocking these molecules. Therefore, it is important to understand the biophysical characteristics of neutrophil migration during sepsis.

3. Digital Volume Correlation

As previous explained, mechanical forces, whether cell generated or externally applied, critically regulate many cellular functions including differentiation, growth, adhesion and ultimately migration. In their native state, all animal cells live within the context of a 3D microenvironment supported architecturally by the extracellular matrix and exert forces through cell-ECM contacts. Over the past three decades, researchers have invented quantitative measurement tools to investigate these force interactions. One such tool is called 2D traction force microscopy (2D TFM), where animal cells are cultured on the surface of a 2D substrate—such as polyacrylamide [57, 58, 59] with embedded fluorescent microspheres. The cells are incubated to allow tractions to develop and a detergent to release the cell tractions. The cellular traction force is calculated from the bead displacements using different mathematical techniques [47, 60].

Since cells are found to behave very differently on a 2D substrate than they do within 3D biomatrices,¹ TFM had to adapt to account for this third dimension, known as 3D TFM. The basic idea behind 3D TFM is similar to that of 2D TFM—consisting of the same two parts (measurement of fluorescent bead displacements caused by the release of cellular traction forces and translation of the bead displacements into a

¹ See Ch 1.1

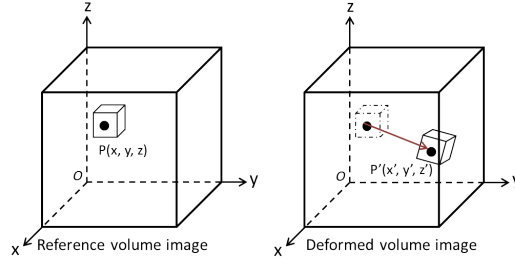


FIGURE 1.6. In DVC a cubic reference sub-volume, centered at the calculation point $P(x, y, z)$ of a reference volume image, is selected and used to track in the deformed volume image. The coordinate difference between the target sub-volume center $P'(x', y', z')$ and reference sub-volume center $P(x, y, z)$ yields the 3D displacement vectors at the specific point.

cellular traction field)—but with the notion of designing an algorithm for all three dimensions.

Since the introduction of 3D TFM, two main computation methods have been established that translate the bead displacements into the traction field: (1) the inverse and (2) the forward computation methods. In the inverse computation method, researchers model the substrate as an infinite half-space of a homogeneous, isotropic, linear, and elastic solid, and use Green’s function approach to derive the relation between cellular traction and bead displacements [61]. This is not trivial since 3D Green’s field is not readily available but can be resolved by using finite element simulations [61]. The complexity of the computational algorithms make this less accessible to the biology and life science community.

The forward approach begins by determining a continuous displacement field from the bead images and directly evaluating the strain and the stress field. Using this approach one could divide the imaging domain into volume elements and correlate the volume elements before and after deformation by comparing image patterns—known as digital volume correlation (DVC). The correlation is realized by the reductions of an objective function showing the match between the volume elements before and after deformation (Figure 1.6). More importantly, this method does not rely on a particular material model to obtain the strain field, and thus can be easily applied

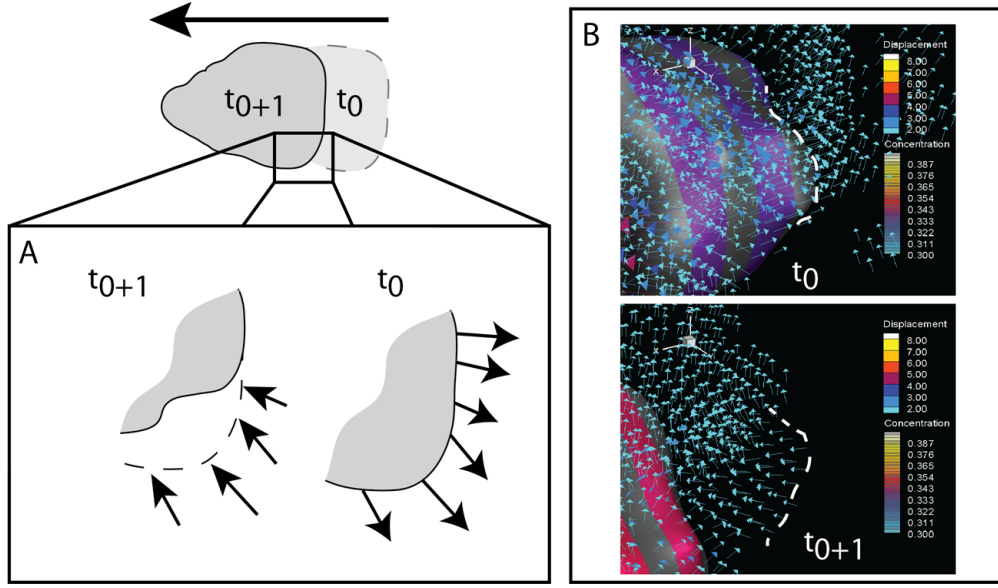


FIGURE 1.7. Schematic of incremental digital volume correlation (IDVC). IDVC compares the displacement at initial time-step (t_0) and time-step + 1 (t_{0+1}) (compared to cumulative digital volume correlation that has a global reference for displacement comparison). For example, when a cell “pushes” from its initial location (t_0) the cell deforms the matrix and then moves to a new location. Once the cell moves to its new location (t_{0+1}) the matrix returns to its original shape reversing the displacement field direction in the initial cell location, which could be incorrectly translated to different cell forces like cell “pulling.” Zoomed in schematic of this can be seen in box (A) and shown in our actual displacement results in box (B) where the dashed line signifies cell surface at t_0 .

when large deformation and/or complex material behaviors are encountered (Figure 1.7). Franck et al. [62] have been able to use this method to an excellent degree and is now beginning to gain traction in the life science community to investigate cell migration. Recently, the Franck lab developed a new fast iterative digital volume correlation (FIDVC) algorithm [63]. The newly developed FIDVC technique provides significant higher spatial resolution, signal-to-noise and faster computational times than previous DVC algorithms. Also, from this information one can investigate a cell’s “pushing” or “pulling” migration tendencies [63]. Using FIDVC would allow us to probe the differences of chemotaxis between health and septic neutrophils.

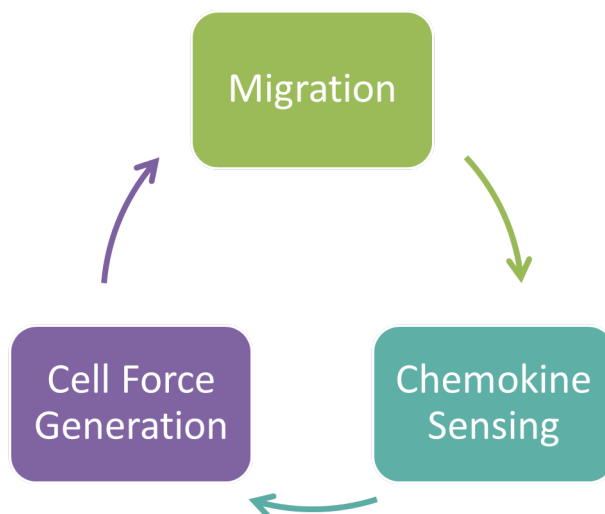


FIGURE 1.8. Cell migration is an important part of human development and life. In chemotaxis, cell migration is regulated by chemokine sensing which results in cell force generation. In order to understand the mechanisms that can alter cell migration, one can study these forces during chemokine sensing.

4. Thesis Overview

The overall purpose of this study is to investigate force generating pathway characteristics and compare the chemotaxis of neutrophils obtained from healthy and diseased donors in a physiologically relevant 3D mechanistically tractable system. This comparison is conducted with the use of 3D FIDVC techniques and a reductionist approach to sepsis. The central hypothesis of this dissertation states that lipopolysaccharide (LPS) activation affects Formyl-Methionyl-Leucyl-Phenylalanine (fMLP) induced chemotactic cell machinery and decrease its directionality. To verify this hypothesis, the biophysical characteristics of migrating naïve and LPS activated neutrophils in a 3D planar gradient system are investigated. This dissertation consists of the following content.

In Chapter 2, a design and investigation of a custom planar gradient diffusion system is presented. The development of a direct-viewing chamber for chemotaxis studies, which allows one to overcome modern chemotaxis chamber obstacles based on time-lapse microscopy allows for measurement of cell forces in a 3D environment during cell migration. Using our system, we were able to successfully model diffusion

through 3D collagen matrices and calculate the coefficient of diffusion of Rhodamine through five different concentrations of collagen. This is important in understanding the biological responses of migrating normal and septic neutrophils.

In Chapter 3, with the use of the planar gradient diffusion system and a custom collagen-bead recipe, the chemotactic characteristics (force & strain generation, speed, chemotactic index and specific concentration) of naïve and LPS activated neutrophils were investigated. Human neutrophils were separated and where either used “as is” or “activated” using accepted protocols and embedded into 3D collagen matrices. With the use of quantitative time-lapse confocal microscopy techniques (including FIDVC), we were able to probe the biophysical chemotactic characteristic differences of naïve and LPS activated neutrophils. We were able to show that LPS activated neutrophils have greater deformation and displacement capabilities, while increasing their speed but decreasing their directionality. When looking at the signaling pathways between fMLP and LPS our research suggests that an investigation of bio-related molecules or chemical compounds that are affected by the activation of both G protein-coupled receptor(GPCR) and Toll-like receptor 4(TLR4) pathways may shed light into new strategies that combat sepsis.

Chapter 4 concludes the entire dissertation and provides recommendations for future work.

CHAPTER 2

Planar Gradient Diffusion System to Investigate Chemotaxis in a 3D Collagen Matrix

1. Introduction

The preferred movement of cells towards a concentration gradient, known as chemotaxis, plays an important role in pathological and physiological processes in the body. Such examples are skin and mucosa wound healing [64], morphogenesis [65], inflammation [16] and tumor growth [66, 67]. It has also been shown that cancer cells can migrate through both individual and collective cell-migration strategies [68]. Moreover, diffusional instability mechanisms can induce the separation of single or clustered cells from a tumorous body/object and then can immigrate towards a source of nutrients and thus invade wider areas and tissues [69].

More importantly, the maintenance of the human body's immunity and tolerance through organs and tissues of the organism are connected by migrating leukocytes (including neutrophils).

Analyses of migrating lymphocytes have been performed both in vivo and in vitro with the aim of increasing the knowledge about the molecular vehicles that control chemokine gradient sensing and migration of immune cells. It was found that different migration mechanisms can be active in 2D and in 3D, due to different roles of adhesion molecules [70]. Therefore, the improvement of physiologically pertinent in vitro assays to study cell motility and chemotaxis in a quantitative way is of growing interest and importance [71]. Due to the difficulty in analyzing cell responses, a comprehensive quantitative chemotaxis assay requires a laborious approach, based on the measurement of objective cell movement and on the application of transport phenomena models.

Past experimental approaches to investigate cell chemotaxis both qualitatively and quantitatively include the Boyden chamber [72] and the under agarose assay [73]. Within these early assays, cell migration could not be monitored as a function of time and concentration gradients were not well defined. They restrict observation of cell migration to two dimensions, do not allow one to monitor the dynamics of migration and do not sustain the signaling gradients for more than a few hours [74]. For example, the Boyden chamber, which is an endpoint assay, does not allow observation of chemotaxis visually and cannot directly distinguish chemotaxis (oriented locomotion) from chemokinesis (stimulated non-directed or random locomotion). Various confounding variables, including variations in the pore size and thickness of membranes, used in this assay further obscure the migratory response of cells to chemokines [75, 6].

Recently, microfluidic devices, usually fabricated with Polydimethylsiloxane (PDMS) by soft lithography [76, 77], have been proposed as a tool to observe cell behavior and motion under chemotaxis or interstitial flow conditions. In addition, the effect of shear stress can be investigated by exposing the cells to static or pulsating flows [78, 79]. Unfortunately, current microfluidic chemotaxis chambers also limit studies of cell migration to 2D substrates.

Another set of chemotaxis chambers, known as direct observation chambers, where the chemoattractant solution is in contact with a 3D gel containing cells have been also reported [68, 45]. A direct observation chamber consists of two compartments, containing chemoattractant and cells, respectively, connected side by side horizontally [80] or as concentric rings [81]. Another alternative technique produces patterns on the surface of a collagen gel via printing with a micropump that dispenses small droplets of solution at controlled rates [74].

Even with these material chemotaxis chambers, the physiologically relevant *in vitro* assay of cell chemotaxis would be achieved in a tissue-like collagen or fibrin gel, allowing for cell tracking and determination of the concentration gradient of the chemotactic factor within the gel, and be relatively simple to set up with significant reproducibility. An additional requirement would be able to measure dimensional

units for quantification (i.e. velocity, force). Most of these criteria have been fulfilled by the in vitro assay of leukocyte chemotaxis reported by Moghe et al. [82], where cells are initially dispersed throughout the gel rather than concentrated on the filter surface as in the Boyden chamber, thus minimizing cell-cell interactions and cell alteration of the chemotactic factor gradient. Furthermore, cell migration can occur over distances many times the cell dimension, as in physiological processes involving chemotaxis, and can be directly observed. Another advantage of this free diffusion assay is the spatial chemotactic factor concentration gradient decays over a few hours.

Researchers have also examined diffusivity through collagen membranes in dialysis cells, as well as the diffusion of tracer molecules through collagen samples subjected to hydrostatic pressure [83, 84, 85]. Some diffusion experiments in collagen gels rely on physical and chemical modifications of the gel using magnetic fields and chemical incorporation [86]. A popular method for modeling diffusivity in collagenous tissues relies on the fluorescence imaging of continuous point photobleaching. This method has revealed anisotropy in the diffusion coefficients of macromolecules in oriented collagenous tissues. Yet, photobleaching has been used in articular cartilage and not collagen matrices. While similar, the necessary modeling experiments must be carried through specifically understanding the diffusion coefficient of collagen gels. More importantly, a gel system that be utilized for TFM/DVC analysis.

The aim of the present work is the development of a novel direct-viewing chamber for chemotaxis studies. To overcome existing limitations of previous mentioned assays, time-lapse microscopy with motorized sample positioning and stage incubation, coupled with DVC image analysis techniques and a custom collagen recipe, we were able to measure cell forces in a 3D environment (See Table 2.1).

In this new assay presented in this work, a chemoattractant planer concentration gradient utilizing a 3D collagen gel sample, was generated via diffusion while the addition of fluorescence beads where dispersed, for the first time, to measure cell forces generated during migration. Mathematical analysis was used to measure the

TABLE 2.1. Obstacles that a 3D chemotaxis assay needs to overcome

Obstacle	References
Simple set up	Wilkinson (1998)
Reproducibility	Boyden (1962) & Moghe (1995)
Simple concentration gradient	Moghe (1995)
Force and dimensional measurements	Bonvin (2010) & LiJeon (2002)
Cell-cell interactions	Moghe (1995)
Direct-viewing chamber	Wells (2002)

diffusion coefficient through the 3D collagen matrix of different collagen densities and to quantitatively describe the diffusion gradient.

2. Methods & Materials

2.1. 3D Mold. The collagen mold was prepared using the Sylgard 184[®] Silicone Elastomer Kit (Dow Corning Corporation, Midland MI) and a Chamlide chamber (Live Cell Instrument, Seoul Korea) for the outer mold surface. In order to create a well in the middle of the mold, a custom machined aluminum metal cube with dimensions 10.07 mm x 3.95 mm x 5.99 mm was used.

2.2. Coverslip Preparation. For easy collagen manipulation hydrophilic and hydrophobic glass coverslips were used. Hydrophilic circular coverslips were created using a multi-step protocol. First, coverslips (Fisher Scientific, Pittsburgh PA) were cleaned with ethanol and immersed for 5 minutes in 0.5% 3-aminopropyltrimethoxysilane (Sigma-Aldrich Co, St. Louis MO) in ethanol. Second, the coverslips were rinsed with ethanol and placed in 0.5% glutaraldehyde (Polysciences Inc., Warrington PA) in distilled water for 30 minutes. Lastly, the coverslips were rinsed with deionized water and dried at room temperature. Hydrophobic square coverslips were created as well. The 22 mm x 22 mm coverslips (Gold Seal, Thomas Scientific, Wedesboro NJ) were

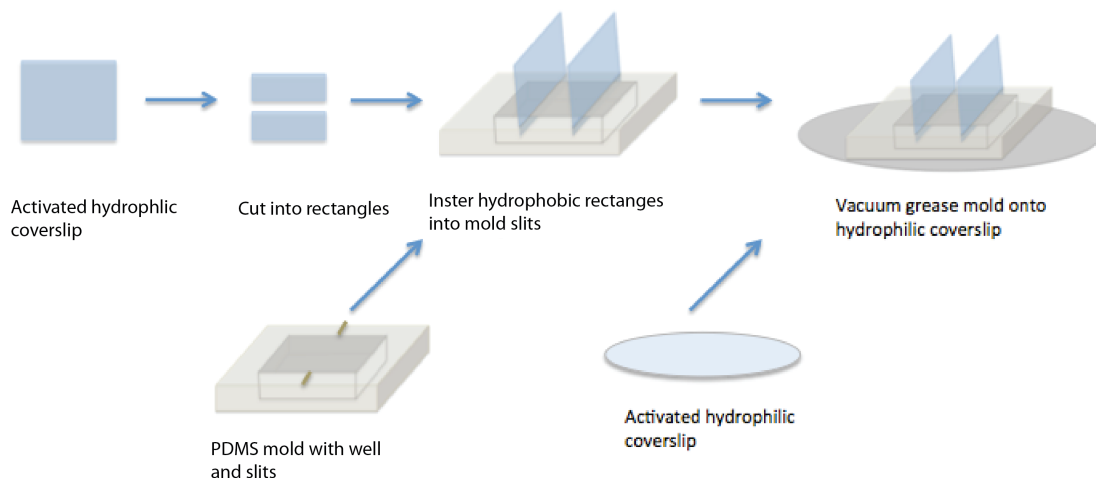


FIGURE 2.1. Activated hydrophobic coverslips were cut into rectangles and inserted into the Sylgard 184[®] Silicone mold. Vacuum grease was added to the bottom of the molds and pressed onto a hydrophilic coverslip.

immersed for 5 minutes in a hexane solution containing 2.5% tridecafluoro-1,1,2,2-tetrahydrooctyl (Gelest, Morrisville PA) and 0.5% acetic acid. The coverslips were then removed from the solution and left to dry at room temperature.

2.3. Mold Assembly. Hydrophilic circular coverslips were used as the base of the mold. The hydrophilic surface attracted the water in the collagen and immobilized the collagen on the slip. Additionally, the attraction between the hydrophilic surface and collagen served to prevent the diffusion of dye or chemotactic factor beneath the collagen. The square hydrophilic coverslips were cut into rectangles slightly larger than 3.95 mm x 5.99 mm using a high-precision diamond-scribing tool (Lunzer, New York NY). The rectangles were slid into slots cut into the silicone mold using an exacto-knife. The slits were spaced 275 mm apart from one another on each side of the silicone mold. The coverslips created a well on each side of the collagen. Their hydrophobicity prevented the collagen from being attracted to the glass and facilitates their removal for imaging and the diffusion experiments. The bottom of the silicone

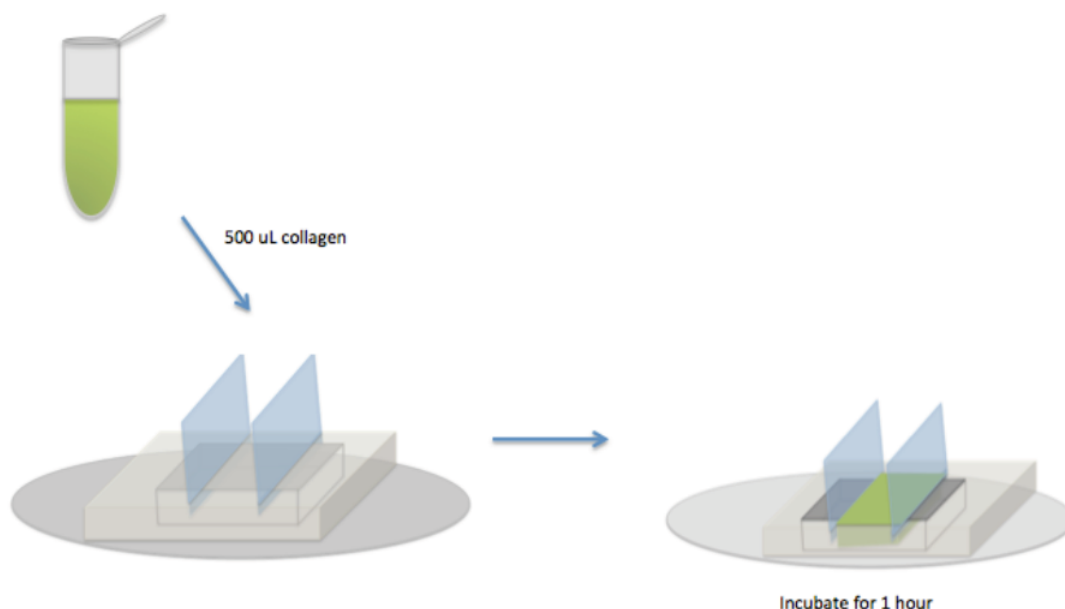


FIGURE 2.2. Collagen solution was prepared according to Table 2.1 where the volume was removed from the tube and pipetted into the center well of the planar gradient diffusion system mold.

mold was coated with a thin layer of vacuum grease and then pressed onto the circular coverslip to create a seal.¹

2.4. Collagen Mixture. Collagen was prepared by following the general protocol provided by BD Sciences. 10x phosphate buffered solution (PBS) (Gibco, Thermo Fisher Scientific Inc, USA) was mixed and vortexed with 1N sodium hydroxide (NaOH) (Sigma-Aldrich Co, St. Louis MO). Collagen I, rat tail (BD Biosciences, San Jose CA) was kept on ice and subsequently added to the solution, which was then vortexed. Next, yellow-green fluorescent carboxylate-modified microspheres (Invitrogen, Thermo Fisher Scientific Inc, USA) were added and vortexed. Lastly, Leibovitz's L-15 (phenol free) with 2 mg/ml glucose (Life Technologies, Thermo Fisher Scientific Inc, USA) was added in and the solution was vortexed. Once the solution was prepared, the volume was removed and placed into the center well of the prepared mold (Figure 2.2) and was then incubated for 40 minutes at 37° Celsius. Summary of collagen mixture for different collagen densities can be found in Table 2.2.

¹ See Figure 2.1 for a step-by-step summary

TABLE 2.2. Custom collagen mixture adapted for the addition of fluorescent beads and cell viability

Collagen Gel Density	PBS	Fluorescent Beads	NaOH	Collagen I Rat-Tail	L-15
2.5 mg/ml	10%	6%	2%	70.2%	11.8%
2.2 mg/ml	10%	6%	2%	61.7%	20.3%
2.0 mg/ml	10%	6%	2%	56.1%	25.9%
1.5 mg/ml	10%	6%	2%	42.1%	39.9%
1.0 mg/ml	10%	6%	2%	28.1%	53.9%

2.5. Diffusion Imaging. In order to image the 3D collagen matrices, a Nikon Eclipse AR1 confocal system mounted on an inverted microscope, equipped with a motorized stage and phase contrast was utilized. An argon laser (488 nm) was used to capture the fluorescence. Fields of view were 512 x 512 pixels with an area of $11,560.5 \mu\text{m}^2$. Each sample was imaged every 2 or 3 seconds for up to 7 hours. A 2 or 3 second increment was selected based on the velocity of dye diffusion through the gel that was obtained through previous experiments. These initial experiments were performed for the optimization of data collection.

Prior to imaging, the hydrophobic cover slips were removed carefully using tweezers, so as to not disrupt the edge of the collagen. Gel did not expand to fill the well because it was polymerized and diffusion could still exist on both sides. After removal of the slips, the collagen was placed under the microscope to ensure that the sides were still straight to allow for a planar diffusion gradient. Any samples in which the side of the collagen was disrupted were discarded. Next, one well was filled with 100 μL of Leibovitz’s L-15 (phenol free) with 2 mg/ml glucose. Afterwards, the other well was filled with 100 μL of 5 μM Rhodamine (Sigma-Aldrich, St. Louis MO) diluted in deionized water. Precaution was taken to ensure that the well was filled with 100 μL and did not overflow onto the top of the collagen (Figure 2.3). Immediately after

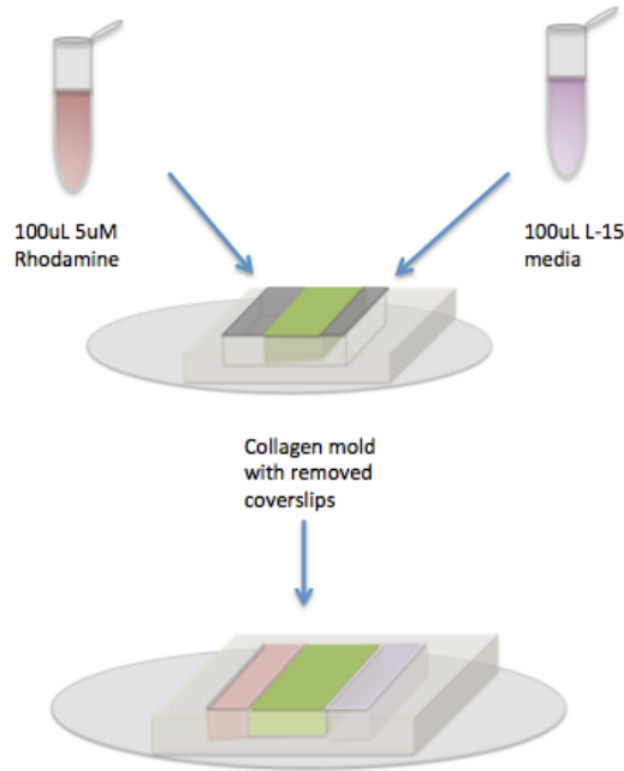


FIGURE 2.3. Planar gradient diffusion system mold assembly with collagen solution ready for the addition of Rhodamine or chemoattractant and L-15 which acted as a concentration sink. System was imaged immediately after using a Nikon Eclipse A1 confocal system with an argon laser (488 nm). Fields of view were 512×512 pixels with an area of $11,560.5 \mu\text{m}^2$ where each sample was imaged every 2 or 3 seconds for up to 7 hours.

adding the Rhodamine, imaging commenced. This procedure was conducted for 5 collagen densities (1.0, 1.5, 2.0, 2.2 and 2.5 mg/ml).

2.6. Mathematical Model & Data Analysis. A mathematical diffusion model was created to find the diffusion coefficient for each gel. Below is a step-by-step calculation for the mathematical model:

Using a partial differential diffusion equation (PDE) to model the diffusion processes and considering the diffusion of molecules through a rectangular matrix, where the

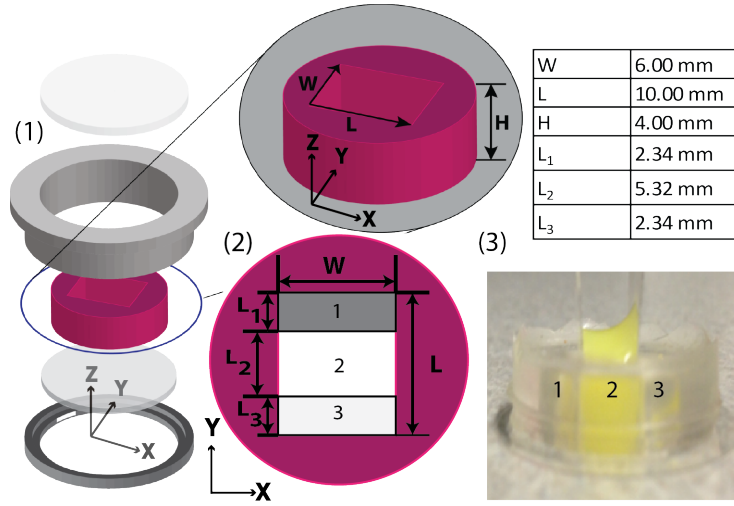


FIGURE 2.4. Schematic of planar gradient diffusion system mold assembly in Chamblide chamber. (1) System consists of (from bottom to top) a bottom housing, a hydrophilic 22 mm coverslip, PDMS mold, top housing, and a glass cover. (2) Dimensions of PDMS mold. (3) Mold with collagen matrix with hydrophobic coverslips on each side to create the 3 well system. Before cell migration experiments, hydrophobic coverslips are removed and well 1 filled with Leibovitz's L-15 (phenol free) with 2 mg/ml glucose (and 40 ng/ml LPS solution for activation experiments), and well 3 filled with fMLP diluted into Leibovitz's L-15 (phenol free) with 2 mg/ml glucose for a final concentration of $1 \mu\text{M}$ (and 40 ng/ml LPS solution for activation experiments).

two ends are kept at constant concentration, C_1 and C_2 , the PDE is:

$$C_t = \alpha^2 C_{xx} \quad 0 < x < L \quad 0 < t < \infty \quad (2.1)$$

where α^2 is the diffusivity of the material. With the following boundary conditions:

$$C(0, t) = C_1 \quad C(L, t) = C_2 \quad 0 < t < \infty \quad (2.2)$$

And initial conditions:

$$C(x, 0) = \phi(x) \quad 0 \leq x \leq L \quad (2.3)$$

However the boundary conditions are not homogeneous; since this is a necessary condition for separation of variables, one can assume the solution will have a steady-state solution that varies linearly between C_1 and C_2 and a transient component with homogeneous boundary conditions. Therefore it is reasonable to describe the

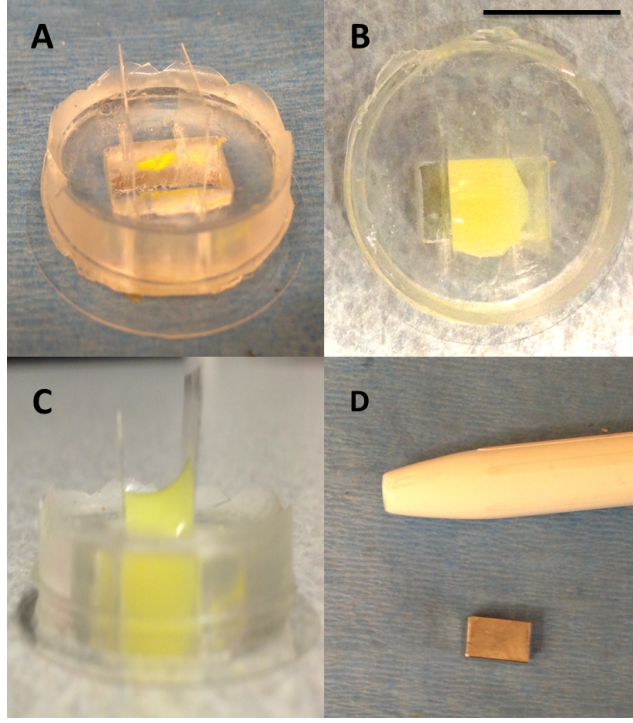


FIGURE 2.5. (A) The planar gradient diffusion system mold fully assembled with a hydrophilic cover slip on the bottom and 2 cut hydrophobic slits to create the three reservoirs. A top (B) and (C) bottom view of the system with polymerized collagen in the center reservoir. Scale bar = 10 mm. (D) Custom machined metal cube to make the negative mold for the reservoirs.

concentration profile as:

$$C(x, t) = \text{steadystate} + \text{transient} \quad (2.4)$$

$$C(x, t) = \left[C_1 + \frac{x}{L} (C_2 - C_1) \right] + \hat{C}(x, t) \quad (2.5)$$

With the goal of finding the transient solution, $\hat{C}(x, t)$, substituting into Equations (2.2 & 2.3). Thus yielding:

$$\alpha^2 \hat{C}_{xx} = \hat{C}_t \quad 0 < x < L \quad (2.6)$$

With now homogeneous boundary conditions:

$$\hat{C}(0, t) = C(0, t) - C_1 \quad \hat{C}(L, t) = C_2 - [C_1 + (C_2 - C_1)] \quad (2.7)$$

$$\hat{C}(0, t) = C_1 - C_1 = 0 \quad \hat{C}(L, t) = C_2 - C_1 - C_2 + C_1 = 0 \quad (2.8)$$

$$\hat{C}(0, t) = 0 \quad \hat{C}(L, t) = 0 \quad (2.9)$$

And,

$$\hat{C}(x, 0) = \phi(x) - \left[C_1 + \frac{x}{L} (C_2 - C_1) \right] \quad (2.10)$$

Assuming that $\hat{C}(x, t)$ is a product of two functions, one depending only on x and the other depending only on t :

$$\hat{C}(x, t) = X(x) T(t) \quad (2.11)$$

Therefore substituting for $\hat{C}$, the differential equation yields:

$$\alpha^2 X'' T = X T' \quad (2.12)$$

For the above equation to be valid, it is necessary that both sides of the equation are equal to the same constant, λ , thus the equation becomes:

$$\frac{X''}{X} = \frac{1}{\alpha^2} \frac{T'}{T} = -\lambda \quad (2.13)$$

This produces two ordinary differential equations:

$$X'' + \lambda X = 0 \quad (2.14)$$

$$T' + \alpha^2 \lambda T = 0 \quad (2.15)$$

Substituting in the boundary conditions the following eigenfunctions are formed:

$$X_n(x) = \sin(n\pi x/L) \quad n = 1, 2, 3... \quad (2.16)$$

which are associated with the following eigenvalues:

$$\lambda_n = \frac{n^2 \pi^2}{L^2} \quad (2.17)$$

and substituting for λ one obtains:

$$T' + (n^2 \pi^2 \alpha^2 / L^2) T = 0 \quad (2.18)$$

By combining each separated solution and neglecting arbitrary constants of proportionality; one can conclude:

$$\hat{C}_n(x, t) = e^{-n^2 \pi^2 \alpha^2 t / L^2} \sin(n\pi x/L), \quad n = 1, 2, 3... \quad (2.19)$$

Using a general linear combination, in the form of the following infinite series:

$$\hat{C}_n(x, t) = \sum_{n=1}^{\infty} a_n u_n(x, t) = \sum_{n=1}^{\infty} a_n e^{-n^2 \pi^2 \alpha^2 t / L^2} \sin\left(\frac{n \pi x}{L}\right) \quad (2.20)$$

Where a_n is:

$$a_n = \frac{2}{L} \int_0^L f(x) \sin\left(\frac{n \pi x}{L}\right) dx \quad (2.21)$$

Therefore the final solution becomes:

$$C(x, t) = \left[C_1 + \frac{x}{L} (C_2 - C_1) \right] + \sum_{n=1}^{\infty} a_n e^{-n^2 \pi^2 \alpha^2 t / L^2} \sin\left(\frac{n \pi x}{L}\right) \quad (2.22)$$

Using the mathematical solution shown above, a MATLAB (Mathworks[®], Natick, MA) code was generated for processing and analysis.² Images (Figure 2.7) were imported into MATLAB and loaded into a movie then rescaled and normalized by the maximum intensity. A filter was used to smooth or blur images that were too pixelated. A gray color-map of the image was utilized to select the edge and the axis along which the diffusion gradient would be calculated. For all concentrations, an axis was picked along the center of the image, to the right of the center and to the left of the center. The normalized intensity was plotted along with the true intensity and the difference between the two. A plot of the data points selected was generated on a gray color-map image of the sample (Figure 2.6) The parameters necessary for fitting were inputted (concentration, time, length). The concentration of the Rhodamine was 5 μ M, the time interval (dt) was either 2 or 3 seconds, the length of the sample was 5.32 mm, and the viewing area was .01 mm². Plots of the normalized concentration versus time was then generated. The concentration profile across x was plotted, and a polynomial was fit to the data (Figure 2.6). This procedure was conducted for all 5 collagen densities (1.0, 1.5, 2.0, 2.2 and 2.5 mg/ml).

² Codes can be found in Appendix A of this work

3. Results & Discussion

Figure 2.8 represents the representative plots of the diffusion of Rhodamine through the five tested collagen concentrations. The shapes of the diffusion plot match the traditional shape of a linear diffusion model. It can be seen diffusion of Rhodamine is inversely proportional to the concentration of collagen; the lower the collagen density the faster the diffusion. This is to be expected, as this concentration of collagen has a higher porosity. The horizontal lines in the collagen diffusion plot at a concentration of 1.5 mg/ml (Figure 2.8B) are simply the result of over saturation of the tracer signal. More importantly, as shown in Figure 2.8, each line represents a specific point (x, y) inside the collagen, to where as time increases the normalized concentration never exceeds the previous point at the same time (dt), indicating no reversal of the chemical. This is an important characteristic of a chemotaxis chamber to allow direct flow of a chemoattractant without reverse flow (a goal set out by the researchers).

Figure 2.8 contains a representative plots of the normalized concentration of the Rhodamine at various locations across the x-axis of the collagen molds. By examining the last ten images at each x location, we were able to generate a plot of the steady-state concentration of Rhodamine at each of the locations. By extrapolating across the entire sample length (Figure 2.8), we were able to look at the whole sample. This indicates that the linear diffusion gradient is consistent across the entire sample length. According the equation 2.22 at steady state, we expect to have a linear concentration profile across the x values. By fitting a linear function to the points, we see that they are all, in fact, linear. The R^2 value of fitting a linear trend line for each of the best fit lines for the three collagen concentrations were at or above 0.98. The norm of the residuals in all extrapolated plots were .0227, indicating a good fit. This linear relationship is in agreement with our analytical solution given by equation 2.22. The concentration does not begin at 100%, as would be expected, because the imaging does not begin directly at the edge of the collagen sample. It is also impossible to perfectly calibrate the imaging.

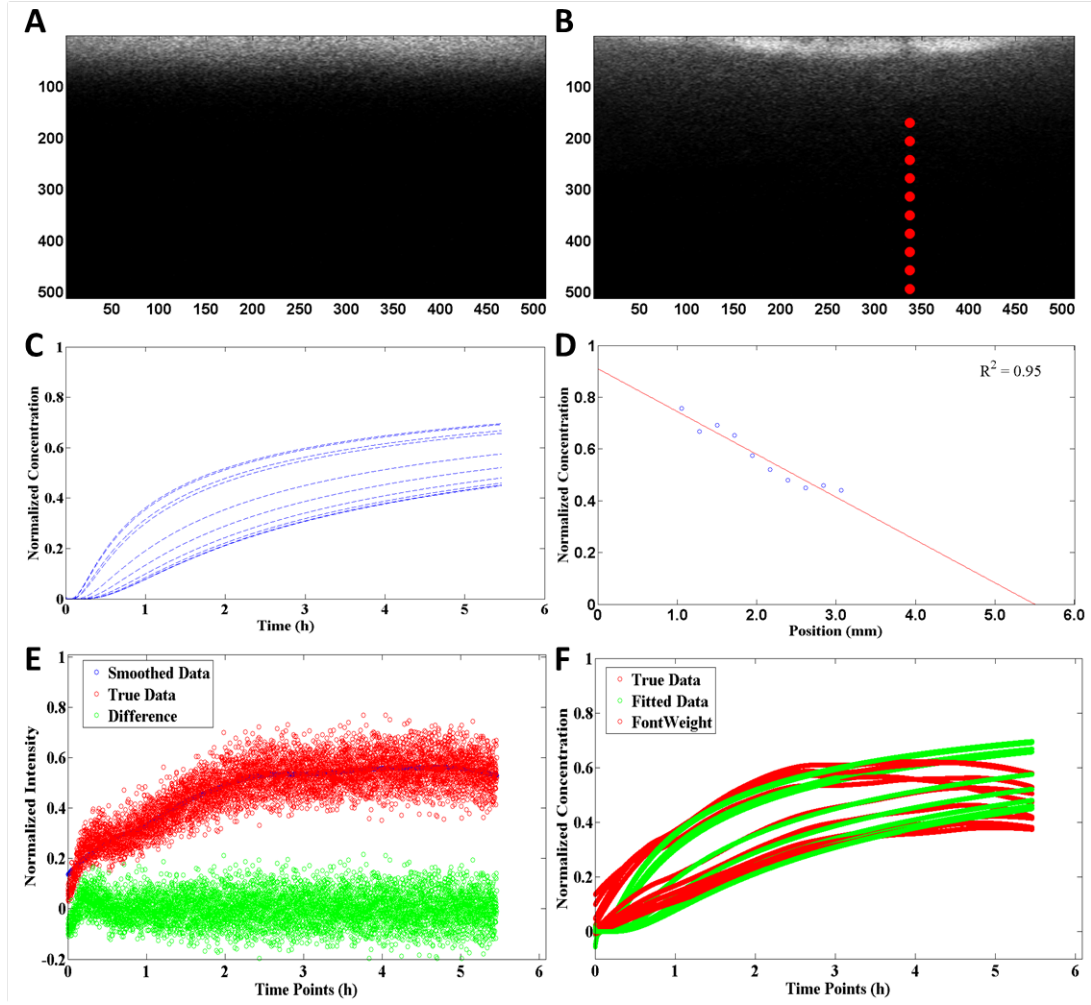


FIGURE 2.6. Analysis and summary of diffusion calculations generated by MATLAB code for determining diffusion characteristics in planar gradient diffusion system with a 2.5 mg/ml collagen concentration. (A) Black and white rendering of initial ($dt = 0$) image uploaded into MATLAB for analysis. White color is the Rhodamine where black indicates the collagen (x and y axes are in pixels). (B) Specific points chosen by the user for intensity comparison and for diffusion calculations (x and y axes are in pixels). (C) Diffusion profile of Rhodamine throughout the collagen matrix where each line correlates to a red point in image B. (D) Concentration profile of Rhodamine throughout the collagen matrix at steady-state. Red line was constructed using data points and extrapolating best line-fit (with R^2 value shown) to show Rhodamine concentration throughout the whole collagen matrix. (E) Comparison between the smoothed data (blue dot) and true data from diffusion experiment (red dot) with difference between smoothed and true (green dot). (F) Normalized concentration of true (red dot) and fitted (green dot) data from MATLAB code throughout the experiment.

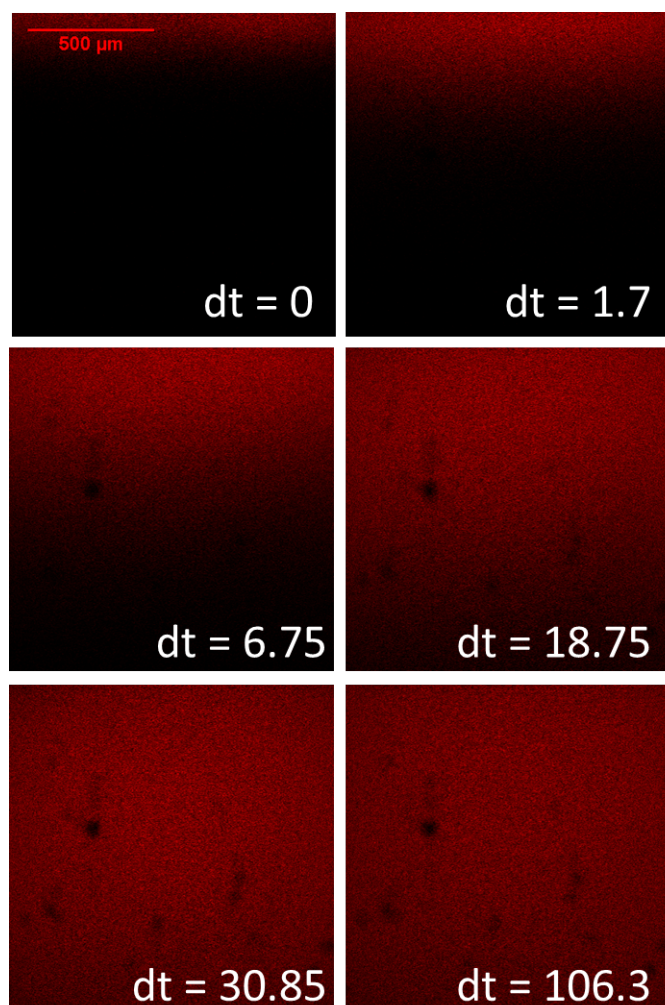


FIGURE 2.7. Images taken during Rhodamine diffusion experiments in the planar gradient diffusion system with a 1.0 mg/ml collagen concentration. A Nikon Eclipse A1 confocal system mounted on an inverted microscope, equipped with a motorized stage and phase contrast was utilized with a diode laser (488 nm) to capture the microsphere fluorescence. Fields of view were 512 x 512 pixels with an area of 11,560.5 μm^2 with imaging capture every 3 seconds. Scale bar = 500 μm where figure dt are represented in minutes.

Running the analysis at several different x-locations on each sample, it is possible to calculate the average coefficient of diffusion for Rhodamine in the various concentrations of collagen, as reported in Table 2.3. These results are consistent with previous research—coefficient of diffusion through the collagen gel decreases as the concentration of collagen decreased due to an increase of porosity in the matrix.

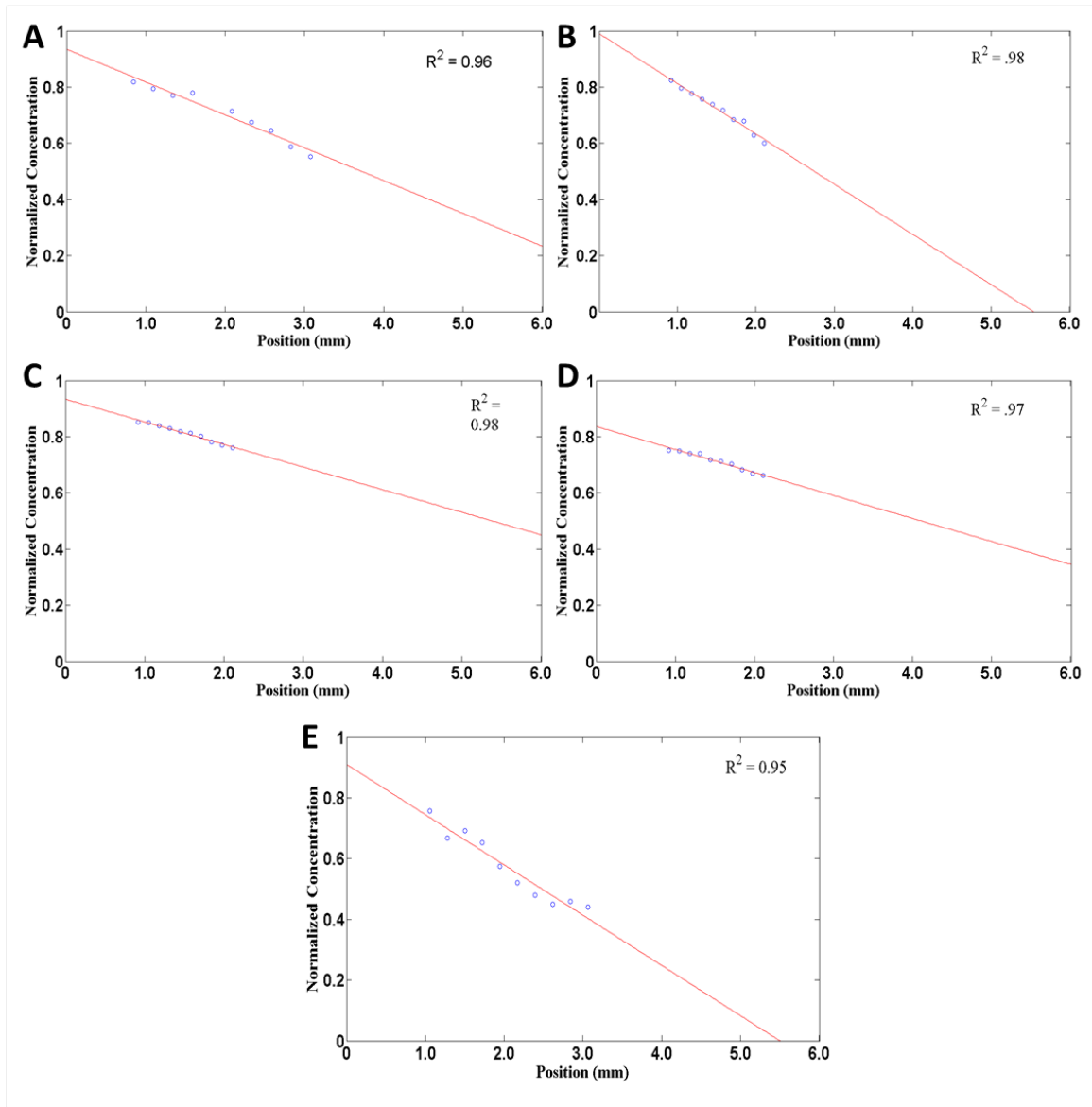


FIGURE 2.8. Concentration (normalized) profile of Rhodamine throughout the collagen matrix using the planar gradient diffusion system at steady-state. Circles are specific data points chosen though the MATLAB code by the user. Red line was constructed using data points and extrapolating best line-fit (with R^2 value shown) to show Rhodamine concentration throughout the whole collagen matrix. Collagen matrix at a density of (A) 1.0 mg/ml, (B) 1.5 mg/ml, (C) 2.0 mg/ml, (D) 2.2 mg/ml and (E) 2.5 mg/ml.

TABLE 2.3. Coefficients of diffusion

Concentration of Collagen Gel	Coefficient of Diffusion ($\frac{cm^2}{sec}$)
2.5 mg/ml	$1.03E-6 \pm 2.54E-7$
2.2 mg/ml	$1.28E-6 \pm 3.53E-7$
2.0 mg/ml	$6.48E-6 \pm 1.66E-7$
1.5 mg/ml	$1.05E-5 \pm 2.04E-7$
1.0 mg/ml	$1.39E-5 \pm 1.06E-7$
Water	$1.50E-5$

Additionally, we were able to reference other papers that looked at diffusion in gels to compare results. Shenoy and Rosenblatt (1995) found the diffusion coefficient of BSA (radius = 4 nm) in a 30 mg/ml succinylated collagen solution to be $2.2 \times 10^{-7} cm^2/sec$. The radius of Rhodamine is .78 nm, which explains its faster diffusion time through collagen [87]. In diffusion studies, it is common to utilize the radius to compare molecules. As radius increases, the molecular weight also increases. Ramanujan et al. (2002) found the diffusion coefficient of Dextran (radius = 6 nm) in polymerized 20% collagen gels (equivalent of 2.0 mg/ml concentration gels) to be $4.72 \times 10^{-8} cm^2/sec$ [84]. Again, this is comparable to the diffusion coefficient we observed for the 2.0 mg/ml concentration gel ($D = 1.28 \times 10^{-6} cm^2/sec$), if we take into account the molecule size difference. Leddy and Guilak (2003) studied the diffusion of Dextrans through articular cartilage. It is possible to compare diffusion through collagen matrices and through articular cartilage because approximately two-thirds of the mass of articular cartilage is polymeric collagen (predominately type II). They found the diffusion coefficient of 3 kDa Dextran through articular cartilage to be between $6-10 \times 10^{-7} cm^2/sec$.

4. Conclusion

The aim of the present work is the development of a well-controlled planar diffusion system to measure cell forces in a 3D environment during chemotaxis. Here we present

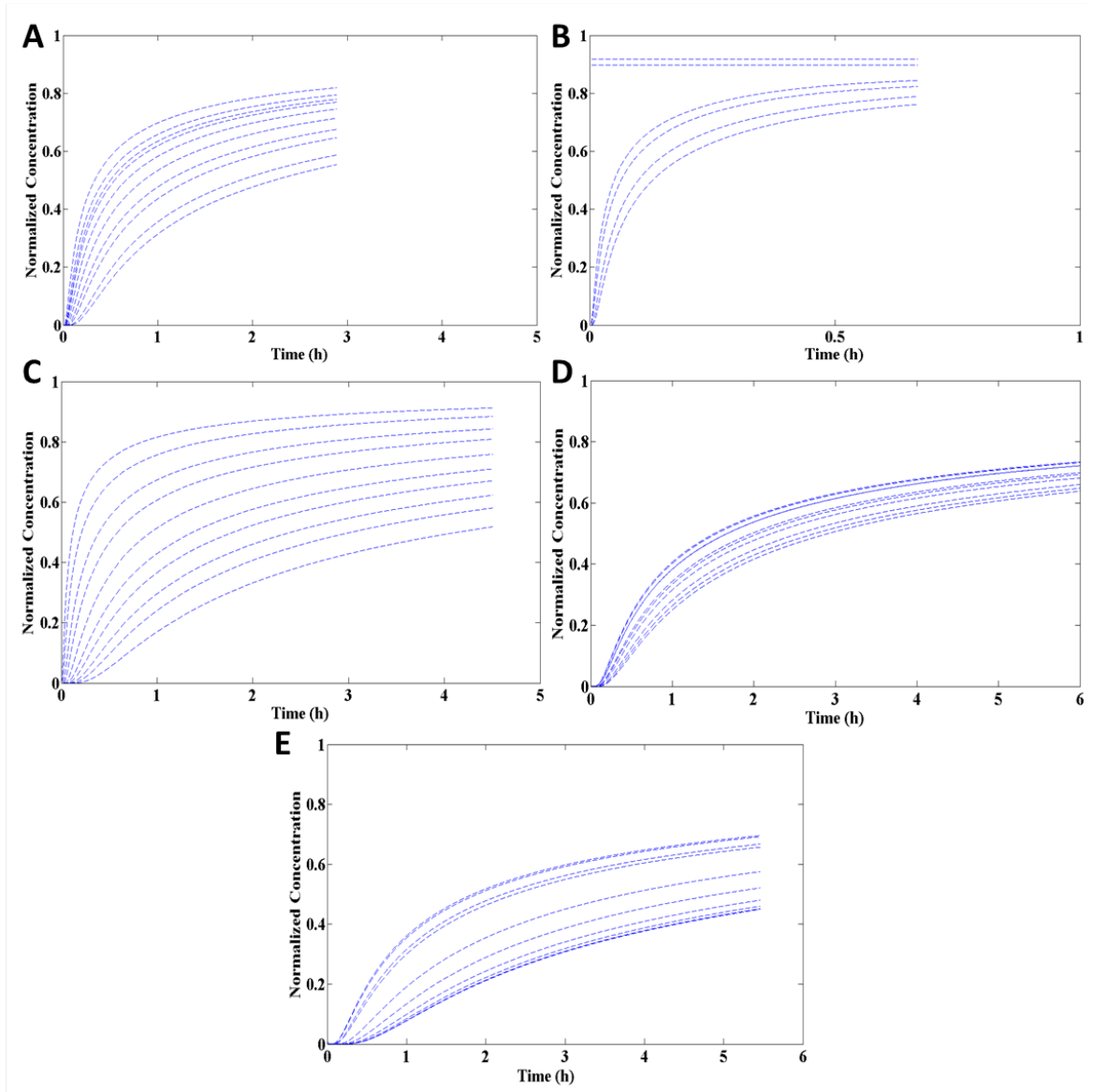


FIGURE 2.9. Diffusion profile of Rhodamine throughout the collagen matrix using the planar gradient diffusion system at different points. Each line indicates a different selected data points chosen from the MATLAB code by the user. Collagen matrix at a density of (A) 1.0 mg/ml, (B) 1.5 mg/ml, (C) 2.0 mg/ml, (D) 2.2 mg/ml and (E) 2.5 mg/ml.

a newly developed simple system that can be used for TFM/FIDVC experiments. Using this system, we were able to successfully model diffusion through 3D collagen matrices. In our experimental set-up, we were able to verify, by fitting the data to analytical expressions, that the planar gradient diffusion system, when seeded with

TABLE 2.4. Obstacles that a 3D chemotaxis assay needs to overcome and how the planar gradient diffusion system meets them

Requirements	Planar gradient diffusion system
Simple set up	A PDMS mold that can be used multiple times over different collagen densities
Reproducibility	Ability to use same mold and system over and over again
Simple gradient	Dual chamber system to create a planar gradient in 3D
Cell-Force and dimensional measurements	Addition of fluorescence beads homogeneously dispersed in 3D collagen matrix to calculate cell traction forces in the X, Y, Z axes
Direct-viewing chamber	Mold is open on all axes during collagen polymerization and imaging

cells, should promote chemotaxis and not chemokinesis. We were able to calculate the coefficient of diffusion for collagen of five different concentrations. The coefficient of diffusion in 1.0 mg/ml collagen was closest to the coefficient of diffusion of water, as expected. The coefficient of diffusion was lowest in 2.5 mg/ml collagen ($D = 1.03 \times 10^{-6} cm^2/sec$).

CHAPTER 3

Characteristics of Naïve & LPS Activated Neutrophil Force Generation During Chemotaxis

1. Introduction

Neutrophils are critical components of the innate immune response to an infectious attack on its host.¹ More importantly, deficiencies of neutrophil function—namely, migration—have been linked to increased frequency and severity of bacterial and fungal infections [88]. In some diseases associated with a high susceptibility to infection, such as diabetes [89] and AIDS [90], an impairment of neutrophil migration is also observed. Since neutrophil function is essential in avoiding bacterial dissemination to the circulation, it can be postulated that neutrophil migration plays a significant role in fighting off these infections.

During migration, neutrophils generate forces on their environment. When a neutrophil adheres to the endothelial wall of a blood vessel, the neutrophil exerts a force into the wall. During transmigration, Rabodzey et al. [91] showed neutrophils generate forces three times higher than the forces generated by an adherent neutrophil not transmigrating. Smith et al. [47] showed neutrophils migrate in a sequence largely reverse of what has been seen in strongly adherent, slow moving cells such as fibroblasts [92].

Furthermore, neutrophil motility seems to be organized and initiated in the uropod, leading to forced lamellipodial polymerization and then the adhesion of the lamellipodia. Other than the leading edge pulling the cell, the rear is anchoring the cell and serving as a locus of force generation. Investigating these forces during migration, may shed light on cell motility during infections (Figure 3.1).

¹ See Ch. 1 & 2

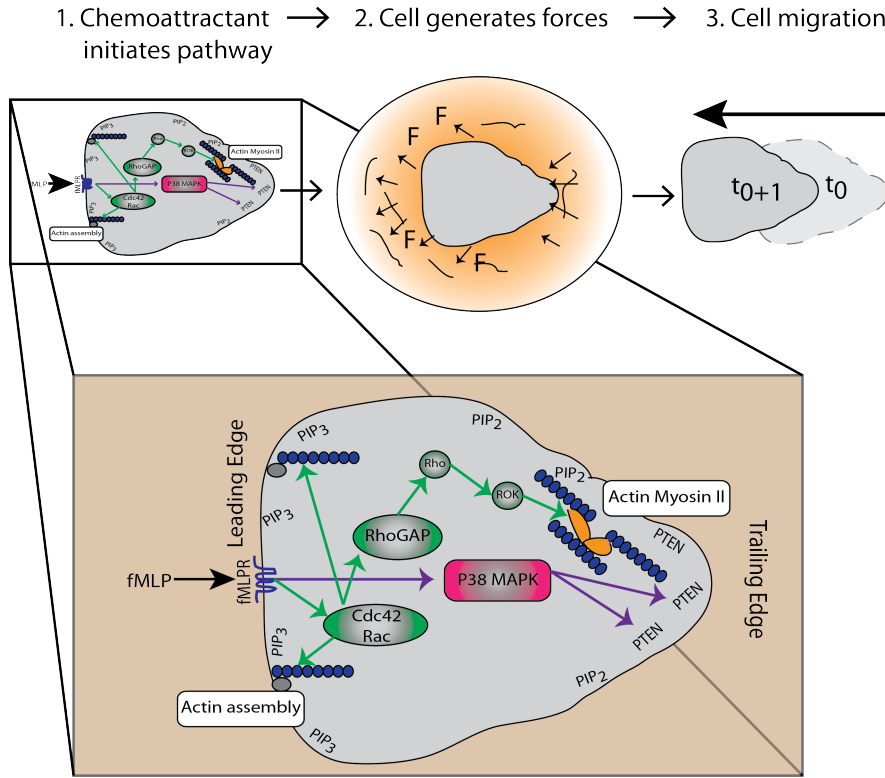


FIGURE 3.1. Through specific biological pathways, a chemoattractant can initiate a process which will ultimately create cell migration through cell force generation. These signaling components may function as the compass responsible for setting up the polarity that drives directed cell movement. Healthy neutrophils can generate forces through the G protein-coupled receptor (GPCR) pathway. For example, at the front, fMLP acts through guanine nucleotide exchange factors (GEFs) to activate both Cdc42 and Rac. The activated Cdc42 and Rac initiates an amplification loop by stimulating phosphatidylinositol 3-kinase (PI3K) which phosphorylates the membrane lipid phosphatidylinositol-3,4-bisphosphate (PIP2) into phosphatidylinositol-3,4,5-triphosphate (PIP3). The localized PIP3 together with Cdc42 and Rac, then act together to assemble actin in the pseudopod. At the rear of the cell, the fMLP receptor activates Rho that then stimulates Rho kinase (ROK) to induce contraction of the uropod. Simultaneously, p38 mitogen-activated protein kinase (MAPK) is initiated to mediate the phosphatase and tensin homolog (PTEN) to the sides and back of the cell, thereby converting PIP3 back to PIP2 in areas of the cell away from the leading edge [37].

Exploring cell force generation in sepsis may assist in understanding how to combat the infection. Recent studies in sepsis show the failure of neutrophil migration is associated with increased number of bacteria in peritoneal exudates and blood,

followed by systemic inflammation and therefore a reduction in the survival rate of patients [93, 94]. This failure of neutrophil migration is not a consequence of chemotactic mediator loss since there are high levels of chemotactic mediator at the infection [93, 95]. Even more frightening, is during sepsis² the neutrophils can injure host tissues and cause organ failure when excessive—a major cause of death in patients with sepsis [95, 96, 97].

Currently, the concept of neutrophil-mediated organ failure can have its origin when high levels of proinflammatory molecules are released due to infectious microbes where the host immune system causes hyperactivation of neutrophils [98]. With systemic activation, neutrophils accumulate in vascular beds remote from the site of infection or tissue injury rather than at the local foci of the infection source. The hyperactive neutrophils release proteolytic enzymes and reactive oxygen species (ROS) upon contact with the tissues they are designed to protect rather than after direct microbial contact. The release of the cytotoxic substances by the hyperactive neutrophils acting away from the source of infection or injury causes tissue and vascular damage which can progress to organ failure and death (Figure 3.2).

Unfortunately, this concept of neutrophil-mediated organ failure is not the complete story. Studies of neutralization of proinflammatory mediator or prevention of hyperactivation by blocking the response of neutrophils to bacterially-derived activating molecules has not produced improvement in the clinical course of the disease or affected morbidity or mortality [99, 100, 101]. Therefore, the complete understanding of how neutrophils are impacted by sepsis and their associated migration is lacking. Furthermore, biophysical involvement in neutrophil migration (healthy or sepsis), particularly in chemotaxis, is not completely understood.

To address these questions, the aim of the present work is to investigate the force generating pathway characteristics of migrating cells between normal and sepsis neutrophil chemotaxis—via a reductionist approach of LPS activation—using a custom

² See Ch. 1.2

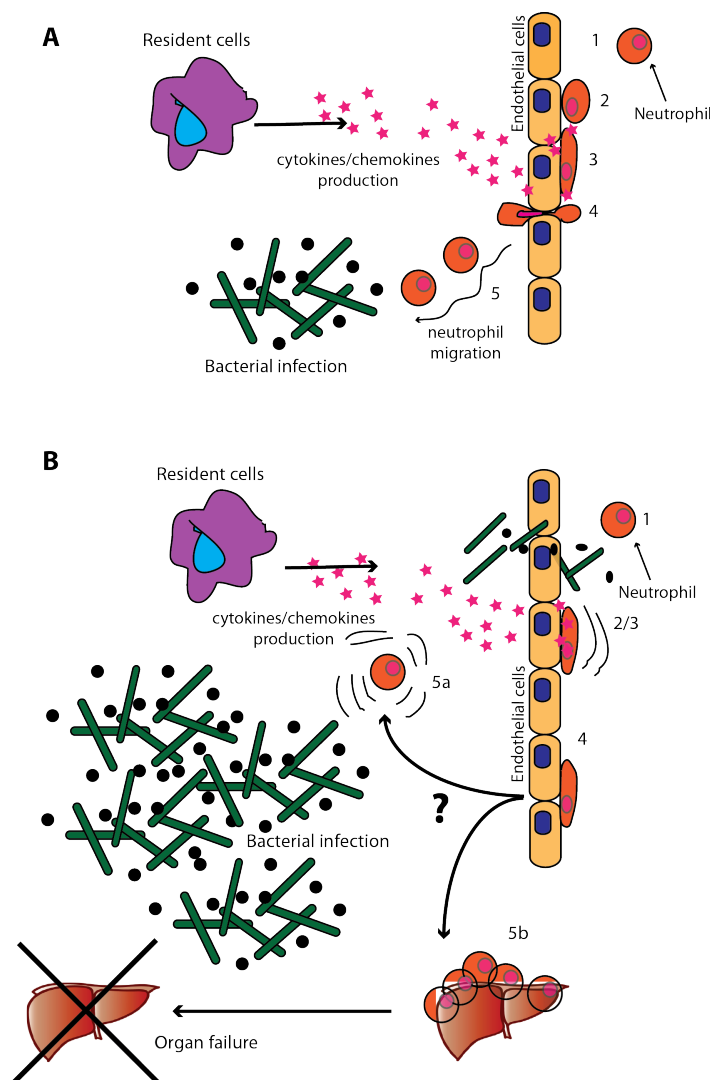


FIGURE 3.2. Before neutrophils can eliminate microorganisms they must migrate from the blood stream to the source of infection. During (A) normal migration, five distinct phases of neutrophil migration to focus site; (A,1) mobilization or capture, (A,2) rolling, (A,3) adhesion and spreading, (A,4) transmigration through the vessel wall and (A,5) extravasation in response to a chemotactic gradient. In (B) sepsis, neutrophil migration is not clearly understood. Neutrophils sense a chemotactic gradient but are also bombarded with foreign bodies in the blood stream. Sensing a gradient or foreign body, (B,1) mobilization or capture will not occur. (B,2/3) Rolling, adhesion and spreading does not always occur. For some neutrophils (B,4) transmigration might not occur once spreading is complete. Once transmigration occurs, dysregulation may occur, resulting in (B,5a) impaired directed migration of neutrophils to infectious foci or (B,5b) deleterious accumulation of neutrophils within remote vital organs, leading to collateral tissue damage and ultimately multiple-organ failure.

planar gradient diffusion system³ coupled with 3D FIDVC techniques for force analysis [63].

2. Methods & Materials

2.1. Reagents. Histopaque 1077, Dextran (average molecular weight, 400 - 500 kDa), formyl-methionyl-leueyl-phenylalanine (fMLP), and dimethyl sulfoxide (DMSO), 0.5% acetic acid, 3-aminopropyltrimethoxysilane, and sodium hydroxide (NaOH) powder were purchased from Sigma-Aldrich; Leibovitz's L-15 medium, Hank Balanced Salt Solutions (HBSS), Phosphate-Buffered Saline (PBS) 10x at 7.4 pH, Vybrant DiI (1,1',di-octadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate), Carboxylate-Modified Microspheres size 0.5 μm (Yellow-Green Fluorescent (505/515) at 2% solids), and glucose were purchased from Invetrogen; PDMS (Sylgard 184 silicone elastomer kit) was purchased from Dow Corning Corporation; Collagen I, rat tail was purchased from BD Biosciences; 2.5% (tridecafluoro-1,1,2,2-tetrahydrooctyl) was purchased from Gelest; 0.5% glutaraldehyde was purchased from Polysciences, Inc.; ultrapure LPS was purchased from Sigma-Aldrich; 10% human serum was purchased from Sigma-Aldrich.

2.2. Cell Preparation. Neutrophils were isolated from healthy human volunteers by collection into ethylenediaminetetraacetic acid-containing Vacutainer tubes (BD Biosciences). Histopaque was used for the initial cell separation followed by gravity sedimentation through 3% Dextran. Contaminating erythrocytes were removed by hypotonic lysis, yielding a neutrophil purity of more than 95%. Neutrophils were suspended Hanks Balanced Salt Solution (without $\text{Ca}^{2+}/\text{Mg}^{2+}$) on ice until use in the experiments. Institutional Review Board approval was obtained from the Rhode Island Hospital's Committee on Protection of Human Subjects to allow donation of venous blood from normal, healthy donors from isolation of peripheral blood neutrophils. In addition, informed consent was obtained in accordance with the Declaration of Helsinki.

³ See Ch. 2

Human neutrophils in HBSS (-/-) at a concentration from $10^6 - 2 \times 10^6$ cells/ml were incubated with membrane dye Vybrant DiI at a final concentration of $2 \mu\text{M}$ for 25 min at room temperature in the dark [102]. After 25 min, the cells were centrifuged for 4 min at 700 RPM and resuspended in Leibovitz's L-15 medium with 2 mg/ml glucose added for a final concentration of 10^6 cells/ml.

Before cell migration experiments, two control experiments were completed to 1) assess Vybrant DiI affect on cell migration, and 2) collagen concentration recipe exactness. When LPS activated cell migration experiments were performed a third and forth control experiments were completed—first two control experiments were performed with normal cells and then again with activated cells (Table 3.1). For the point-tip test, non-stained and Vybrant DiI stained cells were seeded onto a plastic cell culture petri dish and allowed to adhere onto the surface for 3 min. A pipette tip with 10 μl of 100 nM of fMLP was inserted into the cell media. Cells would be imaged every 15 sec for 20 min to see if cells would migrate to the point source gradient. This experiment was completed with non-stained cells to see the comparison between non-stained and stained cell migration towards the chemokine.

In the well plate experiment, Vybrant DiI stained cells were seeded into 2.2 mg/ml collagen concentration with different NaOH amounts (ranging from 2.0 μl to 5.5 μl in 0.5 increments),⁴ with a cell density was 6.5×10^6 cell/ml. After 30 min of incubation samples were imaged to see if cells were still “alive” after incubation. If samples looked viable, then 50 μl of 1 μM of fMLP was added to the well plate, at the top of the collagen surface to see if cells would migrate. Cells would be imaged every 2 min for 10 min to see if cells would migrate to the gradient source.

For LPS activated cell migration experiments, all control experiments were performed the same with the addition of conducting the point-tip test and well plate experiment with “activated” neutrophils.

⁴ See Table 2.2

TABLE 3.1. Summary of experimental controls that were completed prior every cell chemotaxis experiment

Experiment	Cell Type	Set-up
Point-Tip Test	1. Neutrophil	Cells seeded onto a plastic cell culture petri dish and allowed to adhere onto surface for 3 min. Pipette tip with 10 μ l of 100 nM of fMLP inserted into the cell media. Cells imaged every 15 sec for 20 min.
	2. DiI Stained Neutrophil	
	3. Activated Neutrophil	
	4. DiI Stained Neutrophil	
Well-Plate Test	1. DiI Stained Neutrophil	Cells seeded into 2.2 mg/ml collagen concentration with different NaOH amounts (ranging from 2.0 μ l to 5.5 μ l in 0.5 increments). 30 min of incubation. 50 μ l of 1 μ M of fMLP was added to the well plate at top of sample.
	2. DiI Stained Activated Neutrophil	

2.3. 3D Collagen Matrix Preparation. 3D collagen matrices were prepared at room temperature following the method presented in Chapter 2 of this dissertation.⁵ Briefly, PDMS molds were washed with 90% ethyl alcohol and stored in deionized water overnight. Collagen matrices, at a 2.2 mg/ml density, were prepared using an amended protocol from BD Sciences with reformed recipe to account for the addition of fluoresce beads.⁶ Vybrant DiI stained cells were suspended in Leibovitz's

⁵ See Ch. 2.1 – 2.4

⁶ See Table 2.2

L-15 medium with 2 mg/ml glucose added for a final concentration of 10^6 cells/ml, then added to collagen matrix solution. Collagen solution was added to prepared PDMS mold and then incubated for 25 – 30 min at standard conditions (37°C at 5% CO_2) for polymer polymerization (Figure 2.4).

2.4. Microscopy and Live Cell Imaging. Three-dimensional image stacks were acquired using a Nikon A-1 confocal system mounted on a TI Eclipse inverted optical microscope controlled by NI-Elements Nikon Software. A 40x plan fluor air objective mounted on a piezo objective positioner was used for all FIDVC cell experiments, which allowed to image at speeds up to 30 frames per second. Green fluorescent microspheres were used throughout the collagen matrix and excited with an argon (488 nm) laser. DiI stained cells were excited with a diode (561 nm) laser.

512 x 512 x Z voxels ($102\ \mu\text{m}$ x $102\ \mu\text{m}$ x Z) confocal volume stacks were recorded every 2 to 3 min. To ensure physiological imaging conditions within the imaging chamber, temperature was controlled at 37°C during time lapse recording as previously described [103, 104]. Data were analyzed using ImageJ (National Institutes of Health), TecPlot (Tecplot, Inc., Bellevue WA), Mathematica (Wolfram Research, Somerville MA) and MATLAB computational software.

During control experiment, cell outline was estimated from phase contrast microscopy using a 10x and 20x air objective. for reflection microscopy, an Apo LWD 40x WI λS DIC N2 objective, with a numeric aperture = 1.15, a refractive index = 1.33 and a Z-step = $0.05\ \mu\text{m}$ was used.

2.5. Migration Experiments. Once cell-collagen matrix polymerization occurred,⁷ the system was inserted into a Chamlide chamber system and placed into microscope imaging chamber system (Figure 2.4). Hydrophobic cover slips were removed to created the three chamber system. For normal cell migration experiments, one well was filled with 100 μL of Leibovitz’s L-15 (phenol free) with 2 mg/ml glucose, where the other well was filled with 100 μL with fMLP diluted into Leibovitz’s

⁷ See Ch. 2.3

L-15 (phenol free) with 2 mg/ml glucose for a final concentration of 1 μ M. Live cell imaging took place after 5 min of the introduction of chemokine.

For LPS activated cell migration experiments, activation was initiated through the addition of a LPS solution (consisting of 100 ng/ml ultrapure LPS in 10% human serum) to have a final LPS concentration of 40 ng into the experimental system. LPS (endotoxin) has been shown to be a major mediator of neutrophil activation [105, 106, 107]. During the experiment, one well was filled with 100 μ L of Leibovitz's L-15 (phenol free) with 2 mg/ml glucose and 40 ng LPS, where the other well was filled with 100 μ L with fMLP diluted into Leibovitz's L-15 (phenol free) with 2 mg/ml glucose for a final concentration of 1 μ M with an addition of 40 ng/ml LPS. Live cell imaging took place after 5 min of the introduction of chemokine and activation solution.

2.6. Experimental Measurements.

2.6.1. *Concentration.* Referring back to equation 2.22 (found in section 2.6 of this work) one can rewrite the equation to find the specific concentration at any location within the system. In short, using sawtooth and transient system heat transfer models [108], specific concentration and gradient at a specific time and location (x, y, z) can be found throughout the experiment to track cell-concentration understanding and gradient slope (Equation 3.1). During chemotaxis experiment, specific time was recored at moment chemokine was added to system. During cell migration movement and confocal time-lapse recording, specific time and location from edge was recorded. During data analysis, noted time, location and coefficient of diffusion⁸ for the specific cell was plugged into equation 3.1.

$$\frac{c(x, t)}{c_{max}} = \frac{x}{L} + \frac{2}{\pi} \sum_{n=1}^{\infty} \frac{1}{n} \cos(n\pi) \sin\left(\frac{n\pi x}{L}\right) \exp\left[\frac{-Dt \cdot n^2 \pi^2}{L^2}\right] \quad (3.1)$$

⁸ See Table 2.3

2.6.2. *3D Displacements & Principal Strains.* Cell-generated full field displacements were determined using recently developed and new fast iterative digital volume correlation (FIDVC) algorithm to track the motion of the embedded fluorescent beads in all three dimensions between each time increment [63]. In brief, three dimensional time-lapsed volumetric images of fluorescent beads embedded in rat tail collagen type I matrices were recored using laser scanning confocal microscopy (LSCM). Once the images were recorded the FIDVC algorithm was used to find the full field displacements.

Principal strain calculations were determined using developed MATLAB code⁹ appended to full field displacement calculations. In short, time-lapsed displacement results were fed into generated code where E_1, E_2, E_3 and principal strain magnitude (E_P) were calculated.

2.6.3. *Speed.* Speed (U) was calculated first by finding the centroid ($\bar{C}$) of the cell at each time-point. Next, finding the magnitude of the distance traveled between two successive centroids we were able to calculate the speed of the migrating neutrophil (Equation 3.2).

$$U = \frac{\sqrt{(\Delta\bar{C}_{x_{\Delta t}})^2 + (\Delta\bar{C}_{y_{\Delta t}})^2 + (\Delta\bar{C}_{z_{\Delta t}})^2}}{\Delta t} \quad (3.2)$$

2.6.4. *Chemotactic Index.* Chemotactic index (C_i) is the measurement of the accuracy of chemotaxis. We calculated chemotactic index by vector analysis (Equation 3.3). Finding the resultant vector between two time-points and breaking it up into x, y, z components and dividing the resultant vector by the x-component ($\bar{C}_{x_{\Delta t}}$) we can find the chemotactic index (or directionality of the cell). A value of 1 is directly up the gradient.

⁹ Code written by Bar-Kochba E. & Stout D.

$$C_i = \frac{\Delta \bar{C}_{x_{\Delta t}}}{\sqrt{(\Delta \bar{C}_{x_{\Delta t}})^2 + (\Delta \bar{C}_{y_{\Delta t}})^2 + (\Delta \bar{C}_{z_{\Delta t}})^2}} \quad (3.3)$$

2.7. Cell & Force Modeling. Cell image, displacement and principal strain field visualization were rendered using Tecplot 360. In short, data under investigation is converted to Tecplot format using adapted MATLAB code.¹⁰

2.8. Statistical Analysis. Data was subjected to the chi-square goodness-of-fit test to determine if a data sample comes from a normal distribution. Jacobian matrix calculations were computed on principal strain data to increase validity of data. Wilcoxon Rank-Sum and One-Way ANOVA tests were used for comparison of the medians, root-mean-square (RMS) and max of the data and their interactions (Figure 3.3).

3. Results

3.1. fMLP Induces Chemotaxis of Neutrophils in Custom Planar Gradient Diffusion System. To explore neutrophil chemotaxis, naïve and LPS activated neutrophils were embedded into the custom planar gradient diffusion system.¹¹ Both naïve and LPS activated neutrophils demonstrated chemotactic migration when fMLP was introduced into well #1 of the system.¹² Naïve and LPS activated neutrophils moved towards the chemoattractant gradient (Figures 3.5 & 3.8). Interestingly, both naïve and LPS activated neutrophils moved and produced material displacements in all three dimensions (Figures 3.6 & 3.9).

By using equation 3.1, we were able to calculate the specific concentration along the surface of the cell for both naïve and LPS activated neutrophils. Finding the

¹⁰ Code titled “mat2tecplot” downloaded from cfd-online, originally written by Wen Long for the Department of Energy Pacific Northwest National Lab

¹¹ See Ch. 3.2

¹² See Figure 2.4

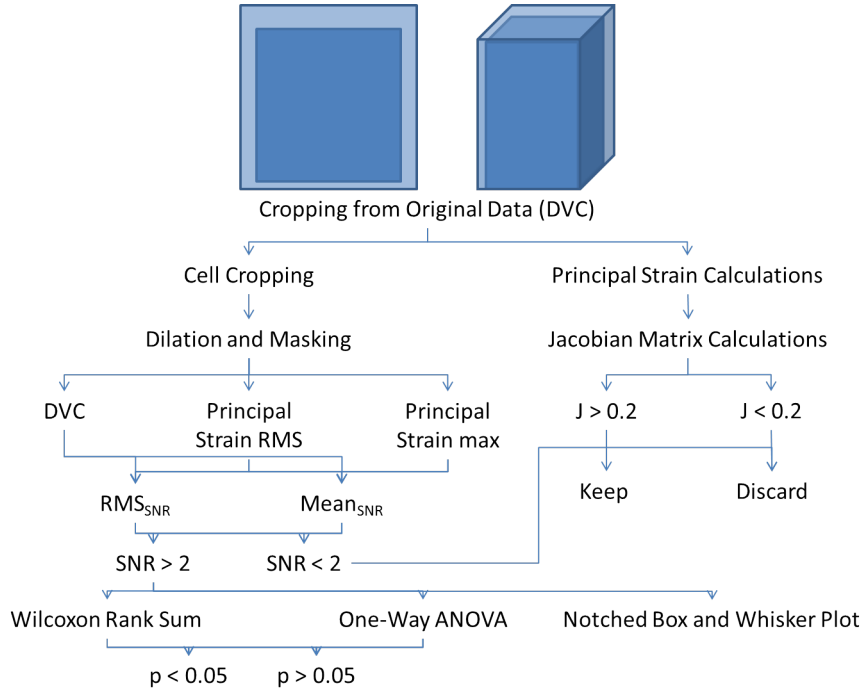


FIGURE 3.3. Flow diagram of raw data to results. DVC and principal strain calculations are computed for each time-point volumetric image. The Jacobian matrix ($J_{mat} < 0.2$) is determined on principal strain data. If time-point data generates a $J_{mat} < 0.2$ it is discarded. Next, each time-point volumetric image DVC data set is cropped around the cell of interest. A dilation mask is placed around the cropped image and signal-to-noise ratio (SNR) is calculated for DVC, principal strain RMS and principal strain max. If the generated SNR is < 2 for any of the three the time-point is discarded. A Wilcoxon Rank-Sum and One-Way ANOVA tests were calculated where a $p < 0.05$ is considered significant. Finally, notched box and whisker plots were generated for visualization of data.

percent difference between the pseudopod and uropod, an average of 3 - 4% difference was shown regardless of neutrophil state (Figure 3.11).

3.2. Neutrophils Activated by LPS Generate Greater Principal Strain and Increase Their Speed. To investigate migration difference between neutrophil states, we computed and evaluated the strains and speeds of neutrophils in chemotaxis. Interestingly, when calculating principal strain, LPS activated neutrophils showed greater deformation compared to their naïve counterparts (Figures 3.7 & 3.10). Significant differences in principal strain root-mean-square (geometric mean) and maximum values (Figure 3.12) indicating LPS activated neutrophils deform their

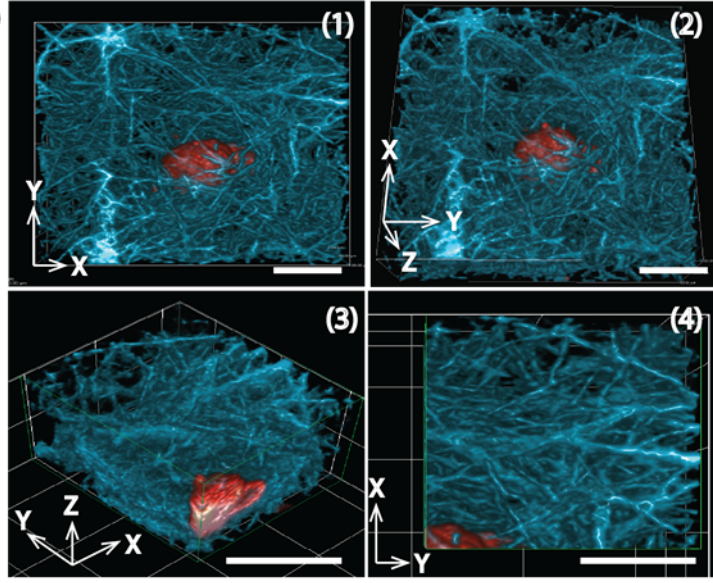


FIGURE 3.4. 3D images of a neutrophil (red) and collagen I fibers (blue) in a 2.2 mg/ml collagen gel imaged using confocal reflectance microscopy. The neutrophil is completely engulfed by the collagen fibers (blue). Scale bar = 10 μm .

surrounding area greater. Investigating neutrophil chemotaxis speed, LPS activated neutrophils showed greater speeds compared to their naïve counterparts (Figure 3.13). Therefore, LPS activation seems to increase neutrophil deformation and speed during chemotaxis.

3.3. Neutrophils Activated by LPS Decrease their “Direct” Movement to Chemoattractant Source. To measure the accuracy of chemotaxis, the chemotactic index was calculated for naïve and LPS activated neutrophils, thus shedding light on the neutrophils “directionality” to the chemoattractant. Interestingly, naïve neutrophil experiments indicated a greater chemotactic index, or directionality than LPS activated neutrophils (Figure 3.14). It seems, LPS activation reduced a neutrophil’s ability to move towards the chemoattractant source.

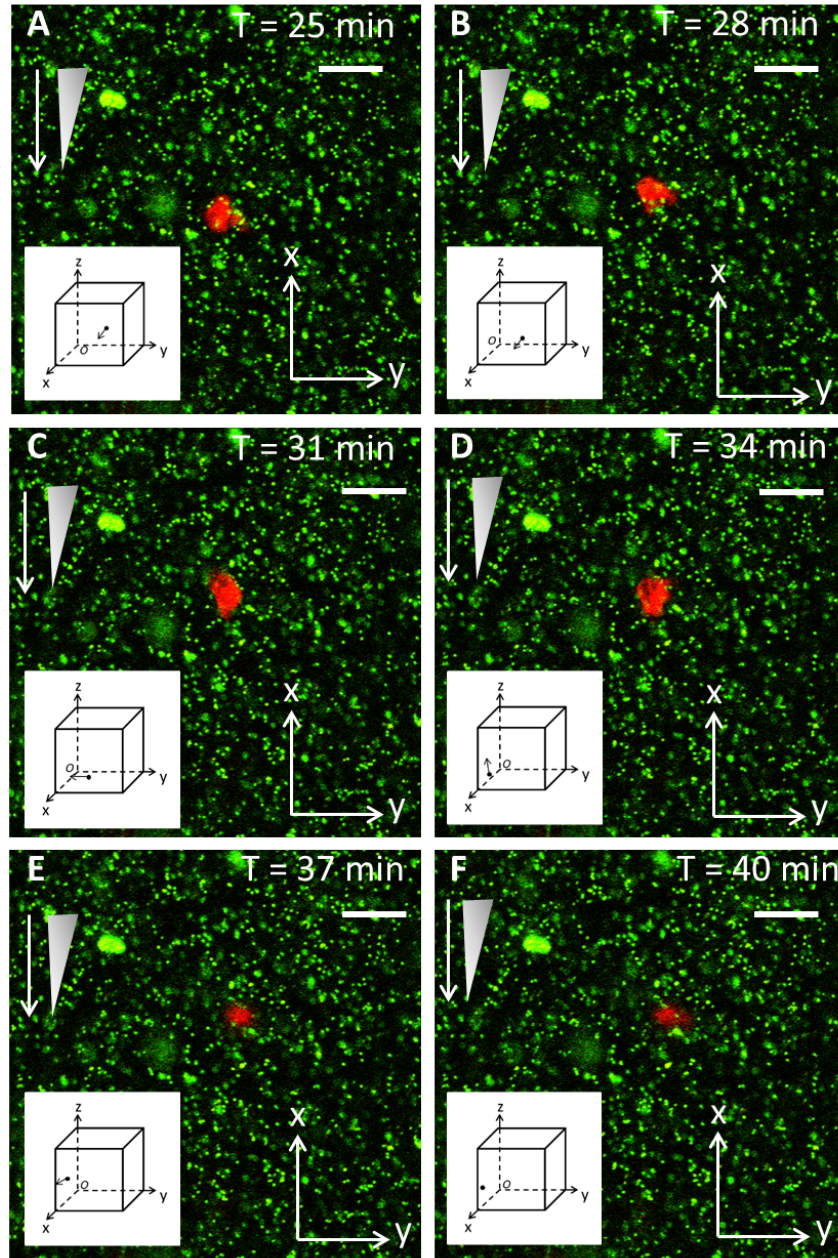


FIGURE 3.5. A Z-slice confocal microscope image of a neutrophil migrating in the 3D planar gradient system. Neutrophil migrates towards the chemoattractant gradient (white arrow depicts direction of gradient). Subplot shows cell in 3D space, where arrow indicates next time-point direction. Scale bar = $10 \mu\text{m}$. Time indicates when image acquisition took place after fMLP addition into system via well #1. $\text{dt} = 3 \text{ min}$.

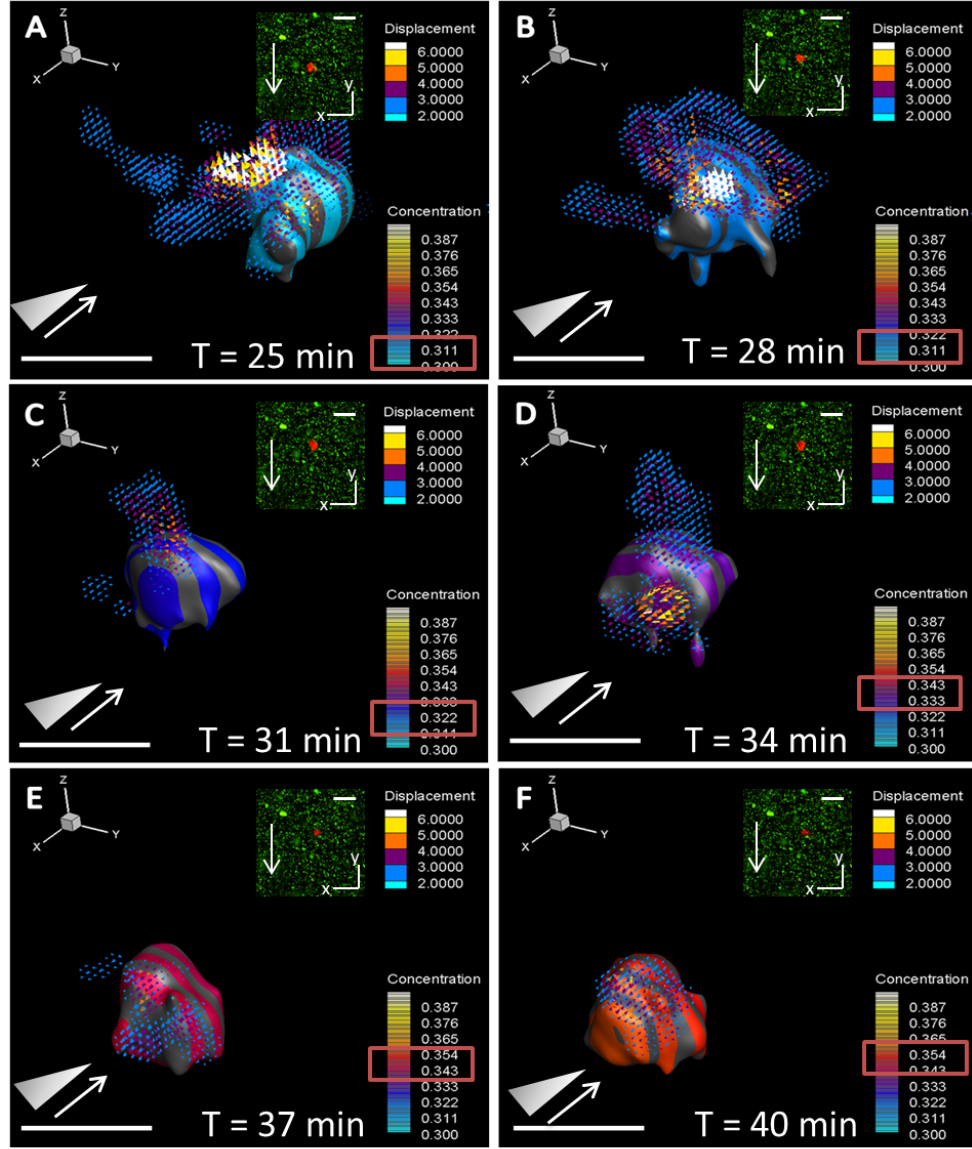


FIGURE 3.6. 3D representation of naïve neutrophil and its displacement fields in chemotaxis. Zebra coloring on the 3D representation of the cell shows the specific concentration difference along on the surface of the cell. The Sub-image in each time-point frame shows one of the accompany Z-slice microscope image of a neutrophil migrating (white arrow depicts direction of gradient). Scale bar = $10\ \mu\text{m}$ for large frame and scale bar = $20\ \mu\text{m}$ for sub-image. Displacement scale factor: $X = 0.21\ \mu\text{m}/\text{px}$; $Y = 0.21\ \mu\text{m}/\text{px}$; $Z = 0.30\ \mu\text{m}/\text{px}$. Time indicates when image acquisition took place after fMLP addition into system via well #1. $dt = 3\ \text{min}$.

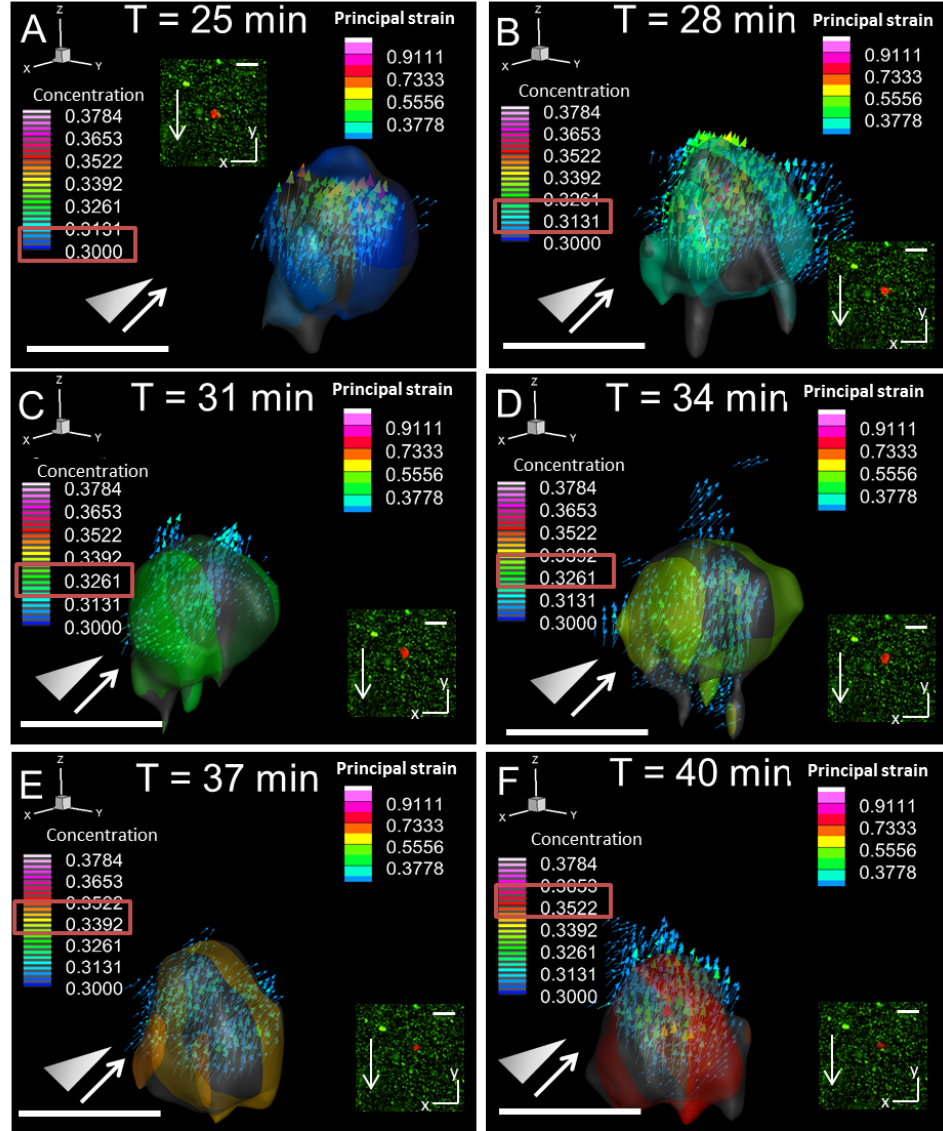


FIGURE 3.7. 3D representation of naïve neutrophil in chemotaxis and its generation of principal strains. Zebra coloring on the 3D representation of the cell shows the specific concentration difference along on the surface of the cell. The Sub-image in each time-point frame shows one of the accompany Z-slice microscope image of a neutrophil migrating (white arrow depicts direction of gradient). Scale bar = $10\ \mu\text{m}$ for large frame and scale bar = $20\ \mu\text{m}$ for sub-image. Displacement scale factor: $X = 0.21\ \mu\text{m}/\text{px}$; $Y = 0.21\ \mu\text{m}/\text{px}$; $Z = 0.30\ \mu\text{m}/\text{px}$. Time indicates when image acquisition took place after fMLP addition into system via well #1. $dt = 3\ \text{min}$.

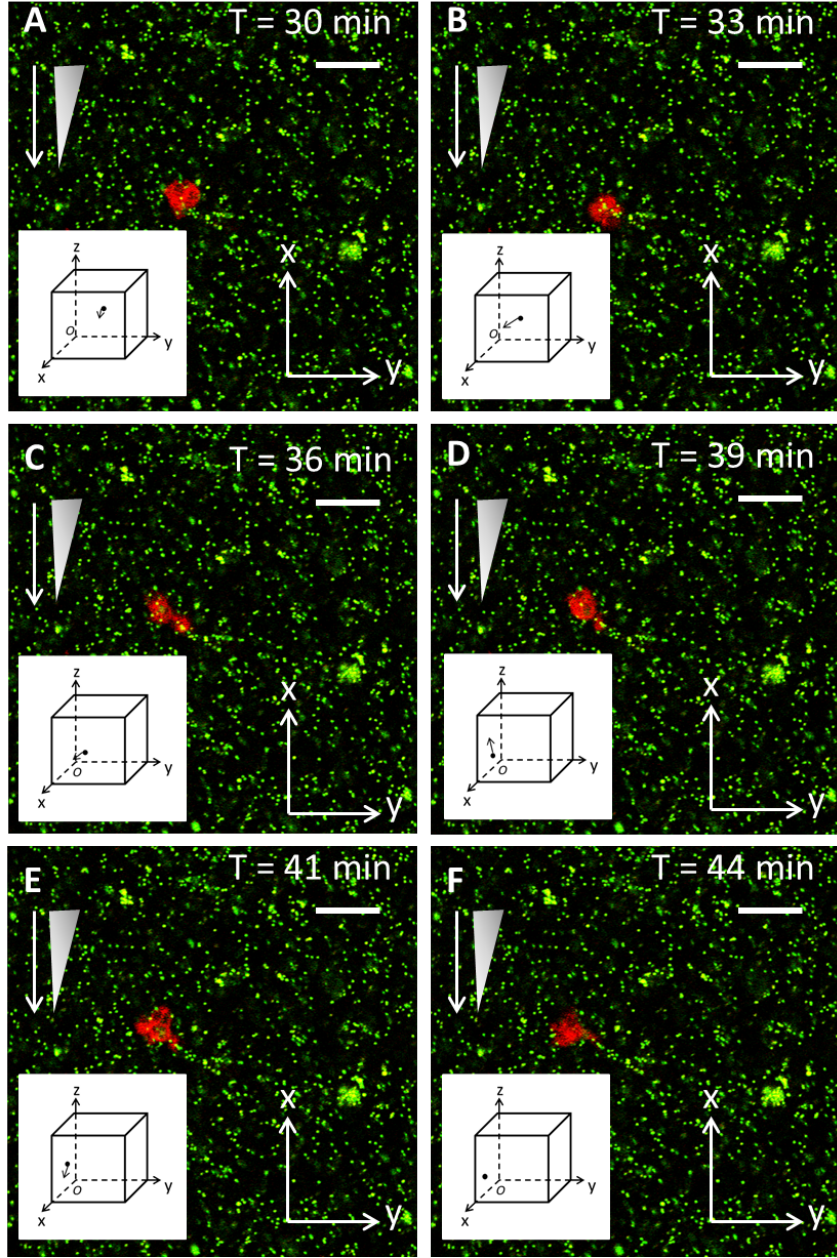


FIGURE 3.8. A Z-slice confocal microscope image of an LPS activated neutrophil migrating in the 3D planar gradient system. Neutrophil migrates towards the chemoattractant gradient (white arrow depicts direction of gradient). Subplot shows cell in 3D space, where arrow indicates next time-point direction. Scale bar = $10 \mu\text{m}$. Time indicates when image acquisition took place after fMLP addition into system via well #1. $\text{dt} = 3 \text{ min}$.

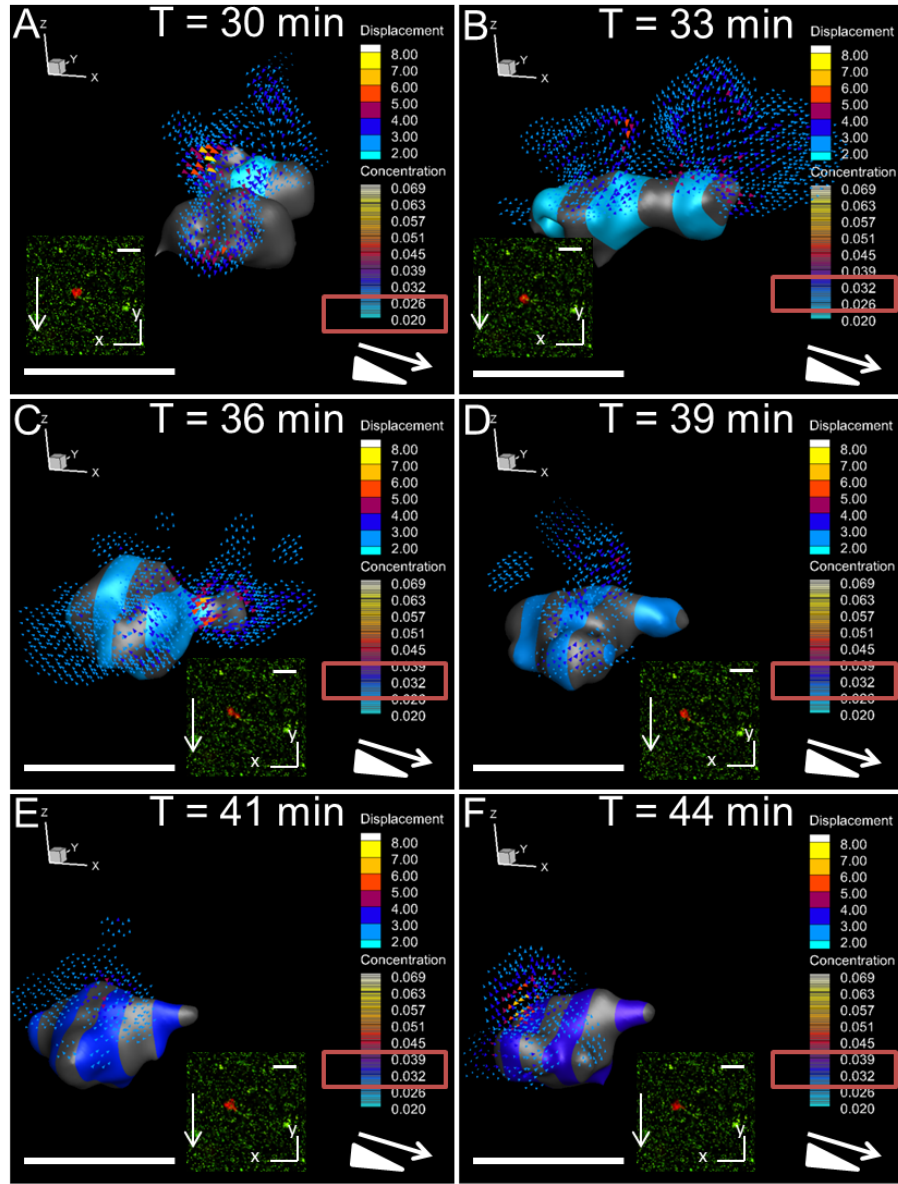


FIGURE 3.9. 3D representation of LPS activated neutrophil and its displacement fields in chemotaxis. The Sub-image in each time-point frame shows one of the accompany Z-slice microscope image of a neutrophil migrating (white arrow depicts direction of gradient). Scale bar = $10 \mu\text{m}$ for large frame and scale bar = $20 \mu\text{m}$ for sub-image. Displacement scale factor: $X = 0.21 \mu\text{m/px}$; $Y = 0.21 \mu\text{m/px}$; $Z = 0.30 \mu\text{m/px}$. Time indicates when image acquisition took place after fMLP addition into the system via well #1. $\text{dt} = 3 \text{ min}$.

4. Discussion

This in vitro study examined neutrophil chemotaxis of two different cell states (naïve and LPS activated) in a new 3D planar gradient system. By initiating chemotaxis, via fMLP, we were able to investigate the force generation of a neutrophil. Interestingly, we were able to show LPS activated neutrophils have greater deformation

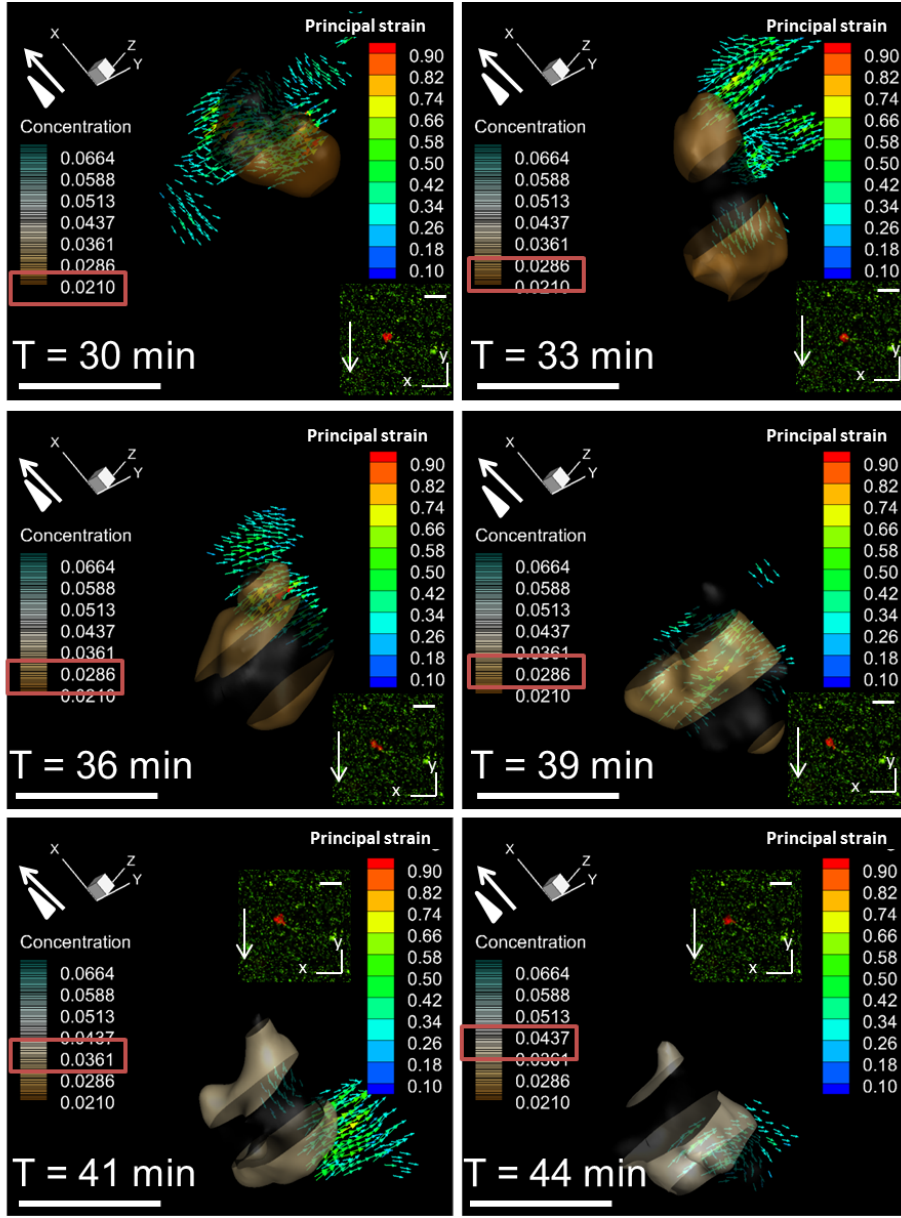


FIGURE 3.10. 3D representation of LPS activated neutrophil in chemotaxis and its generation of principal strains. The Sub-image in each time-point frame shows one of the accompanying Z-slice microscope image of a neutrophil migrating (white arrow depicts direction of gradient). Scale bar = $10\ \mu\text{m}$ for large frame and scale bar = $20\ \mu\text{m}$ for sub-image. Displacement scale factor: $X = 0.21\ \mu\text{m}/\text{px}$; $Y = 0.21\ \mu\text{m}/\text{px}$; $Z = 0.30\ \mu\text{m}/\text{px}$. Time indicates when image acquisition took place after fMLP addition into the system via well #1. $\text{dt} = 3\ \text{min}$.

and displacement capabilities than naïve neutrophils is chemotaxis. This could allow for an LPS activated cell to move faster through the collagen matrix, which is what we saw in our data.

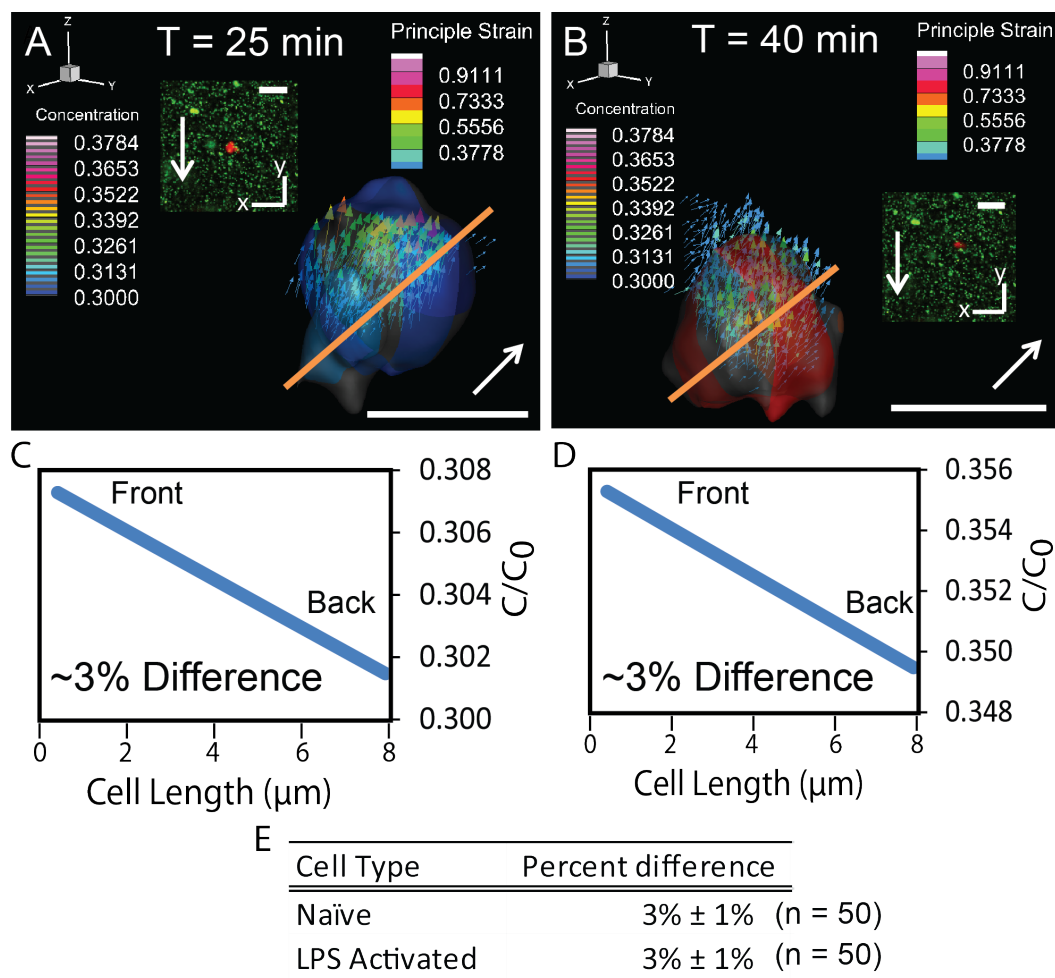


FIGURE 3.11. 3D representation of naïve neutrophil in chemotaxis and its generation of principal strains at time point (A) 25 min and (B) 40 min. Zebra coloring on the 3D representation cell used to show the specific concentration difference along on the surface of the cell. The Sub-image in each time-point frame shows one of the accompanying Z-slice microscope image of a neutrophil migrating (white arrow depicts direction of gradient). Scale bar = $10 \mu\text{m}$ for large frame and scale bar = $20 \mu\text{m}$ for sub-image. Specific concentration along the surface of the cell at the beginning of the experiment (C) at time-point 25 min and at the end of the experiment (D) at time point 40 min where the x-axis depicts the cell length and the y-axis represents the normalized concentration. (E) When comparing between naïve and LPS activated neutrophils the percent different between the pseudopod and uropod showed similar values. Time indicates when image acquisition took place after fMLP addition into the system via well #1. Orange line along the cell surface indicates where specific concentration measurements were taken. $\text{dt} = 3 \text{ min}$.

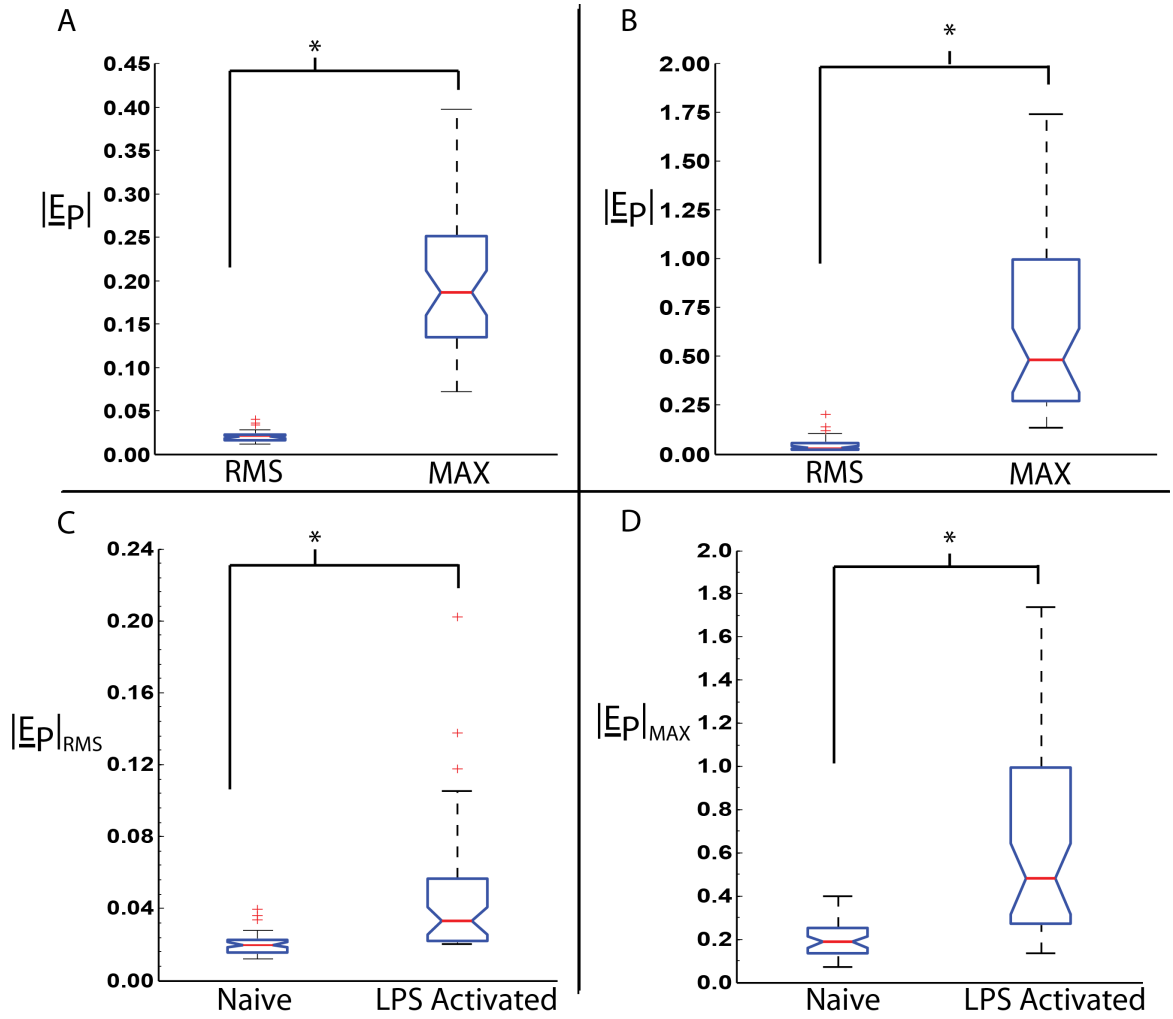


FIGURE 3.12. Calculated root-mean-square (RMS) ($|\underline{E}_P|_{RMS}$) and maximum ($|\underline{E}_P|_{MAX}$) principal strain magnitudes of LPS activated vs naïve neutrophils. (A) Naïve neutrophil; (B) LPS activated neutrophil; (C) RMS comparison; (D) Max comparison. Data indicates LPS activated neutrophils deform greater than their naïve counterparts. Box-and-whisker plot notches indicates two medians are significantly different at the 5% significance level since their intervals do not overlap. Data was subjected to the chi-square goodness-of-fit test to determine if a data sample comes from a normal distribution. * $p < 0.05$ for Wilcoxon and One-Way ANOVA tests. $n = 50$.

When looking at the neutrophil's directionality to the chemokine, its chemotactic index, LPS activated cells had a lower index than its naïve counterpart—meaning its

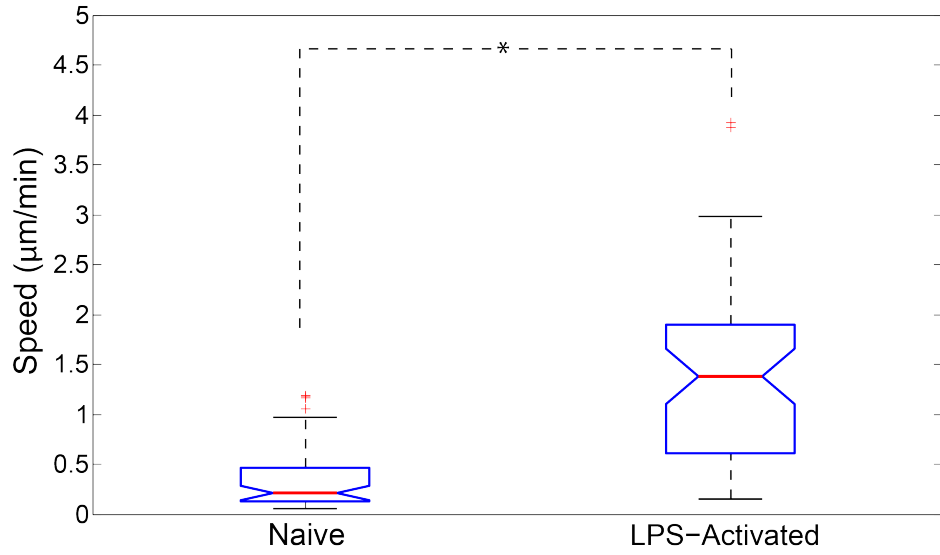


FIGURE 3.13. Calculated speed of LPS activated vs naïve neutrophils. Data indicates LPS activated neutrophils migrate faster than their naïve counterparts. Box-and-whisker plot notches indicates two medians are significantly different at the 5% significance level since their intervals do not overlap. Data was subjected to the chi-square goodness-of-fit test to determine if a data sample comes from a normal distribution. * $p < 0.05$ for Wilcoxon and One-Way ANOVA tests. $n = 50$.

migration was not directed as much or towards the gradient. This seems counterintuitive since LPS activated neutrophils are generating greater deformation and moving faster through the matrix.

Looking at how LPS activation affects the migration pathways, we can come to some interesting points. In normal healthy neutrophils, signaling of a chemoattractant, like fMLP, is through the G protein-coupled receptor (GPCR) pathway (Figure 3.1). For LPS, the transduction pathway is not GPCR, but the Toll-like receptor 4 (TLR4) (Figure 3.15). The mammalian Toll-like receptors (TLRs) are germline-encoded receptors expressed by cells of the innate immune system that are stimulated by structural motifs characteristically expressed by bacteria, viruses and fungi known as pathogen-associated molecular patterns (PAMPs) [109, 110]. Importantly, TLR interactions trigger the expression of proinflammatory cytokines as well as the functional maturation of antigen presenting cells of the innate immune system [110, 111].

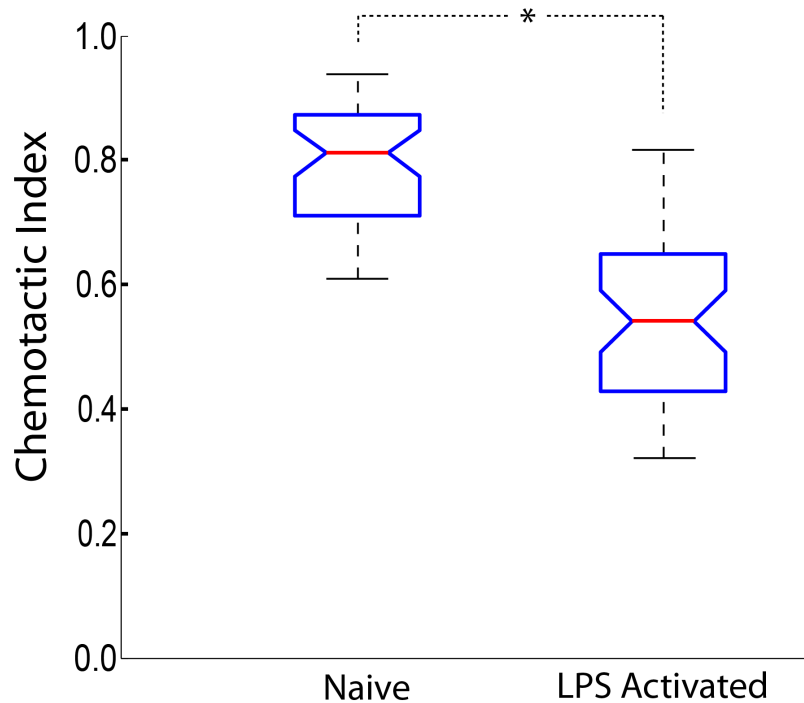


FIGURE 3.14. Calculated chemotactic index of LPS activated vs naïve neutrophils. Data indicates LPS activated neutrophils migrate in a less directional motion to the chemoattractant source than their naïve counterparts. Box-and-whisker plot notches indicates two medians are significantly different at the 5% significance level since their intervals do not overlap. Data was subjected to the chi-square goodness-of-fit test to determine if a data sample comes from a normal distribution. * $p < 0.05$ for Wilcoxon and One-Way ANOVA tests. $n = 50$.

LPS stimulation of mammalian cells occurs through a series of interactions with several proteins, including the LPS binding protein (LBP), CD14, MD-2 and TLR4 [112, 113]. LBP is a soluble shuttle protein which directly binds to LPS and facilitates the association between LPS and CD14 [114, 115]. CD14 is a glycosylphosphatidylinositol-anchored protein, which also exists in a soluble form. CD14 facilitates the transfer of LPS to the TLR4/MD-2 receptor complex and modulates LPS recognition [115]. MD-2 is a soluble protein that non-covalently associates with TLR4 but can directly form a complex with LPS in the absence TLR4 [116]. Although no evidence suggests that TLR4 can bind LPS directly, TLR4 can enhance the binding of LPS to MD-2 [117]. Therefore LPS stimulation of TLR4, includes the participation of several molecules.

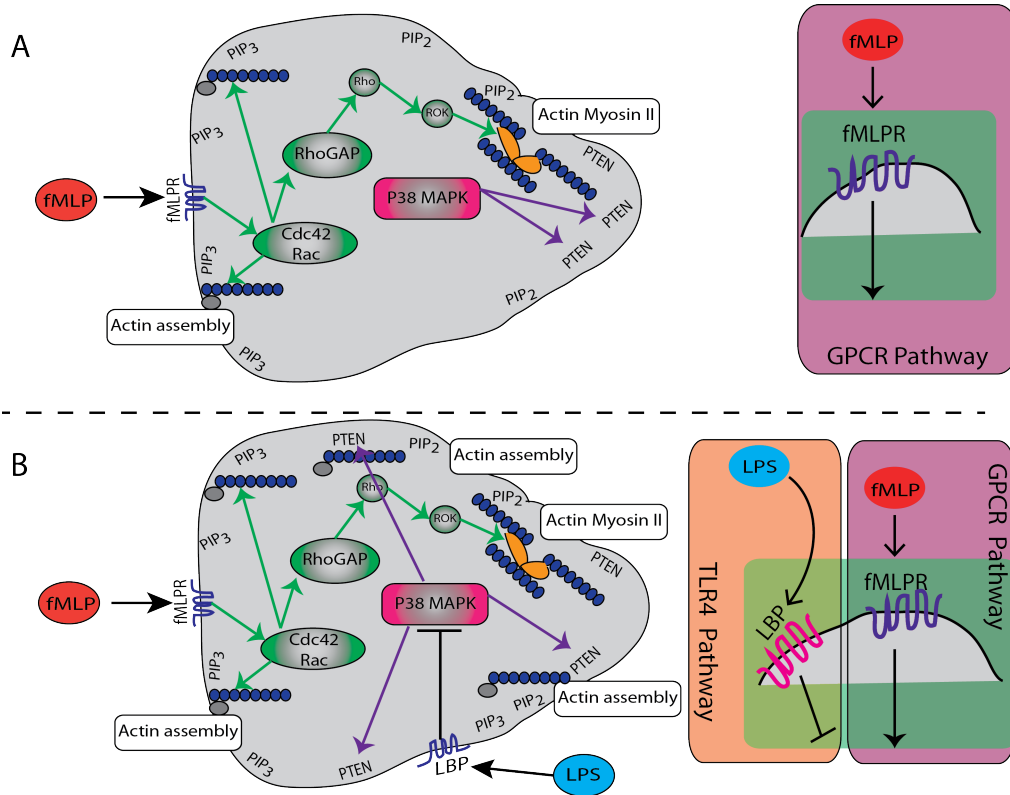


FIGURE 3.15. (A) During fMLP activation, the GPCR pathway is used to initiate and regulate migration. In brief, fMLP acts through GEFs to activate both Cdc42 and Rac. The activated Cdc42 and Rac initiates an amplification loop by stimulating PI3K which phosphorylates the membrane lipid PIP2 into PIP3. The localized PIP3 together with Cdc42 and Rac, then act together to assemble actin in the pseudopod. At the rear of the cell, the fMLP receptor activates Rho that then stimulates the ROK to induce contraction of the uropod. Simultaneously, p38 MAPK is initiated to mediate PTEN to the sides and back of the cell, thereby converting PIP3 back to PIP2 in areas of the cell away from the leading edge. (B) But when LPS is introduced into the system a second simultaneous and competing pathway is initiated, TLR4, which then inhibits p38 MAPK and the cell's ability to mediate both PTEN localization and chemotaxis. In consequence the cell will increase actin polymerization in different location and limit its ability to change shape.

Upon LPS recognition, TLR4 undergoes oligomerization and recruits its downstream adaptors through interactions with the TIR (Toll-interleukin-1 receptor) domains. TIR domains contain three highly conserved regions, which mediate protein-protein interactions between the TLRs and signal transduction adaptor proteins. The

TIR domain of TLR4 is critical for signal transduction, because a single point mutation in the TIR domain can abolish the response to LPS [118]. Interestingly, there are five TIR domain-containing adaptor proteins: MyD88 (myeloid differentiation primary response gene 88), TIRAP (TIR domain-containing adaptor protein), TRIF (TIR domain-containing adaptor inducing IFN- β), TRAM (TRIF-related adaptor molecule), and SARM (sterile α and HEAT-Armadillo motifs-containing protein) [119]. Different TLRs use different combinations of adaptor proteins to determine downstream signaling. TLR4 is the only known TLR which utilizes all these adaptor proteins.

This is very different than the activation of migration via fMLP (Figure 3.1) via a GPCR by activating the class I isoform of PI3K, which phosphorylates the membrane lipid PIP2 into PIP3 at the leading edge of the neutrophil (Figure 3.15). At the same time, the phosphatase and tensin homolog (PTEN) is localized to the sides and back of the migrating cell, thereby converting PIP3 back to PIP2 in areas of the cell away from the leading edge [120]. This mechanism is thought to allow the neutrophil to recognize and amplify shallow gradients of only a few molecules along its length to know that it must move in the direction of PIP3. At the same time fMLP-induced p38 MAPK allows the neutrophil to respond to a bacterial product [121], as well as mediate both PTEN localization and chemotaxis in response to end-target chemoattractants.

Even more interesting, LPS also activates p38 MAPK, the key signaling pathway in fMLP-induced chemotaxis, through TLR4, thus disrupting this chemotactic process (Figure 3.15). Although LPS is sometimes mistaken for a chemotactic molecule, on its own it does not induce chemotaxis but instead functions as a stop signal that overrides chemoattractants [121] and chemotaxis downstream.

Research has shown p38 MAPK inhibition blocked LPS induced actin polymerization and an increase in actin polymerization. This could potentially inhibit recruitment by increasing cell rigidity [122, 123] thus limiting the deformability of the neutrophils which limits their ability to change shape as they undergo transmigration

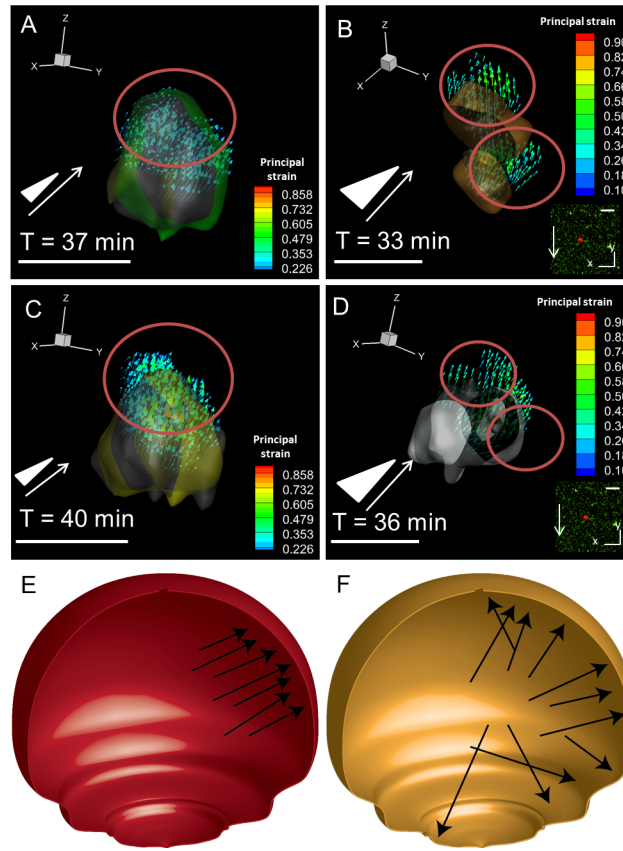


FIGURE 3.16. 3D representation of naïve neutrophil in chemotaxis and its generation of principal strains at time-point (A) 25 min and (C) 40 min. 3D representation of LPS activated neutrophil in chemotaxis and its generation of principal strains at time-point (B) 33 min and (D) 36 min. Zebra coloring on the 3D representation cell used to show the specific concentration difference along on the surface of the cell. Scale bar = 10 μm for large frame. In the naïve neutrophil chemotaxis experiments (A,C) the principal strain are concentrated in the uropod directed to the same vicinity (red circles). (E) depicts a naïve neutrophil 3D drawing where the cell is sliced and the arrow represent principal strain calculations. LPS activated neutrophil chemotaxis experiments (B,D), the principal strains are not concentrated in the back of the uropod and vary with vector direction. (D) depicts a LPS activated neutrophil 3D drawing where the cell is sliced and the arrow represent principal strain calculations, thus supporting LPS's hindrance to mediate PTEN and decrease directionality.

and chemotaxis [124]. This increase in actin polymerization could represent a continued LPS-driven cycling of actin polymerization, which limits additional cytoskeletal changes by other mediators.

The inhibition of p38 MAPK and increase of actin polymerization could account for the increase in speed (Figure 3.13), larger principal strains (Figure 3.12) and decrease in directionality (Figure 3.14). When two competing pathways are activated (GPCR & TRL4; Figure 3.15) the TRL4 hinders p38 MAPK's ability to mediate PTEN localization while increasing actin polymerization started through the GPCR pathway. This could account for increasing speed and principal strains while hindering the neutrophil's ability to migration towards the chemoattractant source in a direct fashion.

This also can be shown in the location of where the deformations are taking place within the neutrophil. Since LPS activates the TRL4 pathway, which hinders p38 MAPK's ability to mediate PTEN, one would expect to see a less concentration location of deformation measurements within the cell. Shown in Figure 3.16, this is exactly what is observed. In the naïve neutrophil chemotaxis experiments the principal strains are concentrated in the uropod and directed towards the same location. LPS activated neutrophil chemotaxis experiments showed a very different deformation picture. Seen in Figure 3.16, the principal strains are not concentrated in the back of the uropod and vary with surface location, thus supporting LPS's hindrance to mediate PTEN and decrease directionality.

Going back to sepsis, by just turning off, or inhibiting, the TLR4 pathway has not shown a decrease in mortality rate among patients. For example, the failure of Eritoran to improve survival in a Phase III trial in patients with severe sepsis by administering a synthetic TLR4 antagonist to block the interaction LPS with its receptor MD2-TLR4. Among the 1961 patients evaluated, who were randomized and treated with either Eritoran or placebo within 12 hours of onset of first organ dysfunction, there was no reduction in 28 day mortality in the group receiving Eritoran [129]. In another trial, Xigris (drotrecogin alfa, a recombinant activated protein C) was pulled from the market following the failure of its worldwide trial, PROWESS Shock, to demonstrate improved outcome [130]. These two recent failures adds to the

TABLE 3.2. Clinical trials and outcomes in patients with severe sepsis

Name	Process	Trial Outcome	References
Meropenem	Inhibits bacterial wall synthesis	No significant difference	[125]
Moxifloxacin + Meropenem	4 th generation synthetic fluoroquinolone antibacterial	No significant difference	[125]
Drotrecogin alfa (activated)	inhibits plasminogen activator inhibitor-1 (PAI-1) generation	No significant difference	[126]
Plasma-derived antithrombin III	Serine protease inhibitor	No significant difference	[127]
Recombinant tissue factor pathway inhibitor (rTFPI)	Multivalent, cationic Kunitz-type plasma protease inhibitor	No significant difference	[128]
Eritoran	synthetic lipid A antagonist that blocks LPS from binding at the cell surface MD2-TLR4 receptor	No significant difference	[129]

long list of ineffective experimental treatments for sepsis (Table 3.2). In consequence, a complete rethinking is needed in the strategies to evaluate novel agents for sepsis.

Our research suggests that an investigation of bio-related molecules or chemical compounds that are affected by the activation of both GPCR and TLR4 pathways may shed light into new strategies to decrease mortality rate in sepsis. Instead of just looking at the pathway of TLR4, one needs to investigate how migratory mediated molecules are affected when both pathways are initiated. For example a recent study showed that cross-talk between TLR4 and monocyte chemotactic protein-1 (MCP-1) in monocytes results in significant augmentation of MCP-1driven monocyte migration

[131]. LPS activation of TLR4 induces phosphorylation of Ser⁶⁷⁰ (which lies within the G $\beta\gamma$ -binding domain) of G protein-coupled receptor kinases-2 (GRK2) through a mechanism mediated by p38 MAPK. This will suppress GRK2 translocation to the cell membrane and the subsequent initiation of MCP-1 internalization and desensitization. As a result, LPS-TLR4 signaling augments monocyte migration by modulating the cell surface expression of chemokine receptors in a GRK2-dependent manner [131].

In short, the activation of both GPCR and TLR4 pathways alter the regulation of PTEN via p38 MAPK and a neutrophil's migration ability (Figure 3.14) through an and increase in actin polymerization (Figure 3.12). This can be seen with an increase principal strain data (Figure 3.12) and a decrease in chemotactic index between naïve and LPS activated neutrophils (Figure 3.14).

Our results indicate that the underlying culprit of neutrophil chemotaxis augmentation during LPS activated sepsis lies between both pathways and an further investigation into these cross-talking molecules may lead to a therapeutic strategy for controlling inflammation during bacterial infection.

5. Conclusion

The aim of the presented work is to investigate the force generating characteristics between normal and septic neutrophil chemotaxis (via a reductionist approach of LPS activation) using a custom planar gradient diffusion system¹³ coupled with 3D imaging techniques for force generation analysis. Using our chamber system we were able to successfully examine neutrophil chemotaxis of two different cell states (naïve and LPS activated) in a new 3D planar gradient system. By initiating chemotaxis, via fMLP, we were able to investigate 3D force generation of a neutrophil.

Our experiments show, that LPS activated neutrophils have greater deformation and displacement capabilities, while increasing their speed but decreasing their chemotactic index. When looking at the signaling pathways between fMLP and LPS our research suggest that an investigation of molecules or chemical compounds

¹³ See Ch. 2

that are affected by the activation of both GPCR and TLR4 pathways may shed light into new strategies to decrease mortality rate in sepsis.

CHAPTER 4

Conclusion

1. Summary of Significant Findings

The importance of cell migration can be seen throughout. When cells migrate, they generate forces and transfer these forces to their surrounding area leading to cell movement and migration. In order to understand the mechanisms that can alter and/or affect cell migration, one can study these forces.

In theory, understanding the fundamental mechanisms and forces underlying cell migration holds the promise of effective approaches for treating diseases and promoting cellular transplantation. One of the main players of this holy grail understanding is the neutrophil. Neutrophils, s PMN, are one of the major members of the immune system that operate during acute inflammation [37, 38, 39].

Furthermore, during sepsis—a complex clinical syndrome that results from a harmful or damaging host response to infection [48]—neutrophils have been linked to dysregulation, resulting in impaired directed migration of neutrophils to infectious foci and inadequate antimicrobial responses of PMNs at these sites [55]. Recent clinical trials to mediate sepsis have failed to improve mortality among patients with sepsis.

Mechanisms of how neutrophils cause tissue damage during sepsis are not well understood. For example, the use of neutralization of proinflammatory mediators blocking in the response of neutrophils to bacterially-derived activating molecules has not produced improvement in the clinical outcomes of the disease. Neither have clinical trials shown an improvement in morbidity or mortality, leading researchers to go-back-to-the-drawing-board and looking at neutrophil migration and sepsis in a different way.

In order to understand how sepsis affects neutrophil migration (mainly chemotaxis), we investigated the force generation, speed, chemotactic index and specific concentration during chemotaxis and compared between normal and septic neutrophils in a physiologically relevant 3D system. With the use of a 3D FIDVC technique and a reductionist approach to sepsis, our hypothesis states LPS-activation affects fMLP induced chemotactic cell machinery and decreases its directionality. To verify this hypothesis, chemotaxis of naïve and LPS activated neutrophils in an in-house 3D planar gradient system were investigated.

1.1. Neutrophil Chemotaxis of naïve vs LPS activated neutrophils.

With the use of the planar gradient diffusion system, chemotaxis characteristics (force generation, speed, chemotactic index and specific concentration) of naïve and LPS activated neutrophils were investigated. We were able to show, for the first time in 3D, LPS activated neutrophils have greater deformation and displacement capabilities, while increasing their speed but decreasing their chemotactic index.

Biophysically, the activation of both GPCR and TLR4 pathways have the ability to affect regulation of PTEN via p38 MAPK and the ability to reduce directionality (chemotactic index) (Figure 3.3) of neutrophil chemotaxis and increase in actin polymerization (Figure 3.12). Unfortunately, clinical research trials of prohibiting TLR4 activation did not promote reduced mortality rate in sepsis (Table 3.2) Therefore, our research promotes the notion of investigating migratory mediated molecules affected by both GPCR and TLR4 pathways, like Ser⁶⁷⁰. This may shed light into new strategies to decrease mortality rate in sepsis.

2. Future Extensions

This research lays the groundwork for further, and more complex experiments to investigate 1) force generation of neutrophil migration and 2) signaling pathways alterations between fMLP and LPS during sepsis.

2.1. Force Generation of Neutrophil Migration. One question that could be answered through our results is the different force generation between neutrophil

chemotaxis and chemokinesis in 3D. Through our FIDVC algorithm and planar gradient system, force generation could be measured and compared to chemotactic results.

Secondly, an investigation of competing chemoattractant gradients and how a neutrophil responds to this could be investigated. Recent competing gradient experiments research in 2D has shown that neutrophils respond to multiple chemoattractants by integrating and prioritizing the signals and that a hierarchy of chemoattractants is found and is dynamic depending on which signaling cascades are triggered [132]. Importantly, the research revealed that a dynamic in vitro platform can provide results that correspond well with in vivo studies; specifically each chemoattractant signal contributes to cellular migration. By using our 3D planar gradient system, we can build upon these findings in 3D and further investigate: 1) does force generation change with different chemoattractants present, 2) is the hierarchy of chemoattractants found in 2D the same in 3D, and 3) which signaling cascades are triggered in competing chemoattractant gradients.

2.2. Signaling Pathway Alterations Between fMLP and LPS During Sepsis. When looking at the signaling pathways between fMLP and LPS our research suggest a bio-related molecule or chemical compound used in both GPCR and TRL4 pathways is being affected. Future experiments of using a HL60 cell model differentiated to neutrophil like phenotype, can be used for genetic modification for investigation of these cross-talk bio-related molecules. For example, a GRK2 interfering RNA could be used to knock down GRK2 and see if chemotaxis is affected—by use of our experimental setup.

3. Last Words

In summary, the work presented in this dissertation serves to provide insight into 1) force generation of neutrophil chemotaxis of two different neutrophil states and 2) looking at neutrophil migration and sepsis in a different way. Despite the extraordinary developments in understanding the immunopathology of sepsis, therapeutic advances have been painfully slow. Interestingly, each therapeutic has been based on

a different aspect of the pathology. For example, therapies aimed at bacterial targets, like anti-endotoxin molecules such as bactericidal/permeability-increasing protein, or modified lipoproteins, both of which absorb and neutralize LPS. Therapies aimed at host molecules, such as PAF-receptor antagonists, and a variety of targets in the coagulation cascade have been studied to decrease the mortality rate.

Unfortunately, these approaches have produced little to no improvement in clinical course of the disease. This has lead to going-back-to-the-drawing-board mentality and looking at neutrophil migration and sepsis in a new way. Introducing the study of mechanics in the investigation of sepsis, we can provide a new vantage point of how sepsis alters cell behavior through physical interactions with its environment. By use of a fast iterative digital volume correlation experimental approach we have presented insight (force generation location and mobility) on how sepsis may alter neutrophil function that may not have been noticed by traditional biological assays.

APPENDIX A

MATLAB and Mathematica codes

1. MATLAB codes for diffusion solutions

```
clear all; clc; close all
%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
%% %%%%%%%%% Diffusion fitting script %%%%%%%%%
%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
%Specify total numbers of fitting points:
npoints = 10;
%% Loading the movie first
% input tif files per time stack
F1 = uigetdir;
cd(F1);
%F2 = [F1,'/sample*.tif'];
F = dir('2_5*.tif');
%F = dir(F2);
H = fspecial('average');
% Here I just rescale the image and normalize by the
% maximum intensity, sometimes the images need blurring or
% smoothing, if it is too pixelated
h = waitbar(0,'Reading in Images... ');
format long
% if matlabpool('size') == 0, matlabpool open; end
ImageD = cell(1,round(length(F)));
for i = 1:round(length(F))
    waitbar(i/length(F),h);
```

```

    filename = F(i).name;
    Image0 = imread(filename,1);
    Image0 = single(Image0(:,:,1));
    Image = imfilter(Image0,H);
    %Image = Image0;
    MaxI = max(Image(:));
    Image = Image/MaxI;
    ImageD{i} = Image; %saves normalized image time-lapse movie
end
close(h);
cd('..');
% Watching Normalized movie
% for j = 1:length(ImageD)
%     B = ImageD{j};
%     imagesc(B); colorbar
%     colormap(gray)
%     grid on
%     Movie(j) = getframe;
% end
%% Find the edge on the image by selecting 5 points,
%change ginput input to change the number of points
imagesc(ImageD{1}); colormap(gray)
[dx,dy] = ginput(6) %The ginput parentheses specify...
... the number of clickable points %
dxi = 1:512;
dyi = spline(dx,dy,dxi);
close all
%% Select the point to be selected away
%% from the edge

```

```

imagesc(ImageD{1}); colormap(gray)

[ya,xa] = ginput(2) % Select a point close to the edge BUT not on the edge
xstep = abs(round(( round(xa(1)) - round(xa(2)) )/ npoints));
xfinal = xa(1) + xstep*npoints;
time = length(ImageD);
Intensity = double(zeros(length(time),npoints));
h = waitbar(0,'Smoothing Data... ');
for k = 1:npoints,
    waitbar(k/npoints,h);
    xpos = round(xa(1))+k*xstep;
    xall(k) = round(xa(1))+k*xstep;
    for ii = 1:time
        I0 = ImageD{ii};
        Intensity(ii,k) = I0(round(xpos),round(ya(1)));
        %% Smooth the signal selected and plot
        %Intensity = double(Intensity);
    end
    Smoothed_Intensity(:,k) = smooth(1:time,Intensity(:,k), 0.3, 'rloess');
end
close(h);
%% %% Smooth the signal selected and plot
% Intensity = double(Intensity);
% Smoothed_Intensity = smooth...
...(1:time,Intensity, 0.3, 'rloess');
figure(3)
scatter(1:time, Smoothed_Intensity(:,1))
hold on
scatter(1:time,Intensity(:,1),'r');
Difference = Intensity(:,1)-Smoothed_Intensity(:,1);

```

```

scatter(1:time,Difference(:,1),'g');
legend('Smoothed Data','True Data', 'Difference');
xlabel('Time Points (dt = 3 seconds)');
ylabel('Normalized Intensity');
%% Plot the point that was chosen to fit data
figure(4)
Image_sample = ImageD{1};
imagesc(Image_sample);colormap(gray)
hold on
%xall = linspace(round(xa(2)),round(xa(1)),npoints);
yall = ya(1)*ones(size(xall));
plot(yall,xall,'ro','MarkerSize',12, ...
     'MarkerFaceColor','r');
%% Put in all the parameters in to pass to the fitting
c0 = 5*10^(-6); % concentration at x=0 ...
(Reservoir concentration of solvent)%%
dt = 3; %time interval (seconds)
tf = time*dt;%time points (total time)
a = 5.32*10^(-3); %the length of the sample (m)
h = waitbar(0,'Calculating Diffusivity constant... ');
for k = 1:npoints,
    waitbar(k/npoints,h);
    y = abs(xall(k)-dyi(round(ya(1))))*2.49*10^(-6); % ...
distance away from edge of the chosen point
    [D, R2,SSE,Concentration] = ...
        fit_Diffusion(c0,tf,dt,y,a,squeeze...
... (Smoothed_Intensity(:,k))');
    D20(k) = D;
    R2_20(k) = R2;

```

```

        Conc(:,k) = Concentration;
        yf(k) = y;
    end
    close(h);
    figure(5); set(gcf,'color','white');
    hist(D20(:),10);
    set(gca,'fontweight','b','FontSize',14);
    xlabel('Diffusivity','fontweight','b','FontSize',14);
    ylabel('Frequency','fontweight','b','FontSize',14);
    title('Collagen Diffusivity (m2/sec)','fontweight','b','FontSize',14);
    D20_mean = median(D20(:))
    D20_std_dev = std(D20(:))
    figure (6); set(gcf,'color','white');
    ttime = [1:time]*dt;
    for i=1:npoints,
        plot(squeeze(ttime),squeeze(Conc(:,i)),'--');
        hold on;
    end
    set(gca,'fontweight','b','FontSize',14);
    xlabel('Time (sec)','fontweight','b','FontSize',14);
    ylabel('Normalized Concentration','fontweight','b','FontSize',14);
    title('Collagen Diffusion Plot at a Concentration of 2.2 uM',...
        ...'fontweight','b','FontSize',16);
    figure(7); set(gcf,'color','white');
    for K = 1:npoints,
        L=length(Conc(:,1))
        C(K)= mean(Conc(L-10:L, K));
        xalltrue(K) =xall(K)*2.49;
        plot (xalltrue(K), C(K), 'o')
    end

```



```

        hold on
    end

    [p,S]=polyfit(xalltrue,C, 1)
    f=polyval(p, xalltrue)
    plot (xalltrue, f, 'r*--', 'LineWidth', 1)
    set(gca,'fontweight','b','FontSize',14);
    xlabel('Position, x','fontweight','b','FontSize',14);
    ylabel('Normalized Concentration','fontweight','b','FontSize',14);
    title('Concentration Profile Across x','fontweight','b','FontSize',16);
    r=1-S.normr^2/norm(C-mean(C))^2;
    gtext(['r^2=',num2str(r)],'fontweight','b','FontSize',13)
    %%%%%%%%%
    %%%%%%%%% Diffusion Equation Function %%%%%%%%%
    %%%%%%%%%
    function [Cnorm] = Diffusion_Equation(c0,tf,dt,D,x,a)
    % BCs: C(0) = C0, C(a) = 0 @ 0<t<inf
    % IC: C(x,0) = 0, 0<x<a

    % Boundary conditions are not homogenous, so we cant use separation of
    % variables, but it is obvious that at steady state the solution will vary
    % linearly between boundary concentrations C0 and 0;

    % So, the concentration C(x,t) can be thought as
    % C(x,t) = steady state + transient
    % C(x,t) = [C(0,t)+(x/a)*(C(a,t)-C(0,t))]+ U(x,t);
    % So, now we can solve for U(x,t) and add it tot he the steady state
    % solution
    % New BCs: U(0,t)= 0, U(a,t) = 0 @ 0<t<inf
    % New IC: U(x,0) = F(x)-(C(0,t)+(x/a)*(C(a,t)-C(0,t)));
    % with this substitution we have a homogenous PDE with homogenous BCs,

```

```

% which is easy to solve with separation of variables.
% a = 5*10(-3);
% D= 10(-9);
% x= 1000;
% x = x*10(-6);
% c0 = 5*10(-6);
t = 1:dt:tf;
format long
for i = 1:length(t)
    for n = 1:100
        Uxt(:,n) = -(2*c0/(n*pi)) *sin(n*pi*x/a)...
            .*exp(-t(i)*(D)*(n*pi/a)^2);
    end
    U(i) = sum(Uxt);
    C(i) = c0-(c0*x/a)+U(i);
end
Cnorm= C/c0;
end
%%%%%%%%%
%%%%%%%%%% Diffusion Fit Function %%%%%%%%%%
%%%%%%%%%%
function [estimates, R2,SSE,Concentration] = ...
    fit_Diffusion(c0,tf,dt,x,a,true_data)
% Call fminsearch with an initial guess
start_point = [5*10(-11)];
[estimates] = fminsearch(@expfun, start_point)
% fminsearch searches for D value that minimizes SSE:Sum of Squared...
%...Errors of Prediction
function [sse] = expfun(start_point)

```

```

D = start_point(1);
[Concentration] = Diffusion_Equation(c0,tf,dt,D,x,a);
ErrorVector = Concentration - true_data; % Residual
sse = (sum(ErrorVector.^ 2)); % Residual Sum of Squares
SSE =sse;
SST = (sum((true_data - mean(true_data(:))).^2));
% Total Sum of Squares
R2 = 1 - SSE/SST % Coefficient of Determination
end
figure(99)
plot(1:length(true_data),true_data,'ro');
hold on
plot(1:length(Concentration),Concentration,'go');
legend('True Data','Fitted Data','FontWeight',12);
xlabel('Time Intervals (dt = 3)', 'FontWeight','bold','FontSize',12);
ylabel('C/C_0', 'FontWeight','bold','FontSize',12);
ylim([-0.1 1.3])
end

```

APPENDIX B

Mechanisms of Greater Cardiomyocyte Functions on Conductive Nanoengineered Composites for Cardiovascular Applications

1. Introduction

¹ Cardiovascular disease is a major epidemic, both in the US and worldwide. Cardiovascular disease refers to a class of diseases that affect the heart and blood vessels of the host, and usually refers to atherosclerosis or arterial disease [133, 134]. Approximately every 25 seconds, an American will have a coronary event, and approximately every minute, someone will die of one [135]. On average, every 40 seconds, someone in the US has a stroke [135]. An estimated 74,500,000 adults in the US have hypertension, with approximately 78 percent aware of their condition and 68 percent using antihypertensive medication, but only 44 percent of those treated have their hypertension controlled [135]. The total direct and indirect costs of cardiovascular disease and stroke in the US for 2010 were estimated at \$503.2 billion [135].

The major culprit of CVD is when an individual has a myocardial infarction, also known as a heart attack. This usually occurs when a blood vessel in the heart is obstructed in the myocardium and the cardiac muscle surrounding the obstruction dies due to oxygen deprivation. This is very important since the myocardium of the heart is composed primarily of specialized muscle cells called cardiac myocytes or cardiomyocytes. Although cardiomyocytes comprise only approximately 25 percent of the total cells in the heart, they account for more than 90 percent of the total

¹A version of this appendix has been published in the International Journal of Nanomedicine [Stout, D.A., J. Yoo, A.N. Santiago-Miranda, and T.J. Webster. “Mechanisms of Greater Cardiomyocyte Functions on Conductive Nanoengineered Composites for Cardiovascular Applications.” *International Journal of Nanomedicine*, 7(1): 5653-5669, 2012]

myocardium volume due to their large size. Therefore, when a blood vessel in the heart is blocked by an obstruction these specialized cells die, known as an infarct. Since cardiomyocytes are terminally differentiated cells, they are usually unable to regenerate after an infarction [136]. Instead, non-contractile fibrous cells gradually begin to replace the dead cardiomyocytes causing a thin and rigid muscular ventricular wall [137]. The scarred tissue formation reduces contractile function of the heart, causing ventricular remodeling, ultimately leading to heart failure [138].

Although changes in environmental exposures, reduction in tobacco use, adjustments in diet, and increased physical activity can all improve patient health, the progression of CVD in the world is growing. New models to detect and treat CVD in asymptomatic patients are needed in order to prevent the first symptoms from being the last as a result of death. A need for improved and safer approaches to coronary and intracranial revascularization is still required, even with advances over the last 10 years.

Unfortunately, no single technology offers the perfect solution. To address these problems, quick technological advancements of cell biology, genomics, and proteomics combined with discoveries in material sciences and bioengineering have created a new field of medicine, nanomedicineutilizing materials and systems which possess at least one physical dimension between 1-100 nm to construct structures, devices, and systems that have novel properties [139, 140].

The advent of advanced novel nano-biomaterials with improved properties capable of being used in several biomedical applications simultaneously, has transformed the field of biomedical research. Nanomaterials are among the most intensively studied materials for a wide range of applications ranging from fuel cells [141] to nanopatches for the myocardium [142, 143]. These nanostructured composite materials are combinations of at least two constituent materials, a matrix (host) and a reinforcement component (i.e., nanofiller) [144]. It is known that the properties of materials change considerably when the size of constituents is significantly small, within the 1100 nm

size range [145, 146, 147]. Since these materials have improved physical, chemical, and mechanical properties, they are significantly versatile for a wide range of applications.

One such example is the use of a poly(lactic-co-glycolic acid) (PLGA) material with embedded carbon nanofibers (CNF) to create a patch for an infarcted area of the heart. Recent research has shown when one adds CNFs to PLGA, cardiomyocytes (specific heart tissue cells responsible for contraction) will adhere and proliferation better than on their non-nano reinforced counterparts, but the mechanism is still largely unknown [143].

To further understand cardiomyocyte functions and elucidate a mechanism for such interactions on PLGA:CNF materials, the purpose of this in vitro study was to alter PLGA density to shed light on material wettability, protein adsorption, conductivity, tensile strength, and cardiomyocyte function, to comprehend why cardiomyocytes favor particular nanocomposites over other composites. The hypothesis of this study is that materials which mimic the conductivity, tensile strength, and topography of the heart tissue itself will maximize cardiomyocyte function.

2. Methods & Materials

PLGA:CNF composites were fabricated as previously described in great detail in prior studies.¹⁵ In short, different PLGA densities (0.1, 0.05, 0.025 and 0.0125 $\frac{g}{ml}$) (50:50 PLA:PGA wt.%; Polysciences, #23986, USA) were created by dilution in a 50 ml flask with 30 ml of tetrahydrofuran (THF; Mallinckrodt Chemicals, #C45763, USA) and were sonicated in a water bath (VWR B3500A-DTH, USA) below 30°C for 30 min. 500 mg of CNFs (99.9% by weight%, Catalytic Materials, USA) with a diameter of 100 nm of different lengths, from 100 to 200 microns, were sonicated (Misonix Sonicator 3000, USA) in a 50 ml beaker with 20 ml of chloroform (Fisher Science, #102591, USA) at 20W for 30 min. After obtaining the separately sonicated PLGA and CNF solutions, various PLGA:CNF weight percent ratios were developed (100:0, 75:25, 50:50, 25:75, and 0:100) by adding the appropriate amount of CNF to PLGA in 20 ml disposable scintillation vials. The CNF weight ratios were measured

using a laboratory balance (Mettler Toledo AL54, USA). When the appropriate ratios were added, each composite material was sonicated (Misonix Sonicator 3000, USA) at 10 W for 20 min each.

For experimental ease, a 22 mm diameter microscope cover glass (Fisher Science circles No. 1 - 0.13 to 0.17mm thick; Size: 22 mm, #12-545-101, USA) was coated with the aforementioned composite. Before the PLGA:CNF composite was positioned onto the glass substrate, the glass substrate was cleaned via soaking in a 70:30 (vol.%) ethanol-deionized solution with shaking (VWR, Advanced Digital Shaker, USA) for 10 min. Next, the substrate was added to a 100% deionized water solution and shaken for 10 min.

Using a disposable pipette (Fisherbrand, #13-711-9AM, USA), 1 ml of the appropriate PLGA:CNF composite solution was placed onto the glass substrate and placed into an oven at 42°C for 15 min. Each composite film was then vacuum dried (Shel Lab, USA) at 20 inches of Hg gauge vacuum pressure for 48 h to allow the tetrahydrofuran (THF) and chloroform to evaporate. All the samples and controls were sterilized using ultraviolet light for 24 h prior to cell experiments.

A Hitachi 2700 scanning electron microscope (SEM) was used to characterize the surface of the PLGA:CNF samples at 5 KV utilizing an InLens system and software with magnification ranging from 1 Kx to 40 Kx.

$$\%SA = \frac{(A_F - A_I)}{A_F} \times 100 \quad (\text{B.1})$$

Atomic force microscopy (AFM) measurements were performed by a scanning probe microscope (Asylum Research, MFP-3D Coax, USA) to create a topographical map of the surface. The analyses were performed in air and under a constant applied force condition (non-contact mode) with a cantilever (Asylum Research, #AC160TS, USA) resonant frequency of 360 kHz and theoretical spring constant $k: 42 \frac{N}{m}$. Images were processed and analyzed using the Image Processing Data Analysis Igor Pro 6 software (WaveMetrics, USA). Material surface roughness was evaluated on topographical AFM images and the root-mean-square (RMS) data averaged as well

as percent surface area (%SA) increase (equation B.1, where A_F is the final area and A_I is the initial area) calculated over a series of six images for each sample ratio with the assistance of AFM software (WaveMetrics, Igor Pro 6, USA).

$$\sigma = \frac{l}{2\pi V} \left(\frac{1}{l_1} + \frac{1}{l_2} - \frac{1}{l_1 + l_2} - \frac{1}{l_2 + l_3} \right) \quad (\text{B.2})$$

Conductivity tests were performed to compare material characteristics to that of the human myocardium. The electrical resistance of the PLGA:CNF dry samples were obtained by measuring sample resistance while taking geometric considerations into account. 1 ml of solution was placed on a glass substrate (Fisher Science circles No. 1 - 0.13 to 0.17mm thick; Size: 22 mm, #12-545-101, USA) and vacuumed dried for 48 h to evaporate all of the chloroform and tetrahydrofuran (THF). Conductivity measurements will be completed by using a 4-point conductivity theorem (equation B.2), where V is voltage, l_1 is the length between leads 2 and 3, l_2 is the length between leads 1 and 2, and l_3 is the length between 3 and 4. In brief, for a four-point probe measurement, one pair of leads was used to inject the measurement current while a second pair of leads, in parallel with the first, was used to measure the potential drop across the device. There was no potential drop across the voltage measurement leads so the contact resistance drop was not included, making a more accurate system. All measurements were conducted on an in house built 4-point conductivity measurement device and completed four times on each sample at different locations.

Tensile tests were performed to compare material characteristics to that of the human myocardium. A negative flat test sample mold was used to create the material samples, made from Sylgard 184 (Dow Corning, #11-1792-01, USA) with sample dimensions of gage length 7.62 mm, width of gage length 3.18 mm, reduced section 9.53 mm, distance between shoulders 26.33 mm, an overall length 63.55 mm, width of grip section 9.53 mm, and a sample thickness 3.20 mm. Each PLGA:CNF composite ratio solution was added to the mold and vacuumed dried (Shel Lab, USA) at 20 inches of Hg gauge vacuum pressure for 72 h to allow for solvent evaporation. After

samples were dried and released from the mold, tensile tests were performed in a Rheometric Scientific minimat 2000 tensile machine (Rheometric, USA) at a speed of 50 mm/min until material failure occurred in accordance to the American Society for Testing and Materials (ASTM) Standard Test Method for Tensile Properties of Plastics Designation D638-10 [148].

The adsorption of proteins on the sample surfaces was performed with fibronectin and vitronectin. First, all samples under investigation were rinsed with phosphate buffer saline (PBS; solution containing 8 g NaCl, 0.2 g KCL, 1.5 g Na₂HPO₄, and 0.2 g KH₂PO₄ in 1000 ml deionized water adjusted to a pH of 7.4; all chemicals from Sigma Aldrich) twice and placed into cell culture well plates (BD Falcon, #353503, USA) for sterility purposes. Two sets of similar well plates were prepared for each experiment. The first set included 1 ml of cardiomyocyte growth media (Celprogen, #M36044-15S, USA) supplemented with 10% fetal bovine serum (FBS, Thermo Scientific, #SH30109, USA). For the second set, 1 ml of cardiomyocyte growth media (Celprogen, #M36044-15S, USA) without FBS was added to each well as a control. Both plates were incubated under standard conditions (at 5% CO₂ 95% humidified air and 37°C) for 24 h to allow for protein adsorption. After 24 h, both plates were taken out and media was aspirated and samples rinsed twice with PBS. Then, the substrate samples were blocked with 500 l of a 2% bovine serum albumin (BSA) solution (BSA and PBS solution mix) for 1 h. The BSA solution was aspirated and samples rinsed twice with PBS. 500 l of the first antibody (fibronectin (Chemicon, #AB19014, USA)) containing solution (3 g antibody in 500 l 1% BSA solution) was added to each well of both plates and was allowed to incubate for 1 h under standard conditions (at 5% CO₂ 95% humidified air and 37°C). After 1 h, the antibody solution was aspirated and rinsed with 0.05% Tween 20 (Sigma Aldrich, #P1379-25ML, USA) three times, each time placed on a table shaker for 5 min. After the last Tween 20 aspiration, 500 l of a goat anti-rabbit secondary antibody conjugated with horseradish peroxidase (Bio-Rad, #170-6515, USA) solution (5 l secondary antibody in 500 l in 1% BSA solution) was added to each well and incubated for 1 h. Then, the secondary antibody

was aspirated and rinsed two times with 0.05% Tween 20, each time placed on a table shaker for 5 min. All samples were transferred into new well plates and rinsed twice with 0.05% Tween 20, each time placed on a table shaker for 5 min. Then, 500 l of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) (Vector Labs, #SK-4500, USA) was added to each well and incubated at room temperature in the dark for 20 min. 200 l of each sample was transferred to a 96 well plate and read at 405 nm using a spectrophotometer. Readings from samples cultured in media without FBS were subtracted from the readings from samples cultured with media with FBS. This was again performed with vitronectin (Chemicon, #AB2047, USA) as a primary antibody. Protein OD readings were normalized to the lowest OD reading whereas the percent surface area data was not normalized.

$$\delta = \frac{\%SA}{\rho} \quad (\text{B.3})$$

Due to changes in surface area between the samples of interest and this study, a unit-less variable (δ) comparison equation (equation B.3) was established between the amount of proteins that absorbed onto the substrate surface (ρ) and surface area percent increases (%SA) assessed by AFM (equation B.1). This allowed for the determination of the protein adsorption capacity for a particular sample with relationship to adding CNFs without regard to a samples surface area.

Contact angle measurements were completed using a Krss EasyDrop system (model DSA20S) with Krss static and dynamic contact angle software (SW4001). This was done by dropping 3 l of distilled water onto the PLGA:CNF composites and measuring the angle between the surface and water droplet (via software). Four measurements were completed on each sample and were completed in triplicate for each PLGA density and PLGA:CNF (wt%:wt%) composite ratio.

$$\%W_L = \frac{(W_O - W_t)}{W_O} \times 100 \quad (\text{B.4})$$

For degradation experiments, initial dry samples of interest were weighed (W_0) and sterilized as previously described. Next, all samples were immersed into PBS

and incubated under standard cell culture conditions (37°C, 5% CO₂ 95% air environment). A glass substrate in PBS and blank PBS without substrates were used as a reference and control, respectively. After 21, 28, and 36 days, samples were removed from PBS, abundantly rinsed with deionized water to remove soluble inorganic salts and dried in an air vacuum chamber (Shel Lab, USA) at 20 inches of Hg gauge vacuum pressure at room temperature for 48 h to reach constant mass. At each time point, samples were weighed (W_t) and the percentage weight loss ($\%W_L$) with respect to incubation time was calculated (equation B.4). Percent weight loss was normalized to the amount of PLGA in the composites since CNFs do not degrade.

For in vitro analysis, human cardiomyocytes (Celprogen, #36044-15, USA) were seeded at a cell density of $5.0 \times 10^4 \frac{\text{cells}}{\text{cm}^2}$ for the cell adhesion assay and $5.0 \times 10^4 \frac{\text{cells}}{\text{cm}^2}$ for the cell proliferation assay on PLGA:CNF composites in complete growth media (Celprogen, #M36044-15S, USA) supplemented with 10% fetal bovine serum (Thermo Scientific, #SH30109, USA) and a 1% Penicillin/Streptomycin mixture (Fisher Scientific, #BP2959-50, USA). Cells were seeded into 12-well human cardiomyocyte stem cell culture extra-cellular matrix plates (Celprogen, #E36044-15-12Well, USA) on top of the various PLGA:CNF samples, and 22 mm diameter microscope cover slips (Fisher Science circles No. 1 - 0.13 to 0.17mm thick; Size: 22 mm, #12-545-101, USA) as controls. The samples were incubated for 4 h for the cell adhesion assay and 24, 72, and 120 h for the cell proliferation assay under standard incubation conditions (at 5% CO₂ 95% humidified air and 37°C, changing the media every other day).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Progenia, CellTiter 96® Non-Radioactive Cell Proliferation Cat. #G-4100) assays were completed to analyze the cytocompatibility properties of the composite. Also, fluorescence microscopy (Leica DM5500B Research Microscope, Lica, Germany) was used to determine cell attachment and proliferation on the PLGA:CNF samples after the prescribed time period and compared to MTT results. At the end of each experiment, samples were rinsed with PBS and saturated with 10% buffer formalin acetate (Fisher Science, #085947, USA) for 15 minutes. For this, the samples were again rinsed with

PBS twice and then dyed with 4-6-Diamidino-2-phenylindole, dihydrochloride (DAPI) (Molecular Probes®, #D1306, USA). The samples were rinsed again and stored in PBS for imaging. Samples were immediately imaged and counted, four images per sample, using a 10x objective lens.

The optical density (OD) data for cardiomyocyte MTT assays were plotted as mean $\pm$ standard error of the mean while OD to cell count was conducted by using a standard curve analysis between OD and cell counts total. For DAPI staining, cardiomyocyte counting was determined in at least 5 randomly chosen microscope fields (magnification at 10X) for each sample.

For cellular viability, cells were seeded into 12-well plates (BD Falcon, #353503, USA) with each sample ratio and a control glass substrate at 5.5×10^4 cells/ml and incubated for 24, 72, and 120 h under standard incubation conditions. A LIVE/DEAD® viability/cytotoxicity assay for mammalian cells kit (Molecular Probes®, #L-3224, USA) was used to qualitatively assess cell viability and morphology. The LIVE/DEAD® assay is a two-color assay simultaneously staining with green-fluorescent calcein-AM indicating intracellular esterase activity and a red-fluorescent ethidium homodimer-1 indicating the loss of plasma membrane integrity. Media were removed from each well and samples were washed with PBS. The cells were then incubated for 30 min with a 500 l solution containing 2M calcein AM and 4M EthD-1 dissolved in PBS and imaged with a fluorescence microscope (Zeiss, Axiovert 200M, Germany). Samples were immediately imaged and counted, four images per sample.

To indicate cellular specificity towards normal cardiomyocyte characteristics, seeded cells were immunofluorescently stained for cardiomyocyte specific biomarkers to demonstrate the integrity of the cells: connexin-43 for gap junction channels (Cx43), α -sarcomeric actin (α -SCA) for cardiac myocytes, and Troponin T (cTnT) for contractile myocytes. Cardiomyocytes are interconnected by gap junction channels that conduct electric impulses and direct intercellular signaling. Cx43 is one of the three proteins that comprise the channels. α -SCA is considered a specific protein found only in alpha skeletal and alpha cardiac muscle actin in cardiomyocytes.

Troponin T is part of the Troponin complex that binds to actin in thin myofilaments indicating the contractibility of cardiomyocytes.

Cells were seeded into 12-well plates (BD Falcon, #353503, USA) with each sample and a control glass substrate at 5×10^4 cells/ml and were incubated under standard incubation conditions for 120 h. Media were removed and cell cultures were rinsed 2x with PBS. Cells were fixed for 15 min with 1-2 ml of 10% formalin acetate (Fisher Scientific, #SF99-20, USA), permeabilized with 0.2% Triton X-100 (Sigma-Aldrich, #X100-1L, USA) diluted in PBS for 5 min, and blocked with Image-iT FX signal enhancer (Invitrogen, #I36933, USA) for 30 min at room temperature with 2x PBS wash steps in between each step. Primary rabbit polyclonal anti-Cx43 (Abcam, #ab11370, USA), mouse monoclonal anti-sarcomeric alpha actin (Abcam, #ab9465, USA), and rabbit polyclonal anti-cardiac troponin T (Abcam, #ab45932, USA) antibodies were used at a dilution of 1:400 in PBS for 1 h at room temperature. Secondary AlexaFluor488 conjugated goat anti-mouse IgM (Invitrogen, #A-21042, USA) and anti-rabbit IgG (Invitrogen, #A-21206, USA) were diluted 1:600 in PBS and incubated for 60 min at room temperature. The nucleus was visualized using a DAPI (Molecular Probes[®], #D1306, USA) stain in a 1:30 dilution for 15 min at room temperature. Samples were then mounted with ProLong Gold Antifade (Invitrogen, #P36930, USA) and imaged with a fluorescence microscope (Zeiss, Axiovert 200M, Germany). Images were analyzed using ImageJ software (National Institute of Health, USA) with the analysis particle tool. Images were converted to a 8-bit color scheme, then adjusted to a red/black threshold and analyzed with analysis particle tool to measure protein color concentration and compared to the total area of the image. This was done for all (DAPI, cTnT, CX43, and α -SCA) stains.

Cardiomyocyte adhesion and proliferation assays were performed at least in triplicate with three repeats each and results were compared to a control glass surface. For determining differences between means, ANOVA software and a student T-test were used. A p-value of < 0.05 was considered to be significant. For correlation graphs, a curve fitting tool (MathWorks, USA) in a sigmoidal curve fit with a Trust-Region

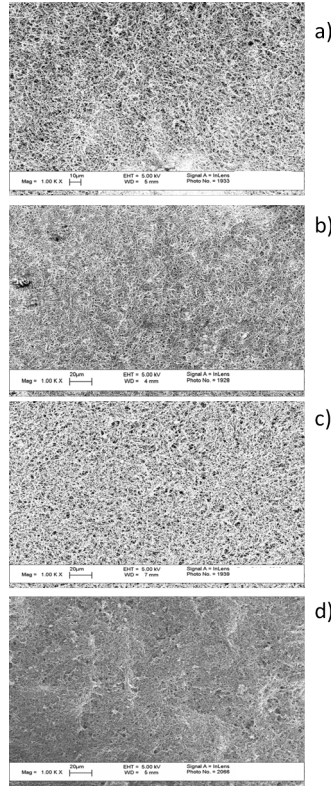


FIGURE B.1. Scanning electron microscopic images at 5000x magnification showing distribution of CNF fibers in the PLGA matrix at (A) 75:25, (B) 50:50, (C) 25:75, and (D) 0:100 (PLGA:CNF [wt:wt]) at a PLGA density of 0.025 g/mL. Note: Scale bar 20 μ m. Abbreviations: CNF, carbon nanofibers; PLGA, poly(lactic-co-glycolic-acid).

Algorithm for nonlinear optimization was used. All experiments within this paper were performed at least in triplicate with a minimum of three repeats each and results were compared to a control glass surface.

3. Results

Representative SEM images of the as-synthesized PLGA:CNF composite surfaces can be seen in Figure B.1. CNFs were uniformly dispersed within the PLGA matrix and, as expected, more CNFs were observed for the higher CNF ratio samples. When looking at the different PLGA densities and similar CNF ratios, uniform CNFs were again uniformly dispersed within the PLGA matrix, suggesting that the materials were properly synthesized (Fig. B.1).

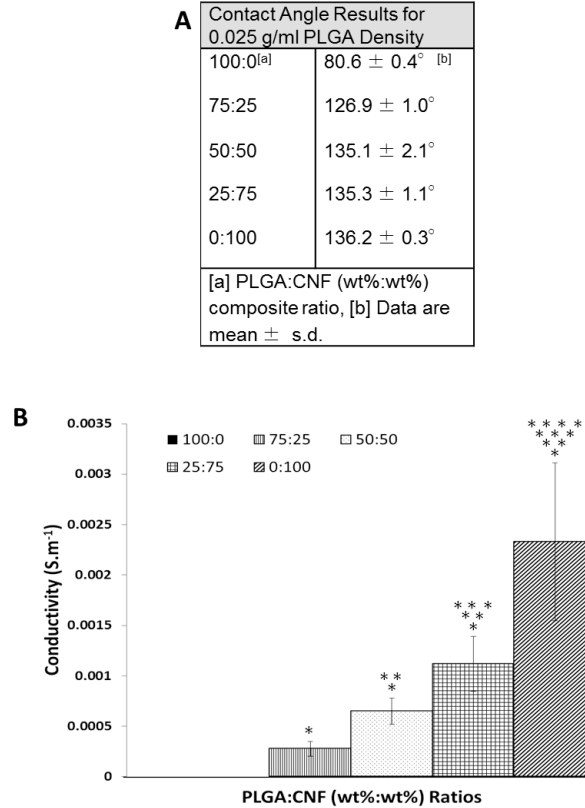


FIGURE B.2. Material characteristics of PLGA:CNF (wt%:wt%) looking at (A) wettability of the material at different PLGA density ratios, indicating that all materials are hydrophobic. (B) Example of material conductivity at a PLGA density of 0.025 g/mL, providing evidence of an inverse relationship between PLGA ratio (wt%) and material conductivity. Notes: Conductivity data are expressed as the mean ± standard deviation (n=3). *P < 0.05 versus 100:0 (PLGA:CNF [wt%:wt%]) ratio; **P < 0.05 versus (PLGA:CNF [wt%:wt%]) ratio; ***P < 0.05 versus 50:50 (PLGA:CNF [wt%:wt%]) ratio, and ****P < 0.05 versus 25:75 (PLGA:CNF [wt%:wt%]) ratio. Abbreviations: CNF, carbon nanofibers; PLGA, poly(lactic-co-glycolic-acid).

Results of this study also provided evidence that increasing the CNF weight ratio in PLGA increased conductivity, as shown in Figure B.2. Conductivity increased as greater amounts of CNFs were added to pure PLGA, from 0 S.m⁻¹ for pure PLGA (100:0 wt.%) to 5.5 x 10⁻³ S.m⁻¹ for pure CNFs (0:100 wt.%), as compared to natural heart tissue (ranging from 0.16 S.m⁻¹ longitudinally to 0.005 S.m⁻¹ transversely 24). Clearly, the increased conductivity of the composites was due to the uniform

distribution of more conductive CNFs within the sample (Fig. B.2). Therefore, as anticipated, CNF addition increased conductivity.

Contact angle experiments suggested that altering PLGA density did not affect the wettability of the composite. For example, contact angle measurements for a 50:50 [PLGA:CNF (wt%:wt%)] sample ranged from 132.0° for a $0.1 \frac{g}{ml}$ PLGA density to 135.0° for a $0.0125 \frac{g}{ml}$ PLGA density. Contact angle measurements also suggested that adding CNFs made the materials more hydrophobic, as compared to pure PLGA samples (Fig. B.2). For example, pure PLGA for all PLGA densities ($0.1 \frac{g}{ml}$, $0.05 \frac{g}{ml}$, $0.025 \frac{g}{ml}$, and $0.0125 \frac{g}{ml}$) were $81.0^\circ \pm 1.0^\circ$, whereas the 75:25 [PLGA:CNF (wt%:wt%)] samples were $125^\circ \pm 3.0^\circ$. This is important since cardiovascular research, particularly with biomaterials and cardiomyocytes, has shown that when a material is more hydrophobic, protein adsorption will occur more readily than their hydrophilic counterparts, to mediate cell attachment and spreading.²⁵

Results of the degradation study showed that the weight loss percentage of pure PLGA was the greatest among all the substrates tested after respective days (Fig. B.3). As expected, no weight loss was observed on pure CNF samples. Specifically for a PLGA density of $0.025 \frac{g}{ml}$, the weight loss of 75:25 samples was 10% less than that for pure PLGA, whereas the 50:50 and 25:75 sample ratios [PLGA:CNF (wt%:wt%)] had a higher percent of weight loss than pure PLGA after 21 days of incubation. Respectively, all sample ratios had 25% or more, which was less than pure PLGA at 28 days of incubation; and 30% or more, which was less than pure PLGA after 36 days of incubation. This indicates that the dispersion status of CNFs in PLGA played an important role in decreasing the degradation behavior of the indicated composites.

Also, the inhibiting effect of CNFs towards PLGA weight loss was more significant after longer time periods of incubation; this correlated to the pH buffering effect of CNFs. Specically, the pH drop was less than 5% for all the composites during the rst 21 days of incubation while it was $\sim 10\%$ for pure PLGA (Fig. B.3). During 22 to 36 days of incubation, the pH drop for all the composites and PLGA was faster than the rst 3 weeks of incubation. For a PLGA density of $0.025 \frac{g}{ml}$, the pH drop was

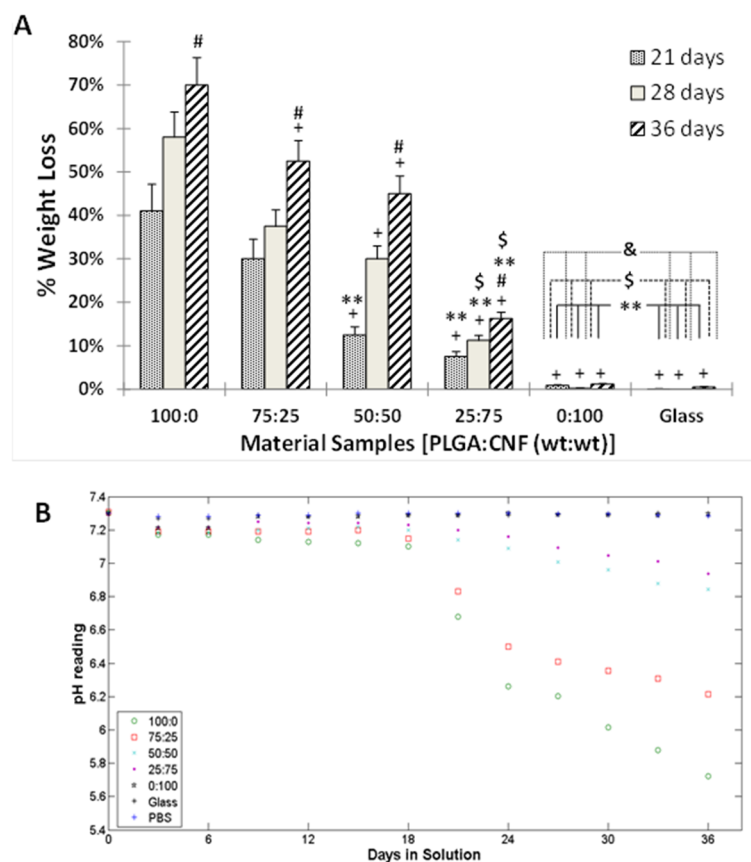


FIGURE B.3. Material characteristics of PLGA:CNF (wt%:wt%) looking at (A) wettability of the material at different PLGA density ratios, indicating that all materials are hydrophobic. (B) Example of material conductivity at a PLGA density of 0.025 g/mL, providing evidence of an inverse relationship between PLGA ratio (wt%) and material conductivity. Notes: Conductivity data are expressed as the mean $\pm$ standard deviation ($n = 3$). * $P < 0.05$ versus 100:0 (PLGA:CNF [wt%:wt%]) ratio; ** $P < 0.05$ versus (PLGA:CNF [wt%:wt%]) ratio; *** $P < 0.05$ versus 50:50 (PLGA:CNF [wt%:wt%]) ratio, and **** $P < 0.05$ versus 25:75 (PLGA:CNF [wt%:wt%]) ratio. Abbreviations: CNF, carbon nanofibers; PLGA, poly(lactic-co-glycolic-acid).

22% for pure PLGA while it was 15% for the 75:25 ratio [PLGA:CNF (wt%:wt%)], 8% for the 50:50 ratio [PLGA:CNF (wt%:wt%)], and only 5% for the 25:75 ratio [PLGA:CNF (wt%:wt%)] after 36 days of incubation. When PLGA degrades, it separates into lactic acid and glycolic acid, which in turn will increase the acidity of the surrounding media. Therefore, higher pH readings were observed in higher CNF

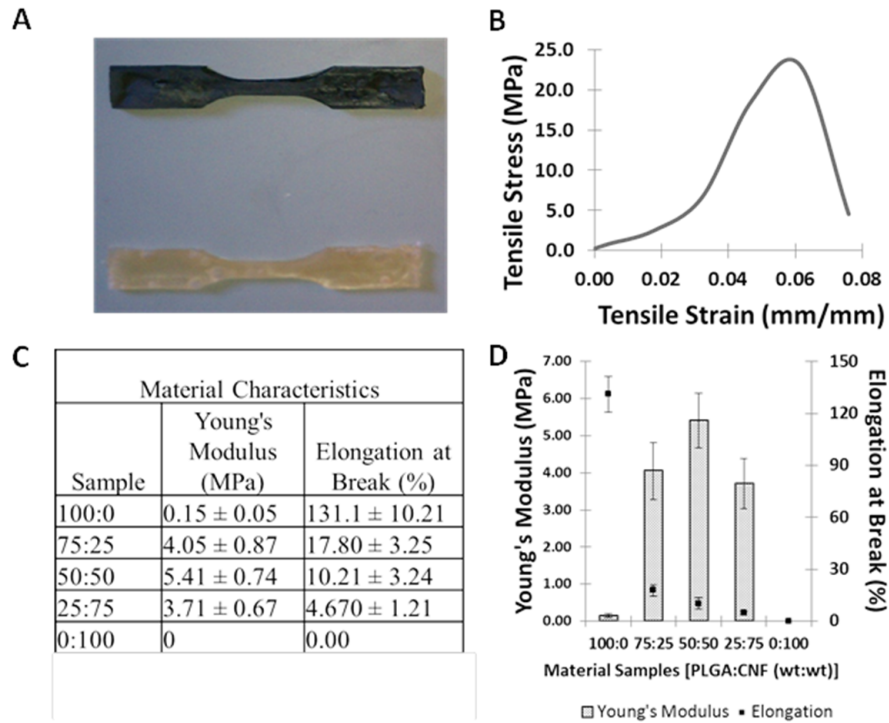


FIGURE B.4. Addition of CNF to PLGA greatly increased the tensile strength and created a material with properties closer to those of normal human myocardium. (A) Photograph of prepared flat test samples. Dimensions of the sample were: gauge length 7.62 mm, width of gauge 3.18 mm, reduced section 9.53 mm, distance between shoulders 26.33 mm, overall length 63.55 mm, width of grip section 9.53 mm, and sample thickness 3.20 mm. (B) Representative stress strain curve graph obtained in tensile experiments used to calculate Youngs modulus of a given sample. Stress strain curve of a 25:75 (PLGA:CNF [wt%:wt%]) sample ratio with a PLGA density of 0.025 g/mL. (C) Summary of sample ratio material tensile characteristics with a PLGA density of 0.025 g/mL as compared with active myocardium. (D) Graphical representation of Youngs modulus and percent elongation at break compared with material sample ratios with a PLGA density of 0.025 g/mL. Note: Data are shown as the mean count of $n=3$. Abbreviations: CNF, carbon nanofibers; PLGA, poly(lactic-co-glycolic-acid).

ratios, as compared to pure PLGA samples, due to less PLGA degradation material in the media.

Results showed that the addition of CNFs to the PLGA composite greatly enhanced composite tensile strength (Fig. B.4). Specifically, the addition of 25% CNFs (percent total weight) to pure PLGA increased the Youngs modulus by over 2500%,

while the addition of 50% CNFs (percent total weight) increased the Young's modulus by 3500% and an addition of 75% CNFs (percent total weight) increased the Young's modulus by $\sim 2100\%$. Compared to native cardiac heart tissue, the composites had mechanical properties that were stiffer than native heart muscle, which is made of collagen and is elastomeric with a stiffness of approximately 50-100 kPa during diastole [149, 150, 151]. However, the modulus of active myocardium during systole is approximately 20-fold higher [152, 153], which more closely resembles PLGA:CNF composites. These results indicated that the addition of CNFs increased composite tensile strength to better mirror the ability to strengthen the myocardium. On the other hand, the addition of CNFs to the PLGA composites decreased the percent elongation at material failure; 86% for the addition of 25% CNFs (percent total weight) to 4% for the addition of 75% CNFs (percent total weight) thus indicating an inverse relationship between the addition of CNFs to PLGA and elongation at failure. This is not surprising since the continual addition of CNFs to a matrix will hinder the ability of PLGA to adhere to the CNFs and strengthen the material.

Increased topographical features were present throughout AFM results on all material compared to an etched glass surface. It was shown that adding CNFs to the PLGA solution increased topographical features and also the surface area of the substrate (Fig. refAFM). Specifically, pure PLGA had a 10% surface area increase compared to an etched glass coverslip of the same dimensions. With the addition of 25% CNF (weight percent) to PLGA, the surface area increased by 45%. A 50% CNF (weight percent) addition to PLGA increased surface area by $\sim 60\%$. With the addition of 75% CNF (weight percent) to PLGA and 100% CNF, a lesser percent surface area increase was observed. Specifically, 75% CNF (weight percent) to PLGA increased surface area by $\sim 28\%$ whereas 100% CNF increased surface area by $\sim 22\%$. This is mainly because when adding too many CNFs to PLGA significant agglomeration occurs which creates more micro features and less nano features which actually

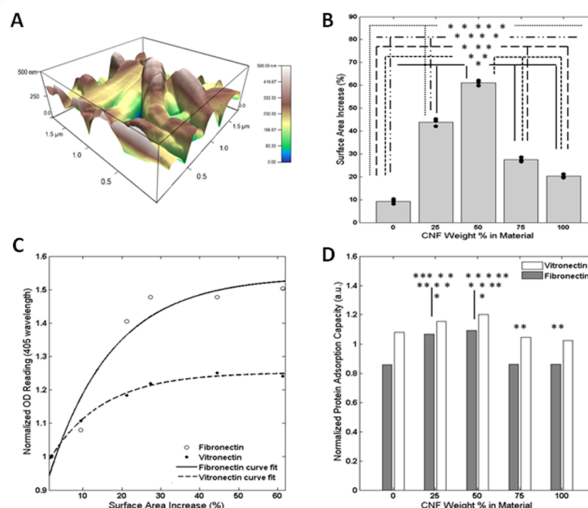


FIGURE B.5. Addition of CNF to the PLGA substrate alters the topography of the material. Using an Asylum MFP three-dimensional atomic force microscope (scan rate 1.0 Hz, AC mode with 256 x 256 scan points and lines), (A) CNF are shown for a 25:75 ratio (PLGA:CNF [wt%:wt%]) sample with a PLGA density of 0.025 g/mL which increases surface nanometer features as well as the total substrate surface area as compared with an etched glass cover slip. (B) It is shown for all PLGA:CNF ratio samples that increasing the CNF weight ratio increased the surface area of the substrate by creating nano, submicron, and micron features. (C) By normalizing the protein adsorption OD readings to the lowest reading (ie, for the controlled etched glass cover slip) and comparing it with the surface area percent increase, the results indicate that protein saturation was around 40% to 55% of amount of increase of the surface area, and provide evidence that increasing the surface area (through addition of CNF) allows more proteins to attach to the surface to promote cell attachment. (D) Calculating the normalized protein adsorption capacity indicates that the 75:25 and 50:50 ratios (PLGA:CNF [wt%:wt%]) had the highest ability to attach the most proteins to a particular surface, indicating that one of the mechanisms regulating cardiomyocyte interactions is increasing material protein capacity through altering topographical features by addition of CNF. Notes: This is evident in the similar cardiomyocyte growth trends reported by Stout et al.¹⁵ * $P < 0.05$ CNF wt% in materials versus 0% CNF wt% in materials; ** $P < 0.05$ CNF wt% in materials versus 75% CNF wt% in materials; *** $P < 0.05$ CNF wt% in materials versus 50% CNF wt% in materials; **** $P < 0.05$ CNF wt% in materials versus 75% CNF wt% in materials, and ***** $P < 0.05$ CNF wt% in materials versus 100% CNF wt% in materials. Data are shown as the mean count of $n=3$. Abbreviations: CNF, carbon nanofibers; OD, optical density; PLGA, poly(lactic-co-glycolic-acid).

decreases the surface area. Importantly, this indicates that the addition of nanomaterials to a system can increase topographical features, which in turn can increase cell attachment and growth [154].

Results of this study indicated that the addition of CNFs to PLGA directly affects initial protein adsorption events. When normalized to pure PLGA samples, the OD reading data for both fibronectin and vitronectin, when compared to the AFM surface area percent increase, results showed significantly greater vitronectin and fibronectin adsorption on composites when adding CNFs up to 50% (weight ratio), then a decrease when adding more CNFs to the matrix (Fig. B.5). These results indicate a direct correlation between surface topography and protein adsorption. This is important since protein adsorption has been correlated to the wound healing process. For example, fibronectin is a known chemoattractant for several cell types which play a role in the wound healing process [155] like fibroblasts, endothelial cells, and macrophages. In addition, fibronectin generates a scaffold to which other extracellular matrix components can attach. During the healing phase after a myocardial infarction, large amounts of plasma proteins, namely fibronectin, are deposited around the area of necrosis to promote cell attachment and growth [156, 157] within one day after infarct [158].

For cardiomyocyte adhesion, after 4 hours of cell culture, results from this study showed that the 0.05 and 0.025 $\frac{g}{ml}$ PLGA densities with the 75:25 and 50:50 ratio [PLGA:CNF (wt%:wt%)] had the highest cardiomyocyte density. The results of the proliferation assay showed the same trend as that observed during the adhesion assay (Fig. B.6). Specifically, the 75:25 and 50:50 sample ratios [PLGA:CNF (wt%:wt%)] at a 0.05 and 0.025 mg/ml PLGA densities had the highest cardiomyocyte densities, whereas the 100:0 sample ratio [PLGA:CNF (wt%:wt%)] for all PLGA density had the lowest cardiomyocyte densities after 24, 72, and 120 h. Due to the slower proliferation of the cardiomyocytes (Celprogen , Cat #36044-15) compared to other cells (around 72 hours for cell doubling times), one would not see a typical doubling time every 48

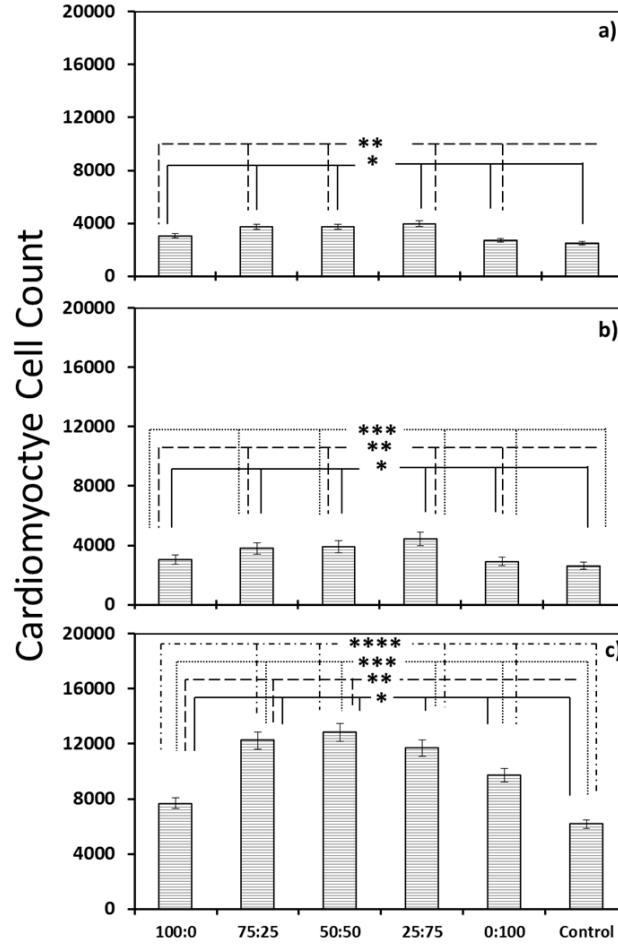


FIGURE B.6. Cardiomyocyte cell proliferation on a PLGA density of 0.025 g/mL after (A) 24, (B) 72, and (C) 120 hours on 100 nm diameter PLGA:CNF composite materials of interest in this study. Notes: Seeding density was 5×10^4 cells/cm². Data are shown as the mean count of n=3. The control was the glass substrate. *P < 0.05 versus control; **P < 0.05 versus similar PLGA:CNF composite ratios and different densities; ***P < 0.05 versus similar PLGA:CNF composite ratios between 24 and 72 hours, and ****P < 0.05 versus PLGA:CNF composite ratios between 72 and 120 hours. Abbreviations: CNF, carbon nanofibers; PLGA, poly(lactic-co-glycolic-acid).

hours. Also, LIVE/DEAD® images showed an increase in cell density over time with 60-80% confluence by day 5 for all PLGA:CNF ratios (Fig. B.7).

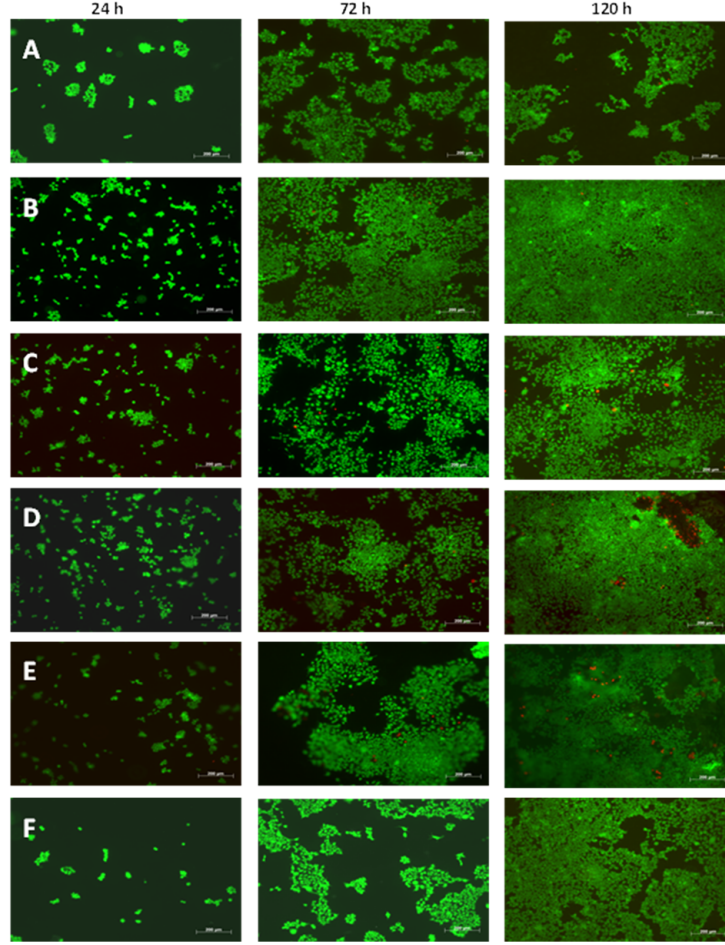


FIGURE B.7. Live/Dead assay images after 24, 72, and 120 hours on the various weight ratios (A) 100:0, (B) 75:25, (C) 50:50, (D) 25:75, (E) 0:100, and (F) glass with a PLGA density of 0.025 g/mL. Note: Green indicates live cells and red indicates dead cells. Seeding density was 5.5×10^4 cells/mL. Scale bar = 200 μm . Abbreviation: PLGA, poly(lactic-co-glycolic-acid).

Furthermore, immunofluorescence staining of specific cardiac biomarkers (cTnT, Cx43, and α -SCA) was conducted in the present study (Fig. B.8), indicating normal cardiomyocyte activity on all materials. When using ImageJ software, cardiac biomarkers staining followed the same patterns showed for all cardiomyocyte adhesion and proliferation experiments (Fig. B.6). Specifically, 50:50 [PLGA:CNF (wt%:wt%)] ratio samples had the highest staining area percent (63% for cTnT, 60% for Cx43, and 61% for α -SCA) for all cardiac biomarkers. Similarly, cells were observed to have

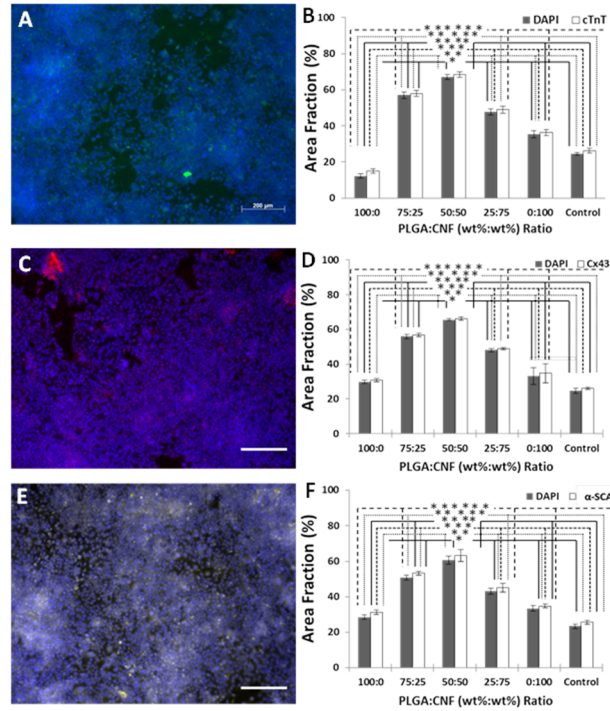


FIGURE B.8. Immunofluorescence staining for cardiomyocyte biomarkers on a 0.025 g/mL PLGA density PLGA:CNF composite after 120 hours of incubation. (A) Cardiac troponin T (green) biomarker with DAPI stain (blue) on a 50:50 (PLGA:CNF [wt%:wt%]) sample. (B) Connexin-43 (red) with DAPI stain (blue) on a 50:50 (PLGA:CNF [wt%:wt%]) sample. (C) α -SCA (yellow) with DAPI stain (blue) on a 50:50 (PLGA:CNF [wt%:wt%]) sample within the cardiomyocytes, indicating cardiomyocyte activity on the materials. Seeding density was 5.5×10^4 cells/mL. Scale bar 200 μ m. Area fraction (%), area of stain on the surface compared with the total area of image, of DAPI (nucleus) and (B) cardiac troponin T, (D) connexin-43, and (F) α -SCA after 120 hours of cell incubation, on all PLGA:CNF (wt%:wt%) ratio samples. Notes: The results indicate trends similar to those of the adhesion and proliferation experiments (Figure B.6), thus verifying those results and indicating cardiomyocyte activity on the materials. Seeding density was 5.5×10^4 cells/mL. Data are shown as the mean count of $n=3$. Control was glass substrate. * $P < 0.05$ versus 100:0 (PLGA:CNF [wt%:wt%]) ratio sample; ** $P < 0.05$ versus 75:25 (PLGA:CNF [wt%:wt%]) ratio sample; *** $P < 0.05$ versus 50:50 (PLGA:CNF [wt%:wt%]) ratio sample; **** $P < 0.05$ versus 25:75 (PLGA:CNF [wt%:wt%]) ratio sample; ***** $P < 0.05$ versus 0:100 (PLGA:CNF [wt%:wt%]) ratio sample, and ***** $P < 0.05$ versus control sample. Abbreviations: CNF, carbon nanofibers; cTnT, cardiac Troponin-T; PLGA, poly(lactic-co-glycolic-acid).

elongated features on all PLGA:CNF ratios. There were no dissimilar morphological features observed among the different weight ratios.

4. Discussion

Due the importance for improved and safer approaches to coronary and intracranial revascularization, many materials (such as alginate [159] and polytetrafluoroethylene (PTFE) [160]) have been developed to promote cardiomyocyte function after a myocardial infarction. Research has shown that composite materials composed of different matrices can stabilize heart tissue to decrease the consequences of a myocardial infarction and such approaches include but are not limited to: culturing patient tissue [161] direct cell injection [162] and biomaterial scaffolds [163]. Recently, the advent of advanced novel nano-biomaterials with improved properties capable of being used in several biomedical applications simultaneously, has transformed the field of biomedical research and the cardiovascular biomaterial world. One such example is the use of a poly(lactic-co-glycolic acid) material with embedded carbon nanofibers to create a patch for an infarcted area of the heart. This present work addresses the underlying mechanism that alters the material-cell interaction on a brand new material and sheds light on cardiomyocyte cellular growth and function, which in turn will help develop alternative bio-inspired systems for cardiovascular applications.

Specifically, the addition of CNFs to a PLGA solution increased material tensile strength to roughly mimic native heart tissue ($\sim 0.5 - 2.00$ MPa^{30,31}) and increases cardiomyocyte growth. It was shown here that a $0.025 \frac{g}{ml}$ PLGA density with 75:25 and 50:50 ratios [PLGA:CNF (wt%:wt%)] had the highest Youngs modulus that can closely mimic the native heart tissue. On the other hand, the 25:75 ratio [PLGA:CNF (wt%:wt%)] was closer to the native heart tissue strength characteristics, but the material had a very limited elongation capability ($\sim 2\%$ at breaking point) this is very important since native heart tissue is continuously expanding and contracting.

Additionally, adding CNFs up to a specific weight ratio (50:50 [PLGA:CNF (wt%:wt%)]) increased surface area, nanoscale roughness, and ultimately vitronectin

and fibronectin adsorption capacity (Fig. B.5). In the past, researchers have found that the adsorption and bioactivity of vitronectin and fibronectin increases on nanophase ceramics and that this enhanced osteoblast functions (from adhesion, proliferation, and differentiation) [164]; these studies reported that the roughness was closer to the nanometer roughness of bone in nano-ceramics, and that led to more bone growth on nanophase ceramics [164]. Even though the myocardium is made up of cardiac muscle, the research here suggests that increasing the nanoroughness of a biomaterial will promote the growth of cardiac specific muscle, which is parallel to the nanoroughness to potentially increase vascular smooth muscle cell and endothelial cell growth [165].

Clearly, CNFs possess nanoscale geometries which imitate the extracellular matrix of various tissues (such as the heart), leading to improved cytocompatibility properties of these materials. This is in direct correlation with the topography of PLGA:CNF composites which can control protein adsorption events via changes in surface energy (Fig. B.5). Importantly, it is also known that a material can be too rough and can hinder cellular activity [154]. For example, Yang et al. used diamond films with nanometer and micron scale topographies, fabricated through microwave plasma enhanced chemical-vapor-deposition and hydrogen plasma treatment and showed that micro-sized diamond topographies decreased osteoblast cell adhesion and proliferation compared to nano-sized diamond topographies [154].

In addition, we were able to show that when adding CNFs to PLGA, the composite became conductive, whereas the PLGA matrix alone was not conductive. The conductivity values measured in the present work were lower than that of the heart tissue (ranging from 0.16 S.m^{-1} longitudinally to 0.005 S.m^{-1} transversely) [166], but future techniques (such as CNF alignment) may increase the anisotropic conductivity [167] to match that of heart tissue. Pedrotty et al. [168] showed that numerous cardiac cell functions (including adhesion, proliferation, and migration) may be modulated by electrical stimulation, hence requiring the use of a conductive material in cardiac

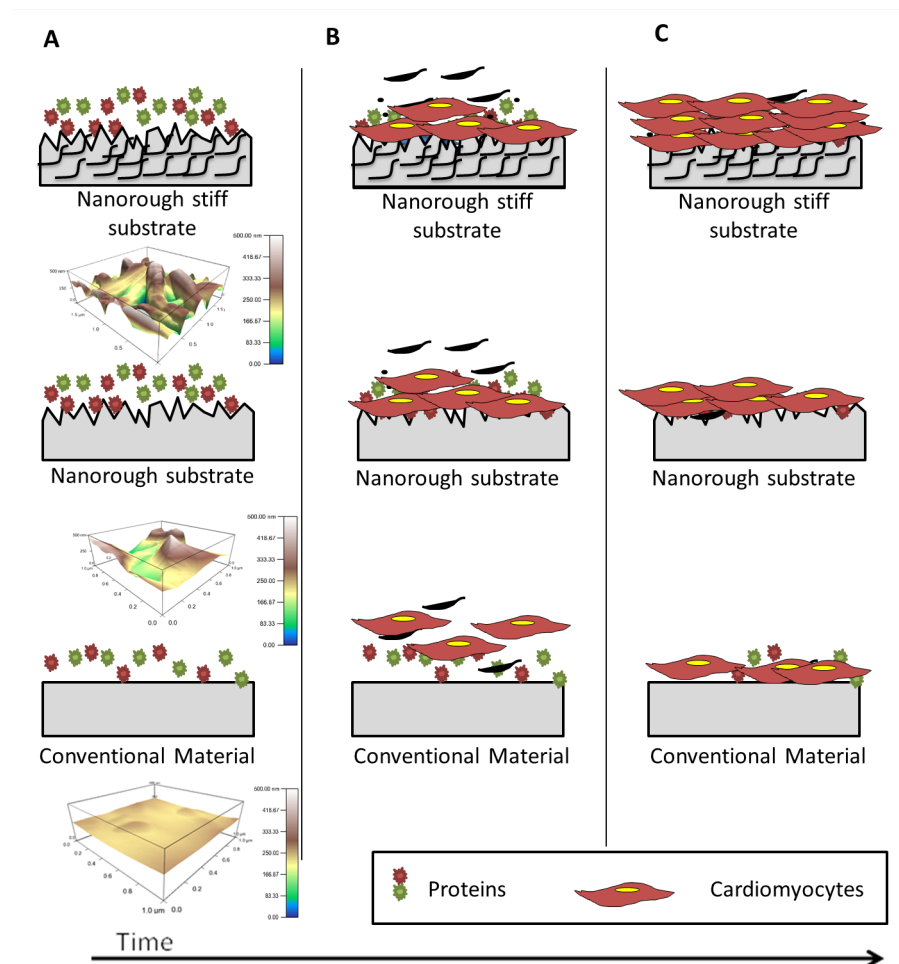


FIGURE B.9. Schematic illustration, with a three-dimensional atomic force microscopy topographical image (scan rate 1.0 Hz, AC mode with a 256 x 256 scan points and lines), of the proposed mechanism by which PLGA:CNF materials may be superior to conventional materials for cardiovascular application. The bioactive surfaces of PLGA:CNF mimic those of the natural myocardium (in stiffness) and increasing protein adsorption capacitance to promote greater amounts of protein adsorption (A) and efficiently stimulate cardiomyocyte growth better than conventional materials and plain nanorough surfaces (B and C). (A) Once a patch is placed into its host, protein adsorption begins on substrates immediately. (B) Cardiomyocyte attachment and growths begins. (C) Due to mimicking the native myocardium (in stiffness) and ability to increase protein adsorption capacitance, more cardiomyocytes adhere and grow on nanorough stiff material as compared with conventional and plain nanorough materials. Abbreviations: CNF, carbon nanofibers; PLGA, poly(lactic-co-glycolic-acid).

applications. Also, Mihardja et al. [169] demonstrated that enhanced myocardial repair following an ischemic injury could be achieved using conductive polymers, such as polypyrrole. The increasing conductivity of a material with greater CNF ratios has the potential to propagate electrical signals from the contraction of the native heart tissue to reduce arrhythmias. Additionally, cellular experiments showed the expression of cardiac differentiation markers (cTnT, Cx43, and α -SMA) as confirmed by fluorescence microscopy, thus, indicating normal cardiomyocyte function.

Interestingly, when combining protein adsorption capacity, surface area percent increases, and tensile testing, all results point to either the 75:25 or a 50:50 material ratio [PLGA:CNF (wt%:wt%)] as the best composites for cardiomyocyte growth, which correlate to previous published results [143]. This research provides the first evidence that the addition of CNFs to PLGA can increase nanosurface roughness which increases material surface area without altering wettability to increase vitronectin and fibronectin adsorption capacity, while at the same time generating tensile strength that can closely resemble that of native heart tissue, to ultimately alter material-cardiomyocyte cell interactions (Fig. B.9).

When considering our cardio-patch material application, future in vivo work must look at CNF compatibility after PLGA degradation. Once the cardio-patch is sutured into place [170, 171, 172], PLGA will begin to degrade and CNF exposure will increase and become embedded into its host, since CNFs are non-biodegradable. In vitro experiments have revealed that cellmatrix interactions limit cell motility, growth, and full scaffold colonization [173] and therefore scaffold degradation is highly desired to allow for cells replacement. Also, research has shown that carbon based nanotubes and fibers have shown to be biocompatible platforms for neuronal growth and cell differentiation. Therefore, our material has the potential to exploit both of these desirable traits in a tissue engineering matrix PLGA degradation to allow for better cell growth and CNF for cell progress and continual development.

5. Conclusion

This present work demonstrates a few underlying mechanisms which elucidate promoted cardiomyocytes growth on PLGA and CNF composites. As one increases CNF weight to specific ratios (50:50 [PLGA:CNF (wt%:wt%)]), this study found that nanoscale roughness increases to promote vitronectin and fibronectin adsorption and ultimately increase cardiomyocyte growth, as well as increase tensile and conductivity properties. Additionally, it was determined that a $0.025 \frac{g}{ml}$ PLGA density at a 50:50 [PLGA:CNF (wt%:wt%)] ratio had the highest cardiomyocyte growth, thus, indicating that PLGA density can alter cardiomyocyte adhesion and proliferation to a certain degree. SEM images showed uniform CNF dispersion within the PLGA matrix and a close interaction between cardiomyocytes and substrates while contact angle experiments suggested that changes in wettability did not occur, thus demonstrating that a simple solution-mixing-drying based synthesis route can be adopted to develop PLGA:CNF hybrid biocomposites over a broad composition range possessing a uniform distribution of CNF without any clustering. In summary, cardiomyocyte growth on PLGA:CNF composites was enhanced by creating nanotopographical features due to the addition of CNFs which in turn increased protein adsorption capacitance, creating a composite which closely resembles native heart tissue.

APPENDIX C

Enhanced Cardiomyocyte Function On Poly-Lactic-Co-Glycolic Acid: Carbon Nanofiber Composites Under Electrical Stimulation

1. Introduction

¹ A major epidemic in the United States and worldwide is cardiovascular disease (CVD). CVD refers to a class of diseases that affect the heart and blood vessels of its host, but usually is used in relationship to atherosclerosis, or arterial disease [133, 134]. The total direct and indirect cost of CVD and stroke in the United States for 2010 was estimated at \$503.2 billion [135]. In recent years, CVD has taken over as the number one killer of American women, surpassing breast cancer. By the time CVD has been detected, it is usually fairly advanced, having developed for decades [174, 175].

Although changes in environmental exposure, reduction in tobacco use, adjustments in diet, and increased physical activity can all improve patient health, the progression of CVD in the world is growing. New models to detect and treat CVD in asymptomatic patients are needed in order to prevent the first symptoms from being the last as a result of death. A need for improved and safer approaches to coronary and intracranial revascularization is still required, even with advances in the last 10 years.

¹Portions of this appendix has been published in Nano Bulletin or in the Material Research Society Smposium Proceedings Vol. 1417E [Stout, DA and TJ Webster. “The Effect of Carbon Nanofiber Density in Poly(lactic-co-glycolic acid) Composites for Myocardial Tissue Engineering Applications.” *Nano Bulletin*, 1(1): 1201021:120102-6, 2012.] or [Stout, DA, E Raimondo, and TJ Webster. “Improved Cardiomyocyte Functions of Carbon Nanofiber Cardiac Patches,” in Biomaterials for Tissue Regeneration, *Mater. Res. Soc. Symp Proc. Volume 1417E*]

Unfortunately, no single technology offers the perfect solution. To address these problems, quick technological advancements of cell biology, genomics, and proteomics combined with discoveries in material sciences and bioengineering have created a new field of medicine, nanomedicineutilizing materials and systems which possess at least one physical dimension between 1-100 nm to construct structures, devices, and systems that have novel properties [139, 140].

The advent of advanced novel nano-biomaterials with improved properties capable of being used in several biomedical applications simultaneously has transformed the field of biomedical research. Nanomaterials are among the most intensively studied materials for a wide range of applications ranging from fuel cells [141, 176] to nanopatches for the myocardium [142, 177]. These nanostructured composites are combinations of at least two constituent materials, a matrix (host) and a reinforcement component (i.e. nanofiller) [144]. It is known that the properties of materials change considerably when the size of constituents is significantly small, within the 1100 nm size range [145, 146, 147]. Since these materials have improved physical, chemical, and mechanical properties, they are significantly versatile for a wide range of applications.

One such example is the use of a poly(lactic-co-glycolic acid) (PLGA) composite with embedded carbon nanofibers (CNF) to create a “patch for an infarcted area of the heart (Fig. C.1). Recent research has shown that when one adds CNFs to PLGA, cardiomyocytes (specific heart tissue cells responsible for contraction) will adhere and proliferate better than on their non-nanoreinforced counterparts, but the mechanism is still largely unknown [177].

To further understand cardiomyocyte function on such novel materials, the purpose of this present in vitro study was to alter the CNF density of the PLGA:CNF composite to determine if CNF density has an effect on a nano-inspired cardiovascular patch, both in a static and electrically stimulated state (Fig. C.2), while analyzing cardiomyocyte function via the Troponin complex (the regulatory proteins that are

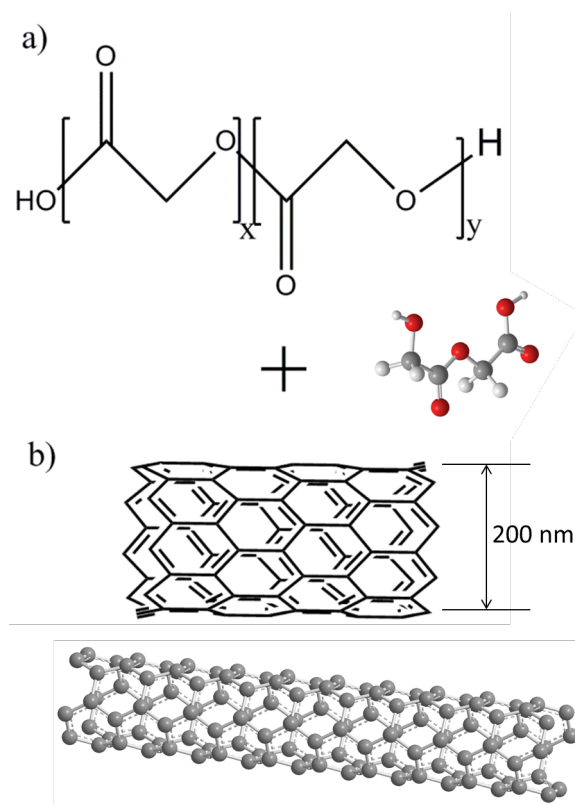


FIGURE C.1. Chemical structure of a) poly(lactic-co-glycolic acid) (PLGA) and b) a carbon nanofiber (CNF). Shapes were created in Chem3D Pro 12.0 (PerkinElmer Informatics).

integral to muscle contraction in the myocardium [178, 179]) (Fig. C.3), and if so, what density would promote cardiomyocyte proliferation the greatest.

2. Methods & Materials

The fabrication procedure for the PLGA:CNF composites have been detailed in previous studies [11]. In short, a PLGA density of 0.025 g/ml (50:50 PLA:PGA wt.%; Polysciences Cat #23986) was created by dilution in a 50 ml flask with 30 ml of tetrahydrofuran (THF; Mallinckrodt Chemicals Lot #C45763) and were sonicated in a water bath (VWR B3500A-DTH) below 30°C for 30 minutes. 500 mg of CNFs (99.9% by weight %, Catalytic Materials, MA) with a diameter of 100 nm and of different lengths, from 100 to 200 microns, were sonicated (Misonix Sonicator 3000) in a 50 ml beaker with 20 ml of chloroform (Fisher Science Lot #102591) at 20W for 30 min. After obtaining the separately sonicated PLGA and CNF solutions, various

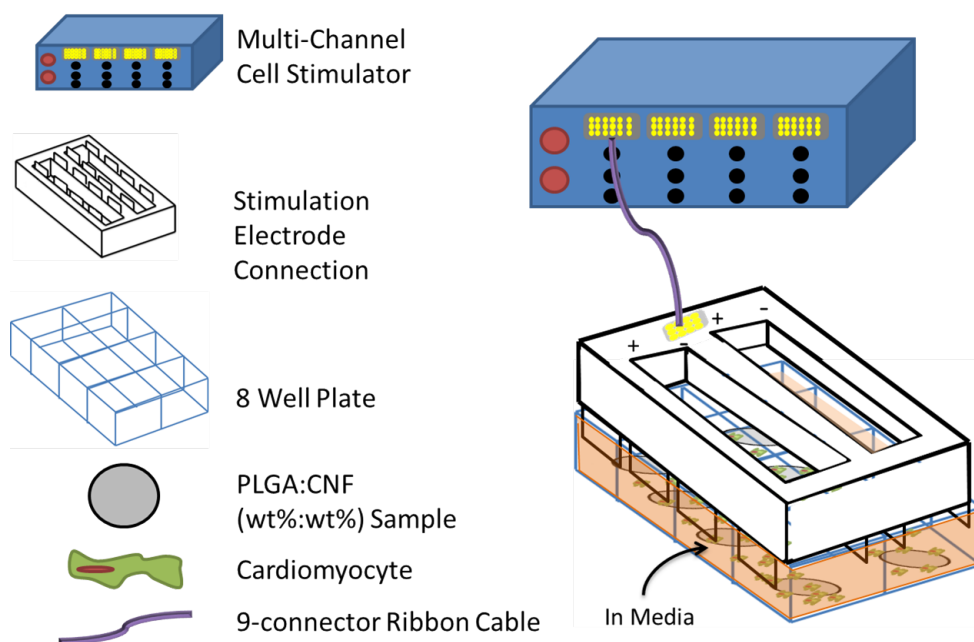


FIGURE C.2. Schematic of electrical stimulation (ES) apparatus (C-Pace EP Culture Pacer (IonOptix LLC, USA)) and experimental design. Human cardiomyocytes were seeded onto PLGA:CNF composites at a density of 10×10^4 cells/cm² and were continuously stimulated (rectangular, 2 nm, 5 V/cm, 1 Hz) for 24, 72 and 120 hours.

PLGA:CNF weight percent ratios were developed by altering the CNF material weight density (100:0, 75:25, 50:50, 25:75, and 0:100) by adding the appropriate amount of CNF to PLGA in 20 ml disposable scintillation vials. The CNF weight ratios were measured using a laboratory balance (Mettler Toledo AL54). When the appropriate ratios were added, each composite material was sonicated (Misonix Sonicator 3000) at 10W for 20 minutes each.

For experimental ease, a 22 mm diameter microscope cover glass (Fisher Science circles No. 1 - 0.13 to 0.17mm thick; Size: 22 mm, Cat #12-545-101) was coated with the aforementioned composite. Before the PLGA:CNF composite was positioned onto the glass substrate, the glass substrate was cleaned via soaking in a 70:30 (vol. %) ethanol-deionized solution shaking (VWR, Advanced Digital Shaker) for 10 minutes. Next, the substrate was added to a 100% deionized water solution and shaken for 10 minutes.

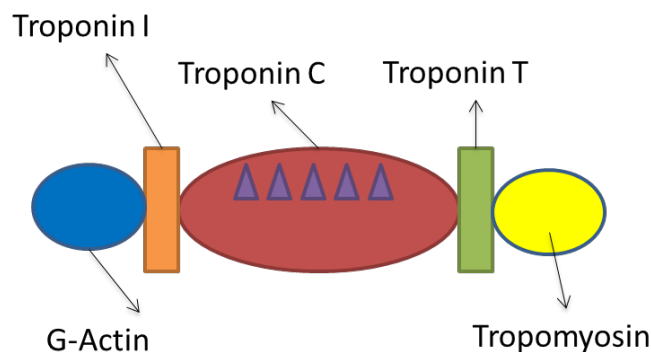


FIGURE C.3. A schematic of the Troponin complex which binds to actin in thin myofilaments to hold the actin-tropomyosin complex in place. Troponin consists of three subunits: Troponin C (TnC) which is the Ca^{2+} -binding subunit; Troponin I (TnI), which is the inhibitory subunit; and Troponin T (TnT) which is the tropomyosin-binding subunit [180]. Tropomyosin is a α -helical coiled-coil protein throughout its entire length, and interacts with adjacent tropomyosin molecules in a head-to-tail manner, forming continuous strands that lie along the thin filament [178]. When calcium binds to the Troponin C, it causes conformational changes which lead to dislocation of Troponin I and finally tropomyosin leaves the binding site for myosin on actin leading to contraction of muscle.

Using a disposable pipette (Fisherbrand #13-711-9AM), 1 ml of the appropriate PLGA:CNF composite solution was placed onto the glass substrate and placed into an oven at 42°C for 15 minutes. Each composite film was then vacuum dried (Shel Lab) at 20 inches of Hg gauge vacuum pressure for 48 hours to allow the tetrahydrofuran (THF) and chloroform to evaporate. All the samples and controls were sterilized using ultraviolet light for 24 hours prior to cell seeding.

A Hitachi 2700 scanning electron microscope was used to characterize the surface of the PLGA:CNF samples at 5 KV with InLens system and software. X-Ray diffraction (Bunker AXS: D8 Focus) was used with settings at 0.5° per 2 minutes between $2\theta = 10\text{--}35^{\circ}$ to characterize the crystallinity of the composites.

For in vitro electrical stimulation analysis, human cardiomyocytes (Celprogen, Cat #36044-15, USA) were seeded onto PLGA:CNF composites (prepared as stated above) in complete growth media supplemented with fetal bovine serum and 1% antibiotics (Celprogen, Cat #M36044-15S, USA) at a density of $10 \times 10^4 \frac{\text{cells}}{\text{cm}^2}$ and

were continuously stimulated (rectangular, 2 nm, 5 $\frac{V}{cm}$, 1 Hz) with a C-Pace EP Culture Pacer (IonOptix LLC, USA) for 24, 72, and 120 hours to mimic normal heart performance [181, 182]. Troponin I (Calbiotech, Cat #TI015C, USA) enzyme-linked immunosorbent assays (ELISA) were completed in tandem to analyze cardiomyocyte viability and protein synthesis on the composites. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Progenia, CellTiter 96®Non-Radioactive Cell Proliferation Cat. #G-4100) assays were completed to analyze cytocompatibility and cell viability on the composite.

Cardiomyocyte proliferation and Troponin I assays were performed at least in triplicate with three repeats each and results were compared to a control glass surface. The optical density (OD) data for cardiomyocyte MTT assays were plotted as the mean $\pm$ standard error of the mean while OD to cell count conversions were conducted by using a stand curve analysis between OD and cell numbers. When data were compared, ANOVA software and a student T-test were used. A p-value of < 0.05 was considered to be significant.

3. Results & Discussion

An SEM image of the as-synthesized PLGA:CNF composite surface is shown in Figure C.4. CNFs were uniformly dispersed within the PLGA matrix and, as expected, more CNFs were observed for the higher CNF samples. When looking at the different PLGA densities and similar CNF ratios, uniform CNFs were again uniformly dispersed within the PLGA matrix, suggesting that materials were properly generated (Fig. C.4).

XRD spectra obtained from the as-synthesized PLGA:CNF composites are shown in Fig. C.4. As expected an extremely broad and flat peak was recorded for the PLGA matrix, confirming its amorphous nature. However, with the addition of CNF to PLGA, the characteristic X-ray peak at $2\theta = 26.5^\circ$ evolved into a rather strong and sharp peak in PLGA:CNF composites for the 25:75 [PLGA:CNF (wt:wt)] ratio. The curves were fitted to the characteristic peak by Gaussian distribution. Clearly,

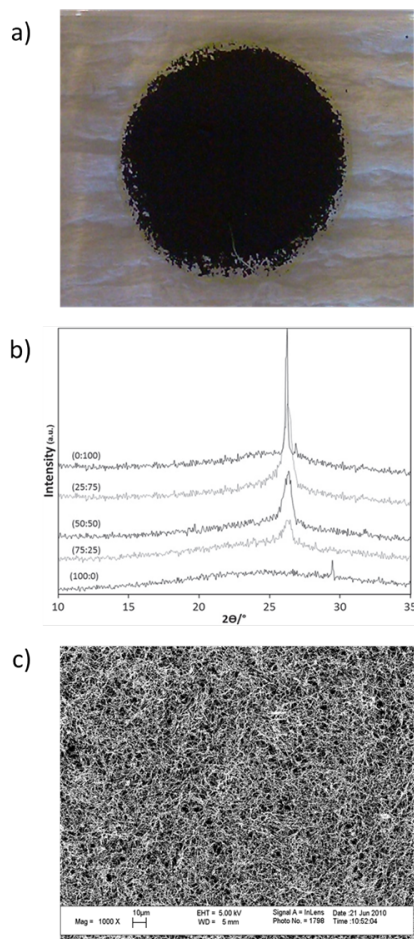


FIGURE C.4. Material analysis of a 50:50 [PLGA:CNF (wt:wt)] composite: a) depicting a visual representation of composite on a 22 mm diameter microscope cover glass (Fisher Science circles No. 1 - 0.13 to 0.17mm thick; Size: 22 mm, Cat #12-545-101) after all synthesis steps were completed; b) X-ray diffraction results of all composites with varying CNF densities; c) scanning electron micrograph at 1K magnification showing the distribution of CNFs in a PLGA matrix Scale bar = 10 μ m.

the intensity increased as full width at half maximum (FWHM) decreased as CNFs were added to PLGA (Fig. C.4).

For cardiomyocyte proliferation experiments, results indicated that PLGA:CNF composites promoted human cardiomyocyte proliferation more when electrically stimulated compared to pure PLGA. General trends were seen between each ratio and supported published results [177]. For 100 nm diameter CNFs, it was determined that the 50:50 ratio [PLGA:CNF; (wt:wt)] had the highest cardiomyocyte density

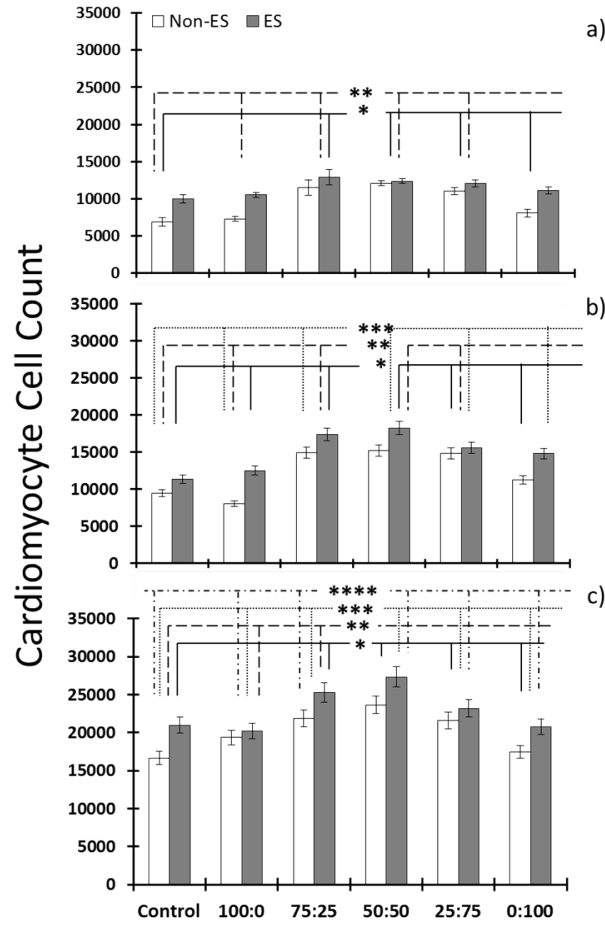


FIGURE C.5. Cardiomyocyte cell proliferation between non-electrical stimulation (Non-ES) and electrical stimulation (ES) after a) 24, b) 72 and c) 120 hrs on 100 nm diameter PLGA:CNF composite materials of interest to this study. Seeding density = 10×10^4 cells/cm². Data are mean counts $\pm$ s.d. to n=3. Control was a glass substrate. * $p < 0.05$ compared to the control, ** $p < 0.05$ compared to similar PLGA:CNF composite ratios and different densities. *** $p < 0.05$ compared to similar PLGA:CNF composite ratios between 24 and 72 hrs. **** $p < 0.05$ compared to similar PLGA:CNF composite ratios between 72 and 120 hrs.

at all three time points, whereas the lowest density was observed on the 100:0 ratio sample [PLGA:CNF; (wt:wt)] (Fig. C.5).

Statistical analysis using ANOVA showed that the cardiomyocyte results were significant at the 5% significance level for both adhesion and proliferation assays for all densities and composite ratios. Due to the slower proliferation of the cardiomyocytes

(Celprogen, Cat #36044-15) compared to other cells (around 72 hours for cell doubling times), one would not see a typical doubling time every 48 hours.

Looking at cardiomyocyte function, Troponin-I assays indicated that cardiomyocytes had a functional Troponin Complex throughout the cytocompatibility assays. All CNF experiments for all time points showed a slight increase of Troponin-I indicating better productivity [183]. As discovered, the Troponin-I assays presented the same trends seen in the proliferation cytocompatibility assays (Fig. C.6).

It is worth speculating why greater cardiomyocyte attachment and growth were observed on such composites with increasing CNF density in PLGA. In the past, researchers have found that the adsorption and bioactivity of vitronectin increases on nanophase ceramics and that this enhanced osteoblast function (including adhesion, proliferation, and differentiation) was due to roughness values closer to the nanometer roughness of bone itself [164].

Clearly, CNFs possess nanoscale geometries which imitate the extracellular matrix of various tissues (such as the heart), potentially leading to improved cytocompatibility properties of these materials [184]. Although requiring further study, nanotechnology (or the use of CNFs) can play a similar important role towards promoting cardiomyocyte density by increasing platelet-derived growth factor-BB (PDGF-BB) adsorption, which in turn, will induce cardiomyocyte adhesion and proliferation [185].

While the mechanism of enhanced cardiomyocyte density is still not thoroughly known at this time, it could have to do with the topography of PLGA:CNF composites which dictates surface energy to control protein adsorption events. Importantly, it is also known that a material can be too rough and can hinder cellular activity [26]. Yang et al. showed that diamond films with nanometer and micron scale topographies, fabricated through microwave plasma enhanced chemical-vapor-deposition and hydrogen plasma treatment, influenced osteoblast adhesion and proliferation, with the nano-sized diamond topographies better than micron topographies [154].

Even though the mechanism is not fully understood, it is shown here that CNF density does play a role in mediating cardiomyocyte growth and functionality. This

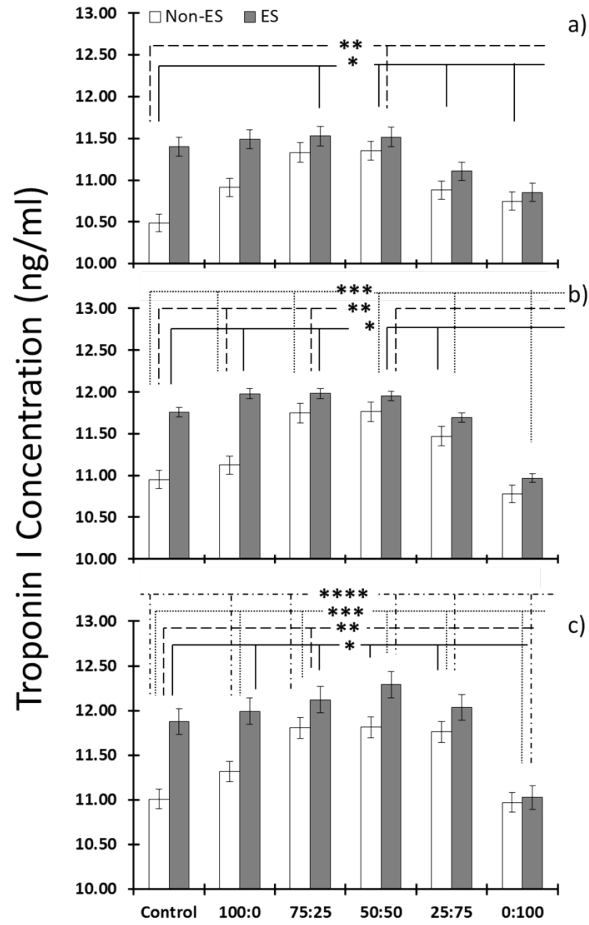


FIGURE C.6. Comparison of Troponin I concentration from cardiomyocyte cell proliferation between non-electrical stimulation (Non-ES) and electrical stimulation (ES) after a) 24, b) 72 and c) 120 hrs on 100 nm diameter PLGA:CNF composite materials of interest to this study. Seeding density = 10×10^4 cells/cm². Data are mean counts $\pm$ s.d. to $n=3$. Control was a glass substrate. * $p < 0.05$ compared to the control of all Non-ES samples, ** $p < 0.05$ compared to the control of all ES samples. *** $p < 0.05$ compared to similar PLGA:CNF composite ratios between 24 and 72 hrs. **** $p < 0.05$ compared to similar PLGA:CNF composite ratios between 72 and 120 hrs.

may be due to the creation of a material which resembles normal heart tissue material characteristics better (such as topography, conductivity, mechanical properties, etc.). Altering CNF density in the composites was able to provide a more conducive environment for cardiomyocyte functions.

4. Conclusion

In the present work it was demonstrate that a simple solution-mixing-drying based synthesis route can be adopted to develop PLGA:CNF hybrid biocomposites over a broad CNF density range possessing a uniform distribution of CNF without any clustering and using electrical stimulation to mimic heart conditions to promote cardiomyocyte growth. With the use of an electrical stimulation system we were able to demonstrate that increased cardiomyocyte density on all PLGA:CNF composite ratios as well as cardiac protein biomarker Troponin I (with the 50:50 PLGA:CNF (wt:wt)) composite with the highest cell density and Troponin I levels) when compared to non-electrical stimulation data as well normal glass substrates. These results indicated that CNF density does have an effect on PLGA:CNF composite cytocompatibility and should be further studied for myocardial tissue engineering applications, where specifically 100 nm CNF 50:50 PLGA:CNF (wt:wt) composites would be the best of those studied.

Bibliography

- [1] A. J. Ridley, M. A. Schwartz, K. Burridge, R. A. Firtel, M. H. Ginsberg, G. Borisy, J. T. Parsons, and A. R. Horwitz, “Cell migration: integrating signals from front to back.,” *Science (New York, N.Y.)*, vol. 302, pp. 1704–9, Dec. 2003.
- [2] R. W. Williams and K. Herrup, “The control of neuron number.,” *Annual review of neuroscience*, vol. 11, pp. 423–53, Jan. 1988.
- [3] E. W. Dent, A. V. Kwiatkowski, L. M. Mebane, U. Philippar, M. Barzik, D. A. Robinson, S. Gupton, J. E. Van Veen, C. Furman, J. Zhang, A. S. Alberts, S. Mori, and F. B. Gertler, “Filopodia are required for cortical neurite initiation.,” *Nature cell biology*, vol. 9, pp. 1347–59, Dec. 2007.
- [4] A. K. Mongiui, E. L. Weitzke, O. Y. Chaga, and G. G. Borisy, “Kinetic-structural analysis of neuronal growth cone veil motility.,” *Journal of cell science*, vol. 120, pp. 1113–25, Mar. 2007.
- [5] C. W. Pak, K. C. Flynn, and J. R. Bamberg, “Actin-binding proteins take the reins in growth cones.,” *Nature reviews. Neuroscience*, vol. 9, pp. 136–47, Feb. 2008.
- [6] P. C. Wilkinson, “Assays of leukocyte locomotion and chemotaxis.,” *Journal of immunological methods*, vol. 216, pp. 139–53, July 1998.
- [7] J. B. McCarthy, S. L. Palm, and L. T. Furcht, “Migration by haptotaxis of a Schwann cell tumor line to the basement membrane glycoprotein laminin.,” *The Journal of cell biology*, vol. 97, pp. 772–7, Sept. 1983.
- [8] S. Li, P. Butler, Y. Wang, Y. Hu, D. C. Han, S. Usami, J.-L. Guan, and S. Chien, “The role of the dynamics of focal adhesion kinase in the mechanotaxis of endothelial cells.,” *Proceedings of the National Academy of Sciences of the United States of America*, vol. 99, pp. 3546–51, Mar. 2002.
- [9] C.-M. Lo, H.-B. Wang, M. Dembo, and Y.-l. Wang, “Cell Movement Is Guided by the Rigidity of the Substrate,” *Biophysical Journal*, vol. 79, no. 1, pp. 144–152, 2000.
- [10] N. Gov, “Cell mechanics: moving under peer pressure.,” *Nature materials*, vol. 10, pp. 412–4, June 2011.

- [11] D. T. Tambe, C. C. Hardin, T. E. Angelini, K. Rajendran, C. Y. Park, X. Serra-Picamal, E. H. Zhou, M. H. Zaman, J. P. Butler, D. A. Weitz, J. J. Fredberg, and X. Trepat, "Collective cell guidance by cooperative intercellular forces.," *Nature materials*, vol. 10, pp. 469–75, June 2011.
- [12] P. A. Beachy, S. S. Karhadkar, and D. M. Berman, "Tissue repair and stem cell renewal in carcinogenesis.," *Nature*, vol. 432, pp. 324–31, Nov. 2004.
- [13] J. Banchereau and R. M. Steinman, "Dendritic cells and the control of immunity.," *Nature*, vol. 392, pp. 245–52, Mar. 1998.
- [14] J. G. Wagner and R. A. Roth, "Neutrophil Migration Mechanisms, with an Emphasis on the Pulmonary Vasculature," *Pharmacol. Rev.*, vol. 52, pp. 349–374, Sept. 2000.
- [15] E. W. Raines, "The extracellular matrix can regulate vascular cell migration, proliferation, and survival: relationships to vascular disease," *International Journal of Experimental Pathology*, vol. 81, pp. 173–182, Dec. 2001.
- [16] L. M. Coussens and Z. Werb, "Inflammation and cancer.," *Nature*, vol. 420, pp. 860–7, Jan. 2002.
- [17] P. Friedl and D. Gilmour, "Collective cell migration in morphogenesis, regeneration and cancer.," *Nature reviews. Molecular cell biology*, vol. 10, pp. 445–57, July 2009.
- [18] O. C. Rodriguez, A. W. Schaefer, C. A. Mandato, P. Forscher, W. M. Bement, and C. M. Waterman-Storer, "Conserved microtubule-actin interactions in cell movement and morphogenesis.," *Nature cell biology*, vol. 5, pp. 599–609, July 2003.
- [19] S. D. Conner and S. L. Schmid, "Regulated portals of entry into the cell," *Nature*, vol. 422, pp. 37–44, Mar. 2003.
- [20] O. du Roure, A. Saez, A. Buguin, R. H. Austin, P. Chavrier, P. Silberzan, P. Silberzan, and B. Ladoux, "Force mapping in epithelial cell migration.," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, pp. 2390–5, Feb. 2005.
- [21] D. A. Lauffenburger and A. F. Horwitz, "Cell Migration: A Physically Integrated Molecular Process," *Cell*, vol. 84, no. 3, pp. 359–369, 1996.
- [22] T. D. Pollard and G. G. Borisy, "Cellular motility driven by assembly and disassembly of actin filaments.," *Cell*, vol. 112, pp. 453–65, Feb. 2003.
- [23] M. P. Sheetz, D. Felsenfeld, C. G. Galbraith, and D. Choquet, "Cell migration as a five-step cycle.," *Biochemical Society symposium*, vol. 65, pp. 233–43, Jan. 1999.
- [24] K. A. Beningo, M. Dembo, I. Kaverina, J. V. Small, and Y.-l. Wang, "Nascent Focal Adhesions Are Responsible for the Generation of Strong Propulsive Forces in Migrating Fibroblasts," *The Journal of Cell Biology*, vol. 153, pp. 881–888, May 2001.

- [25] M. Dembo and Y. L. Wang, “Stresses at the cell-to-substrate interface during locomotion of fibroblasts.,” *Biophysical journal*, vol. 76, pp. 2307–16, Apr. 1999.
- [26] K. VON DER MARK, V. GAUSS, H. VON DER MARK, and P. MÜLLER, “Relationship between cell shape and type of collagen synthesised as chondrocytes lose their cartilage phenotype in culture,” *Nature*, vol. 267, pp. 531–532, June 1977.
- [27] O. W. Petersen, L. Ronnov-Jessen, A. R. Howlett, and M. J. Bissell, “Interaction with basement membrane serves to rapidly distinguish growth and differentiation pattern of normal and malignant human breast epithelial cells.,” *Proceedings of the National Academy of Sciences*, vol. 89, pp. 9064–9068, Oct. 1992.
- [28] J. EMERMAN, “MAINTENANCE AND INDUCTION OF MORPHOLOGICAL-DIFFERENTIATION IN DISSOCIATED MAMMARY EPITHELIUM ON FLOATING COLLAGEN MEMBRANES,” *In vitro*, vol. 13, no. 5, 1977.
- [29] E. Y. Lee, “Modulation of secreted proteins of mouse mammary epithelial cells by the collagenous substrata,” *The Journal of Cell Biology*, vol. 98, pp. 146–155, Jan. 1984.
- [30] R. O. Hynes, “Integrins: A family of cell surface receptors,” *Cell*, vol. 48, no. 4, pp. 549–554, 1987.
- [31] K. Burridge, K. Fath, T. Kelly, G. Nuckolls, and C. Turner, “Focal adhesions: transmembrane junctions between the extracellular matrix and the cytoskeleton,” *Annual review of cell biology*, vol. 4, pp. 487–525, Jan. 1988.
- [32] C. A. Reinhart-King, M. Dembo, and D. A. Hammer, “The Dynamics and Mechanics of Endothelial Cell Spreading,” *Biophysical Journal*, vol. 89, no. 1, pp. 676–689, 2005.
- [33] S. Khetan and J. A. Burdick, “Patterning network structure to spatially control cellular remodeling and stem cell fate within 3-dimensional hydrogels,” *Biomaterials*, vol. 31, no. 32, pp. 8228–8234, 2010.
- [34] A. D. Doyle, F. W. Wang, K. Matsumoto, and K. M. Yamada, “One-dimensional topography underlies three-dimensional fibrillar cell migration.,” *The Journal of cell biology*, vol. 184, pp. 481–90, Feb. 2009.
- [35] M. H. Zaman, L. M. Trapani, A. L. Sieminski, A. Siemeski, D. Mackellar, H. Gong, R. D. Kamm, A. Wells, D. A. Lauffenburger, and P. Matsudaira, “Migration of tumor cells in 3D matrices is governed by matrix stiffness along with cell-matrix adhesion and proteolysis.,” *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, pp. 10889–94, July 2006.

- [36] G. Raeber, M. Lutolf, and J. Hubbell, "Molecularly Engineered PEG Hydrogels: A Novel Model System for Proteolytically Mediated Cell Migration," *Biophysical Journal*, vol. 89, no. 2, pp. 1374–1388, 2005.
- [37] M. Phillipson and P. Kubes, "The neutrophil in vascular inflammation.," *Nature medicine*, vol. 17, pp. 1381–90, Jan. 2011.
- [38] A. Mantovani, M. A. Cassatella, C. Costantini, and S. Jaillon, "Neutrophils in the activation and regulation of innate and adaptive immunity.," *Nature reviews. Immunology*, vol. 11, pp. 519–31, Aug. 2011.
- [39] O. Soehnlein and L. Lindbom, "Phagocyte partnership during the onset and resolution of inflammation.," *Nature reviews. Immunology*, vol. 10, pp. 427–39, June 2010.
- [40] G. J. Lieschke, D. Grail, G. Hodgson, D. Metcalf, E. Stanley, C. Cheers, K. J. Fowler, S. Basu, Y. F. Zhan, and A. R. Dunn, "Mice lacking granulocyte colony-stimulating factor have chronic neutropenia, granulocyte and macrophage progenitor cell deficiency, and impaired neutrophil mobilization.," *Blood*, vol. 84, pp. 1737–46, Sept. 1994.
- [41] R. C. Furze and S. M. Rankin, "Neutrophil mobilization and clearance in the bone marrow.," *Immunology*, vol. 125, pp. 281–8, Nov. 2008.
- [42] N. Borregaard, "Neutrophils, from Marrow to Microbes," *Immunity*, vol. 33, no. 5, pp. 657–670, 2010.
- [43] M. Häger, J. B. Cowland, and N. Borregaard, "Neutrophil granules in health and disease.," *Journal of internal medicine*, vol. 268, pp. 25–34, July 2010.
- [44] V. Brinkmann, U. Reichard, C. Goosmann, B. Fauler, Y. Uhlemann, D. S. Weiss, Y. Weinrauch, and A. Zychlinsky, "Neutrophil extracellular traps kill bacteria.," *Science (New York, N.Y.)*, vol. 303, pp. 1532–5, Mar. 2004.
- [45] M. Sixt and T. Lämmermann, "In vitro analysis of chemotactic leukocyte migration in 3D environments.," *Methods in molecular biology (Clifton, N.J.)*, vol. 769, pp. 149–65, Jan. 2011.
- [46] P. W. Oakes, D. C. Patel, N. a. Morin, D. P. Zitterbart, B. Fabry, J. S. Reichner, and J. X. Tang, "Neutrophil morphology and migration are affected by substrate elasticity.," *Blood*, vol. 114, pp. 1387–95, Aug. 2009.
- [47] L. A. Smith, H. Aranda-Espinoza, J. B. Haun, M. Dembo, and D. A. Hammer, "Neutrophil traction stresses are concentrated in the uropod during migration.," *Biophysical journal*, vol. 92, pp. L58–60, Apr. 2007.
- [48] J. Cohen, "The immunopathogenesis of sepsis.," *Nature*, vol. 420, no. 6917, pp. 885–91.
- [49] G. S. Kansas, "Selectins and their ligands: current concepts and controversies.," *Blood*, vol. 88, pp. 3259–87, Nov. 1996.

- [50] M. Adib-Conquy and J.-M. Cavaillon, "Compensatory anti-inflammatory response syndrome.," *Thrombosis and haemostasis*, vol. 101, pp. 36–47, Jan. 2009.
- [51] P. A. Ward, "Fatal sequence: The killer within," *Journal of Clinical Investigation*, vol. 115, pp. 3304–3304, Dec. 2005.
- [52] D. C. Angus, W. T. Linde-Zwirble, J. Lidicker, G. Clermont, J. Carcillo, and M. R. Pinsky, "Epidemiology of severe sepsis in the United States: analysis of incidence, outcome, and associated costs of care.," *Critical care medicine*, vol. 29, pp. 1303–10, July 2001.
- [53] G. S. Martin, D. M. Mannino, S. Eaton, and M. Moss, "The epidemiology of sepsis in the United States from 1979 through 2000.," *The New England journal of medicine*, vol. 348, pp. 1546–54, Apr. 2003.
- [54] W. T. Linde-Zwirble and D. C. Angus, "Severe sepsis epidemiology: sampling, selection, and society.," *Critical care (London, England)*, vol. 8, pp. 222–6, Aug. 2004.
- [55] A. C. Muller Kobold, J. E. Tulleken, J. G. Zijlstra, W. Sluiter, J. Hermans, C. G. Kallenberg, and J. W. Tervaert, "Leukocyte activation in sepsis; correlations with disease state and mortality.," *Intensive care medicine*, vol. 26, pp. 883–92, July 2000.
- [56] D. D. Danikas, M. Karakantza, G. L. Theodorou, G. C. Sakellariopoulos, and C. A. Gogos, "Prognostic value of phagocytic activity of neutrophils and monocytes in sepsis. Correlation to CD64 and CD14 antigen expression.," *Clinical and experimental immunology*, vol. 154, pp. 87–97, Oct. 2008.
- [57] J. Lee, "Traction forces generated by locomoting keratocytes," *The Journal of Cell Biology*, vol. 127, pp. 1957–1964, Dec. 1994.
- [58] T. Oliver, K. Jacobson, and M. Dembo, "Design and use of substrata to measure traction forces exerted by cultured cells," *Methods in Enzymology*, vol. 298, pp. 497–521, 1998.
- [59] R. Merkel, N. Kirchgeßner, C. M. Cesa, and B. Hoffmann, "Cell Force Microscopy on Elastic Layers of Finite Thickness," *Biophysical Journal*, vol. 93, no. 9, pp. 3314–3323, 2007.
- [60] R. J. Pelham and Y.-l. Wang, "Cell locomotion and focal adhesions are regulated by substrate flexibility," *Proceedings of the National Academy of Sciences*, vol. 94, pp. 13661–13665, Dec. 1997.
- [61] W. R. Legant, J. S. Miller, B. L. Blakely, D. M. Cohen, G. M. Genin, and C. S. Chen, "Measurement of mechanical tractions exerted by cells in three-dimensional matrices.," *Nature methods*, vol. 7, pp. 969–71, Dec. 2010.
- [62] C. Franck, S. Hong, S. A. Maskarinec, D. A. Tirrell, and G. Ravichandran, "Three-dimensional Full-field Measurements of Large Deformations in Soft Materials Using Confocal Microscopy and Digital Volume Correlation," *Experimental Mechanics*, vol. 47, pp. 427–438, Mar. 2007.

- [63] E. Bar-Kochba, J. Toyjanova, E. Andrews, K.-S. Kim, and C. Franck, "A Fast Iterative Digital Volume Correlation Algorithm for Large Deformations," *Experimental Mechanics*, no. In press, 2014.
- [64] N. S. Adzick, "The Molecular and Cellular Biology of Wound Repair, 2nd Edition," Feb. 1997.
- [65] A. H. Reddi, "Bone morphogenetic proteins: an unconventional approach to isolation of first mammalian morphogens.," *Cytokine & growth factor reviews*, vol. 8, pp. 11–20, Mar. 1997.
- [66] F. G. Vital-Lopez, A. Armaou, M. Hutnik, and C. D. Maranas, "Modeling the effect of chemotaxis on glioblastoma tumor progression," *AIChE Journal*, vol. 57, pp. 778–792, Mar. 2011.
- [67] S. K. Hughes-Alford and D. A. Lauffenburger, "Quantitative analysis of gradient sensing: towards building predictive models of chemotaxis in cancer.," *Current opinion in cell biology*, vol. 24, pp. 284–91, Apr. 2012.
- [68] P. Friedl and K. Wolf, "Tumour-cell invasion and migration: diversity and escape mechanisms.," *Nature reviews. Cancer*, vol. 3, pp. 362–74, May 2003.
- [69] V. Cristini, "Morphologic Instability and Cancer Invasion," *Clinical Cancer Research*, vol. 11, pp. 6772–6779, Oct. 2005.
- [70] T. Lammermann, B. L. Bader, S. J. Monkley, T. Worbs, R. Wedlich-Soldner, K. Hirsch, M. Keller, R. Forster, D. R. Critchley, R. Fassler, and M. Sixt, "Rapid leukocyte migration by integrin-independent flowing and squeezing," *Nature*, vol. 453, no. 7191, pp. 51–55, 2008.
- [71] C. A. Parent and P. N. Devreotes, "A cell's sense of direction.," *Science (New York, N.Y.)*, vol. 284, pp. 765–70, Apr. 1999.
- [72] S. BOYDEN, "The chemotactic effect of mixtures of antibody and antigen on polymorphonuclear leucocytes.," *The Journal of experimental medicine*, vol. 115, pp. 453–66, Mar. 1962.
- [73] R. D. Nelson, P. G. Quie, and R. L. Simmons, "Chemotaxis under agarose: a new and simple method for measuring chemotaxis and spontaneous migration of human polymorphonuclear leukocytes and monocytes.," *Journal of immunology (Baltimore, Md. : 1950)*, vol. 115, pp. 1650–6, Dec. 1975.
- [74] W. J. Rosoff, R. McAllister, M. A. Esrick, G. J. Goodhill, and J. S. Urbach, "Generating controlled molecular gradients in 3D gels.," *Biotechnology and bioengineering*, vol. 91, pp. 754–9, Sept. 2005.
- [75] A. Wells, J. Kassis, J. Solava, T. Turner, and D. A. Lauffenburger, "Growth factor-induced cell motility in tumor invasion.," *Acta oncologica (Stockholm, Sweden)*, vol. 41, pp. 124–30, Jan. 2002.

- [76] C. Bonvin, J. Overney, A. C. Shieh, J. B. Dixon, and M. A. Swartz, "A multichamber fluidic device for 3D cultures under interstitial flow with live imaging: development, characterization, and applications.," *Biotechnology and bioengineering*, vol. 105, pp. 982–91, Apr. 2010.
- [77] N. Li Jeon, H. Baskaran, S. K. W. Dertinger, G. M. Whitesides, L. Van de Water, and M. Toner, "Neutrophil chemotaxis in linear and complex gradients of interleukin-8 formed in a microfabricated device.," *Nature biotechnology*, vol. 20, pp. 826–30, Aug. 2002.
- [78] J. Shao, L. Wu, J. Wu, Y. Zheng, H. Zhao, Q. Jin, and J. Zhao, "Integrated microfluidic chip for endothelial cells culture and analysis exposed to a pulsatile and oscillatory shear stress.," *Lab on a chip*, vol. 9, pp. 3118–25, Nov. 2009.
- [79] U. Haessler, Y. Kalinin, M. A. Swartz, and M. Wu, "An agarose-based microfluidic platform with a gradient buffer for 3D chemotaxis studies.," *Biomedical microdevices*, vol. 11, pp. 827–35, Aug. 2009.
- [80] S. H. Zigmond, "Ability of polymorphonuclear leukocytes to orient in gradients of chemotactic factors.," *The Journal of cell biology*, vol. 75, pp. 606–16, Nov. 1977.
- [81] D. Zicha, G. A. Dunn, and A. F. Brown, "A new direct-viewing chemotaxis chamber.," *Journal of cell science*, vol. 99 (Pt 4), pp. 769–75, Aug. 1991.
- [82] P. V. Moghe, R. D. Nelson, and R. T. Tranquillo, "Cytokine-stimulated chemotaxis of human neutrophils in a 3-D conjoined fibrin gel assay.," *Journal of immunological methods*, vol. 180, pp. 193–211, Mar. 1995.
- [83] D. L. Gilbert, "Macromolecular diffusion through collagen membranes," *International Journal of Pharmaceutics*, vol. 47, pp. 79–88, Nov. 1988.
- [84] S. Ramanujan, A. Pluen, T. D. McKee, E. B. Brown, Y. Boucher, and R. K. Jain, "Diffusion and convection in collagen gels: implications for transport in the tumor interstitium.," *Biophysical journal*, vol. 83, pp. 1650–60, Sept. 2002.
- [85] K. Weadock, F. H. Silver, and D. Wolff, "Diffusivity of 125I-calmodulin through collagen membranes: effect of source concentration and membrane swelling ratio.," *Biomaterials*, vol. 7, pp. 263–7, July 1986.
- [86] A. Erikson, H. N. Andersen, S. N. Naess, P. Sikorski, and C. d. L. Davies, "Physical and chemical modifications of collagen gels: impact on diffusion.," *Biopolymers*, vol. 89, pp. 135–43, Feb. 2008.
- [87] S. Ramanujan, A. Pluen, T. D. McKee, E. B. Brown, Y. Boucher, and R. K. Jain, "Diffusion and convection in collagen gels: implications for transport in the tumor interstitium.," *Biophysical journal*, vol. 83, pp. 1650–60, Sept. 2002.

- [88] H. L. Malech and J. I. Gallin, "Current concepts: immunology. Neutrophils in human diseases.," *The New England journal of medicine*, vol. 317, pp. 687–94, Sept. 1987.
- [89] M. A. Pereira, P. Sannomiya, and J. G. Leme, "Inhibition of leukocyte chemotaxis by factor in alloxan-induced diabetic rat plasma.," *Diabetes*, vol. 36, pp. 1307–14, Nov. 1987.
- [90] C. M. Mastroianni, M. Lichtner, F. Mengoni, C. D'Agostino, G. Forcina, G. D'Ettorre, P. Santopadre, and V. Vullo, "Improvement in neutrophil and monocyte function during highly active antiretroviral treatment of HIV-1-infected patients.," *AIDS (London, England)*, vol. 13, pp. 883–90, May 1999.
- [91] A. Rabodzey, P. Alcaide, F. W. Lusinskas, and B. Ladoux, "Mechanical forces induced by the transendothelial migration of human neutrophils.," *Biophysical journal*, vol. 95, pp. 1428–38, Aug. 2008.
- [92] S. Munevar, Y. Wang, and M. Dembo, "Traction force microscopy of migrating normal and H-ras transformed 3T3 fibroblasts.," *Biophysical journal*, vol. 80, pp. 1744–57, Apr. 2001.
- [93] C. F. Benjamim, S. H. Ferreira, and F. Q. Cunha, "Role of nitric oxide in the failure of neutrophil migration in sepsis.," *The Journal of infectious diseases*, vol. 182, pp. 214–23, July 2000.
- [94] D. P. Crosara-Alberto, A. L. C. Darini, R. Y. Inoue, J. S. Silva, S. H. Ferreira, and F. Q. Cunha, "Involvement of NO in the failure of neutrophil migration in sepsis induced by *Staphylococcus aureus*.," *British journal of pharmacology*, vol. 136, pp. 645–58, July 2002.
- [95] J. C. Alves-Filho, A. de Freitas, M. Russo, and F. Q. Cunha, "Toll-like receptor 4 signaling leads to neutrophil migration impairment in polymicrobial sepsis.," *Critical care medicine*, vol. 34, pp. 461–70, Feb. 2006.
- [96] A. J. Aldridge, "Role of the neutrophil in septic shock and the adult respiratory distress syndrome.," *The European journal of surgery = Acta chirurgica*, vol. 168, pp. 204–14, Jan. 2002.
- [97] J. A. Russel, "The current management of septic shock.," *Minerva medica*, vol. 99, pp. 431–58, Oct. 2008.
- [98] C. R. B. Welbourn, G. Goldman, I. S. Paterson, C. R. Valeri, D. Shepro, and H. B. Hechtman, "Pathophysiology of ischaemia reperfusion injury: Central role of the neutrophil," *British Journal of Surgery*, vol. 78, pp. 651–655, June 1991.
- [99] E. Abraham, P.-F. Laterre, R. Garg, H. Levy, D. Talwar, B. L. Trzaskoma, B. François, J. S. Guy, M. Brückmann, A. Rea-Neto, R. Rossaint, D. Perrotin, A. Sablotzki, N. Arkins, B. G. Utterback, and W. L. Macias, "Drotrecogin alfa (activated) for adults with severe sepsis and a low risk of death.," *The New England journal of medicine*, vol. 353, pp. 1332–41, Sept. 2005.

- [100] K. Reinhart and W. Karzai, "Anti-tumor necrosis factor therapy in sepsis: update on clinical trials and lessons learned.," *Critical care medicine*, vol. 29, pp. S121–5, July 2001.
- [101] A. J. Martí-Carvajal, I. Solà, D. Lathyris, and A. F. Cardona, "Human recombinant activated protein C for severe sepsis.," *The Cochrane database of systematic reviews*, vol. 3, p. CD004388, Jan. 2012.
- [102] P. Sundd, E. Gutierrez, M. K. Pospieszalska, H. Zhang, A. Groisman, and K. Ley, "Quantitative dynamic footprinting microscopy reveals mechanisms of neutrophil rolling.," *Nature methods*, vol. 7, pp. 821–4, Oct. 2010.
- [103] C. Franck, S. a. Maskarinec, D. a. Tirrell, and G. Ravichandran, "Three-dimensional traction force microscopy: a new tool for quantifying cell-matrix interactions.," *PloS one*, vol. 6, p. e17833, Jan. 2011.
- [104] S. a. Maskarinec, C. Franck, D. a. Tirrell, and G. Ravichandran, "Quantifying cellular traction forces in three dimensions.," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 106, pp. 22108–13, Dec. 2009.
- [105] L. A. Guthrie, "Priming of neutrophils for enhanced release of oxygen metabolites by bacterial lipopolysaccharide. Evidence for increased activity of the superoxide-producing enzyme.," *Journal of Experimental Medicine*, vol. 160, pp. 1656–1671, Dec. 1984.
- [106] M. E. Doerfler, R. L. Danner, J. H. Shelhamer, and J. E. Parrillo, "Bacterial lipopolysaccharides prime human neutrophils for enhanced production of leukotriene B4.," *The Journal of clinical investigation*, vol. 83, pp. 970–7, Mar. 1989.
- [107] Y. Aida and M. J. Pabst, "Neutrophil responses to lipopolysaccharide. Effect of adherence on triggering and priming of the respiratory burst.," *Journal of immunology (Baltimore, Md. : 1950)*, vol. 146, pp. 1271–6, Feb. 1991.
- [108] H. Carslaw and J. Jaeger, *CONDUCTION OF HEAT IN SOLIDS. 2nde edition, Edition anglaise*. Oxford Science Publications, Clarendon Press, 1986.
- [109] C. A. Janeway and R. Medzhitov, "Innate immune recognition.," *Annual review of immunology*, vol. 20, pp. 197–216, Jan. 2002.
- [110] S. Akira, S. Uematsu, and O. Takeuchi, "Pathogen recognition and innate immunity.," *Cell*, vol. 124, pp. 783–801, Feb. 2006.
- [111] H. K. Lee and A. Iwasaki, "Innate control of adaptive immunity: dendritic cells and beyond.," *Seminars in immunology*, vol. 19, pp. 48–55, Feb. 2007.
- [112] T. L. Gioannini and J. P. Weiss, "Regulation of interactions of Gram-negative bacterial endotoxins with mammalian cells," *Immunologic Research*, vol. 39, pp. 249–260, June 2007.

- [113] K. Miyake, "Innate immune sensing of pathogens and danger signals by cell surface Toll-like receptors.," *Seminars in immunology*, vol. 19, pp. 3–10, Feb. 2007.
- [114] P. S. Tobias, K. Soldau, and R. J. Ulevitch, "Isolation of a lipopolysaccharide-binding acute phase reactant from rabbit serum.," *The Journal of experimental medicine*, vol. 164, pp. 777–93, Sept. 1986.
- [115] S. D. Wright, P. S. Tobias, R. J. Ulevitch, and R. A. Ramos, "Lipopolysaccharide (LPS) binding protein opsonizes LPS-bearing particles for recognition by a novel receptor on macrophages.," *The Journal of experimental medicine*, vol. 170, pp. 1231–41, Oct. 1989.
- [116] R. Shimazu, S. Akashi, H. Ogata, Y. Nagai, K. Fukudome, K. Miyake, and M. Kimoto, "MD-2, a molecule that confers lipopolysaccharide responsiveness on Toll-like receptor 4.," *The Journal of experimental medicine*, vol. 189, pp. 1777–82, June 1999.
- [117] H. Mitsuzawa, C. Nishitani, N. Hyakushima, T. Shimizu, H. Sano, N. Matsushima, K. Fukase, and Y. Kuroki, "Recombinant soluble forms of extracellular TLR4 domain and MD-2 inhibit lipopolysaccharide binding on cell surface and dampen lipopolysaccharide-induced pulmonary inflammation in mice.," *Journal of immunology (Baltimore, Md. : 1950)*, vol. 177, pp. 8133–9, Dec. 2006.
- [118] A. Poltorak, X. He, I. Smirnova, M. Y. Liu, C. Van Huffel, X. Du, D. Birdwell, E. Alejos, M. Silva, C. Galanos, M. Freudenberg, P. Ricciardi-Castagnoli, B. Layton, and B. Beutler, "Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene.," *Science (New York, N.Y.)*, vol. 282, pp. 2085–8, Dec. 1998.
- [119] L. A. J. O'Neill and A. G. Bowie, "The family of five: TIR-domain-containing adaptors in Toll-like receptor signalling.," *Nature reviews. Immunology*, vol. 7, pp. 353–64, May 2007.
- [120] S. Funamoto, R. Meili, S. Lee, L. Parry, and R. A. Firtel, "Spatial and temporal regulation of 3-phosphoinositides by PI 3-kinase and PTEN mediates chemotaxis.," *Cell*, vol. 109, pp. 611–23, May 2002.
- [121] B. Heit, S. M. Robbins, C. M. Downey, Z. Guan, P. Colarusso, B. J. Miller, F. R. Jirik, and P. Kubes, "PTEN functions to 'prioritize' chemotactic cues and prevent 'distraction' in migrating neutrophils.," *Nature immunology*, vol. 9, pp. 743–52, July 2008.
- [122] D. E. Doherty, G. P. Downey, B. Schwab, E. Elson, and G. S. Worthen, "Lipolysaccharide-induced monocyte retention in the lung. Role of monocyte stiffness, actin assembly, and CD18-dependent adherence.," *Journal of immunology (Baltimore, Md. : 1950)*, vol. 153, pp. 241–55, July 1994.

- [123] G. S. Worthen, B. Schwab, E. L. Elson, and G. P. Downey, "Mechanics of stimulated neutrophils: cell stiffening induces retention in capillaries.," *Science (New York, N.Y.)*, vol. 245, pp. 183–6, July 1989.
- [124] S. Sheikh, W. B. Gratzner, J. C. Pinder, and G. B. Nash, "Actin polymerisation regulates integrin-mediated adhesion as well as rigidity of neutrophils.," *Biochemical and biophysical research communications*, vol. 238, pp. 910–5, Sept. 1997.
- [125] F. M. Brunkhorst, M. Oppert, G. Marx, F. Bloos, K. Ludwig, C. Putensen, A. Nierhaus, U. Jaschinski, A. Meier-Hellmann, A. Weyland, M. Gründling, O. Moerer, R. Riessen, A. Seibel, M. Ragaller, M. W. Büchler, S. John, F. Bach, C. Spies, L. Reill, H. Fritz, M. Kiehn-topf, E. Kuhnt, H. Bogatsch, C. Engel, M. Loeffler, M. H. Kollef, K. Reinhart, and T. Welte, "Effect of empirical treatment with moxifloxacin and meropenem vs meropenem on sepsis-related organ dysfunction in patients with severe sepsis: a randomized trial.," *JAMA : the journal of the American Medical Association*, vol. 307, pp. 2390–9, June 2012.
- [126] S. P. LaRosa and S. M. Opal, "Clinical trials of novel anticoagulants for severe sepsis: A tale of three molecules," *endothelium*, vol. 14, p. 16.
- [127] B. L. Warren, A. Eid, P. Singer, S. S. Pillay, P. Carl, I. Novak, P. Chalupa, A. Atherstone, I. Péntzes, A. Kübler, S. Knaub, H. O. Keinecke, H. Heinrichs, F. Schindel, M. Juers, R. C. Bone, and S. M. Opal, "Caring for the critically ill patient. High-dose antithrombin III in severe sepsis: a randomized controlled trial.," *JAMA : the journal of the American Medical Association*, vol. 286, pp. 1869–78, Oct. 2001.
- [128] E. Abraham, K. Reinhart, S. Opal, I. Demeyer, C. Doig, A. L. Rodriguez, R. Beale, P. Svoboda, P. F. Laterre, S. Simon, B. Light, H. Spapen, J. Stone, A. Seibert, C. Peckelsen, C. De Deyne, R. Postier, V. Pettilä, A. Artigas, S. R. Percell, V. Shu, C. Zwingelstein, J. Tobias, L. Poole, J. C. Stolzenbach, and A. A. Creasey, "Efficacy and safety of tifacogin (recombinant tissue factor pathway inhibitor) in severe sepsis: a randomized controlled trial.," *JAMA : the journal of the American Medical Association*, vol. 290, pp. 238–47, July 2003.
- [129] S. M. Opal, P.-F. Laterre, B. Francois, S. P. LaRosa, D. C. Angus, J.-P. Mira, X. Wittebole, T. Dugernier, D. Perrotin, M. Tidswell, L. Jauregui, K. Krell, J. Pacht, T. Takahashi, C. Peckelsen, E. Cordasco, C.-S. Chang, S. Oeyen, N. Aikawa, T. Maruyama, R. Schein, A. C. Kalil, M. Van Nuffelen, M. Lynn, D. P. Rossignol, J. Gogate, M. B. Roberts, J. L. Wheeler, and J.-L. Vincent, "Effect of eritoran, an antagonist of MD2-TLR4, on mortality in patients with severe sepsis: the ACCESS randomized trial.," *JAMA : the journal of the American Medical Association*, vol. 309, pp. 1154–62, Mar. 2013.

- [130] G. R. Bernard, J. L. Vincent, P. F. Laterre, S. P. LaRosa, J. F. Dhainaut, A. Lopez-Rodriguez, J. S. Steingrub, G. E. Garber, J. D. Helterbrand, E. W. Ely, and C. J. Fisher, "Efficacy and safety of recombinant human activated protein C for severe sepsis.," *The New England journal of medicine*, vol. 344, pp. 699–709, Mar. 2001.
- [131] Z. Liu, Y. Jiang, Y. Li, J. Wang, L. Fan, M. J. Scott, G. Xiao, S. Li, T. R. Billiar, M. A. Wilson, and J. Fan, "TLR4 Signaling augments monocyte chemotaxis by regulating G protein-coupled receptor kinase 2 translocation.," *Journal of immunology (Baltimore, Md. : 1950)*, vol. 191, pp. 857–64, July 2013.
- [132] D. Kim and C. L. Haynes, "Neutrophil chemotaxis within a competing gradient of chemoattractants.," *Analytical chemistry*, vol. 84, pp. 6070–8, July 2012.
- [133] K. Thygesen, J. S. Alpert, and H. D. White, "Universal Definition of Myocardial Infarction.," *Journal of the American College of Cardiology*, vol. 50, no. 22, pp. 2173–2195, 2007.
- [134] A. Maton, *Human biology and health*. Englewood Cliffs, N.J.: Prentice Hall, 1993.
- [135] M. Writing Group, D. Lloyd-Jones, R. Adams, M. Carnethon, G. De Simone, T. B. Ferguson, K. Flegal, E. Ford, K. Furie, A. Go, K. Greenlund, N. Haase, S. Hailpern, M. Ho, V. Howard, B. Kissela, S. Kittner, D. Lackland, L. Lisabeth, A. Marelli, M. McDermott, J. Meigs, D. Mozaffarian, G. Nichol, C. O'Donnell, V. Roger, W. Rosamond, R. Sacco, P. Sorlie, R. Stafford, J. Steinberger, T. Thom, S. Wasserthiel-Smoller, N. Wong, J. Wylie-Rosett, Y. Hong, f. t. A. H. A. S. Committee, and S. Stroke Statistics, "Heart Disease and Stroke Statistics–2009 Update: A Report From the American Heart Association Statistics Committee and Stroke Statistics Subcommittee," *Circulation*, vol. 119, no. 3, pp. e21–181, 2009.
- [136] M. A. Laflamme and C. E. Murry, "Regenerating the heart," *Nat Biotech*, vol. 23, no. 7, pp. 845–856, 2005.
- [137] S. Cohen and J. Leor, "REBUILDING BROKEN HEARTS," *Scientific American*, vol. 291, no. 5, pp. 45–51, 2004.
- [138] M. Radisic, H. Park, H. Shing, T. Consi, F. J. Schoen, R. Langer, L. E. Freed, and G. Vunjak-Novakovic, "Functional assembly of engineered myocardium by electrical stimulation of cardiac myocytes cultured on scaffolds," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 101, no. 52, pp. 18129–18134, 2004.
- [139] S. H. Saw, K. Wang, T. Yong, and S. Ramakrishna, "Polymeric Nanofibers in Tissue Engineering," in *Nanotechnologies for the Life Sciences*, Wiley-VCH Verlag GmbH & Co. KGaA, 2007.
- [140] M. Cappelletti, N. Muggleton, and V. Walsh, "Quantity without numbers and numbers without quantity in the parietal cortex," *Neuroimage*, vol. 46, no. 2, pp. 522–529, 2009.

- [141] W. Shuangyin, K. Noel, J. Sanping, and W. Xin, "Controlled synthesis of dendritic Au@Pt coreshell nanomaterials for use as an effective fuel cell electrocatalyst," *Nanotechnology*, vol. 20, no. 2, p. 25605, 2009.
- [142] T. Dvir, B. P. Timko, M. D. Brigham, S. R. Naik, S. S. Karajanagi, O. Levy, H. Jin, K. K. Parker, R. Langer, and D. S. Kohane, "Nanowired three-dimensional cardiac patches," *Nat Nano*, vol. 6, no. 11, pp. 720–725, 2011.
- [143] D. A. Stout, B. Basu, and T. J. Webster, "Cytocompatible Poly-Lactic-co-Glycolic Acid: Carbon Nanofiber Composite Analysis for Cardiovascular Applications," vol. 11, IEEE.
- [144] S. Komarneni, "Nanocomposites," *Journal of Materials Chemistry*, vol. 2, no. 12, pp. 1219–1230, 1992.
- [145] C. C.A, "Nanomechanical and nanotribological properties of carbon-based thin films: A review," *International Journal of Refractory Metals and Hard Materials*, vol. 28, no. 1, pp. 51–70, 2010.
- [146] H. Liu and T. J. Webster, "Nanomedicine for implants: A review of studies and necessary experimental tools," *Cellular and Molecular Biology Techniques for Biomaterials Evaluation*, vol. 28, no. 2, pp. 354–369, 2007.
- [147] A. Verma and F. Stellacci, "Effect of Surface Properties on NanoparticleCell Interactions," *Small*, vol. 6, no. 1, pp. 12–21, 2010.
- [148] "Standard Test Method for Tensile Properties of Plastics," 2010.
- [149] G. C. Engelmayer, M. Cheng, C. J. Bettinger, J. T. Borenstein, R. Langer, and L. E. Freed, "Accordion-like honeycombs for tissue engineering of cardiac anisotropy," *Nat Mater*, vol. 7, no. 12, pp. 1003–1010, 2008.
- [150] I. MIRSKY and W. W. PARMLEY, "Assessment of Passive Elastic Stiffness for Isolated Heart Muscle and the Intact Heart," *Circulation Research*, vol. 33, no. 2, pp. 233–243, 1973.
- [151] J. Boublik, H. Park, M. Radisic, E. Tognana, F. Chen, M. Pei, G. Vunjak-Novakovic, and L. E. Freed, "Mechanical properties and remodeling of hybrid cardiac constructs made from heart cells, fibrin, and biodegradable, elastomeric knitted fabric," *Tissue Engineering*, vol. 11, no. 7-8, pp. 1122–1132, 2005.
- [152] C. A. Phillips and J. S. Petrofsky, "Myocardial material mechanics: Characteristic variation of the circumferential and longitudinal systolic moduli in left ventricular dysfunction," *Journal of Biomechanics*, vol. 17, no. 8, pp. 561–568, 1984.
- [153] N. Westerhof, N. Stergiopoulos, and M. I. M. Noble, *Snapshots of Hemodynamics: An Aid for Clinical Research and Graduate Education*. Springer, 2010.

- [154] L. Yang, B. W. Sheldon, and T. J. Webster, "The impact of diamond nanocrystallinity on osteoblast functions," *Biomaterials*, vol. 30, no. 20, pp. 3458–3465, 2009.
- [155] F. Grinnell, "Fibronectin and wound healing," *Journal of Cellular Biochemistry*, vol. 26, no. 2, pp. 107–116, 1984.
- [156] S. P. Kent, "Diffusion of Plasma Proteins into Cells - a Manifestation of Cell Injury in Human Myocardial Ischemia," *American Journal of Pathology*, vol. 50, no. 4, pp. 623–&, 1967.
- [157] W. Casscells, H. Kimura, J. A. Sanchez, Z. X. Yu, and V. J. Ferrans, "Immunohistochemical study of fibronectin in experimental myocardial infarction," *American Journal of Pathology*, vol. 137, no. 4, pp. 801–810, 1990.
- [158] I. E. M. G. Willems, J.-W. Arends, and M. J. A. P. Daemen, "TENASCIN AND FIBRONECTIN EXPRESSION IN HEALING HUMAN MYOCARDIAL SCARS," *The Journal of Pathology*, vol. 179, no. 3, pp. 321–325, 1996.
- [159] N. Landa, L. Miller, M. S. Feinberg, R. Holbova, M. Shachar, I. Freeman, S. Cohen, and J. Leor, "Effect of Injectable Alginate Implant on Cardiac Remodeling and Function After Recent and Old Infarcts in Rat," *Circulation*, vol. 117, no. 11, pp. 1388–1396, 2008.
- [160] A. S. Krupnick, D. Kreisel, F. H. Engels, W. Y. Szeto, T. Plappert, S. H. Popma, A. W. Flake, and B. R. Rosengard, "A novel small animal model of left ventricular tissue engineering," *The Journal of Heart and Lung Transplantation*, vol. 21, no. 2, pp. 233–243, 2002.
- [161] H.-J. Wei, C.-H. Chen, W.-Y. Lee, I. Chiu, S.-M. Hwang, W.-W. Lin, C.-C. Huang, Y.-C. Yeh, Y. Chang, and H.-W. Sung, "Bioengineered cardiac patch constructed from multilayered mesenchymal stem cells for myocardial repair," *Biomaterials*, vol. 29, no. 26, pp. 3547–3556, 2008.
- [162] Y. Shintani, S. Fukushima, A. Varela-Carver, J. Lee, S. R. Coppen, K. Takahashi, S. W. Brouillette, K. Yashiro, C. M. N. Terracciano, M. H. Yacoub, and K. Suzuki, "Donor cell-type specific paracrine effects of cell transplantation for post-infarction heart failure," *Journal of Molecular and Cellular Cardiology*, vol. 47, no. 2, pp. 288–295, 2009.
- [163] M. Siepe, M.-N. A. Giraud, M. Pavlovic, C. Receptuto, F. Beyersdorf, P. Menasché, T. Carrel, and H. T. Tevæarai, "Myoblast-seeded biodegradable scaffolds to prevent post-myocardial infarction evolution toward heart failure," *The Journal of Thoracic and Cardiovascular Surgery*, vol. 132, no. 1, pp. 124–131, 2006.
- [164] T. J. Webster, C. Ergun, R. H. Doremus, R. W. Siegel, and R. Bizios, "Specific proteins mediate enhanced osteoblast adhesion on nanophase ceramics," *Journal of Biomedical Materials Research*, vol. 51, no. 3, pp. 475–483, 2000.

- [165] D. C. Miller, A. Thapa, K. M. Haberstroh, and T. J. Webster, "Endothelial and vascular smooth muscle cell function on poly(lactic-co-glycolic acid) with nano-structured surface features," *Biomaterials*, vol. 25, no. 1, pp. 53–61, 2004.
- [166] K. C. Roberts-Thomson, P. M. Kistler, P. Sanders, J. B. Morton, H. M. Haqqani, I. Stevenson, J. K. Vohra, P. B. Sparks, and J. M. Kalman, "Fractionated atrial electrograms during sinus rhythm: Relationship to age, voltage, and conduction velocity," *Heart Rhythm*, vol. 6, no. 5, pp. 587–591, 2009.
- [167] B. Smrutisikha, "Experimental study of mechanical and electrical properties of carbon nanofiber/epoxy composites," *Materials & Design*, vol. 31, no. 5, pp. 2406–2413, 2010.
- [168] D. M. Pedrotty, J. Koh, B. H. Davis, D. A. Taylor, P. Wolf, and L. E. Niklason, "Engineering skeletal myoblasts: roles of three-dimensional culture and electrical stimulation," *American Journal of Physiology - Heart and Circulatory Physiology*, vol. 288, no. 4, pp. H1620–H1626, 2005.
- [169] S. S. Mihadja, R. E. Sievers, and R. J. Lee, "The effect of polypyrrole on arteriogenesis in an acute rat infarct model," *Biomaterials*, vol. 29, no. 31, pp. 4205–4210, 2008.
- [170] A. C. Nicolosi, Z. C. Weng, P. W. Detwiler, and H. M. Spotnitz, "Simulated left ventricular aneurysm and aneurysm repair in swine," *J Thorac Cardiovasc Surg*, vol. 100, no. 5, pp. 745–755, 1990.
- [171] Y. Sakamoto, A. Mizuno, G. D. Buckberg, R. Baretti, J. S. Child, and G. Fonarow, "Restoring the Remodeled Enlarged Left Ventricle: Experimental Benefits of In Vivo Porcine Cardioreduction in the Beating Open Heart," *Journal of cardiac surgery*, vol. 13, no. 6, pp. 429–439, 1998.
- [172] J. Cui, J. Li, M. Mathison, F. Tondato, S. P. Mulkey, C. Micko, N. A. F. Chronos, and K. A. Robinson, "A clinically relevant large-animal model for evaluation of tissue-engineered cardiac surgical patch materials," *Cardiovascular Revascularization Medicine*, vol. 6, no. 3, pp. 113–120.
- [173] M. P. Lutolf and J. A. Hubbell, "Synthetic biomaterials as instructive extracellular microenvironments for morphogenesis in tissue engineering," *Nat Biotech*, vol. 23, no. 1, pp. 47–55, 2005.
- [174] D. Björn, "Cardiovascular Disease Risk Factors: Epidemiology and Risk Assessment," *The American Journal of Cardiology*, vol. 105, no. 1, Supplement, pp. 3A–9A, 2010.
- [175] V. J. Dzau, E. M. Antman, H. R. Black, D. L. Hayes, J. E. Manson, J. Plutzky, J. J. Popma, and W. Stevenson, "The Cardiovascular Disease Continuum Validated: Clinical Evidence of Improved Patient Outcomes," *Circulation*, vol. 114, no. 25, pp. 2850–2870, 2006.

- [176] Y. Mu, H. Liang, J. Hu, L. Jiang, and L. Wan, "Controllable Pt Nanoparticle Deposition on Carbon Nanotubes as an Anode Catalyst for Direct Methanol Fuel Cells," *The Journal of Physical Chemistry B*, vol. 109, no. 47, pp. 22212–22216, 2005.
- [177] D. A. Stout, B. Basu, and T. J. Webster, "Poly(lactico-glycolic acid): Carbon nanofiber composites for myocardial tissue engineering applications," *Acta Biomaterialia*, vol. 7, no. 8, pp. 3101–3112, 2011.
- [178] D. G. Vassilyev, S. Takeda, S. Wakatsuki, K. Maeda, and Y. Maéda, "Crystal structure of troponin C in complex with troponin I fragment at 2.3-Å resolution," *Proceedings of the National Academy of Sciences*, vol. 95, no. 9, pp. 4847–4852, 1998.
- [179] G. M. C. Gasmi-Seabrook, J. W. Howarth, N. Finley, E. Abusamhadneh, V. Gaponenko, R. M. M. Brito, R. J. Solaro, and P. R. Rosevear, "Solution Structures of the C-Terminal Domain of Cardiac Troponin C Free and Bound to the N-Terminal Domain of Cardiac Troponin I," *Biochemistry*, vol. 38, no. 26, pp. 8313–8322, 1999.
- [180] C. S. Farah and F. C. Reinach, "The troponin complex and regulation of muscle contraction," *The FASEB Journal*, vol. 9, no. 9, pp. 755–767, 1995.
- [181] G. Yong, W. Yan, Z. Xi-zheng, Y. Yu-xian, L. Rui-xin, and Z. Chun-qiu, "Effects of Electrical Stimulation on Growth and Metabolism of Cardiomyocytes In Vitro," in *Biomedical Engineering and Informatics, 2009. BMEI '09. 2nd International Conference on*, pp. 1–4, 2009.
- [182] Y. Xia, L. M. Buja, and J. B. McMillin, "Change in Expression of Heart Carnitine Palmitoyltransferase I Isoforms with Electrical Stimulation of Cultured Rat Neonatal Cardiac Myocytes," *Journal of Biological Chemistry*, vol. 271, no. 20, pp. 12082–12087, 1996.
- [183] A. T. Soufan, G. van den Berg, J. M. Ruijter, P. A. J. de Boer, M. J. B. van den Hoff, and A. F. M. Moorman, "Regionalized Sequence of Myocardial Cell Growth and Proliferation Characterizes Early Chamber Formation," *Circulation Research*, vol. 99, no. 5, pp. 545–552, 2006.
- [184] P. A. Tran, L. Zhang, and T. J. Webster, "Carbon nanofibers and carbon nanotubes in regenerative medicine," *Advanced Drug Delivery Reviews*, vol. 61, no. 12, pp. 1097–1114, 2009.
- [185] T. Shimizu, K.-i. Kinugawa, A. Yao, Y. Sugishita, K. Sugishita, K. Harada, H. Matsui, O. Kohmoto, T. Serizawa, and T. Takahashi, "Platelet-derived growth factor induces cellular growth in cultured chick ventricular myocytes," *Cardiovascular Research*, vol. 41, no. 3, pp. 641–653, 1999.

Index

- α -sarcomeric actin, 86
- AIDS, 36
- ANOVA One-Way, 46
- Atomic force microscopy, 81, 93
- Cardiomyocyte, 80, 96–98, 105, 112
- Cardiovascular disease, 78, 104
- CD14, 58
- Cell migration, 1, 3, 17, 66
 - 2D vs 3D, 6
 - Cancer, 17
 - Embryonic, 1
 - Human development, 1, 2
 - Inflammatory response, 3
 - Mesenchymal, 1
 - Neurites, 1
 - Neutrophil, 3, 8, 9, 39
 - Process, 5
- Chamblide chamber, 20, 25, 43
- Chemoattractant, 18, 19
- Chemokine, 3
- chemotactic index, 56
- Chemotatic Index, 45
- Chemotatic index, 45, 48, 58
- Chemotaxis, 2, 3, 17–20, 35, 47, 48, 53, 60
 - Microfluid, 18
 - Neutrophil, 46
- Collagen, 18, 19, 22
 - 3D matrix, 19, 21, 23, 29, 33, 34, 42
 - Density, 23, 24, 29, 34
- Conductivity theorem, 82
- Confocal microscopy, 23, 30, 31
 - Live cell image, 43
- Connexin-43, 86
- Degradation, 84, 91
- Diffusion, 17, 19
 - Coefficient, 20, 31, 33, 35
 - Collagen membrane, 19
 - Concentration, 32
 - Gradient, 20, 44
 - Profile, 34
 - Specific concentration, 44
- Durotaxis, 3
- Electrial stimulation, 108
- Embryonic cells, 1
- Extracellular matrix, 3, 5, 12

fMLP, 37, 40, 41, 43, 44, 53, 57, 60, 64, 68
 G protein-coupled receptor, 16, 37, 57, 60, 62, 63, 65, 67, 68
 Haptotaxis, 3
 Hydrophilicity, 20
 Hydrophobicity, 20
 ImageJ, 43
 Immunofluorescence, 86, 97, 98
 Jacobian matrix, 46
 lamellipodial, 36
 Leukocyte, 3, 8, 17, 66
 Lipopolysaccharide, 16, 38, 46, 47, 53, 57, 58, 62, 64, 67–69
 LPS binding protein, 58
 Lymphocyte, 17
 Mathematica, 43
 mechanotaxis, 3
 Mesenchymal cells, 1
 Migration
 Displacements, 46
 Speed, 45, 48
 Nanomaterial, 80, 99, 105
 Nanomedicine, 79, 105
 Neuron, 2
 Neutrophil, 3, 7, 8, 17, 36, 38, 40, 53, 66
 Hyperactivation, 38
 Isolation, 40
 Transmigrations, 39
 Ordinary differential equation, 27
 p38 MAPK, 37, 59, 60, 62
 Partial differential equation, 24
 PIP2, 37
 PIP3, 37, 59, 60
 Plithotaxis, 3
 Principal strain, 16, 45, 47, 56, 62, 64
 Prinicipal strain, 46
 Pseudopod, 47
 PTEN, 37, 59, 60, 62
 Reactive oxygen species, 38
 Rhodamine, 23, 28, 29, 32, 33
 ROK, 37
 Scanning electron microscopy, 81, 88
 Sepsis, 10, 16, 37, 38, 41, 44, 62–65, 67
 1st phase, 10
 Neutrophil, 11, 12, 38, 39, 41
 Neutrophil-mediated organ failure, 38
 Surface area, 81, 93, 94, 100, 101

TecPlot, 43	Fast iterative digital volume
Tecplot, 46	correlation, 14, 45
Toll-like recetpor 4, 16, 57–60, 62, 63,	Troponin, 86, 108
65, 67, 68	Troponin I, 108, 109, 112
Traction force microscopy	Troponin T, 86, 87, 108, 113
2D, 12	Uropod, 36, 47, 59, 62
3D, 13, 40, 64	Wilcoxon Rank-Sum, 46
Digital volume correlation, 13, 14,	X-ray diffraction, 109
40, 45, 64	