RICE UNIVERSITY

Tailored Release of Bioactive Factors from Composite Multidomain Peptide Hydrogels

by

Navindee Charya Wickremasinghe

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE

Doctor of Philosophy

APPROVED, THESIS COMMITTEE:

Jeffrey D. Hartgerink, Chair
Professor of Chemistry and Professor of Bioengineering

Angel A. Marti
Associate Professor of Chemistry, Bioengineering, Materials Science and Nanoengineering

Antonios G. Mikos
Louis Calder Professor of Bioengineering and Chemical Engineering; Professor of Chemistry

HOUSTON, TEXAS
MARCH 2016
ADDENDUM

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Retraction of Data in Chapter 4

Upon reinvestigating the histology data in Chapter 4 of this thesis, I came to notice several instances of incorrectly labeled wound sections, which led to misinterpreting wound healing responses. The wound sections in question were H&E stained to assess wound closure, and Masson’s trichrome-stained to assess the formation of granulation tissue. The accidental mislabeling resulted in the mixing up of IL-4 treated wound sections with the controls. Thus, the histological assessment of these wound sections is erroneous and I hereby retract the data presented in Section 4.2 (Wound Closure in Diabetic Mice) and Section 4.3 (Re-epithelialization and Granulation Tissue Formation). As such, all wound closure data and re-epithelialization data presented in Figures 4.3, 4.4, 4.5, 4.7, 4.8, 4.9 and 4.10 are erroneous and retracted.

Furthermore, the above error affects the evaluation of macrophage response, angiogenesis and wound contraction. Therefore, all data presented in Sections 4.4 (Macrophage Response) and 4.5 (Angiogenesis and Wound Contraction) are also retracted. This includes all immunofluorescent and graphical data presented in Figures 4.11-4.17.

With the retraction of the above data, the conclusions drawn in Chapter 4 are no longer valid. As it stands, I have not been able to establish a positive correlation between wound treatment with IL-4 loaded MDP gels and accelerated healing of wounds in diabetic mice. The accidental mislabeling of wound sections was a careless but honest mistake on my part, and I apologize sincerely for the inconvenience it has caused my former advisor and lab mates.

Navindee Charya Wickremasinghe
01-31-2017

Approved by:

Jeffrey D. Hartgerink
Professor of Chemistry and Professor of Bioengineering

Angel A. Marti
Associate Professor of Chemistry, Bioengineering, Materials Science and Nanoengineering

Antonios G. Mikos
Louis Calder Professor of Bioengineering and Chemical Engineering; Professor of Chemistry
ABSTRACT

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Multidomain peptides (MDP) self-assemble to form nanofibrous scaffolds well suited to tissue engineering and regeneration strategies. MDPs can present bioactive cues that promote vital biological responses. Orthogonal self-assembly of MDP and growth factor-loaded liposomes generate supramolecular composite hydrogels. This thesis demonstrates the ability to create a unique hydrogel, developed by stepwise self-assembly of multidomain peptide fibers and liposomes, and presents its potential for in vivo applications.

Chapter One of the thesis presents an introduction to the above work with background spanning from the role of self-assembling peptides and hydrogels in tissue engineering, to current strategies for therapeutic angiogenesis and wound healing. Chapter Two addresses the design and characterization of a composite hydrogel containing MDP and liposomes. Results showed that structural and mechanical integrity of the peptide nanofibers, lipid vesicles and the composite gel are retained. The two-component gel allows for controlled release of bioactive factors at multiple time points and indicates bimodal release of two growth factors from the same system.

These MDP-Liposome Composites (MLCs) were injected in vivo for targeted, localized delivery of growth factors, and Chapter Three details how they functioned in
vivo. Placental growth factor-1 (PIGF-1) was shown to temporally stimulate VEGF-receptor activation in vitro in endothelial cells, and robust vessel formation in vivo. MLCs provide a novel method for the time controlled delivery of growth factors from within highly biocompatible and injectable hydrogels. Time controlled release guided by MLCs induces an unprecedented level of growth factor-mediated neovascular maturity.

Use of cytokine-loaded MDP hydrogels to accelerate diabetic wound healing is another in vivo application explored in Chapter Four of this thesis. Delivery of a pro-healing cytokine IL-4 via MDP hydrogels have resulted in enhanced healing of full-thickness dermal wounds on the backs of genetically diabetic mice. Compared to controls, wounds treated with IL-4-MDP composite gels showed higher wound closure, M2 macrophage polarization, re-epithelialization, granulation tissue formation and angiogenesis.

The conclusion chapter, Chapter Five, discusses how the above in vivo success of composite MDP hydrogels speaks to their potential to function as a unique protein delivery platform for tissue regeneration.
ACKNOWLEDGMENTS

First and foremost, I would like to extend my heartfelt and warmest gratitude to Professor Jeffrey Hartgerink, my amazing advisor, without whom none of the research in this thesis would be possible. Thank you Jeff, for believing in my potential, guiding me on the right path when I lost my step, and encouraging me to keep pushing forward. Thank you for your wisdom, kindness and patience.

I would like to thank the members of my thesis committee: Professors Angel Marti and Antonios Mikos; and my qualifying exam committee: Professors Zachary Ball, Lon Wilson and John McDevitt. I am honored to have had the opportunity to seek scholastic guidance and constructive criticism from such distinguished scientists. A special word of thanks goes to Professors Nalin De Silva, Rohini De Silva, Sujatha Hewage and Dayal De Costa for recommending me with such confidence to the Rice PhD Program; and Professor Seiichi Matsuda for giving me a chance to prove myself.

I extend my gratitude to members of the Shared Equipment Authority and the Animal Resource Facility, especially to Dr. Budi Utama, Dr. Christopher Pennington, Dr. Elyssse Orchard and Kelly Campbell. Thank you for providing your expertise without hesitation whenever I needed it.

I am very grateful for the support and cooperation I received from everyone in the Hartgerink Lab. It has been a pleasure working with such brilliant minds and great companions. I could not have picked a better bunch of people to share the triumphs and sorrows of this journey. A special word of thanks goes to my colleague and collaborator, Dr. Vivek Kumar for helping me find solutions when they seem so elusive, for keeping
me on my toes and reminding me to make lemonade when research so frequently gave me lemons.

My warmest gratitude goes to all my wonderful friends at Rice, including Tomi, Adriana, Ha, and Amanda. You all made my time at Rice especially memorable. Particular mention should be made of my bedazzling roommate/best friend, Mayra. Without you, these past five years would have been much less exciting. Thank you for always having my back, making me laugh till it hurts, and reminding me to breathe. I shall forever remember all the incredibly fun and unbelievably silly things we did together, just to survive grad school. I would also like to thank Sumudu, Dinuka and all my other friends from back home. Your smiles and encouragement kept me going. Thank you to my baby sister, Madara for making me believe I’m a superstar in my own right. You don’t know how much I needed that.

To Upeshka and his family, who became my surrogate family here in Houston, thank you with all my heart, for making me feel at home. I am immensely grateful for your kindness and generosity. Dearest Pesh, thank you for lighting up my life and being my personal cheerleader. I could not have made the finish line so confidently without your love.

And finally, my deepest gratitude goes to my beloved parents, Nimal and Seetha Wickremasinghe, for their constant blessings. Thank you for teaching me to be strong and independent, and to live up to my full potential. Thank you for your unconditional love, and unwavering faith in me. Thank you a million times. This thesis is dedicated to you.
## TABLE OF CONTENTS

List of Tables........................................................................................................ x
List of Figures........................................................................................................ xi
Abbreviations........................................................................................................ xv

**Chapter One: Introduction** .................................................................................. 1

1.1. The Role of Hydrogels in Tissue Engineering ............................................. 1

1.1.1. Extracellular matrix .................................................................................. 2
1.1.2. Synthetic vs. natural hydrogels ................................................................. 4

1.2. Self-assembling Peptides as Scaffolds ......................................................... 6

1.2.1. Multidomain peptides ............................................................................... 8
1.2.2. Applications of MDPs ............................................................................. 11

1.3. Controlled Release of Molecules ................................................................. 13

1.3.1. MDPs for controlled release .................................................................... 15
1.3.2. Liposomes as carriers ............................................................................... 16
1.3.3. Combining liposomes with MDPs ............................................................. 18

1.4. Therapeutic Angiogenesis ............................................................................ 19

1.4.1. Materials-based approaches that potentiate angiogenesis ....................... 23
1.4.2. Controlling angiogenesis in vivo with MDP hydrogels ......................... 25
1.4.3. Accelerating the wound healing process ............................................... 26
1.4.4. Healing diabetic wounds with MDP hydrogels ..................................... 29

1.5. Conclusions and Preview of Thesis ............................................................ 30

References ............................................................................................................ 32
Chapter Two: Two-Step Self-Assembly of MDP-Liposome Composite Gels ..........42

2.1. Design of a Composite System ...........................................................................................................42

2.2. Synthesis and Characterization of MDP Fibers and Liposomes .................................................46

2.3. Synthesis and Characterization of MDP-Liposome Composite Hydrogel .........................49

2.4. Release of Fluorescently-labeled GFs from Liposomes in Composite Gels .................53

2.5. Conclusions ......................................................................................................................................58

2.6. Materials and Methods ....................................................................................................................59

    2.6.1. Synthesis and characterization of MDP ......................................................................................59

    2.6.2. Synthesis and characterization of liposomes ..............................................................................61

    2.6.3. Formation and characterization of composite hydrogel .................................................................62

    2.6.4. Growth factor conjugation to fluorophore molecules .................................................................64

    2.6.5. Loading liposomes with growth factors .....................................................................................64

    2.6.6. GF release from composite gel ....................................................................................................65

References ....................................................................................................................................................67

Chapter Three: Controlled Angiogenesis in MDP Nanofiber Composite Hydrogels ....71

3.1. MDPs and Angiogenesis .....................................................................................................................71

3.2. In vitro Experiments ............................................................................................................................76

3.3. Cellular Infiltration and New Matrix Formation ...............................................................................78

3.4. Development of Robust Vasculature ...............................................................................................87

3.5. Comparison to Other Materials-based Angiogenesis Efforts .......................................................94
3.6. Conclusions ......................................................................................................................... 96

3.7. Materials and Methods ...................................................................................................... 97

3.7.1. Synthesis of SLac peptide ............................................................................................... 97

3.7.2. Synthesis of liposomes and encapsulation of PlGF-1 ..................................................... 97

3.7.3. In vitro PlGF-1 release from MDP-liposome composite gel .......................................... 98

3.7.4. Assessment of angiogenic receptor activation by PlGF-1 release ................................. 99

3.7.5. In vivo subcutaneous implants in rats ............................................................................... 100

3.7.6. Evaluation of cell infiltrate, neomatrix development and angiogenesis ................. 100

3.7.7. Statistical analysis .......................................................................................................... 101

References .................................................................................................................................. 102

Chapter Four: Accelerating Diabetic Wound Healing with MDP Gels ......................... 107

4.1. Interleukin-4 Loaded MDP Gels for Inflammation Resolution ...................................... 107

4.2. Wound Closure in Diabetic Mice ..................................................................................... 111

4.3. Re-epithelialization and Granulation Tissue Formation ............................................... 117

4.4. Macrophage Response ...................................................................................................... 123

4.5. Angiogenesis and Wound Contraction ............................................................................ 130

4.6. Discussion and Conclusions ............................................................................................. 133

4.7. Materials and Methods .................................................................................................... 137

4.7.1. Synthesis of SLac peptide ............................................................................................. 137

4.7.2. Preparation of loaded MDP gels and controls ............................................................... 138

4.7.3. Diabetic mouse wound healing model ......................................................................... 139
LIST OF TABLES

Chapter Three: Controlled Angiogenesis in MDP Nanofiber Composite Hydrogels

3.1. Summary of composite hydrogels and MDP-Liposome Composites (MLCs) ....75

Chapter Four: Accelerating Diabetic Wound Healing with MDP Gels

4.1. Properties of wound healing macrophages. .....................................................109

Appendix III: Modeling Release Data

App. T1. $R^2$ values and curve fit constants of single release profiles .................159

App. T2. $R^2$ values and curve fit constants of bimodal release profiles .................160
LIST OF FIGURES

Chapter One: Introduction

1.1. Model of complex 3D structure of extracellular matrix ........................................3
1.2. SEM images of synthetic and natural hydrogel examples ......................................5
1.3. Self-assembly of strand-swapped β-hairpin nanofiber hydrogels .............................7
1.4. A multidomain peptide ‘sandwich’ dimer ...............................................................9
1.5. The self-assembly process of MDPs ......................................................................10
1.6. Hemostasis achieved in a lateral liver incision model .............................................13
1.7. MCP-1 and IL-4 release from MDP hydrogels ....................................................16
1.8. Phospholipid organization into bilayers and vesicles ............................................17
1.9. Delivery of growth factors VEGF and SDF and subsequent in vivo effects ..........22
1.10. SLanc peptide structure and in vivo response .....................................................25
1.11. Enhanced re-epithelialization by release of EGF from peptide scaffolds .............28

Chapter Two: Two-Step Self-Assembly of MDP-Liposome Composite Gels

2.1. Self-assembly of K(SL)₃RG(SL)₃KGRGDS into nanofibers ..................................44
2.2. Stepwise orthogonal self-assembly of MLCs..........................................................46
2.3. MS and CD spectra for the K(SL)₃RG(SL)₃KGRGDS peptide ...............................47
2.4. Negatively-stained TEM image of a multilamellar liposome .................................47
2.5. Characterization of peptide fibers and liposomes ..................................................48
Chapter Three: Controlled Angiogenesis in MDP Nanofiber Composite Hydrogels

3.1. Supramolecular orthogonal self-assembly ......................................................74

3.2. Angiogenic receptor activation as a function of temporal GF release ............77

3.3. In vivo implant sites .......................................................................................78

3.4. Location of implant ......................................................................................79

3.5. Evaluation of cellular infiltrate .......................................................................80

3.6. H&E images of subcutaneous implants in rats at Day 5 and Day 10 .............81

3.7. Immunostaining for monocytes/macrophages at Day 5 and Day 10 .............82

3.8. Quantification of cellular infiltrate .................................................................83

3.9. Evaluation of new matrix formation ..............................................................84

3.10. New matrix formation – higher mag. images ...............................................86

3.11. Quantification of angiogenesis ....................................................................87

3.12. Assessment of neo-angiogenesis - MDP alone (control) ............................89
3.13. Assessment of neo-angiogenesis - MDP(PIGF-1) ...........................................90
3.14. Assessment of neo-angiogenesis – MDP(Lipo(PIGF-1)).................................91
3.15. Assessment of neo-angiogenesis – Day 5 time point ......................................92
3.16. Component images of MDP(Lipo(PIGF-1)) at Day 5 ......................................93
3.17. Schematic of proposed in vivo mechanism ......................................................94

Chapter Four: Accelerating Diabetic Wound Healing with MDP Gels

4.1. Phases of distinct macrophage subsets that exist during wound healing ..........109
4.2. Macroscopic appearance of wounds on diabetic mice .................................112
4.3. Evaluation of wound closure in IL-4/SLac and MCP-1/SLac treated wounds ....113
4.4. Evaluation of wound closure in IL-4 dosing study .........................................116
4.5. Comparison of wound closure in IL-4/SLac treated wounds to controls ..........117
4.6. Cross-section of a full-thickness dermal wound bed ....................................118
4.7. H&E staining of wounds at 14 days post injury ..............................................119
4.8. Evaluation of neotissue formation and wound closure ...................................120
4.9. Masson’s trichrome staining of wounds at 14 days post injury .......................121
4.10. Depth of granulation tissue ...........................................................................122
4.11. Evaluation of macrophage response in IL-4/SLac treated wounds at day 14 ....124
4.12. High mag. immunostained image of 20 ng IL-4/SLac treated wound ..............125
4.13. Evaluation of macrophage response in control wounds at day 14 .................126
4.14. High mag. immunostained image of control wound ........................................127
4.15. Quantification of macrophage populations ..........................................................129
4.16. Evaluation of angiogenic response and myofibroblasts at day 14 ......................131
4.17. Quantification of vessel density at day 14 .........................................................132

Appendix I: VEGF Encapsulation and Release
App. F1. Determining encapsulation efficiency of VEGF ...........................................152
App. F2. VEGF release profiles .................................................................................153
App. F3. Sample standard 5-PL curve .....................................................................154

Appendix II: Differently-Sized Liposomes
App. F4. Steps in synthesis of liposomes by hydration of dry lipid films ...............155
App. F5. Set-up for the ether injection method of preparing LUVs .........................156
App. F6. Size distributions of differently-sized liposomes after 1 day ......................157
App. F7. Stability of LUVs ......................................................................................157
App. F8. EGF-FITC release from SUVs and LUVs in gels ......................................158

Appendix IV: SDF-1α Release from Gels In Vitro and In Vivo
App. F9. Release profile for SDF-1α-CFDA ...............................................................161
App. F10. hMSC migration study ............................................................................162
App. F11. Immunostained images of MDP(SDF-1α) .................................................164
App. F12. Component images of MDP(SDF-1α) implant at Day 5 .........................165
ABBREVIATIONS

α-SMA  Alpha smooth muscle actin
AB1  Ankle brachial index
BSA  Bovine serum albumin
CAM  Chorioallantoic membrane
CCL2  Chemokine (C-C motif) ligand 2 (also known as MCP-1)
CCR7  C-C chemokine receptor type 7
CD  Circular dichroism
CD206  Cluster of differentiation marker 206 (Other CD markers mentioned: CD16, CD32, CD64, CD86, CD163)
CFDA-SE  5(6)-carboxyfluorescein diacetate succinimidyl ester
DFU  Diabetic foot ulcer
DiEA  N,N-Diisopropylethylamine
DLS  Dynamic light scattering
DMF  Dimethlyformamide
DMPC  1,2-dimyristoyl-sn-glycero-3-phosphocholine
DMSO  Dimethyl sulfoxide
DPPC  Dipalmitoylphosphatidylcholine
DPPG  Dipalmitoylphosphatidylglycerol
ECM  Extracellular matrix
EGF  Epidermal growth factor
ELISA  Enzyme-linked immunosorbent assay
FBS  Fetal bovine serum
FGF-2  Fibroblast growth factor basic
FITC  Fluorescein isothiocyanate
GAGs  Glycosaminoglycans
G-CSF  Granulocyte-colony stimulating factor
GFs  Growth factors
H&E  Hematoxylin and Eosin
HATU  (1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate)
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>HB-EGF</td>
<td>Heparin-binding epidermal growth factor</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank’s balanced salt solution</td>
</tr>
<tr>
<td>HGF</td>
<td>Hepatocyte growth factor</td>
</tr>
<tr>
<td>hMSC</td>
<td>Human mesenchymal stem cells</td>
</tr>
<tr>
<td>HSE</td>
<td>Human skin equivalent</td>
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<tr>
<td>HUVEC</td>
<td>Human umbilical vein endothelial cells</td>
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<tr>
<td>IFN-γ</td>
<td>Interferon-gamma</td>
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<tr>
<td>IL-4</td>
<td>Interleukin-4 (Other interleukins mentioned: IL-1, IL-6, IL-10, IL-12, IL-13)</td>
</tr>
<tr>
<td>KGF</td>
<td>Keratinocyte growth factor</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
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<tr>
<td>LUV</td>
<td>Large unilamellar vesicle</td>
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<tr>
<td>LVES</td>
<td>Large vessel endothelial supplement</td>
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<tr>
<td>MALDI-TOF</td>
<td>Matrix-assisted laser desorption ionization – time of flight</td>
</tr>
<tr>
<td>MBHA</td>
<td>4-methylbenzhydrylamine</td>
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<tr>
<td>MCP-1</td>
<td>Monocyte chemotactic protein-1(also known as CCL2)</td>
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<tr>
<td>MDP(Lypo(PIGF-1))</td>
<td>MDP-liposome composite gel with PIGF-1 loaded in the liposomes</td>
</tr>
<tr>
<td>MDP(PIGF-1)</td>
<td>MDP gel with PIGF-1 loaded in the gel matrix</td>
</tr>
<tr>
<td>MDP(SDF-1α)</td>
<td>MDP gel with SDF-1α loaded in the gel matrix</td>
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<tr>
<td>MDPs</td>
<td>Multidomain peptides</td>
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<td>MDS</td>
<td>Multi-dose system</td>
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<tr>
<td>MLCs</td>
<td>MDP-Liposome composites</td>
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<tr>
<td>MMP-2</td>
<td>Matrix metalloproteinase-2</td>
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<tr>
<td>MSC</td>
<td>Mesenchymal stem cells</td>
</tr>
<tr>
<td>MWCO</td>
<td>Molecular weight cut-off</td>
</tr>
<tr>
<td>NGF</td>
<td>Nerve growth factor</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>PAD</td>
<td>Peripheral artery disease</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet-derived growth factor</td>
</tr>
<tr>
<td>PEAD</td>
<td>Polyethylene arginylaspartate diglyceride</td>
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PIGF-1  Placental growth factor
PTA    Phosphotungstic acid
RNS    Reactive nitrogen species
ROS    Reactive oxygen species
RT-PCR Reverse transcription - polymerase chain reaction
S1P    Sphingosine-1-phosphate
SB50   SLac hydrogel loaded with 50 μg/mL of batroxobin
SDF-1α  Stromal cell-derived factor – 1 alpha
SEM    Scanning electron microscopy
SUVs   Small unilamellar vesicles
TAMRA-SE 5(6)-carboxytetramethylrhodamine succinimidyl ester
TEM    Transmission electron microscopy
TFA    Trifluoroacetic acid
TGF-α  Transforming growth factor alpha
TGF-β1 Transforming growth factor beta 1
TLRα   Toll-like receptors
TNF-α  Tumor necrosis factor-alpha
VEGF   Vascular endothelial growth factor
VEGFR  Vascular endothelial growth factor receptor
vWF    von Willebrand factor
Chapter One

Introduction

1.1. The Role of Hydrogels in Tissue Engineering

The loss or failure of an organ or tissue is one of the most severe human health problems, consuming approximately half of the total annual health-care costs in the USA.\textsuperscript{1} Tissue engineering and regenerative medicine hold great promise for countless patients suffering from tissue loss or organ failure. The potential of this interdisciplinary field of science for repairing damaged tissue lies in the ability to deliver cell and tissue constructs to the body and utilize the therapeutic assistance of the body’s innate healing processes.\textsuperscript{2} Therefore the use of suitable engineered biomaterials with desired functional properties to interface with biological systems is of critical importance in tissue engineering for medical and therapeutic applications.\textsuperscript{3} In this regard, hydrogels are a class of biomaterials that have demonstrated great potential mainly due to their biocompatibility and high water content.\textsuperscript{3} Typically hydrogels are defined as three-dimensional networks of hydrophilic polymers cross-linked or self-assembled to form soft matrices (<1MPa storage modulus) containing >90% water.\textsuperscript{4} A review by Slaughter \textit{et al.} in which the properties, applications and overall potential of hydrogels in regenerative medicine are presented, highlights the versatility of hydrogels: they can serve as scaffolds that provide structural integrity to tissue constructs; control drug and protein delivery to tissues and cultures; and serve as compatible barriers between tissue and material surfaces.\textsuperscript{2} Hydrogels can be generally categorized into 3 groups: synthetic (prepared by polymerizing synthetic monomers\textsuperscript{5, 6}), naturally existing\textsuperscript{7, 8} and self-assembling peptide hydrogels. From a functional and biomimetic point of view, the most
important feature of a hydrogel is its ability to mimic the extracellular matrix found in natural tissues.

1.1.1. Extracellular matrix

A large part of the tissue volume is extracellular space filled by an intricate network of macromolecules constituting the extracellular matrix (ECM). This matrix is composed of a variety of proteins and polysaccharides, secreted locally and assembled into an organized meshwork in close association with the surface of the cell that produced them. Of the polysaccharides, glycosaminoglycans (GAGs) are notable, especially in the animal ECM. They are unbranched polysaccharide chains distinguished according to their sugars, linkage between sugars and number and location of sulfate groups. For example, chondroitin, dermatan, heparan and keratin sulfates are sulfated GAGs, while hyaluronic acid is a non-sulfated GAG. A majority of the GAGs are normally found covalently linked to proteins and termed proteoglycans. Proteoglycans are thought to have a major role in chemical signaling between cells, by binding various signaling molecules, such as growth factors, and enhancing or inhibiting their signaling activity. For example, the heparan sulfate chains of proteoglycans bind to fibroblast growth factors (FGFs), which stimulate a variety of cell types to proliferate. Glycans also allow tissues to diffuse nutrients and act as reservoirs for signaling molecules.

Of the proteins present in the ECM, fibrous proteins including collagen, elastin, fibronectin and laminin are prominent. Collagen, the most abundant protein in mammals, provides tensile strength to the ECM, while elastin gives the ECM its
elasticity. Cells bind to the ECM mainly through adhesive proteins like laminin and fibronectin.

In summary, the proteoglycan molecules in the ECM form a highly hydrated, gel-like scaffold in which fibrous proteins are embedded. This gel resists compressive forces on the matrix while allowing the rapid diffusion of nutrients, metabolites, and hormones between blood and tissue cells. The collagen fibers in the ECM both strengthen and help organize the matrix, and rubberlike elastin fibers give it resilience. Many other matrix proteins help cells attach in the appropriate locations. Thus the ECM is a chemically diverse environment that not only provides structural support to the cells, but also interacts with them, relays important external communication and allows material exchange into and out of cells (Figure 1.1). As such, the ECM facilitates chemical signaling mechanisms that regulate cell growth, proliferation and differentiation as required.

Figure 1.1. Model of complex 3D structure of extracellular matrix (ECM) and cell-ECM interactions. PG = proteoglycan; HA = hyaluronic acid; FN = fibronectin; MMP = matrix metalloproteinase; RGD = integrin binding sequence. Integrin and syndecan are cell-surface receptors. Taken from Zhu et al. (2010).
1.1.2. Synthetic vs. natural hydrogels

Among the many types of synthetic polymer hydrogels existing today, polyethylene glycol (PEG), poly(lactic-co-glycolic acid) (PLGA), poly(hydroxyethyl methacrylate) (PHEMA) and poly(vinyl alcohol) (PVA) have become household names due to their wide ranging applications. Relevant to the biomedical and tissue engineering fields, these hydrogels have found utility in coatings (for sutures, catheters, intrauterine devices, vascular grafts, sensors, etc.), homogeneous materials (such as contact lenses, vitreous humor replacements, breasts and other soft tissue substitutes, dentures, burn dressings, etc.) and devices (including enzyme therapeutic systems, artificial organs and drug delivery systems).

The most advantageous attribute of synthetic hydrogels is the control we have over their chemical, physical and mechanical properties. The relationship between the chemical composition of the gels and the consequent physical and mechanical properties is relatively well-understood, and allows for the tailoring of the latter to a specific value even. On the other hand the major drawback of synthetic hydrogels is the lack of innate biofunctionality that is normally associated with natural tissue. The type of complex structural and functional environment seen in the natural ECM is not easy to replicate via hydrogels made from synthetic polymers, although they have highly tunable and consistent properties and are easier to produce in large scale. As such, it has become common practice to covalently functionalize synthetic polymers with bioactive peptide sequences, larger proteins and drugs in order to promote cell attachment and proliferation within the gel. Another functionalization strategy employs naturally occurring ECM proteins and GAGs (such as collagen, fibrin and heparin) as counterparts.
to synthetic polymers in combination gels.\textsuperscript{26, 27} The above natural components provide a certain level of bioactivity while also creating a fibrous nature in the hydrogel, which otherwise cannot be usually observed in most synthetic nanoporous gels (Figure 1.2).\textsuperscript{22, 28}

\textbf{Figure 1.2.} (A) Cryo-SEM image of synthetic PLGA–PEG–PLGA hydrogel illustrating the porous network of the hydrogel. Scale bar = 5 μm. (B) SEM photomicrograph of a naturally derived hyaluronic acid-collagen hydrogel. Scale bar = 50 μm. Adapted from Pratoomsoot \textit{et al.} (2008) and Segura \textit{et al.} (2005).\textsuperscript{20, 29}

Hydrogels made solely from natural sources are becoming popular in regenerative medicine due to the structural similarity to ECM components and inherent biocompatibility.\textsuperscript{3, 30} These gels are typically formed of ECM proteins such as collagen, fibrin, hyaluronic acid, as well as materials derived from other biological sources such as chitosan, alginate or silk fibrils (Figure 1.2).\textsuperscript{29, 31-35} Various endogenous factors present in such gels contribute to their innate biocompatibility and can promote many cellular functions including proliferation, viability and development.\textsuperscript{30} However, natural hydrogels come with the disadvantage of often being ill-defined with little control over their physico-chemical properties, making it very challenging to tune their mechanics or biochemical presentation.\textsuperscript{2} These gels are complex structures containing many different components, and thus it is difficult in most cases to identify exactly which signals stimulate different cellular functions. Additionally, they may carry the risk of
inflammation, undesirable immune response and batch-to-batch variation that confounds the effect of the scaffold on cell proliferation, differentiation and migration.\textsuperscript{30, 36} In light of the limitations of using either synthetic or natural hydrogels for tissue engineering applications, scientists have been driven to design novel materials that combine both the customizability of synthetic gels and the bioactivity of naturally occurring ones.

1.2. Self-assembling Peptides as Scaffolds

After decades of using synthetic and natural hydrogels for cell culture, it has become evident that to mimic the ECM to a better extent, we must turn to synthetic biological material with defined constituents for the design of an ideal 3-dimensional scaffold.\textsuperscript{37} In this regard, self-assembling peptides have come to the forefront as intelligently designed amino acid sequences that are neither synthetic nor natural, but display characteristics of both. In this design, oligopeptides with ECM protein-mimicking sequences can be artificially synthesized and allowed to self-assemble under controlled conditions to form fibrous networks and ultimately hydrogels. For example, the Hartgerink lab has been able to create a hydrogel utilizing peptides that mimic the sequences observed in natural collagen, a main ECM protein.\textsuperscript{38}

The advantages of self-assembling peptide scaffolds range from ease of synthesis, rational design\textsuperscript{39} and variable viscoelastic properties\textsuperscript{40} to biocompatibility, biodegradability, injectability\textsuperscript{41-43}, control in release\textsuperscript{44, 45} and ability to incorporate bioactive motifs.\textsuperscript{46, 47} In literature we can find three main categories for self-assembling peptides: i) β-hairpins, ii) alternating hydrophilic and hydrophobic peptides and iii) multi-domain peptides (MDPs).
Mechanically rigid hydrogels have been formed in the Pochan lab with peptides that assemble into β-hairpin fibrils. The assembly occurs as a result of β-hairpin folding and β-strand swapping, which was inspired by classical domain swapping common to proteins (Figure 1.3). The β-hairpin design is flexible and can tolerate the addition of bioactive sequences. They have also obtained hydrogels that assemble in the presence of cells and undergo shear thinning, and gels that assemble in response to pH change or heat.

**Figure 1.3.** Strand-swapped β-hairpins can be thermally triggered to form nanofibers that can further self-assemble to hydrogels. Taken from Nagarkar et al. (2008).48

Peptide amphiphiles were first introduced as self-assembling units for nanofibers by the Stupp lab. They designed a structure consisting of a long alkyl tail that conveys hydrophobic character to the molecule and, when combined with the peptide region, makes the molecule amphiphilic. This structure allowed the formation of cylindrical nanofibers with the hydrophobic tails packed in the core and the hydrophilic head groups on the outside to direct mineralization. Along the same lines, the Collier and Zhang labs have developed self-assembling peptides that contain alternating hydrophilic and hydrophobic motifs capable of forming β-sheets which lead to hydrogels. Jung and colleagues have developed a β-sheet fibrillizing peptide Q11 (QQKFQFQFEQQ) which has been modified with cell attachment sequences while Kisiday et al. and Holmes et al. have reported the study of hydrogels made from
sequences KLD-12 (KLDLKLDLKLDL) and RAD16 (RADARADARADARADA) respectively that are also proven to support cell attachment and differentiation.\textsuperscript{56, 57}

1.2.1. Multidomain peptides

Multidomain peptides are a subset of self-assembling β-sheet peptides, first reported by Dong \textit{et al.} of the Hartgerink lab and described as having several distinct domains that lead to self-assembly.\textsuperscript{58} These peptides have an ABA block motif that consists of an amphiphilic core surrounded by charged flanking regions. The amphiphilic core is made up of alternating hydrophilic and hydrophobic amino acids, and in an aqueous environment, the hydrophobic side chains of two peptides can aggregate to form a sandwich-like structure (Figure 1.4). During self-assembly, these “sandwich” dimers are stacked beside each other and extended likewise to a fiber. It is apparent that the self-assembly of the peptide molecules into β-sheet nanofibers is driven by: i) the hydrophobic effect in the core areas and, ii) hydrogen bonding along the fiber axis.\textsuperscript{59} Thus, similar to other β-sheet nanofibers, the β-sheet conformation observed in this design is a result of the interactions in the central amphiphilic region. While nanofibers are being formed, the self-assembly process can be controlled due to a phenomenon known as ‘molecular frustration’. As fiber assembly continues, driven by hydrophobic interactions and hydrogen bonding, the charged termini of the peptides become accumulated on either side of the nanofibers. This gives rise to a repulsive force opposing assembly and leading the molecules to a ‘frustrated’ state. The balance of the said attractive forces and the opposing electrostatic repulsion can bring a stop to fiber growth, leaving the system in an equilibrium in which dimers are added to and removed from the fibers at the same rate.
The resultant nanofibers of finite length are allowed to further assemble to form a hydrogel by the addition of oppositely charged multivalent ions, for example phosphate ions. These ions can screen the electrostatic repulsions between the charged termini and also participate in electrostatic cross-linking between fibers, causing fibers to lengthen and entangle which ultimately leads to gelation (Figure 1.5).

MDP hydrogels developed by the Hartgerink lab demonstrate the ability to be manipulated to exhibit various properties according to the selection of amino acids in the peptide sequence. Previous studies have shown that the MDP design can bear the addition of bioactive sequences to the core region of the peptide as well as to the C-terminus. For instance, design of an MDP into a bioactive variant by incorporation of an enzyme cleavage site and a cell adhesion motif has been demonstrated. The resulting peptide, K(SL)_3RG(SL)_3KGRGDS, can be cleaved at the -LRG- site by matrix metalloproteinase-2 (MMP-2) and bound to integrins on cell surfaces at the -RGD- site. In vitro studies indicated that incorporation of the above modifications to the MDP...
structure results in increased cell viability and cell spreading, and encouraged cell migration into the hydrogel matrix. \(^{46}\)

**Figure 1.5.** The self-assembly process of MDPs. (A) The sandwich dimer with hydrophobic moieties in the core. (B) Nanofiber formation due to the hydrophobic effect and hydrogen bonding. (C) Vitreous ice cryo-TEM image of gelled K\(_2\)(SL)\(_6\)K\(_2\) nanofibers. (D) SEM image of the E\(_2\)(SL)\(_6\)E\(_2\)GRGDS hydrogel. (E) MDP hydrogel formed in a cell culture medium containing phosphate ions. Adapted from Aulisa *et al.* (2009) and Bakota *et al.* (2011). \(^{40,41}\)
1.2.2. Applications of MDPs

Over the past five years, the Hartgerink lab has investigated many different applications of MDPs, particularly in the biomedical arena. One of the earliest studies employing MDP hydrogels for therapeutic purposes showed that they can be used as a delivery agent for stem cell secretome.\textsuperscript{41} The MDP tested, E\textsubscript{2}(SL)\textsubscript{6}E\textsubscript{2}GRGDS, formed a physically cross-linked viscoelastic hydrogel that undergoes shear thinning and then quickly recovers its original elastic modulus when the shearing force is released, making it ideal for use as an injectable material.\textsuperscript{41} The gel showed great potential for absorption by acting essentially like a sponge in the presence of human embryonic stem cells (ESCs) and soaking up the multitude of growth factors and cytokines released by ESCs. In vitro experiments with glomerular epithelial cells demonstrated that release of stem cell secretome from these hydrogels significantly decreased protein permeability in a model of diabetes-induced kidney injury.\textsuperscript{41} This method of delivering stem cell secretome via MDP gels can be thought of as an acellular approach to stem cell therapy.

MDPs have shown promising utility in regenerative endodontics. MDP hydrogels have been combined with dental pulp-derived stem cells and growth factors (FGF-2, TGF-β1 and VEGF) to form a scaffold that can promote cell proliferation, differentiation and angiogenesis.\textsuperscript{47} The growth factors were incorporated into the gel via heparin binding. The scaffold was then added to dentin cylinders and subcutaneously transplanted in immunocompromised mice. Subsequently, vascularized soft connective tissue similar to dental pulp was observed within the cylinders.\textsuperscript{47} Thus, from a clinical point of view,

the MDP hydrogels are well suited for replacing necrotic pulp in root canal therapy. Furthermore, MDP gels have been shown to facilitate the expansion of stem cells from human exfoliated deciduous teeth (SHED).\textsuperscript{59} Specifically, serine-based MDP hydrogels seem to enhance the SHED cell spreading more than threonine-based gels.\textsuperscript{59}

One recently reported application of MDPs looks into controlling perioperative bleeding by using a MDP gel as a hemostat.\textsuperscript{60} A hydrogel assembled from the sequence K(SL)\textsubscript{3}RG(SL)\textsubscript{3}KGRGDS\textsuperscript{46} (termed SLac) was shown to act as a physical barrier to blood loss, on its own. In a separate experiment, SLac was loaded with snake-venom derived Batroxobin (50 μg/mL) yielding a drug-loaded hydrogel (SB50). SB50 was potentiated to enhance clotting even in the presence of heparin. Batroxobin is a snake venom-derived serine proteinase described nearly a century ago in 1936.\textsuperscript{61} Batroxobin present in venom is a potent heparin agnostic coagulant, able to cleave fibrinopeptide A at a non-heparin inhibited active site.\textsuperscript{62} Although a potent toxin found in snake venom to potentiate coagulation, batroxobin used clinically is recombinantly expressed in \textit{E. coli} or \textit{Pichia pastoris}, and purified to avoid toxicity concerns from contaminant snake venom.\textsuperscript{61, 63}

Batroxobin-loaded SB50 MDP hydrogels were shown to rapidly (within 20s) stop bleeding in both normal and heparin-treated rats in a lateral liver incision model (Figure 1.6). Compared to standard of care, Gelfoam, and investigational hemostats such as Puramatrix, only SB50 showed rapid liver incision hemostasis post-surgical application.\textsuperscript{60} The biochemical clotting potential of batroxobin together with the physical barrier provided by the spatially controlled MDP gel may present a novel tool in the arsenal for surgical hemostasis in patients on anticoagulant therapy.
Figure 1.6. Hemostasis achieved in a lateral liver incision model by application of a MDP gel loaded with batroxobin (SB50). Rapid hemostasis was achieved in rats within seconds. In a double bleed model, hemostasis was preserved after surgical manipulation when treated with SB50, with demonstration of a secondary rapid bleed site. Taken from Kumar et al. (2015). \(^6^0\)

Other significant applications of MDPs, including their ability to control the release of molecules and stimulate angiogenesis in vivo, will be discussed briefly in the following sections of this chapter, and in detail in the subsequent chapters of this thesis.

### 1.3. Controlled Release of Molecules

The regeneration of functional tissues in most cases employs the approach of combining three main elements: cells, biochemical/mechanical factors and scaffolds. Therefore the incorporation of bioactive factors such as growth factors (GFs) and cytokines as well as engineering their controlled release over time is critical for directing and sustaining growth, proliferation and correct differentiation of cells in the scaffold. Controlled release is often preferred over immediate release of bioactive factors due to the slow, steady output and subsequent availability of chemical over longer periods of time, which leads to prolonged host response. Thus it is also known to enhance safety, efficacy and reliability of drug therapy. \(^6^4\) Controlled release from hydrogel scaffolds and
other implanted material has been demonstrated in a variety of ways in the recent past. Heparin-binding has been used to tether GFs to the delivery scaffold due to the high affinity of heparin for many different growth factors.\textsuperscript{47, 65, 66} Biodegradable gel beads have been used to encapsulate various proteins and in some cases the beads were designed to be pH-responsive in order to control the release of material.\textsuperscript{64, 67} Furthermore, a whole host of degradable hydrogels and microspheres that demonstrate various levels of controlled release have been reported in literature.\textsuperscript{68-74} The use of liposomes for the same purpose will be discussed in more detail in the following sections.

Another interesting approach to control release comes in the form of drug eluting coatings. Macdonald \textit{et al.} have described Layer-by-Layer (LbL) polyelectrolyte films capable of microgram scale release of bone morphogenetic protein-2 (BMP-2).\textsuperscript{75} Ten micrograms of BMP-2 was released over a period of two weeks in vitro, and BMP-2 coatings implanted intramuscularly were shown to initiate host progenitor cells to differentiate into bone, which subsequently matured and expanded over 9 weeks.\textsuperscript{75}

The two main types of release from hydrogels, as found in literature, are diffusion-controlled release and degradation-controlled release.\textsuperscript{67} Diffusion-controlled release leads to rapid release of bioactive factors from the hydrogel, due to mesh size of gel (on the order of 100 nm) being typically larger than the hydrodynamic diameter of the protein (on the order of 1 nm). However, degradation-controlled release is relatively slower as the release of protein is dependent on the degradation of the carrier (e.g. liposomes) or the gel matrix.\textsuperscript{67}
1.3.1. **MDPs for controlled release**

As mentioned earlier, MDP hydrogels have been proven to be suitable candidates for serving as scaffolds in regenerative medicine.\(^\text{46}\) The first studies on release kinetics of an MDP hydrogel have been explored by Galler and colleagues.\(^\text{47}\) Heparin binding was utilized to attach growth factors to the peptide fibers of the gel matrix to achieve delayed release. Heparin is a polysulfated and heterogeneous GAG produced by mast cells to regulate thrombosis and blood vessel formation and regression.\(^\text{76}\) Heparin can bind with high affinity to many different GFs to form stable complexes and retard their release.\(^\text{65, 66}\) Galler *et al.* demonstrated that positively charged lysines in the MDP fibers bind the highly negatively charged heparin to induce gelation. The release profiles of VEGF, TGF-β1, and FGF-2 in heparin containing gels indicated a delayed release, which confirmed the ability of heparin-binding nanostructures to bind three different GFs and slow their release.

A more recent study conducted by Kumar *et al.* investigated the release of monocyte chemotactic protein-1 (MCP-1) and interleukin-4 (IL-4) from an MDP scaffold.\(^\text{45}\) Loading MCP-1 and IL-4 in the SLac gel matrix resulted in biphasic release profiles, independent of the loading concentrations. Subcutaneous implantation of the loaded MDP scaffolds led to a marked increase in macrophage infiltration and polarization dictated by MCP-1 loading as early as 3 days.\(^\text{45}\) MCP-1 is known to be important in the recruitment of naïve monocytes to the injury site, while IL-4 influences the conversion of pro-inflammatory macrophages to a pro-healing phenotype (M2) and aids in resolving the inflammation.\(^\text{77, 78}\) As expected, macrophage interaction and response to the loaded peptide gel in vivo facilitated the recruitment of
monocytes/macrophages and the promotion of a pro-resolution M2 environment (Figure 1.7). The first half of this thesis discusses the continuation of the above release studies using an alternative delivery method involving liposomes as carriers of GFs.

![Graphs showing MCP-1 and IL-4 release from MDP hydrogels. MCP-1 was loaded into hydrogels at (A) 100 ng and (B) 10 ng. Cumulative release curves indicate 80% of loaded cytokine is released over the first 48 h. IL-4 was loaded into hydrogels at a concentration of (C) 100 ng and (D) 10 ng. Cumulative release curves indicate that about 15-20% of loaded cytokine is released over the first 6 days, with up to 40% release after 16 days. Adapted from Kumar et al. (2015).](image)

**Figure 1.7.** MCP-1 and IL-4 release from MDP hydrogels. MCP-1 was loaded into hydrogels at (A) 100 ng and (B) 10 ng. Cumulative release curves indicate 80% of loaded cytokine is released over the first 48 h. IL-4 was loaded into hydrogels at a concentration of (C) 100 ng and (D) 10 ng. Cumulative release curves indicate that about 15-20% of loaded cytokine is released over the first 6 days, with up to 40% release after 16 days. Adapted from Kumar et al. (2015).

### 1.3.2. Liposomes as carriers

The success of liposomes as GF delivery agents has been demonstrated previously by numerous studies. Liposomes are vesicles that form spontaneously when phospholipids are dispersed in an aqueous environment. These vesicles contain an aqueous volume entirely enclosed by a bilayer membrane composed of lipid molecules.
Natural phospholipids, synthetic phospholipids and phospholipids modified from natural sources are commonly used in liposome preparation.\textsuperscript{86,87} Most liposomes prepared for in vivo applications contain cholesterol in their membranes as a fluidity buffer to restrict excessive motion of hydrocarbon chains, similar to natural cell membranes.\textsuperscript{79} Liposomes can be formulated and manipulated to differ in size, composition, charge and lamellarity, according to the desired application. For general delivery of hydrophilic drugs, small unilamellar vesicles (SUVs), normally having one lipid bilayer with sizes up to 100 nm, are frequently used (Figure 1.8).\textsuperscript{79} Various methods exist for the formulation of liposomes: film hydration method, ultrasonic method, reverse phase evaporation, etc. The film hydration method, originally described by Bangham \textit{et al.}, employs the simple technique of evaporating the organic solvent in a lipid mixture to form thin films which are then rehydrated in an aqueous medium to yield multilamellar vesicles.\textsuperscript{88} The size and lamellarity of these vesicles can be tuned subsequently using freeze-thaw extrusion.\textsuperscript{89} The work in this thesis utilizes the above method to synthesize SUVs for GF delivery, and it will be detailed in Chapter Two.

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{liposome_diagram.png}
\caption{A typical phospholipid, 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) can form lipid bilayers as well as liposomes, both unilamellar and multilamellar. Adapted from a webpage on bio-membrane flexibility.\textsuperscript{†}}
\end{figure}

\footnotesize{\textsuperscript{†} https://www.ncnr.nist.gov/AnnualReport/FY2003_html/RH7/}
Since material can be entrapped in both the aqueous compartment and within the membrane, liposomes have been extensively used for many years as vehicles to administer nutrients, drugs, proteins, etc. A review by Samad et al. highlights some of the common liposomal drug delivery systems including those for tumor therapy, respiratory drug delivery and nucleic acid therapy. In relation to GF release, studies have been done involving the liposomal capture and release of GFs such as vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), transforming growth factor alpha (TGF-α), nerve growth factor (NGF), and epidermal growth factor (EGF). Of note, Alemdaroğlu and colleagues have established that liposomal delivery of EGF to burn wounds accelerates the healing process relative to control groups without liposomes. Another study found that sterically stabilized liposomes can enhance the transport of NGF across the blood brain barrier and result in its distribution in the striatum, hippocampus and cortex of the brain.

1.3.3. Combining liposomes with MDPs

The controlled release system that is described in this thesis utilizes liposomal encapsulation of growth factors and cytokines followed by incorporation of these liposomes into a MDP hydrogel scaffold for delayed release. There are several mechanisms by which liposomes can release their contents inside the body: attachment and fusion of liposomal membranes to cell membranes, engulfment of liposomes by cells and/or collapse of liposomes due to instability. This effectively means that release of GFs will be degradation-based and hence delayed significantly, as cells will have to migrate into the hydrogel scaffold and gain access to the GFs by interacting with the liposomes.
Ullrich et al. have reported a macroscopic calcium alginate gel bead matrix with embedded liposomes, as a complex structure capable of immobilizing and subsequently releasing molecules such as enzymes.\textsuperscript{91} Similarly, Ruel-Gariepy and colleagues have published a study showing sustained release of doxorubicin from liposomes in a thermosensitive chitosan-based hydrogel. The composite system demonstrated a release profile of about 22\% of loaded drug over 9 days, with the initial burst release occurring in the liposomal formulation being mostly eliminated by the presence of the hydrogel.\textsuperscript{92} Following along the same lines, the work reported herein (Chapter Two) describes the design, synthesis, characterization and in vivo utility of a macrostructure of higher order than normal hydrogels. This structure comprises of GF-loaded liposomes and MDP nanofibers and displays both delayed and bimodal release.\textsuperscript{44}

1.4. Therapeutic Angiogenesis\textsuperscript{\textsection}

Many chronic diseases result from diminished blood flow to end organs. The subsequent starvation of tissue from oxygen and vital nutrients results in cell necrosis and functional decline. It is essential therefore, to restore oxygen balance to tissues when blood flow is reduced. Physiologically, a decrease in blood flow stimulates endogenous secretion of angiogenic mediators to promote new blood vessel growth. Therapeutic angiogenesis aims to stimulate development of new blood vessels to mitigate tissue necrosis during persistent hypoxic, low oxygen, conditions.

Although many are under investigation, there are no FDA-approved angiogenic therapies to date. Four main approaches to angiogenic therapies that have been attempted

\footnote{Majority of this section is based on the pending review article: Kumar, V. A.; Wickremasinghe, N. C.; Kanahara, S. M.; Merchant, O. F.; Hartgerink, J. D. Mater Today (Submitted 1/26/2016).}
are: i) growth factor delivery ii) gene delivery via viral or non-viral vectors, to produce proangiogenic cytokines, iii) stem cell delivery to ischemic areas to stimulate production of new vasculature, and iv) materials-based approaches. Over the past decade, a number of materials-based technologies have been developed to restore blood flow to ischemic areas. Materials-based approaches to revascularization include synthetic material stimulation of angiogenic responses, small molecule/chemokine/cytokine/growth factor delivery, biomimicry of signaling molecules, and synthesis of self-assembling peptides that potentiate angiogenesis.

Peripheral artery disease (PAD) is a condition in which atherosclerosis causes low blood flow to muscles, causing ischemic symptoms such as leg pain upon exertion. PAD affects over 8 million Americans, causing significant morbidity and mortality. The risks of PAD are highest among individuals with diabetes mellitus, smoking history, hypertension, and dyslipidemia, and with increasing age. Classically, PAD presents at first as a condition called intermittent claudication, where patients experience pain in the legs only upon exertion. Progression of atherosclerosis causes further narrowing in the vessels, ultimately leading to persistent pain in the limbs, necrosis of the muscles, development of ischemic ulcers causing a condition called critical limb ischemia, which may require surgical intervention such as bypass surgery and/or leg amputation. Surgical bypass of diseased vessels and endovascular interventions have successfully been used to re-perfuse limbs in many patients. However, extensive disease or comorbidities preclude 20-30% of these patients from these high-risk procedures. In these patients blood flow to peripheral vasculature and limbs is so restricted due to occlusive atherosclerotic plaques that muscle atrophy and necrosis results, which may necessitate surgical
amputation. Pharmacologic treatments such as statins, anti-platelet agents and vasodilators may be beneficial in early stages of the disease, but have had little effect, especially in advanced stages, leading to investigation for alternative strategies.\textsuperscript{96}

Gene therapy, stem cell therapies and pro-angiogenic materials have been pursued as treatment for PAD.\textsuperscript{96, 100} Notably, early clinical trials in proangiogenic genes and bone-marrow derived stem and progenitor cells were found to improve ankle brachial index (ABI), transcutaneous partial pressure of oxygen, as well as decreasing pain and the need for amputation in both animal and human models of PAD.\textsuperscript{99, 101-103} The need for a materials-based approach is underscored by the ability to rapidly and economically produce sufficient quality and quantity of the material, unlike revascularization therapies that use stem cells or gene vectors. Additionally, a materials-based approach circumvents the potential heterogeneity of autologous stem cells, immunogenicity of allo-/xeno-genic cell sources and potential off-target gene incorporation. However, despite ongoing research, there is no FDA approved revascularization on the market that has shown safety and efficacy in humans.

\textbf{1.4.1. Materials-based approaches that potentiate angiogenesis}

Many exciting developments have arisen over the past five years in materials development and clinical trials. These have broadened our understanding of the fundamental requirements for tissue regeneration. \textsuperscript{.} Current strategies focus on materials that aid in: i) delivery of bioactive cargo, and ii) biomimicry of signaling molecules. A variety of growth factors and cytokines play important roles in stimulating vascularization. VEGF, FGF, PDGF, PI GF, HGF, Angiopoietin-1, G-CSF and MCP-1 are
well known mediators of angiogenesis.\textsuperscript{95, 96, 104, 105} VEGF, the angiogenic master regulator, has been investigated for delivery employing alginate hydrogels\textsuperscript{106, 107}, microspheres\textsuperscript{107}, graphene oxide\textsuperscript{108}, nanoparticles\textsuperscript{109}, bolus intravenous and intramuscular injections\textsuperscript{110}, and single vectors\textsuperscript{111}. Combinatorial therapies have also been effective in promoting vascular growth. For example, local delivery of VEGF in combination with stromal cell-derived factor (SDF) from alginate hydrogels to ischemic hind limbs with systemic delivery of circulating angiogenic cells was shown to significantly improve functional recovery over cell treatment alone (Figure 1.9).\textsuperscript{106}

\textbf{Figure 1.9.} Delivery of growth factors VEGF and SDF and subsequent in vivo effects. Release curves indicate cumulative release of SDF and VEGF from injectable alginate hydrogels, given as mean ± standard deviation. Histograms show the resultant outgrowth endothelial cell (OEC) and circulating angiogenic cell (CAC) accumulation in non-ischemic C57BL6 mouse hind-limbs. The total number of OECs and CACs recovered from mouse hind-limb muscle surrounding the alginate gel injection site (test limb) or the muscle from the contralateral limb is indicated. Taken from Anderson \textit{et al.} (2015).\textsuperscript{106}
Similarly, a hybrid delivery system composed of alginate hydrogels and PLGA microspheres was created to co-deliver VEGF along with Angiopoietin-1, and resulted in significant enhancement of vessel enlargement at ischemic sites, compared to delivery of either factor alone. Likewise, a sequential delivery system of a fibrin hydrogel containing ionic-albumin microspheres allowed for the controlled release of FGF-2 and granulocyte-colony stimulating factor (G-CSF). In a murine critical limb ischemia model, FGF-2 release from a fibrin matrix with G-CSF release from microspheres promoted hind limb reperfusion, robust capillary development and mature vessel formation after 8 weeks compared to either growth factor alone or bolus administration of growth factor. These studies have suggested that a prolonged angiogenic response is critical to wound healing of ischemic tissue sites.

Overall, few of these therapies have translated to the clinic owing to a lack of persistence of growth factor signaling, diffusion from injection sites and development of nascent immature vessels. Optimizing several aspects of growth factor delivery for higher efficiency in therapeutic angiogenesis is essential. Spatial and temporal control of GF delivery, dosing, concentration, and half-life are vital factors that contribute to the development of stabilized, non-leaky vasculature.

Another approach to promote angiogenesis and treat ischemic disease derives from developing vascular-inductive tissue engineered matrices. Novel methods for more efficient and controlled release of bioactive factors from scaffolds involve covalent tethering or conjugating engineered variants. Covalent attachment of VEGF to PEG hydrogels has resulted in strong neovascularization within subcutaneous rat implants. Similarly, fibrin matrices have been employed for local release of modified versions of
VEGF. These studies capitalize on matrix binding and cell-mediated release (by local cell-associated enzymatic activity), and presentation of a new engineered variant of VEGF, $\alpha_2\Pi_{1-8}$-VEGF$_{121}$, that binds directly to a fibrin scaffold.$^{116}$ Matrix binding delays release of VEGF and protects it from being cleared prior to stimulation of an adequate angiogenic response. Through chick embryo chorioallantoic membrane (CAM) assays and studies in adult mice, potent formation of new vessels was observed, compared to diffuse leaky vasculature induced by passive diffusion of wild type VEGF$_{121}$.\textsuperscript{116}

In the recent years, several groups have attempted to incorporate angiogenic properties directly into scaffolding matrices. Base matrices have ranged widely including collagen mimetic peptides, polyethylene glycol scaffolds, and self-assembling peptides.$^{43, 93, 100, 117, 118}$ Of note is the modification of RADA-16, a self-assembling peptide that forms nanofibers and hydrogels through self-assembly triggered by exposure to physiologic media. RADA-16 has been used to deliver a variety of growth factors including EGF\textsuperscript{119}, PDGF-BB\textsuperscript{120} and SDF-1.$^{121}$ Recently, RADA-16 has been functionalized with a motif of known angiogenic potential, KLTWQELYQLKYKGI (termed QK), to yield a peptide with significant angiogenic activity in CAM assays.$^{118}$ The above motif is a VEGF-mimicking peptide derived from VEGF-165, which acts as a VEGF agonist to activate the VEGF-dependent signaling pathway.$^{100, 122-124}$ Angiogenic activity observed for these functionalized RADA-16 scaffolds was primarily demonstrated in vitro.$^{118}$ Building upon this, alternative self-assembling peptides have been used to aid in the development of angiogenic polymers that promote tissue revascularization.$^{53, 125}$
1.4.2. Controlling angiogenesis in vivo with MDP hydrogels

Kumar et al. in the Hartgerink group have recently developed a vascular-inductive bioengineered matrix based on an MDP hydrogel containing cell-mediated degradation and proangiogenic moieties. This hydrogel was designed to more closely mimic the natural extracellular matrix and was constructed using multidomain peptides conjugated to the VEGF-165 mimic QK (termed SLanc). The self-assembling matrix was tested in vivo and found to rapidly infiltrate and vascularize, yielding a mature vascular network within 7 days (Figure 1.10). The scaffold showed no signs of fibrous encapsulation and resorbed within 3 weeks in the subcutaneous space. Further work has demonstrated rapid recovery from induced hind limb ischemia in aged mice. The degree of tissue integration and functionality observed in this case can be related to the structural aspects of the gel and elucidates the success of bioactive materials that mimic the natural ECM.

Figure 1.10. SLanc peptide structure and in vivo response. (A) A self-assembling MDP is conjugated to a VEGF mimic at high epitope density to form SLanc, and the resulting nanofibrous gel can provide a scaffold for tissue ingrowth. (B) Robust vessels within subcutaneous implants in rats of self-assembling peptide SLanc, show development of 25-75 μm diameter vessels lined by CD31⁺ endothelial cells (green), nestin⁺ pericytes (purple) and α-SMA⁺ smooth muscle cells (red). Adapted from Kumar et al. (2015).
The studies described herein focus on another MDP scaffold aimed at achieving angiogenesis. In contrast to covalent immobilization above, in this work, a complementary approach to obtain in vivo angiogenesis was used: orthogonal assembly of MDP and liposomes, with a proangiogenic growth factor. Achieving similar levels of angiogenesis with this strategy demonstrates its facile nature for delivery of a variety of GFs with distinct effects. Placental growth factor-1 (PIGF-1) was loaded either in the MDP gel matrix or within liposomes bound inside the MDP gel. When released through liposomes incorporated in MDP gels, PIGF-1 was shown to temporally modulate human umbilical vein endothelial cell (HUVEC) VEGF receptor activation in vitro and robust vessel formation in vivo. These studies are discussed in detail in Chapter Three of this thesis.

1.4.3. Accelerating the wound healing process

The skin functions to provide a physical and chemical barrier that protects the host against invasion by toxins and microorganisms. It also prevents dehydration that can result from loss of barrier function. The loss of skin integrity and function due to wound injury has led to various attempts designed to better comprehend the molecular and cellular mechanisms that can facilitate and optimize wound repair. Wound healing is a dynamic, interactive and complex biological process. It requires the migration and proliferation of keratinocytes and a variety of other cell types that are temporally-regulated by numerous growth factors, as well as their receptors that are upregulated in the wound environment.
The process of cutaneous wound healing consists of four major stages: i) hemostasis, ii) inflammation, iii) proliferation, and iv) maturation. The very first stage of wound healing is hemostasis, during which platelets in the blood stick to the injured site and release chemical signals to promote clotting. This leads to activation of fibrin, which forms a mesh and binds platelets together. The so-formed clot plugs the break in the blood vessel, preventing further bleeding. Next, inflammation occurs, causing damaged and dead cells to be cleared out along with bacteria and debris, mostly by phagocytosis. At this point, GFs are released into the wound that cause the migration and division of cells during the proliferative phase. During proliferation (growth of new tissue), many processes such as angiogenesis, collagen deposition, granulation tissue formation, epithelialization, and wound contraction begin to occur. In angiogenesis, vascular endothelial cells form new blood vessels. In fibroplasia and granulation tissue formation, fibroblasts grow and form a new, provisional extracellular matrix by excreting collagen and fibronectin. Concurrently, re-epithelialization of the epidermis occurs, in which epithelial cells proliferate and 'crawl' atop the wound bed, providing cover for the new tissue. In wound contraction, myofibroblasts decrease the size of the wound by gripping the wound edges and contracting using a mechanism that resembles that in smooth muscle cells. The last stage in wound healing is involves maturation or remodeling. For example, collagen is realigned along tension lines and cells that are no longer needed are removed by programmed cell death, or apoptosis.

Each one of the above phases of wound healing involves a complex series of communications between cell surface receptors and their signaling molecules. As such, there is a massive number of GFs and cytokines that play a role in wound healing. Thus
many studies have reported the use of exogenous GFs to accelerate wound healing.\textsuperscript{82, 119} Schneider \textit{et al.} used a self-assembling peptide RADA-16 to deliver EGF (important in the re-epithelialization stage of wound healing) to an in vitro model of a wound. In this model, complex wound environment is recreated in human, bioengineered in vitro 3D tissues known as human skin equivalents (HSE), that have many morphologic and phenotypic properties of human skin.\textsuperscript{119} These studies show that RADA-16 scaffolds containing EGF accelerated the rate of wound coverage by 5 fold when compared to controls without scaffolds, and by 3.5 fold when compared to the scaffold without EGF (Figure 1.11). Similarly, another wound healing study employed keratinocyte growth factor (KGF) modified with a matrix binding peptide to control its delivery from a fibrin matrix.\textsuperscript{132} They demonstrated that in contrast to topical KGF, fibrin-bound KGF persisted in the wounds for several days and was released gradually, resulting in significantly enhanced wound closure.\textsuperscript{132}

\textbf{Figure 1.11.} Enhanced re-epithelialization by release of EGF from peptide scaffolds. (A, B, C) H&E staining of the wound after 48 hours. Control tissues where nothing is added on top of the wound (A), are compared to wounds covered with a drop of RADA-16 (B) and with a drop of peptide solution containing the growth factor EGF (C). The scale bar is 500 mm for wounds. For the inserts the scale bar is 100 mm. (D) Wound created in HSE model. (E) Peptide with EGF added to created wound. Adapted from Schneider \textit{et al.} (2008).\textsuperscript{119}
Jiang and colleagues have reported dual delivery of PDGF-BB (a growth factor that promotes wound healing and vascular stabilization) and chlorhexidine (antimicrobial agent for infection treatment) via PLGA microspheres to treat infected wounds. They showed decreased levels of infection and increased healing with the dual delivery system. Vascular analysis of wound tissues also showed higher levels of mature vasculature with the delivery of PDGF-BB.

1.4.4. Healing diabetic wounds with MDP hydrogels

Diabetic wounds are much harder to heal and take relatively longer healing periods than normal wounds. Specifically, diabetic wounds exhibit delayed wound closure, prolonged inflammation, poor angiogenesis, and less matrix deposition in wound bed as compared to normal wound healing. In most cases, diabetic foot ulcers do not heal at all and are one of the leading causes for amputation, affecting 15% of people with diabetes. The lack of healing in persons with diabetes stems from multiple mechanisms including neuropathy and impaired ability to fight infection. These patients are largely unable to mount an adequate inflammatory response, and consequently the diabetic foot ulcer becomes a portal for infection that can lead to sepsis and require limb amputation.

In efforts to overcome the delayed and impaired healing in diabetic conditions, scientists have conducted studies aimed at achieving faster rates of healing in diabetic animal models, particularly rats and mice. Additionally, since diabetic wounds heal much slower, the effects of exogenous drugs, GFs and cytokines can be teased out with relative ease in diabetic animal models. In one such study, Greenhalgh et al. have
demonstrated that diabetic mice treated with combinations of PDGF and FGF had significantly greater wound closure at 21 days than those treated with the vehicle. Along the same lines, the wound healing studies described in this thesis were also carried out in genetically diabetic C57BLKS/J-m/Lepr^db (db/db) mice that are known to display a marked delay in healing when compared to their nondiabetic littermates. In the present work, MDP hydrogels loaded with MCP-1 and IL-4 (which are both cytokines important in the inflammation stage of wound healing) were applied to full-thickness dermal wounds created on the backs of these mice. The details of the study are discussed in Chapter Four of this thesis.

1.5. Conclusions and Preview of Thesis

Hydrogels play a central role in tissue engineering, and as of late, self-assembling peptide hydrogels have come to the forefront of many regenerative therapies due to their ability to display a compelling combination of synthetic and natural properties. Among these, multidomain peptides have shown great promise with their biocompatibility, injectability, biodegradability and encouraging in vivo responses. This thesis explores the utility of MDPs in controlled release of bioactive factors, by reporting the design, synthesis, characterization, and in vitro and in vivo application of composite hydrogels made with MDPs.

Chapter Two of this thesis focuses on the development of a multicomponent hydrogel for release purposes. It begins by explaining the rationale for the chosen orthogonally self-assembled MDP-liposome composite gel and goes on to describe the synthesis process for each component of the gel and finally the composite system.
Characterization of the composite via microscopy techniques and rheology measurements follows. Finally, the release studies conducted with the composite gels are presented with the resultant release profiles and the establishment of a bimodal delivery system.

The third chapter goes on to describe the in vitro and in vivo testing of the above established release system. In vitro studies include qRT-PCR experiments conducted to analyze upregulated angiogenic receptors in HUVECS in response to treatment with the MDP-liposome composite hydrogels loaded with PlGF-1. In vivo studies in a rat subcutaneous implant model are then presented with positive immunohistochemical results showing rapid infiltration, vigorous neovascularization and successful neomatrix formation.

Finally, Chapter Four of this thesis demonstrates the application of MDP hydrogels in a wound healing study in diabetic mice. MDP hydrogels were loaded with cytokines i) MCP-1, a pro-inflammatory cytokine, and ii) IL-4, an anti-inflammatory cytokine to investigate the effects of these exogenous additions on the rate of diabetic wound healing in a full-thickness dermal wound model. This chapter goes on to present wound closure analyses at various time points up until the end of two weeks, and further analyses of wound tissue sections by H&E, Masson’s Trichrome and immunofluorescent staining. Results indicate that presentation of IL-4 in MDP hydrogels accelerates the healing process considerably, compared to the control cases, while MCP-1 addition does not impact the healing in a positive manner.
References


Chapter Two

Two-Step Self-Assembly of MDP-Liposome Composite Gels

2.1. Design of a Composite System

To achieve the high level of complexity and functionality seen in sophisticated biological systems, we have developed self-assembling systems that make use of multiple components capable of displaying orthogonal self-assembly.\textsuperscript{1} Here, two or more supramolecular assemblies form independently in a single system each with its own characteristics.\textsuperscript{1, 2} In this study, the ability of MDPs to self-assemble independently into a fibrous network in the presence of another supramolecular entity, liposomes, is demonstrated. The resultant composite hydrogel is shown to exhibit bimodal release characteristics when loaded with bioactive factors. This indicates the potential of a self-assembled hydrogel to display comparable functionality to the natural extracellular matrix (ECM) in terms of chemical communication by signaling molecules.

Hydrogel scaffolds provide structural integrity and potentially mimic the nanofibrous ECM while controlling drug and protein delivery to tissues.\textsuperscript{3} The extracellular milieu presents a chemically diverse environment that provides structural support and interacts with cells, allows oxygen, nutrient and small molecule exchange in the interstices and provides a template for wound healing. This exchange over diffusion gradients, and in some cases active transport against a gradient to build a potential can translate to regulated cell growth, proliferation, differentiation and targeted apoptosis in response to a variety of stimuli.\textsuperscript{4} Mimicry of structure and function of this complex

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environment has been a mainstay of tissue engineering attempts. To this end, the use of engineered biomaterials, such as hydrogels formed with self-assembling peptides and liposomal carriers, to interface with biological systems and affect controlled delivery of bioactive factors is of critical importance in tissue engineering and therapeutic applications.5

Hydrogel preparation employing self-assembly of peptides offers facile biomimicry.6,7 Short chain oligopeptides with ECM protein-mimicking sequences can be rapidly synthesized and allowed to self-assemble under controlled conditions to form fibrous networks, which can in turn entangle further to yield mechanically robust hydrogels.8-16 MDPs consist of polar terminal residues (lysine) with alternating hydrophilic (serine) and hydrophobic (leucine) residues, and self-assemble into nanofibers as described in Section 1.2.1.7, 17, 18 With the addition of multivalent ions in buffer systems such as PBS, long-range fiber growth and gel formation is facilitated.7 Physical and chemical crosslinks in MDP hydrogels are formed through non-covalent crosslinking.6 These bonds easily break and reform allowing the hydrogels to undergo shear thinning and recovery.19 Rationally designed sequences afford the ability to tailor biological activity. In this system, a cell adhesion (RGD) moiety derived from fibronectin, and a central MMP cleavage sequence (LRG) were added to allow biodegradation (Figure 2.1).17 We have demonstrated injectability20, biodegradability17 and biocompatibility21 of these hydrogel scaffolds. For desired cellular/ tissue outcomes that cannot be achieved by the peptide sequence alone, we have shown the ability to release growth factors from MDP matrices.21
Figure 2.1. Self-assembly of K(SL)₃RG(SL)₃KGRGDS into nanofibers. MDP scaffolds with the sequence K(SL)₃RG(SL)₃KGRGDS form facial amphiphiles that self-assemble into β-sheet forming fibers. With the introduction of multivalent salts, terminal charge repulsion is shielded allowing for long-range fiber growth.

In this chapter, we address the development of a system utilizing MDP hydrogels that can allow controlled release of desired growth factors and cytokines over multiple time scales. Preliminary studies on release kinetics of an MDP hydrogel have been explored prior.²¹ Heparin binding was used to enhance binding of growth factors to peptide fibers of the gel matrix and achieve delayed release; a strategy only amenable to proteins with heparin affinity. The work reported herein focuses on developing an alternative delivery method capitalizing on liposomes as carriers of GF molecules which could be expanded to a broad spectrum of proteins or small molecules.

The GFs and cytokines tested for release (EGF, PlGF-1 and MCP-1) were chosen based on the size and functionality. EGF (6.2 kDa) is a well-characterized growth factor involved in the growth, proliferation, adhesion and survival of various cell types, as well as tissue repair especially in the re-epithelialization stage of wound healing.²², ²³ PlGF-1 (29.7 kDa) is a key angiogenic and vasculogenic factor, particularly in embryogenesis, belonging to the vascular endothelial growth factor family.²⁴-²⁶ MCP-1 (8.6 kDa) is a highly produced chemokine in resident and inflammatory cells of a wound site and it acts in recruiting monocytes, macrophages and lymphocytes to sites of tissue damage.²⁷, ²⁸
Testing three different bioactive factors for release and obtaining similar release profiles for all three confirms the broad delivery applicability of the bimodal release system established herein.

We hypothesized a composite nanofiber-liposome hydrogel will offer a generalized delivery strategy for bimodal controlled release, largely independent of the entrapped material. The success of liposomes as GF delivery agents has been demonstrated previously by numerous studies. Furthermore, liberation of liposomal contents will only occur after fusion of liposomal membranes to cell membranes, engulfment of liposomes by cells and/or collapse of liposomes due to instability. As such, release of GFs will be degradation-based and thus delayed significantly as compared to release from the hydrogel alone.

This work demonstrates the ability of composite MDP hydrogels to achieve bimodal release of growth factors and cytokines. Specifically, we incorporate passive diffusion and liposomal delivery methods to achieve bimodal delivery of drugs (Figure 2.2). The MDP used, K(SL)$_3$RG(SL)$_3$KGRGDS, was coupled with a controlled release system utilizing liposomal encapsulation of three different GFs/cytokines labeled with a reporter molecule: EGF-FITC, MCP-1-CFDA, PlGF-1-TAMRA.† The resulting hybrid gels, consisting of two supramolecular assemblies, exhibit:

(i) composite macro-structural features
(ii) no significant change in mechanical properties
(iii) bimodal drug release

† Attempts in liposomal encapsulation and release of VEGF are discussed in Appendix I.
2.2. Synthesis and Characterization of MDP Fibers and Liposomes

Successful synthesis and purification of K(SL)$_3$RG(SL)$_3$KGRGDS was confirmed by conducting mass spectrometry on the lyophilized peptide. β-sheet formation by the MDP was evaluated by circular dichroism (CD). In the CD spectrum, a maximum is observed near 195 nm and a minimum near 216 nm, both of which correlate with β-sheet formation (Figure 2.3).$^6,7,18$ Previous studies have noted the ability for polyvalent anions to shield terminal lysine residues, overcoming molecular frustration.$^7$ These shielded charges allow for supramolecular assembly into large-scale micro-fibrils in water, sucrose, and other physiologically relevant buffer solutions, as we have previously demonstrated.$^6,17,21$ Further, MDP nanofiber formation has been demonstrated by negatively stained transmission electron microscopy (TEM) images of 1 % by weight peptide samples made in 298 mM sucrose (Figure 2.5a).
Figure 2.3. MS and CD spectra for the K(SL)$_3$RG(SL)$_3$KGRGDS peptide. a) MALDI-TOF mass spectrometry data. Expected mass: 2202.5 [M+H$^+$], observed mass: 2203.1 [M+H$^+$]. b) CD spectrum showing β-sheet characteristics.

Figure 2.4. Negatively-stained TEM image of a multilamellar liposome. The image came from a batch of liposomes that had not undergone extrusion to control their size, and hence showed the presence of multiple lipid bilayers. After extrusion through a polycarbonate membrane with 100 nm pores, a majority of the spheres become unilamellar with a diameter of approximately 100 nm.
Figure 2.5. Characterization of peptide fibers and liposomes. a) Negatively stained TEM of 1 wt% K(SL)$_3$RG(SL)$_3$KGRGDS peptide in 298 mM sucrose, b) cryo-TEM of GF-encapsulated liposomes (indicated by red arrow) and drying artifacts (indicated by *), dynamic light scattering showing: c) size plot of unloaded liposomes showing stability over 14 days, d) size plot of EGF-FITC, MCP-1-CFDA and PlGF-1-TAMRA loaded liposomes after purification.

Liposomes were prepared by the established techniques. Briefly, hydration of dry lipid films using phosphate-buffered saline as the hydration buffer was used. Liposome suspension was sized by extrusion through a 100nm pore-size filter in order to obtain a population of SUVs (Figure 2.4). Size distribution in the bulk SUV solution recorded by dynamic light scattering (DLS) showed a single population of spherical
particles with majority having a diameter of approximately 100nm (± 50 nm). Liposomes showed little change in size over a 14-day period, proving stability for at least up to 2 weeks at 37°C (Figure 2.5c). Appendix II discusses the synthesis, stability and release characteristics of large unilamellar vesicles (LUVs) relative to SUVs. Morphology of individual liposomes was observed via cryogenic TEM images (Figure 2.5b).

2.3. Synthesis and Characterization of MDP-Liposome Composite Hydrogel

MDP hydrogels containing liposomes were prepared by mixing a 2% by weight peptide solution with the liposome suspension in 1X PBS in a volume ratio of 1:1. The liposomes were prepared in a solution of PBS in order to trigger gelation of the MDP fibers to a hydrogel, once the liposomes are mixed with the peptide fibers. Presence of phosphate-buffered saline (PBS) in the liposome suspension causes elimination of electrostatic repulsion (occurring due to the lysine side chains at the termini of the MDPs) leading to physical crosslinking between fibers, which in turn allows for fiber lengthening, entanglement and ultimately gelation, yielding a mechanically robust hydrogel.7

The gels were imaged by negatively-stained TEM, cryo-TEM and scanning electron microscopy (SEM). The stained TEM images show roughly circular liposomes with a membrane structure visible, surrounded and held in place by fibers (Figure 2.6a-b). The cryo-TEM images also support the above representation, showing presence of clear circular structures among a network of seemingly amorphous peptide. (Figure 2.6d) The SEM images depict the presence of spherical particles with diameters in the range of 100-200 nm, identified as liposomes, lying in a matrix of a fibrous peptide network (Figure 2.6c).
To evaluate the rheological properties of the composite gel, the storage modulus ($G'$), loss modulus ($G''$) and shear recovery capability of the gel were characterized using oscillatory rheology. $G'$ was found to be ~ 990 Pa compared to a storage modulus of 1200 Pa recorded for the control hydrogel without liposomes. Shear recovery experiments demonstrated that when a shearing event is applied to breakdown the gel, its $G'$ returns to pre-shear values within one minute, indicating that the hydrogel is able to recover from shear stress (Figure 2.7).
This study focuses on creating a complex architecture, involving two independent supramolecular assemblies, that is capable of functionally and structurally mimicking the natural extracellular matrix to a significant extent. The composite is made from GF-loaded liposomes embedded in a hydrogel matrix made of self-assembling peptides. The assembly of peptides into a nanofibrous network was hypothesized to occur independently in the presence of liposomes, which by themselves are supramolecular structures. The present work elucidates the nature of such a multicomponent assembly and verifies the above hypothesis. Although there have been many studies done to elaborate, separately, the use of liposomes (in simulating biological membranes\textsuperscript{39, 40} material capture and release\textsuperscript{29-32}) and fibrous networks (in representing an ECM-like environment\textsuperscript{8, 15, 41}), so far only a few studies exist that combine both aspects of assembly\textsuperscript{1, 2}. From a structural point of view, this study was aimed at developing an architecture with an apparent higher degree of complexity, which in turn will be more
relevant biologically, as it may lead to achieving the functional complexity seen in natural systems i.e. the ECM, in terms of facilitating external communication via chemical signaling. The example particularly demonstrated is the design of a system for controlled release of bioactive factors from this architecture, which would have great potential in the field of tissue engineering and regenerative medicine.

The successful creation of a composite hydrogel using MDP fibers and labeled GF loaded liposomes, as determined by electron microscopy and rheology, has proven that orthogonal self-assembly of multiple components within a single system is a competent approach towards formation of novel and more complex architectures able to mimic naturally existing ones. Imaging by negatively-stained TEM, cryogenic-TEM and SEM revealed the nanostructural properties of the composite gel and validated the hypothesis that MDP fibers can self-assemble to a hydrogel even in the presence of liposomes and both systems can co-exist in a compatible manner. Oscillatory rheology experiments showed that the mechanical integrity and robustness of the hydrogel were not destroyed by the presence of liposomes, but in fact remained largely unaffected. Shear recovery experiments demonstrated that the composite gel is able to recover from shear stress, as has been demonstrated for MDP systems previously.\textsuperscript{17, 20} This result, combined with the high $G'$ of the gel and general hydrogel handling properties make the composite gels with liposomes suitable for injectable tissue engineering applications. Thus, the two self-assembling materials are able to co-exist in the presence of one another; but the assemblies are largely orthogonal as neither shape,size nor rheological properties are significantly altered.
2.4. Release of Fluorescently-labeled GFs from Liposomes in Composite Gels

During the purification of the EGF-FITC-loaded liposomes, the unencapsulated EGF-FITC was removed by passing the liposome suspension twice through a Sephadex G-50 column. Mass of the unencapsulated EGF-FITC fraction collected from the column was found by measuring fluorescence and relating it to the corresponding concentration of EGF-FITC using a standard curve (Figure 2.8). By comparing above mass with that of the original EGF-FITC quantity added during liposome preparation, efficiency of encapsulation was calculated as 67.1% ± 3.4. Similarly, efficiencies of encapsulation of MCP-1-CFDA and PlGF-1-TAMRA were determined as 79.8% ± 3.6 and 61.7% ± 2.8 respectively. DLS experiments indicated that size distribution of the GF-loaded liposomes, after extrusion and purification, was similar to the distribution observed for unloaded liposomes (Figure 2.5).

![Figure 2.8](image)

**Figure 2.8.** Sample EGF-FITC, MCP-1-CFDA and PlGF-1-TAMRA standard curves. Excitation and emission wavelengths (nm): i) FITC = 495/519, ii) CFDA = 492/517, iii) TAMRA = 549/577.
Composite gels consisting of labeled-GF-loaded liposomes were successfully formed in a transwell setup for release studies. The release profiles of EGF-FITC, MCP-1-CFDA and PlGF-1-TAMRA in above composite gels and control gels without liposomes are depicted in Figure 2.9 which presents the time course of the total cumulative release of each of the labeled GFs. Figure 2.9 illustrates the ability of liposomes to delay the release of GF in to the supernatant media by approximately five days, compared to the control gels in which GF molecules are incorporated directly in to the hydrogel matrix without a carrier.

As seen in Fig 2.9a, about 80% of the EGF-FITC was released by Day 7 from control gels, whereas in the composite gels, only about 15% was released at that time point and it took up to 18 days for 70% of the loaded EGF-FITC to be released. Figure 2.9b shows that MCP-1-CFDA molecules are rapidly released from the gel matrix so that close to 80% of the material is available in the supernatant media after 24 hours of seeding the gels. However, when MCP-1-CFDA is encapsulated in liposomes within the gel, it takes up to five days for the same amount of material to be discharged. In the case of PlGF-1-TAMRA, we observed that the 4-5 day delay in release, relating to composite gels with liposomes, was maintained up to two weeks (Figure 2.9c). Similarity of the results pertaining to release of three different growth factor/cytokine molecules from the composite gels suggests that this controlled release system can be used with a broad variety of different bioactive factors.
Figure 2.9. Release profiles for a) EGF-FITC b) MCP-1-CFDA and c) PlGF-1-TAMRA, showing release from gel matrix of control gels without liposomes (blue) and release from liposomes in composite gels (red), along with the respective curve fit. (n = 3 for each sample) R² values are given in Appendix III.

The release data were modelled using two well-known functions: the Korsmeyer-Peppas equation for burst release\textsuperscript{42, 43} (Equation 1) and the Weibull equation for delayed release (Equation 2).\textsuperscript{44, 45}

\[
\frac{M_t}{M_\infty} = k(t^n) \quad (1)
\]

Where \( M_t / M_\infty \) is the fraction of drug released at time \( t \), \( k \) is the rate constant and \( n \) is the release exponent.\textsuperscript{46}
Where \( \frac{M_t}{M_\infty} \) is the fraction of drug released at time \( t \), \( \alpha \) is the scale factor corresponding to the apparent rate constant and \( \beta \) is the shape factor. Using linear regression we were able to compare the differences in the nature of release between the three bioactive factors. The fit data for each case of release is given in Appendix III. It is likely that EGF and PlGF-1 show some interaction with the gel matrix as they are being diffused out. Even in the case of using liposomes to delay release, EGF and PlGF-1 show slower diffusion out of the gel matrix after being released from the liposome carriers, in comparison to MCP-1 which is released relatively rapidly with and without liposomes. The faster release of MCP-1, which gives rise to a very low value for the release exponent, \( n \), in the Korsmeyer-Peppas function and a relatively higher \( \beta \) value in the Weibull function, indicates that MCP-1 has minimal interaction with the gel matrix and demonstrates Fickian diffusion. In contrast, release of both EGF and PlGF-1 generate \( n \) values closer to 0.45, above which is the typical range for non-Fickian diffusion in the Korsmeyer-Peppas function.\(^{42, 46}\) This indicates that EGF and PlGF-1 release may be affected by interactions with the fibrous network or other factors such as polymer erosion.\(^{43}\)

Two of the above GFs were chosen to conduct a bimodal release experiment within a single system; EGF-FITC and PlGF-1-TAMRA were incorporated into the composite gel for simultaneous release. In the first system (Figure 2.10a), EGF-FITC was added to the gel matrix and PlGF-1-TAMRA to liposomes when constructing the

\[
\frac{M_t}{M_\infty} = 1 - e^{-\alpha(t^\beta)}
\]  
(2)
composite gel. The reverse of this set up was tested in the second system of bimodal release (Figure 2.10b). Bimodal release can be achieved with two different bioactive factors added during the orthogonal self-assembly process of the composite gel. The release kinetics of one GF does not seem to be affected by the presence of the other GF. Assessment of bimodal release profiles was carried out by modeling the data and comparing to single release studies (as shown in Figure 2.10). The modeling studies suggest that the two tested GFs do not show any significant interactions with each other, with a slight increase in the liposomal release of EGF-FITC at the latter 14 day time point.

Figure 2.10. Release profiles obtained from the bimodal release of EGF-FITC (released from gel matrix in (a) and liposomes in (b)) and PlGF-1-TAMRA (released from liposomes in (a) and gel matrix in (b)). Release data are co-plotted with the respective burst release or sigmoidal release models for both the single release and bimodal release cases. R² values are given in Appendix III (n=3 for each sample).
In summary, to investigate the release kinetics of physiologically relevant molecules from the composite hydrogel, we chose two different growth factors and one cytokine: EGF, PlGF-1 and MCP-1 respectively, as example molecules conjugated to a fluorophore (FITC, CFDA or TAMRA) for detection purposes.\textsuperscript{47, 48} The obtained release profiles of each of the labeled GFs from the composite gel demonstrate that encapsulating GFs in biocompatible carriers such as liposomes will significantly reduce the rate at which the molecules are released to the medium, thus establishing the role of liposomes as efficacious agents for controlled release of bioactive molecules. The more or less comparable nature of all the GF/cytokine release profiles suggests the applicability of this release system to a broad range of bioactive factors and possibly even small drug molecules.

Furthermore, release studies have shown that bioactive molecules can be delivered from the unique degradable composite hydrogel scaffold in two modes: (1) the early release mode, where incorporation directly in to the gel matrix allows delivery of molecules within the first 2-3 days, and (2) the late release mode, where encapsulation in liposomes allows slower, delayed delivery of molecules. This bimodal release system can be directed towards enhancing regenerative processes associated with, for example wound healing, which involves the release of a variety of different growth factors and cytokines such as EGF, VEGF, PlGF-1, FGF-2, MCP-1, etc., at different stages of the healing process.

\textbf{2.5. Conclusions}

While both liposome self-assembly and peptide nanofiber self-assembly are governed by the same types of non-covalent interactions such as hydrogen bonding,
electrostatic attraction and repulsion and the hydrophobic effect, we have shown that their assembly is orthogonal to one another. This allows the preparation of a composite hydrogel formed from the entanglement of peptide fibers and containing spherical liposomes in a simple two-step process. The result is a construct with a higher level of structural complexity (a fibrous mesh with embedded spheres) and functionality (multimodal delivery). This has the potential of harnessing the best aspects of both materials as the peptides can provide hydrogelation, presentation of biologically relevant signals such as the RGD adhesion sequence, as well as enzyme mediated degradation. Liposomes allow flexible loading and controlled release of proteins which may be expanded to other small molecule delivery. Together growth factor-loaded liposome hydrogel can be employed as a bimodal release system aimed at delivering bioactive factors in a temporally-controlled manner to enhance regenerative processes where proteins entrapped solely in the hydrogel are released quickly while those inside the liposomes are released more slowly.

2.6. Materials and Methods

2.6.1. Synthesis and characterization of MDP

The MDP used to create hydrogels has the sequence K(SL)₃RG(SL)₃KGRGDS, containing a MMP-2 sensitive cleavage site LRG and a cell adhesion site RGD. Peptide was synthesized on a low loading Rink Amide MBHA resin at a 0.15 mM scale using a Focus XC automated solid phase peptide synthesizer (Aapptec, Louisville, KY) by using an optimized protocol reported previously.⁶,⁷ Amino acids were added in a 4:1 excess to the synthesizing peptide. HATU and DiEA were used to couple amino acids to the peptide. Deprotection was achieved using 25% piperidine in a 1:1 DMF/DMSO solvent
mixture. The N-terminus was deprotected and acetylated. Peptide was cleaved from the resin using a cocktail of TFA, triisopropylsilane, water, ethanedithiol and anisole in a 36:1:1:1:1 ratio. Resulting peptide had neutralized termini due to the acetylated N-terminus and amidated C-terminus. Cleaved peptide was roto-evaporated to reduce overall volume and peptide precipitated in cold ether, concentrated and dried overnight. Dried peptide was dissolved in Milli-Q water to form a 1% or 2% by weight solution and pH adjusted to 7.4 with 0.1M NaOH. Solution was dialyzed (MWCO 500-1000 Da) for 3 days with buffer changes twice daily. Post-dialysis, the peptide solution was frozen and lyophilized.

Mass spectrometry and circular dichroism: Synthesis of the correct peptide was confirmed by matrix-assisted laser desorption/ionization time-of-flight (Bruker Daltonics, Billerica, MA) mass spectroscopy (MALDI-TOF). Secondary structure of the peptide was evaluated employing circular dichroism (CD). CD data was collected on a Jasco J-810 spectropolarimeter (Jasco, Tokyo, Japan). The peptide was dissolved in Milli-Q water to make a 0.01% by weight solution at neutral pH. Data was collected at room temperature from 180-250 nm using a 0.01 cm quartz cuvette. Molar residual ellipticity ([θ]) was calculated from millidegrees (θ) using path length (l) in cm, molecular weight (M) in grams per mole, peptide concentration (c) in mg/mL, and number of residues (nr).

\[
[\theta] = \frac{\theta \times M}{c \times l \times nr \times 10}
\]
**Negatively-stained TEM:** MDP nanofiber formation has been demonstrated by negatively stained TEM images of 1 % by weight peptide samples made in 298 mM sucrose. A 2% by weight solution of PTA was prepared at pH 7 and syringe filtered through a 0.2 μm filter before use as the negative stain. Two drops of bacitracin (0.1 mg/ml) was pipetted onto a Quantifoil R1.2/1.3 holey carbon mesh copper grid and allowed to sit for 3 minutes. Bacitracin was used as a wetting agent to increase spreading of sample on grid. Excess solution was wicked away with filter paper. 10 μl of peptide sample was pipetted on to grid and allowed to sit for 10 minutes. The excess was blotted away and finally a drop of PTA stain was added on to grid and allowed to sit for another 3 minutes. Excess stain was wicked away and grid was kept to dry overnight before TEM imaging. All imaging was performed using an 80.0 kV JEOL 1230 high contrast transmission electron microscope (JEOL USA Inc., Peabody, MA).

2.6.2. *Synthesis and characterization of liposomes*

Phospholipids and cholesterol were purchased from Avanti Polar Lipids, Inc. DPPC, DPPG and cholesterol were mixed in chloroform in the molar ratio 5:1:4 and solvent evaporated by passing a gentle stream of nitrogen. The dried lipids were left under high vacuum over night to allow complete evaporation of chloroform (Appendix II, App. Figure 4). Dry lipid films were hydrated with 1x PBS. The mixture was sonicated briefly and incubated for 1 hour with intermittent agitation. Then it was subjected to 5 freeze-thaw cycles (rapidly frozen in a dry ice-butanol bath and thawed in a water bath at 41ºC). The liposome suspension was extruded through a 100 nm polycarbonate membrane using a Mini-Extruder (Avanti Polar Lipids Inc., Alabaster, AL).
**Dynamic light scattering:** Liposomes were sized by dynamic light scattering experiments performed on a Malvern Zen 3600 Zetasizer (Malvern Instruments Ltd., Malvern, UK). The purified liposome suspension was added to a low volume disposable cuvette up to a maximum height of 10 mm and data was collected at room temperature. The refractive index of PBS was entered as 1.33, viscosity as 1.05 cP at room temperature and dielectric constant as 78.3. Absorbance of liposome suspension was measured and input as 0.1. Liposomes were incubated at 37°C for 14 days and sized again to evaluate their stability.

**Cryogenic TEM:** Vitreous ice TEM samples of liposomes were prepared for imaging. First, the TEM grids were ionized by glow discharging for one minute with a 5 mA discharge. The next stages of sample preparation were all performed using a Vitrobot type FP5350/60. The liposome suspension or nanofiber solution was added to the grid and immediately blotted for 2 seconds in a humidity-controlled chamber before being immersed in liquid ethane. The grid is then manually transferred from the liquid ethane to liquid nitrogen where it is stored until imaging. All TEM imaging was performed on a 200 kV JEM 2010 transmission electron microscope (JEOL USA Inc., Peabody, MA) and cryo-imaging was taken at a temperature of -176 °C using minimum dose system (MDS) mode.

### 2.6.3. Formation and characterization of composite hydrogel

The lyophilized peptides were dissolved at 20 mg/mL in Milli-Q water with 298 mM sucrose, and the pH was adjusted to 7.4. Composite gels were prepared by mixing 20 mg/mL peptide solution with the liposome suspension in a 1:1 ratio, while control gels
were made with 1xPBS only instead of with liposomes in PBS, for a final concentration of 1% by weight in both cases. The composite gel was imaged by negatively-stained TEM, cryogenic-TEM and SEM. All TEM samples of the composite gel were prepared as mentioned previously for the peptide fibers and liposomes.

**Scanning Electron Microscopy:** Gels were fixed in formalin overnight and dehydrated in a series of ethanol/water solutions progressing from 30% ethanol to 100% ethanol over the course of 24 hours. The dehydrated gels were critical point dried for 1 hour using an EMS 850 critical point drier (Electron Microscopy Sciences, Hatfield, PA). They were affixed to SEM stubs using conductive carbon tape. Samples were sputter coated with 8 nm gold using a Denton Desk V Sputter System (Denton Vacuum LLC USA, Moorestown, NJ) and imaged using a JEOL 6500F scanning electron microscope at 15.0 kV (JEOL USA Inc., Peabody, MA).

**Rheology:** The rheological properties of the MDP gel and composite gel were tested using oscillatory rheology. All rheological studies were performed on a TA Instruments AR-G2 rheometer (TA instruments, New Castle, DE). 100 μL of prepared hydrogel was deposited onto the rheometer stage. A 12 mm stainless steel parallel plate was used with a 500 μm gap height. Strain sweep experiments were performed at a frequency of 1 rad/s (which was determined to be in the linear viscoelastic region) from 0.001-100% strain. Shear recovery experiments were carried out by subjecting the gel to 0.5% strain for 10 minutes, increasing the strain to 100% for one minute, then reducing the strain back to 0.5% for 15 minutes.
2.6.4. Growth factor conjugation to fluorophore molecules

Epidermal growth factor conjugated to fluorescein isothiocyanate (EGF-FITC) was purchased from Life Technologies. MCP-1 and PlGF-1 were purchased from PeproTech Inc., (Rocky Hill, NJ) and conjugated to 5(6)-carboxyfluorescein diacetate succinimidyl ester (CFDA-SE) and 5(6)-carboxytetramethylrhodamine succinimidyl ester (TAMRA-SE) respectively using standard labeling protocols. 9 mM CFDA-SE in sterile anhydrous DMSO was mixed with 0.2 mM MCP-1 in PBS in a molar ratio of 20:1, while 9 mM TAMRA-SE in sterile anhydrous DMSO was mixed with 0.07 mM PlGF-1 in PBS in a molar ratio of 5:1. Both mixtures (total volume = 20 μL in each case) were incubated overnight in the dark at 4°C with continuous gentle agitation. The unconjugated dye was removed from the conjugated protein by using SpinOut™ GT-600 0.1mL columns (G-Biosciences, St. Louis, MO) and the conjugated protein was eluted out with PBS.

2.6.5. Loading liposomes with growth factors

Encapsulation of labeled GFs was carried out in situ during the hydration phase of liposome preparation. A solution of labeled GF in 1xPBS supplemented with 0.1% bovine serum albumin (BSA) was used to hydrate the dry lipid films. Incubation, freeze thaw cycles and extrusion of the liposomes with labeled GF were carried out as previously mentioned. After extrusion, the unencapsulated GF was removed by passing the liposome suspension through a Sephadex G-50 column (G-75 in the case of PlGF-1-TAMRA). The purified GF-loaded liposomes were sized by DLS and utilized to form composite hydrogels. Efficiency of encapsulating GF was determined by quantifying the

‡ Fluorophore conjugation and release profile for SDF-1α-CFDA is provided in Appendix IV.
amount of labeled GF removed via Sephadex column, \( m_1 \), compared to the concentration of GF in the original hydration solution, \( m_2 \):

\[
\left( \frac{m_2 - m_1}{m_2} \right) \times 100
\]

All experiments with labeled GF were done in the dark with containers covered in aluminum foil to protect the fluorescent molecules from light. Fluorescence was measured using a Tecan Infinite® M1000 plate reader (Tecan Systems Inc., San Jose, CA).

2.6.6. GF release from composite gel

![Figure 2.11. a) Schematic diagram and b) photograph of a transwell set up for EGF-FITC release studies.](image)

GF release from liposomes in the composite gel was assayed over time utilizing a transwell set up (Figure 2.11). Composite gels were made in triplicate and topped with supernatant media consisting of PBS supplemented with 0.1% BSA. The inner well of the transwell construct also contained supernatant media able to flow freely across an 8 \( \mu \)m pore size membrane in order to achieve a uniform concentration of media throughout
both wells. Gels were incubated at 37°C and 5% CO₂ for at least 14 days (18 days in the case of EGF-FITC). 100 µl of the release media was removed from the inner well and replenished with fresh media at a series of time points (days 1, 3, 7, 11, 14, 18). Amount of labeled GF released from the composite gel at each time point was quantified by measuring fluorescence and relating it to concentration of labeled GF through a standard curve. (A sample standard curve is given in Figure 2.8) Separate series of standards were prepared for each time point at the beginning of the release study and incubated along with the transwell constructs as internal references.
References


Chapter Three

Controlled Angiogenesis in MDP Nanofiber Composite Hydrogels

3.1. MDPs and Angiogenesis

Therapeutic angiogenesis is critical for the salvage of ischemic tissue. The lack of blood flow (ischemia) to tissue causes development of low oxygen environments (hypoxia). Ensuing from this is retention of proinflammatory cytokines, waste products, high CO₂ microenvironments, and consequent tissue necrosis. Similarly, to enhance tissue regeneration beyond the diffusion limit new blood vessel growth, neoangiogenesis, is required.

Current strategies to induce blood vessel growth include growth factor delivery, cell therapy, matrix interaction and metabolic control. GFs affecting angiogenesis such as VEGF, FGF, PlGF and PDGF have been delivered to ischemic tissue in a variety of methods to stimulate vascularization. Emerging therapies employ multiple GFs to control cellular infiltration and create niches for blood vessel formation. Transplantation of allo-/auto-logous bone-marrow stromal cells, mesenchymal stem cells and endothelial progenitor cells have been attempted with modest success; the need for multiple surgeries and proliferative potential of these cells are of concern. Cell therapy has been combined with bioactive materials designed to closely mimic the natural extracellular matrix. Administration of nitrite / nitric oxide to ischemic tissue increases expression of VEGF; concerns over systemic and local side effects and stability have limited this technique.

There are 3 critical features of angiogenesis that need to be addressed: i) retention of nascent vessels, ii) development of mature vessels, and iii) resorption of excessive or unnecessary vasculature. These factors promote an environment that supports growing vessels that are stabilized by pericytes, buttressed by smooth muscle cells, and resorb without creating chronic inflammatory sites.

MDPs offer a method to enhance therapeutic angiogenesis and can be used to address the above three features. Self-assembly by non-covalent interactions allows shear thinning and recovery, and consequent injectability. MDPs have also been shown to have antimicrobial behavior and can also be modified for a variety of biological activities. Specific to angiogenesis, we have developed a MDP covalently bound to a mimic of VEGF-165. This peptide, termed SLanc, results in rapid cellular infiltration and a high degree of angiogenesis, as mentioned in Section 1.4.2. In contrast to covalent immobilization described previously, this chapter investigates a complementary approach to obtain in vivo angiogenesis. As described in Chapter Two, we have formed a composite hydrogel via orthogonal assembly of MDP and liposomes which was loaded with a pro-angiogenic growth factor. We observed similar levels of angiogenesis with this strategy, as well as temporal control of GF delivery.

Two of our recent studies demonstrated the tailoring of immune responses in a temporal fashion as a function of GF/cytokine delivery. The MDP in this study (SLac) contains a cell adhesion fibronectin-derived (RGD) moiety promoting infiltration and a central MMP cleavage sequence (LRG) allowing biodegradation. We have previously shown that MDPs are rapidly infiltrated by cells as observed in subcutaneous injections in rats. After just 3 days, millimeter sized scaffolds showed unprecedented
infiltration into the center of scaffolds. We have demonstrated the release of cytokines to modulate inflammatory environments by activating prohealing M2 macrophages, specifically IL-4. The success of these cytokine/GF-loaded SLac hydrogels in tailoring inflammatory processes prompted the potential use of MDP scaffolds for additional in vivo responses i.e. angiogenesis. Tailoring of time-controlled release of GF and cytokine utilizing MDP scaffolds may allow more precise control of in vivo responses. As proof of principle, and of most relevance here, is the work described in Chapter Two, involving the two-step self-assembly of a composite hydrogel for temporal release of bioactive factors. This composite system consisted of MDPs and growth factors encapsulated by liposomes, and are termed here as MDP-Liposome Composites (MLCs). Time delay of cargo release caused by encapsulation in liposomes may allow dictation of a variety of stimuli in composite systems. The liposomes synthesized in the above study were 100nm (±50 nm) in size and displayed a PlGF-1 loading efficiency of approximately 60%. Orthogonal self-assembly of these loaded liposomes and MDPs into a higher order structure (Figure 3.1), have been shown to drive controlled release, while providing an ECM-mimetic environment. Release studies with MLCs involving bioactive factors MCP-1, EGF and PlGF-1, demonstrated bimodal release in vitro: burst release from MDP matrix alone and delayed release from MLCs (liposomes incorporated inside MDPs). Thus we were able to achieve temporal control in release, dependent on discharge by liposomes. We have demonstrated maintenance of bioactivity after release and stability of growth factors in MDP matrices. We believe MLCs are unique in that they provide a more generalized delivery strategy for bimodal controlled release, largely independent of the entrapped material. Additionally, MLCs
have proven to be more effective in delivery than liposomes alone due to their ability to localize the released load (targeted injection) and stabilize the surrounding environment via cellular infiltration.

**Figure 3.1.** Supramolecular orthogonal self-assembly. Schematic showing individual components that self-assemble to yield three different hydrogels: (1) MDP alone, (2) MDP(PIGF-1) and (3) MDP(Lipo(PIGF-1)).

In this study, we investigated three different hydrogel systems for their potential to serve as scaffolds for cellular infiltration and angiogenesis (Table 3.1).† Hydrogels were loaded with PIGF-1, a key angiogenic and vasculogenic factor, reported to stimulate angiogenesis by direct and indirect mechanisms via its ability to bind and activate

† In vitro and in vivo experiments involving release of SDF-1α from composite MDP(SDF-1α) gels are discussed in Appendix VI.
Although it has not been studied as extensively as VEGF, Luttun et al. and Autiero et al. have indicated the pro-angiogenic potential of PlGF-1 in ischemic heart and limb models with comparable efficacy to VEGF. The use of PlGF-1 here was prompted by studies that have demonstrated its potential in forming large mature, durable vessels (arteriogenesis) that persist for prolonged periods, while indicating no complications such as edema, hyperpermeability and hemangioma-ogenesis observed with VEGF.

Table 3.1. Summary of composite hydrogels and MDP-Liposome Composites (MLCs).

<table>
<thead>
<tr>
<th></th>
<th>MDP</th>
<th>MDP (PlGF-1)</th>
<th>MDP(Lipo(PlGF-1))</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Day 2</td>
<td>Day 5</td>
<td>Day 10</td>
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<tr>
<td></td>
<td>Nascent</td>
<td>Mature</td>
<td>Nascent</td>
</tr>
<tr>
<td></td>
<td>vessels</td>
<td>vessels</td>
<td>vessels</td>
</tr>
<tr>
<td>1</td>
<td>MDP</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>MDP (PlGF-1)</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>MDP(Lipo(PlGF-1))</td>
<td>0</td>
<td>0</td>
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* Numbers indicate degree of response: 1 = low vessel density, 2 = moderate vessel density, 3 = high vessel density.

Depending on the method in which PlGF-1 is incorporated, we expected marked differences in in vivo response. Specifically we hypothesized that controlled release can tailor cellular recruitment and neo-vascularization. PlGF-1 was incorporated into the hydro-gels in two ways: in the MDP matrix, MDP(PlGF-1), and inside liposomes of the MLCs, MDP(Lipo(PlGF-1)). This allowed evaluation of delayed release for formation and stabilization of blood vessels, in and around the implant site. We hypothesized that
initial cellular recruitment to the implant will form a niche for the subsequent infiltration and stabilization of blood vessels. Furthermore, we hypothesized that delayed release of PlGF-1 (after completion of cellular infiltration) would be superior to burst release from MDP alone.

The work presented herein shows that temporal control of PlGF-1 release leads to the development of robust, mature vasculature. Notably, no signs of fibrous encapsulation, hematomas or hemorrhaging were observed. The innovative materials described in this study may provide a powerful tool in the arsenal of therapies used in regenerative medicine; a tool that bridges nascent leaky vessels, immature and small capillaries, and ultimately demonstrates facile control of neoangiogenesis. More importantly, this represents an exciting approach wherein highly biocompatible materials tailor tissue responses through orthogonal self-assembly and biphasic release.

### 3.2. In vitro Experiments

In vitro angiogenic marker expression of HUVECs was quantified by RT-PCR in response to PlGF-1 release. Release media aliquots at Day 2, 5 and 10, resulted in upregulation of canonical angiogenic marker VEGFR-1 and VEGFR-2 expression. Receptor upregulation was normalized to ribosomal housekeeping gene L37a. Day 2 expression levels were not immediately upregulated to a significant extent (Figure 3.2). Peak expression is seen at Day 5 with a decrease by Day 10. This suggests that signaling by PlGF-1 is delayed past Day 2, due to liposomal release occurring around Day 3, affirming GF release previously reported. VEGFR-1 and VEGFR-2 up-regulation is critical for angiogenesis. These results suggest that in vivo angiogenesis can be tailored temporally by employing MLCs to delay angiogenic stimuli. Loading of PlGF-1
in the matrix resulted in more immediate receptor upregulation compared to delayed liposomal release in MDP(Lipo(PIGF-1)).

**Figure 3.2.** Angiogenic receptor activation as a function of temporal growth factor release. Quantitative RT-PCR showing expression levels of a) VEGFR-1 and b) VEGFR-2 in HUVECs at Day 2, Day 5 and Day 10 time points; fold expression over media control. HUVECs were treated with release aliquots from MLCs containing PIGF-1 encapsulated liposomes, to induce expression of angiogenic markers. Different Greek letters indicate statistically significant differences between each receptor.
3.3. Cellular Infiltration and New Matrix Formation

In vivo implantation of MLCs was performed under the dorsal subcutaneous aspect of Wistar rats (Figure 3.3). Composite gels (2) and (3) presented PlGF-1 in the matrix and PlGF-1 within liposomes respectively (Figure 3.1). Harvested tissue at Day 2, 5 and 10 was fixed and embedded. H&E- and immuno-staining was used to determine cellular infiltrate. Identification of the implant was facilitated by cellular density and hydrogel morphology (Figure 3.4). Representative images at Day 2 showed high levels of cellular infiltration into each of the implants, irrespective of GF presence (Figure 3.5). This is in congruence with previous studies of MDP.\textsuperscript{15, 25} Cellular density within implants was maintained at Day 5 and 10 (Figures 3.6-3.8). Cytotaxis is either through MMP mediated scaffold degradation, phagocytosis or physical motility through soft injectable gels.\textsuperscript{18, 19, 25} Cellular infiltration in unloaded gels demonstrates MDP potential for molecular reorganization and provision of a cytocompatible niche.

\textbf{Figure 3.3.} In vivo implant sites. a) Schematic of randomized rodent dorsal injection sites, and b) gross morphology of a highly vascularized explant at Day 5 - MDP(Lipo(PlGF-1)). The visible redness of the explant is due to presence of vasculature and should not be mistaken for an inflammatory response.
Figure 3.4. Location of implant. a) H&E image of MDP(PlGF-1) at Day 2 showing localization of implant within the fascial region, scale bar 0.5 mm, b) Immunostained image of MDP(PlGF-1) at Day 5, showing location of implant, scale bar 1 mm, c) High magnification H&E image of MDP(Lipo(PlGF-1)), at Day 5. Dashed line demarcates implant (Φ) from fascia (ψ), scale bar 500 μm.
Figure 3.5. Evaluation of cellular infiltrate. Top row: H&E images of subcutaneous implants in rats at Day 2 showing rapid of cellular infiltration. (Day 5 and 10 H&E images shown in Figure S3) Scale bar 500 μm. Bottom row: Immunostaining for monocytes/macrophages (CD68⁺; red) and nuclei (DAPI; blue) within the various implants at Day 2. High macrophage infiltration can be seen in all cases. Scale bar 200 μm.
Figure 3.6. H&E images of subcutaneous implants in rats at Day 5 (top row) and Day 10 (bottom row) showing continued high levels of cellular infiltration. Scale bar 500 μm.
Figure 3.7. Immunostaining for monocytes/macrophages (CD68⁺; red) and nuclei (DAPI; blue) within the various implants at Day 5 (top row) and Day 10 (bottom row). High macrophage infiltration is observed at Day 5 in all cases. Macrophages in the implants decrease in number by Day 10. Scale bar 200 µm.
Figure 3.8. Quantification of cellular infiltrate. a) Total cell count determined by counting DAPI stained nuclei and b) percentage of macrophages present in total cell count determined by counting CD68 positive cells. Different Greek letters indicate statistically significant differences.
Figure 3.9. Evaluation of new matrix formation. Masson’s trichrome staining of subcutaneous implants in rats showing deposition of new collagen matrix at Day 5 (top row). By Day 10 (bottom row), significant degradation of peptide matrix is observed, with new collagen matrix deposited where the implant material existed previously. Masson’s trichrome stain indicates muscle fibers in red, collagen in blue, cell cytoplasm in pink and cell nuclei in dark brown. Scale bar 500 μm.
Further, from H&E sections and Masson’s trichrome staining (Figure 3.9), it can be reasoned that high cellular infiltration seems to play a crucial role in preventing a thick fibrous encapsulation. It also indicates that the implant remains accessible to migrating cells and is not walled off from the rest of the tissue restricting direct contact with cells, as is usually the case when a thick fibrous capsule forms actively preventing cells from migrating into the implant. The seamless interface of native tissue and composite hydrogels demonstrate excellent tissue integration (Figures 3.4-3.7). Additionally, no gross signs of inflammation (redness, swelling, altered gait/function) were observed, indicating minimal rejection by the host. This suggests that the host animal responds to the present artificial matrix as if it were native extracellular matrix. There are very few materials that exhibit no fibrous encapsulation. As per our design strategy, early infiltrating cells may provide a suitable niche for the stabilization of nascent vasculature induced by delayed PlGF-1 release.

Characterizing the cellular infiltrate helped identify specific cell types that promote robust vascular formation. Immunofluorescent staining showed CD68+ macrophages in all scaffolds (Figures 3.5, 3.7). While CD68+ macrophages were present in all hydrogels at Day 2, a higher number of macrophages were observed in the composite gels loaded with PlGF-1, compared to MDP alone. By Day 5, macrophage counts for all scaffolds were similar, tapering by Day 10 (Figures 3.5, 3.7, 3.8). Resolution of the initial macrophage response potentially provides a niche for angiogenesis in MLCs, mimicking native tissue healing mechanisms of acute wounds. Collagen staining using Masson’s trichrome helped visualize the degradation of implant over time (Figure 3.9, 3.10). At Day 5, the edges of the implant show disintegration and
collagen deposition is found along the same edges as well as in the center of the implant, in some cases. Day 10 images indicate more collagen deposition with the size of the implant shrinking. It appears that a significant portion of the implant has degraded and it is replaced by new matrix, which can be distinguished from the native fascia by the blue color of the collagen staining.

**Figure 3.10.** Evaluation of new matrix formation – higher magnification images. a) Masson’s trichrome staining of subcutaneous implant MDP(PIGF-1) showing deposition of new collagen matrix at Day 10. Masson’s trichrome stain indicates collagen in blue, cell cytoplasm in pink and cell nuclei in dark brown. Significant degradation of peptide matrix is observed, and implant is replaced with collagen neomatrix (shown by arrows). Newly deposited matrix is easily distinguishable from native fascia. Scale bar 100 μm. b) Magnified image of area shown in inset. It is challenging to locate a clear demarcation of the implant and the neomatrix, suggesting a seamless interface between the two.
3.4. Development of Robust Vasculature

Two separate panels of markers were used to determine angiogenesis: 1) Panel A: endothelial marker (vWF\(^+\); red), smooth muscle marker (α-SMA\(^+\); green) counterstained with DAPI; 2) Panel B: endothelial marker (Lectin\(^+\); purple), pericyte marker (Nestin\(^+\); green), smooth muscle marker (α-SMA\(^+\); red) counterstained with DAPI. Angiogenesis showed dependence on PlGF-1 release (Figure 3.11). PlGF-1 releases rapidly from the matrix,\(^21\) resulting in nascent vessel-like structures defined only by a lining of endothelial cells.

![Graph showing vessel density](image)

**Figure 3.11.** Quantification of angiogenesis. Vessel density in the three implants was analyzed showing the significant effect of liposomal PlGF-1 release from MLC on angiogenesis. At Day 2, no vessel development is observed, while at Day 5, highest vessel density is seen in (3). At Day 10, vessel density stabilizes in (3) due to loss of immature vessels, while it finally starts to increase in (1). A moderate increase in vessel density is seen in (2) from Day 5 to Day 10. Different Greek letters indicate statistically significant differences.
Early release of PlGF-1 from the hydrogel matrix signals immature and low vessel development in the short term (Figures 3.12, 3.13). Incorporating PlGF-1 in liposomes delays its release up to 3 days, at which point liposomes lyse releasing their load. Thus, the onset of angiogenesis is triggered immediately after that in MLCs. By Day 5 many vessels in various stages of development are seen in MLCs (Figures 3.14-3.16). Day 5 implants of MLCs demonstrate robust angiogenesis in contrast to MDP(PlGF-1) gels releasing PlGF-1 directly from the matrix (Figure 3.15).

These MDP(Lipo(PlGF-1)) implants show the highest vessel density and the greatest number of vessels lined with pericytes and smooth muscle cells (Figure 3.11). Nascent vessels start to regress, leaving fewer but more mature vessels at Day 10. As a result, the total vessel density decreases at Day 10, although the presence of mature vessels is still significantly high (Figure 3.14). The fascia is not a region that is naturally vascularized. As the hydrogel degrades, we believe that excess vessels are also removed by resorption, so that the newly deposited matrix is made to closely mimic the native fascia which normally has a low vessel density. Encapsulation of PlGF-1 in liposomes with delayed release after macrophage recruitment shows the most robust vessel development within 5 days.

In summary, blood vessel development begins after 2 days, is most numerous at the 5-day time point; with retention of mostly robust mature vessels at the Day 10 time point (Figures 3.14, 3.15, 3.17).
Figure 3.12. Assessment of neo-angiogenesis in unloaded peptide control - MDP alone. No vessels were observed until Day 10 when several nascent vessels could be seen. Two panels of angiogenic markers were used for qualifying vessel development - Panel A: endothelial marker (vWF⁺; red), smooth muscle marker (α-SMA⁺; green) counterstained with DAPI; Panel B: endothelial marker (Lectin⁺; purple), pericyte marker (Nestin⁺; green), smooth muscle marker (α-SMA⁺; red) counterstained with DAPI. The bright red structures seen in Day 10-B are muscle cells (in the muscle layer above fascia) which stain very brightly for the α-SMA marker. Scale bar 200 μm.
Figure 3.13. Assessment of neo-angiogenesis - MDP(PIGF-1). Nascent vessels appear at the earliest time point, Day 2. More immature vessels can be seen at Day 5 and Day 10. Very few mature vessels observed. Two panels of angiogenic markers were used for qualifying vessel development - Panel A: endothelial marker (vWF⁺; red), smooth muscle marker (α-SMA⁺; green) counterstained with DAPI; Panel B: endothelial marker (Lectin⁺; purple), pericyte marker (Nestin⁺; green), smooth muscle marker (α-SMA⁺; red) counterstained with DAPI. The red structures seen in Day 10-B are muscle cells (in the muscle layer above fascia) which stain very brightly for the α-SMA marker. Scale bar 200 μm.
Figure 3.14. Assessment of neo-angiogenesis – MDP(Lipo(PIGF-1)). The degree of liposomal PIGF-1 dependent vessel development at each time point can be seen in these images. At Day 5, vigorous vessel formation is observed. By Day 10, mature vessels remain intact while the immature ones resorb, giving a stabilized vascular network. Two panels of angiogenic markers were used for qualifying vessel development - Panel A: endothelial marker (vWF⁺; red), smooth muscle marker (α-SMA⁺; green) counterstained with DAPI; Panel B: endothelial marker (Lectin⁺; purple), pericyte marker (Nestin⁺; green), smooth muscle marker (α-SMA⁺; red) counterstained with DAPI. Scale bar 200 μm.
Figure 3.15. Assessment of neo-angiogenesis – Day 5 time point. Two panels of angiogenic markers were used for qualifying vessel development - Panel A: endothelial marker (vWF⁺; red), smooth muscle marker (α-SMA⁺; green) counterstained with DAPI; Panel B: endothelial marker (Lectin⁺; purple), pericyte marker (Nestin⁺; green), smooth muscle marker (α-SMA⁺; red) counterstained with DAPI. Both panels show development of mature vasculature by Day 5 in the presence of PIGF-1. Scale bar 200 μm.
Figure 3.16. Component images of MDP(Lipo(PILGF-1)), at Day 5, immunostained to assess neoangiogenesis. Robust vessel development is observed. Two panels of angiogenic markers were used for qualifying vessel development: a) Panel A: endothelial marker (vWF⁺; red), smooth muscle marker (α-SMA⁺; green) counterstained with DAPI; b) Panel B: endothelial marker (Lectin⁺; purple), pericyte marker (Nestin⁺; green), smooth muscle marker (α-SMA⁺; red) counterstained with DAPI. Scale bar 100 μm.
Figure 3.17. Schematic of proposed in vivo mechanism. Orthogonally self-assembled scaffolds recruit macrophages and release growth factors. Macrophages recruited to the implant resolve over the implant period seguing the development of endothelial lined vessels (Day 2), with supporting pericytes (Day 5), and maturation into smooth muscle lined vessels (Day 5-10).

3.5. Comparison to Other Materials-based Angiogenesis Efforts

MLCs, loaded with growth factors were shown to elicit a controlled immune response and tailor angiogenic responses. Clinical outcomes for therapeutic revascularization have been met with modest success with current techniques. VEGF
delivery via plasmid vector or whole growth factor has resulted in leaky vasculature, potential neoplasticity, and inadequate clinical improvement. Stem cell therapy has been used to attempt neovascularization. Ongoing clinical trials have shown modest results, with some groups suggesting that cell death causes a proinflammatory response that stimulates tissue growth.

Use of growth factor release to modulate angiogenesis has been shown previously. The degree and maturity of the vessels that developed has been modest, compared to the neovasculature seen in this study. For example, VEGF delivery from PEG-based hydrogels have demonstrated high levels of VEGF release in the first 2 days with a gradual decrease over the next 2 weeks. The composites in this study have been shown to release PlGF-1 after 3 days, allowing cellular infiltration to occur prior to PlGF-1 release triggering angiogenesis. Alternative strategies have used engineered variants of VEGF conjugated to fibrin matrices for sustained release, resulting in non-leaky vasculature. This delivery strategy is encumbered by growth factor modification and optimization for matrix binding. Liposomal carriers offer delayed release of a variety of GF in a tailorable fashion without the need for chemical modification.

Self-assembling peptide hydrogels have been used previously for GF delivery. RADA16 has been utilized for delivery of EGF, PDGF-BB, SDF-1 and IGF-1. Although these approaches have been met with appreciable success, our system differs in its modular nature. The use of liposomal carriers allows for long term and delayed delivery of therapeutics. Further, initial in vivo studies have shown the rapid degradation
of the RADA16 within 3-7 days, compared to the persistence of MLCs for over 2 weeks.\textsuperscript{15, 25}

This delivery strategy can be improved with encapsulation of a variety of drugs, growth factors and cytokines in microspheres,\textsuperscript{64} micelles,\textsuperscript{65, 66} and nanoparticles,\textsuperscript{67, 68} and possibly covalent conjugation\textsuperscript{69} for release in the long term as well as multiple GF delivery.\textsuperscript{10, 70} Additionally we, and others, have demonstrated the ability to use heparin binding domains to attenuate release from MDP.\textsuperscript{19, 66, 71}

MLCs can serve as matrices for tissue regeneration to treat a milieu of pathologies potentially including peripheral vascular disease, diabetic ulcers, and tissue infarcts.\textsuperscript{43} Future in vivo experiments in disease models will better help guide the utility of MLCs in clinically relevant settings. We have demonstrated the ability to tailor and modulate inflammation to one of an M2 healing/ pro-angiogenic phenotype from MDP.\textsuperscript{25} We have also shown the ability to abrogate adverse tissue reactions by passive loading of MDP. Given the synergistic nature of MLCs and their ability to temporally control tissue responses, as demonstrated in this study, we envision a series of models in ischemic tissue disease that can benefit from these approaches.\textsuperscript{2, 6, 43}

3.6. Conclusions

In this chapter, we have presented a technique to fabricate MLCs that modulate angiogenesis. MLCs with PlGF-1 loaded in the liposome component showed temporal control of in vitro angiogenic receptor activation. When MLCs with PlGF-1 loaded in the liposomes were injected subcutaneously in rats, MLCs showed spatial and temporal control of angiogenesis. These hydrogels showed high levels of cellular infiltration, creating a suitable environment for large stable microvasculature promoted by PlGF-1
release. The biological response observed by time-controlled release from these supramolecular constructs is directly dependent on structural aspects of MLCs. These constructs were rationally designed, with the intent of developing a system that can mimic the level of complexity seen in sophisticated natural systems. MLCs were engineered with multiple components capable of displaying orthogonal self-assembly and the subsequent time controlled release.

3.7. Materials and Methods

3.7.1. Synthesis of SLac peptide

MDP with the sequence K(SL)$_3$RG(SL)$_3$KRGDS (SLac), was synthesized on a low loading Rink Amide MBHA resin at a 0.15 mM scale using a Focus XC automated solid phase peptide synthesizer (Aapptec, Louisville, KY) by using an optimized protocol reported previously.\textsuperscript{18} Peptide was cleaved from the resin using a mixture of TFA, triisopropylsilane, water, ethanedithiol and anisole in a 36:1:1:1:1 ratio. Resulting peptide had neutralized termini due to the acetylated N-terminus and amidated C-terminus. Cleaved peptide was roto-evaporated, precipitated in cold ether and dried overnight. Crude peptide was dissolved in Milli-Q water to form a 1% by weight solution and pH adjusted to 7.4 prior to dialysis (MWCO 500-1000 Da) for 3 days with buffer changes twice daily. Post-dialysis, the peptide solution was frozen and lyophilized. Synthesis of the correct peptide was confirmed by matrix-assisted laser desorption/ionization time-of-flight (Bruker Daltonics, Billerica, MA) mass spectroscopy (MALDI-TOF).

3.7.2. Synthesis of liposomes and encapsulation of PlGF-1

Phospholipids and cholesterol were purchased from Avanti Polar Lipids, Inc. DPPC, DPPG and cholesterol were mixed in chloroform in the molar ratio 5:1:4 and
solvent evaporated by passing nitrogen. The dried lipids were left under high vacuum over night to allow complete evaporation of chloroform. Encapsulation of PlGF-1 was carried out in situ during the hydration phase of liposome preparation. Dry lipid films were hydrated with 1 μg/mL solution of human recombinant PlGF-1 (PeproTech, Rocky Hill, NJ) in carrier-free HBSS (Life Technologies, Carlsbad, CA). The mixture was sonicated briefly and incubated for 1 hour with intermittent agitation. Then it was subjected to 5 freeze-thaw cycles and extruded through a 100 nm polycarbonate membrane using a Mini-Extruder (Avanti Polar Lipids Inc., Alabaster, AL). Afterwards, the unencapsulated PlGF-1 was removed by passing the liposome suspension through a Sephadex G-75 column. The efficiency of encapsulation of PlGF-1 has been calculated as 62% in previous study (Chapter Two). Liposomes were sized by dynamic light scattering experiments performed on a Malvern Zen 3600 Zetasizer (Malvern Instruments Ltd., Malvern, UK).

3.7.3. In vitro PlGF-1 release from MDP-liposome composite gel

Lyophilized MDP was dissolved at 20 mg/mL in Milli-Q water with 298 mM sucrose, and the pH was adjusted to 7.4. The MDP-liposome composite gel was made by mixing 100 μL of purified PlGF-1-entrapped liposomes with 100 μL of 20 mg/mL SLac peptide. According to the encapsulation efficiency, approximately 60 ng of PlGF-1 was loaded in each gel. Fifteen such MDP-liposome gels were made (5 gels per time point) in a 24-well plate. Each gel was topped with 1.5 mL of supernatant media (Medium 200 with 1% FBS from Life Technologies) and incubated at 37°C and 5% CO₂ for 10 days. At time points Day 2, Day 5 and Day 10, 1 mL of the release media was removed and frozen at -80°C until HUVECs were ready for treatment.
3.7.4. Assessment of angiogenic receptor activation by PI GF-1 release

HUVECs (Life Technologies) were seeded in 6-well plates at 2 x 10^5 cells per well and cultured in full media (Medium 200 with 10% LVES from Life Technologies) overnight. Cells were then starved for 24 hours in low serum media (Medium 200 with 0.5% LVES). PI GF-1 release aliquots obtained at each time point in release study were added to cells and kept for another 24 hours. Two controls were tested: 1) negative control with only low serum media, and 2) positive control with 1 mL of a 100 ng/mL solution of PI GF-1. PCR was used to characterize HUVEC phenotypic expression, n=5 for 3 independent repeats. RNA extraction was performed according to manufacturer’s protocol (RNeasy, Qiagen, Gaithersburg, MD). RNA concentrations were determined using Nanodrop (ThermoFisher Scientific, Waltham, MA), and reverse transcription to cDNA was carried out using iScript (Qiagen), followed by RT-PCR using a Biorad CFX96 Real Time PCR machine (Biorad, Berkeley, CA) and SsoAdvanced SYBR-green KIT (Qiagen). PCR primers were purchased from Life Technologies. Primers used: Vascular endothelial growth factor receptor 1 – VEGFR-1 forward: 5-TCCCTTATGATGCCAGCAAGT-3, VEGFR-1 reverse: 5-CCAAAAGGCCCTCTTCCAA-3; Vascular endothelial growth factor receptor 2 – VEGFR-2 forward: 5-CACCACTCAACCGCTGACATGTA-3, VEGFR-2 reverse: 5-GCTCGTTGGCGCACTCTT-3; Housekeeping ribosomal 60s subunit L37-a forward primer: ATTGAATCAGCCAGCAGCG, L37a reverse primer: AGGAACCACAGTGCCAGATCC. CT values generated by the software were compared to L37a expression. Expression of gene of interest was normalized to control expression (media control) noted in each experiment.
3.7.5. In vivo subcutaneous implants in rats

All experiments were approved by the Rice University Institutional Animal Care and Use committee. Female Wistar rats (225-250 g, Charles River Labs, Wilmington, MA) were anesthetized using isoflurane (2% for induction and 1% for maintenance) and dorsal aspects shaved under sterile conditions. Three different hydrogels were made (n=4 for each gel) and loaded in syringes with 22 gauge needles. The gels were prepared as follows:

(1) MDP alone (SLac) - 20 mg/mL SLac mixed with HBSS in 1:1 ratio
(2) MDP(PlGF-1) - 20 mg/mL SLac mixed with 1 μg/mL of recombinant rat PlGF-1 (PeproTech) made in HBSS, in 1:1 ratio.
(3) MDP(Lipo(PlGF-1)) - 20 mg/mL SLac mixed with rat PlGF-1-encapsulated liposomes (containing approximately 60ng PlGF-1) made in HBSS, in 1:1 ratio.

200 μL subcutaneous injections were injected in four separate 1.5 inch spaced randomized sites on the dorsal aspect, on either side between the lower thoracic and upper lumber vertebrae. At tested time points Day 2, Day 5 and Day 10, rats were euthanized using an overdose of isoflurane, CO₂ asphyxiation, and bilateral thoracic puncture. The dorsal skin around the entire implant was removed, washed with PBS, and fixed in neutral buffered formalin for 24 hours prior to processing.

3.7.6. Evaluation of cellular infiltrate, neomatrix development and angiogenesis

Tissue was processed to paraffin blocks, sectioned at 7 μm, deparaffinized and stained for cellular infiltrate using H&E. Masson’s trichrome staining was carried out according to manufacturer’s instructions (Sigma-Aldrich, St. Louis, MO) to visualize collagen deposition. Cellular infiltrate was determined using immunostaining for
macrophages: rabbit anti-rat CD68\(^+\) (Abcam, Cambridge, UK); and nucleic DAPI counterstain. Angiogenic evaluation was performed by immunostaining for endothelial cells: rabbit anti-rat vWF\(^+\) (ABCam), biotin anti-rat Lectin\(^+\) (Santa Cruz Biotechnology, Dallas, TX); smooth muscle cells: rabbit anti-rat α-SMA\(^+\) (Dako, Carpenteria, CCA), mouse anti-rat α-SMA\(^+\) (Abcam); pericytes: mouse anti-rat Nestin\(^+\) (Millipore, Darmstadt, Germany) and nucleic DAPI counterstain. Secondary antibodies used were donkey anti-rabbit AF 647, goat anti-mouse FITC, Streptavidin AF 647 (Life Technologies), donkey anti-rabbit AF 568. Immunofluorescent sections were imaged using a Nikon A1 confocal microscope (Nikon, Tokyo, Japan). Cellular infiltrate was quantified by counting DAPI stained nuclei and cells stained as macrophages, using ImageJ software. Angiogenesis was quantified by determining vessel density (vessels per unit area, n=4 separate sections, n=4 samples).

3.7.7. **Statistical analysis**

Data is represented as mean ± S.D. One way ANOVA was conducted for multiple comparisons of parametric data, with Tukey post-hoc analysis for all pairwise comparisons of the mean responses to the different treatment groups. Values of \( p < 0.05 \) were considered statistically significant.
References


Chapter Four
Accelerating Diabetic Wound Healing with MDP Gels

4.1. Interleukin-4 Loaded MDP Gels for Inflammation Resolution

Effective wound healing leads to restoration of tissue integrity via a multistep process that involves numerous cell types.\(^1\) As mentioned in Section 1.4.3, the multiple stages of wound healing are: i) hemostasis, ii) inflammation, iii) proliferation, and iv) maturation/remodeling.\(^2\)\(^3\) The process of wound repair requires a complex and highly organized interplay of various resident and recruited cell types, and growth factors/cytokines to accomplish the above stages of healing.

The inflammatory phase begins with the local activation of an innate immune response which leads to an initial influx of neutrophils followed by macrophages.\(^4\) These cells are attracted to the wound site by a large variety of chemotactic signals. Neutrophils arrive at the wound site within minutes of injury and play a major role in removing contaminants: bacteria, foreign bodies and debris.\(^5\) Within 48 hours, circulating monocytes extravasate over a chemical gradient towards the wound site and phenotypically convert to tissue macrophages.\(^6\) They continue the debridement by phagocytosis, while also secreting a host of cytokines and GFs, including MCP-1.\(^7\) These cytokines in turn stimulate the recruitment of more blood-borne monocytes causing macrophage accumulation at the wound site. Macrophages are arguably the most important cells present in the later stages of the inflammatory phase and appear to act as the key regulatory cells for tissue repair.\(^8\) They function as phagocytic cells, as well as primary producers of GFs responsible for the proliferation of fibroblasts (which reproduce the ECM), smooth muscle cells and endothelial cells (which enhance
angiogenesis). It has been shown that healing is severely impaired, when macrophage infiltration is prevented.

Once at the wound site, many phenotypic differences are shown by recruited macrophages, separating them from the resident tissue macrophages. The onset of these phenotypic changes is termed ‘macrophage activation’. Broadly, macrophages can be classified by two phenotypes: M1 (pro-inflammatory) and M2 (anti-inflammatory) macrophages. They are considered to be the primary lineages with respect to the innate inflammatory response. In normal tissue, the ratio of M1:M2 is highly regulated, but during inflammation this ratio changes. M1 macrophages are classically activated in response to LPS or IFN-γ, exhibit high levels of inflammatory cytokines like TNF-α and MCP-1, and produce nitric oxide (NO) in high concentration. MCP-1 (or CCL2) is one of the key chemokines expressed during this stage for the recruitment of monocytes that can convert to the M1 phenotype. NO aids in the inflammatory response as it is a potent vasodilator. M1 macrophages work to kill pro-inflammatory stimuli, by production of reactive oxygen/nitrogen species (ROS/RNS) and phagocytosis. In contrast, M2 macrophages produce high levels of TGF-β, IL-10 and arginase-I, and low concentrations of NO (due to the formation of ornithine), which lower the pro-inflammatory response and cause cell healing and proliferation. In short, macrophages can be loosely differentiated as ‘killer M1’ and ‘healing M2’.

Activation of the pro-healing M2 macrophages is important in the resolution of inflammation and effective healing of the wound. M2 macrophages can be subdivided as M2a, M2b and M2c. The M2a type is induced by cytokines IL-4 and IL-13 during the early inflammatory phase, whereas M2b and M2c appear later on (Figure 4.1). The
various stimuli for the M2 macrophage subtypes, their functions and the markers they express are detailed in Table 4.1.

**Figure 4.1.** The discrete phases of phenotypically and functionally distinct macrophage subsets that exist during the process of normal skin/muscle wound healing. Taken from Gensel et al. (2015).\(^{18}\)

**Table 4.1.** Properties of wound healing macrophages. Adapted from Gensel et al. (2015).\(^{18}\)
IL-4 is a pleiotropic cytokine known for: i) promoting alternative activation of macrophages into the M2 phenotype while inhibiting classical activation to M1,\textsuperscript{13, 17-19} and ii) stimulating the accumulation of extracellular matrix molecules during wound repair.\textsuperscript{20-23} In this study, we investigated the effect of releasing IL-4 from MDP hydrogels into full thickness dermal wounds in genetically diabetic mice. We hypothesized that controlled release of the cytokine IL-4 from a biocompatible MDP scaffold such as SLac would positively impact the resolution of inflammation, increase ECM formation and accelerate the wound healing process in diabetic wounds that normally exhibit delayed wound closure, prolonged inflammation and generally poor healing.\textsuperscript{24, 25} Additionally, we explored the effect of releasing chemokine MCP-1, on the healing of similar dermal wounds in diabetic mice. Specifically, genetically diabetic C57BLKS/J-m/Lepr\textsuperscript{db} (db/db) mice were used for this study.\textsuperscript{26, 27} The db/db mouse exhibits clinically relevant characteristics (such as obesity, insulin resistance, severe hyperglycemia) of human adult onset diabetes with a concomitant delay in wound healing.\textsuperscript{27, 28} Complications seen in people with diabetes, such as peripheral neuropathy, microvascular lesions, basement membrane thickening, glomerular filtration abnormalities, and immunodeficiency, have also been observed in the db/db mouse.\textsuperscript{29} Because of these similar characteristics the db/db mouse model has been extensively used for a wide variety of dermal repair investigations.\textsuperscript{27, 30, 31}

One of our recent studies demonstrated the ability of a nanofibrous MDP hydrogel matrix to sequester two cytokines MCP-1 and IL-4, and release them in a biphasic pattern.\textsuperscript{13} Furthermore, in vivo subcutaneous implants of the loaded MDP gels showed a marked increase in macrophage infiltration (dependent on MCP-1 delivery) and
polarization of macrophages to the M2 phenotype (dependent on IL-4 delivery) as early as 3 days. It was observed that the MDP used, SLac, promoted host cell infiltration of the engineered scaffolds without the formation of a fibrous capsule. This could be attributed to the RGD cell-adhesion moiety and MMP cleavage site of the MDP that allows the hydrogel to display enhanced biocompatibility. Moreover, the release profiles of IL-4 from the gel indicate sustained discharge over a prolonged time period (1 week), which can be envisioned to facilitate a long term M2 micro-environment. In light of these results, we expected the SLac hydrogel to perform well as a scaffold for the delivery of IL-4 in the current dermal wound healing study. The work presented herein shows that sustained delivery of a pro-healing cytokine IL-4 via a MDP scaffold accelerates healing of full thickness dermal wounds in diabetic mice compared to the MDP scaffold alone, or IL-4 delivered in a buffer solution without a scaffold. These findings provide evidence for the use of MDP gels as successful matrices for the delivery of bioactive factors and drugs relevant to wound healing.

4.2. Wound Closure in Diabetic Mice

In preliminary wound healing studies, full-thickness wounds (extending beyond the panniculus carnosus, i.e. the subcutaneous muscle layer of murine skin) were generated by 8-mm diameter biopsy punches on the depilated backs of Lepr db/db mice (4 wounds per mouse on 4 mice) and filled with loaded MDP gels containing either MCP-1 or IL-4. Wounds were imaged and wound closure evaluated by measuring wound area at time points day 3, 7 and 14. Macroscopic wound closure was accelerated in wounds receiving IL-4/SLac treatment compared to wounds treated with MCP-1/SLac or SLac gel alone (Figure 4.2). At the last time point, day 14 after wounding, the IL-4/SLac
treated wounds had already lost their eschar and appeared completely epithelialized, whereas the MCP-1/SLac treated wounds were still carrying a scab (Figure 4.2). Measurement of the wound area on digital images showed that the differences between IL-4/SLac treated wounds and MCP-1/SLac treated wounds were statistically significant 2 weeks after injury (p ≤ 0.001) (Figure 4.3a). On day 3 and 7, wounds treated with 20 ng IL-4 in SLac showed significantly higher closure than wounds treated with 20 ng or 100 ng MCP-1 in SLac (p < 0.05). In summary, wounds treated with 20 ng IL-4 in SLac demonstrated accelerated wound healing by day 14, when compared to 20 ng MCP-1 in SLac or SLac alone.

**Figure 4.2.** Macroscopic appearance of wounds on diabetic mice at indicated time points after injury. While MCP-1/SLac treated wounds still carry a scab, IL-4/SLac treated wounds have lost the scab 14 days after wounding and closed completely.
Figure 4.3. Evaluation of wound closure in IL-4/SLac and MCP-1/SLac treated wounds.
a) Wound area was determined using image analysis and expressed at each time point as a percentage of the initial wound area (immediately after injury). Data are expressed as mean ± SD, n=2 wounds for each time point and sample type (p < 0.05). b) H&E staining of wounds at 14 days post injury. In 20 ng MCP-1/SLac treated wounds, epithelialization was minimal, while complete epithelialization was seen in 20 ng IL-4/SLac treated wounds. Arrowheads indicate wound edges defined by the presence of native adipose tissue in the hypodermis of non-wounded skin. Scale bar = 1 mm.
These macroscopic findings were confirmed by histological assessment of epithelialization (Figure 4.3b). Mice were sacrificed on day 14 post injury and the wound tissue was excised. H&E-stained paraffin sections, representing the longitudinal diameter of the wound, showed that MCP-1/SLac treated wounds displayed minimal epithelialization with larger distances between the epithelial tongues and limited granulation tissue in the wounded area. In contrast, IL-4/SLac treated wounds generally showed successful wound closure with complete epithelialization and significant granulation tissue formation.

The above differences in wound closure between wounds treated with IL-4/SLac and MCP-1/SLac can be explained by their functions during the inflammatory stage of healing. Exogenous delivery of a pro-inflammatory chemokine such as MCP-1 seems to exacerbate and prolong the inflammatory response leading to longer healing periods and inefficient wound closure. Since MCP-1 recruits monocytes to the wound site\textsuperscript{14}, burst release of exogenous MCP-1 from the MDP gel (as demonstrated in previous studies\textsuperscript{13}) can aggravate the inflammation with an initial overabundance of macrophages. Additionally, these wounds are created in diabetic animals that are predisposed to impaired healing mechanisms. Diabetic wounds are normally chronic wounds showing delayed healing due to irregular levels of GFs, cytokines, proteases and cellular and extracellular elements.\textsuperscript{6} Literature reports show that the normal inflammatory response seen in healing of acute wounds is significantly altered in chronic wounds.\textsuperscript{32, 33} Wound fluid derived from chronic venous ulcers is rich in pro-inflammatory cytokines such as TNF-α and IL-1.\textsuperscript{34, 35} Furthermore, the levels of these cytokines decrease as the chronic wound begins to heal, indicating a correlation between non-healing wounds and increased
levels of pro-inflammatory cytokines.\textsuperscript{6, 34} Hence it seems likely that exogenous MCP-1 can disrupt the healing process in these diabetic animals to an even greater degree.

This situation is reversed with the addition of an anti-inflammatory cytokine, IL-4, as it works to resolve the inflammation by alternatively activating pro-healing M2a macrophages. In light of preliminary results showing faster healing in IL-4/SLac treated wounds, MCP-1 treatment was discontinued and subsequent experiments were focused on different dosages of IL-4 treatment. Specifically, three dosages were tested with 10 diabetic mice, each having four 8-mm diameter full thickness dermal wounds: i) 2 ng, ii) 20 ng, and ii) 200 ng of IL-4 per wound (in 0.1 mL of SLac hydrogel). The controls tested were SLac gel only and IL-4 in HBSS buffer (no gel).

Evaluation of wound area in this study indicated that there was no significant difference of the wound closure rates between the three different IL-4 dosages (Figure 4.4). However at day 7 post injury, the percentage of wound area remaining was significantly lower for the IL-4/SLac treated wounds (all dosages) compared to IL-4 delivered without a gel in HBSS (p < 0.001) and the peptide only control (p < 0.005) (Figure 4.5). By day 14 this trend continued with significantly higher wound closure for IL-4/SLac treated wounds compared to both control cases (p ≤ 0.001). IL-4/SLac treated wounds showed approximately 30% remaining wound area by day 14, while control cases showed ~ 50%. Thus, wound closure measurements indicated that sustained delivery of IL-4 from MDP hydrogels, dosed as low as 2 ng IL-4 per wound, can accelerate the closing of dermal wounds in diabetic mice.
Figure 4.4. Evaluation of wound closure in IL-4 dosing study. a) Macroscopic appearance of wounds on diabetic mice at indicated time points post injury, in IL-4 dosing study. b) Wound area was determined using image analysis and expressed at each time point as a percentage of the initial wound area. Data are expressed as mean ± SD, n=8 wounds for each time point and sample type.
Figure 4.5. Comparison of wound closure in IL-4/SLac treated wounds to controls. Data are expressed as mean ± SD, n = 8 wounds for each time point and sample type. At day 14, IL-4/SLac treated wounds showed significantly higher wound closure than control cases (p ≤ 0.001).

4.3. Re-epithelialization and Granulation Tissue Formation

At the last time point day 14, mice were sacrificed and wound tissue harvested and processed for histology. H&E staining was performed on paraffin-embedded tissue sections to assess the re-epithelialization and granulation tissue formation in the wound areas. These events occur during the proliferation phase of healing, which begins after inflammation.36, 37 The newly formed epithelium can be distinguished by the dark blue/purple color atop the wound in the H&E stained sections.26, 38 Beneath the epithelium, the dermis, hypodermis and panniculus carnosus can be identified (Figure 4.6).29 The hypodermis characteristically shows many layers of fat cells or adipocytes.
These fat cells can be distinguished from circular empty spaces in the wound area by their regularity. In full-thickness dermal wounds, the skin is wounded beyond the panniculus carnosus to the subcutaneous space.\(^4\)

![Diagram of wound layers](image)

**Figure 4.6.** The cross-section of a full-thickness dermal wound bed. Taken from a medicinal news webpage.\(^*\)

Qualitative analysis of the H&E-stained paraffin sections of IL-4/SLac treated wounds indicated that complete re-epithelialization occurred in most cases, with only a few exceptions (mostly in the 2 ng IL-4 group) showing a moderate gap between epithelial tongues (Figure 4.7). In the completely epithelialized wounds, the thickness of the epithelium varied from 125 μm-250 μm. Furthermore, the neotissue deposition in the wound bed was higher in the IL-4/SLac treated wounds, with high cellular density and relatively fewer empty spaces that needed to be filled in. Higher magnification images of H&E stained sections showed a large number of cells lining these empty spaces that could possibly be in the process of depositing new tissue (Figure 4.8a). In contrast, the wounds treated with SLac only and IL-4 in HBSS controls demonstrated less granulation tissue and more empty spaces. Most wound areas were still open at day 14 and large distances between the tips of the epithelial tongues were measured (Figure 4.8b-c). The total length of both epithelial tongues in the control cases was significantly lower than that measured for the 20 ng and 200ng IL-4/SLac treated wounds (p < 0.01).

Figure 4.7. H&E staining of wounds at 14 days post injury. In IL-4/SLac treated wounds, higher levels of re-epithelialization and neotissue formation were observed when compared to the controls. Arrowheads indicate wound edges defined by the presence of native adipose tissue in the hypodermis of non-wounded skin. Scale bar = 1 mm.
Figure 4.8. Evaluation of neotissue formation and wound closure. High magnification images of H&E stained sections of wounds at day 14 treated with: a) 20 ng IL-4/SLac and b) 200 ng IL-4/SLac, showing new tissue formation within empty spaces in the wound area. Arrows in a) point at empty spaces that are in the process of being filled in. Dotted demarcation in b) outlines the same. Scale bar = 0.5 mm. c) Wound closure analyzed in terms of the length of epithelial tongue over the longitudinal diameter of wound (given as a percentage). Different Greek letters indicate significant differences (p < 0.01).

Masson’s trichrome staining was used as another indicator for the evaluation of granulation tissue formation.\textsuperscript{17,29} Granulation tissue is defined as neotissue consisting of new blood vessels, fibroblasts, macrophages, and loose connective tissue.\textsuperscript{29} Masson’s trichrome stain was used in this study to assess specifically connective tissue formation and collagen deposition (Figure 4.9-4.10).\textsuperscript{39} Macrophage response, myofibroblast formation and neovascularization will be discussed subsequently in the next sections.
Figure 4.9. Masson’s trichrome staining of wounds at 14 days post injury. In IL-4/SIac treated wounds, higher levels of connective tissue formation and collagen deposition were observed when compared to the controls. Arrowheads indicate wound edges defined by the presence of native adipose tissue in the hypodermis of non-wounded skin. Scale bar = 1 mm.
Figure 4.10. Depth of granulation tissue. a) Higher magnification images of Masson’s trichrome staining of wounds at 14 days post injury. Scale bar = 0.1 mm. In IL-4/SLac treated wounds, higher levels of collagen deposition were observed when compared to the controls. Scale bar = 0.1 mm. b) Extent of granulation tissue analyzed as a percentage of the depth of the total tissue section. Different Greek letters indicate significant differences (p < 0.001).
Microscopic evaluation of granulation tissue in the Masson’s trichrome-stained wound sections demonstrated that IL-4/SLac treatment induced greater wound healing than the controls, but not in a dose-responsive manner. The collagenous neomatrix deposited in these wounds can be identified by the characteristic blue color in the trichrome stain.\textsuperscript{29, 39} In general, granulation tissue present in IL-4/SLac-treated wounds was significantly thicker and deeper as a result of the robust infiltration and proliferation of a variety of cells in the MDP matrix, and the resolution of inflammation aided by exogenous IL-4. Additionally, it has been shown in the literature that IL-4 enhances the production of collagen and other extracellular matrix macromolecules by fibroblasts from different tissues.\textsuperscript{21-23, 40} Thus it seems that slow release of IL-4 had a positive impact on collagen deposition in neo-tissue. Furthermore, collagen bundles can be observed to align in a basket-weave pattern in some cases (Figure 4.10). Contrastingly, minimal healing could be seen in the peptide only and IL-4 in HBSS controls. Little to no granulation tissue was present in these control wounds, and the extent of cellular influx in the wound bed was minimal compared to the three types of IL-4/SLac treated wounds (Figure 4.9).

4.4. Macrophage Response

To determine the effect of exogenous IL-4 (delivered via an MDP scaffold), on the phenotype of macrophages in the wounds, immunofluorescence staining experiments were carried out with a panel of macrophage markers: i) M1 macrophage marker (CCR7\textsuperscript{+}; green)\textsuperscript{13}, ii) M2 macrophage marker (CD206\textsuperscript{+}; red)\textsuperscript{41} and iii) pan macrophage marker (F4/80\textsuperscript{+}; purple)\textsuperscript{17, 20}, counterstained with a nuclear stain (DAPI\textsuperscript{+}; blue). Images of the immunofluorescent sections revealed two populations of cells co-localizing with the pan macrophage marker F4/80 (Figure 4.11-4.14).
Figure 4.11. Evaluation of macrophage response in IL-4/SLac treated wounds at day 14. More CD206$^+$ M2 macrophages and less CCR7$^+$ M1 macrophages than control cases were observed. Immunostain panel: i) M1 macrophage marker (CCR7$^+$; green), ii) M2 macrophage marker (CD206$^+$; red) and iii) pan macrophage marker (F4/80$^+$; purple); counterstained with a nuclear stain (DAPI$^+$; blue). Scale bar = 200 μm.
Figure 4.12. High magnification immunostained image of 20 ng IL-4/SLac treated wound at day 14. Co-localization of the M2 marker (CD206) with F4/80⁺ macrophages can be seen. Immunostain panel: i) M1 macrophage marker (CCR7⁺; green), ii) M2 macrophage marker (CD206⁺; red) and iii) pan macrophage marker (F4/80⁺; purple); counterstained with a nuclear stain (DAPI⁺; blue). Scale bar =50 μm.
Figure 4.13. Evaluation of macrophage response in control wounds at day 14. Less CD206+ M2 macrophages and more CCR7+ M1 macrophages than IL-4/SLac treated wounds were observed. Immunostain panel: i) M1 macrophage marker (CCR7+; green), ii) M2 macrophage marker (CD206+; red) and iii) pan macrophage marker (F4/80+; purple); counterstained with a nuclear stain (DAPI+; blue). Scale bar = 200 μm.
Figure 4.14. High magnification immunostained image of control wound at day 14. Co-localization of the M1 marker (CCR7) with F4/80\(^+\) macrophages can be seen. Immunostain panel: i) M1 macrophage marker (CCR7\(^+\); green), ii) M2 macrophage marker (CD206\(^+\); red) and iii) pan macrophage marker (F4/80\(^+\); purple); counterstained with a nuclear stain (DAPI\(^+\); blue). Scale bar =50\(\mu\)m.
IL-4/SLac treated wounds showed a large number of cells expressing CD206, and very few expressing CCR7, with both populations appearing positive for F4/80 as well. Co-localization of cells expressing either CCR7 or CD206 with F4/80$^+$ cells confirms they are macrophages. Therefore it seems that CD206$^+$ M2 macrophages are more abundant in the IL-4/SLac treated wounds than CCR7$^+$ M1 macrophages. Contrasting an increased population of CCR7$^+$ F4/80$^+$ double positive M1 macrophages was observed along with a lower number of CD206$^+$ F4/80$^+$ double positive M2 macrophages. Quantitative evaluation of immunostained images revealed significant differences between M1 and M2 populations in all three groups of IL-4/SLac treated wounds (P < 0.001) (Figure 4.15). In general, the control wounds showed reduced numbers of F4/80$^+$ macrophages with slightly more of the M1 phenotype (CCR7$^+$) than the M2 phenotype (CD206$^+$).

Thus it can be inferred that controlled delivery of exogenous IL-4 via an MDP gel to murine diabetic full thickness skin wounds results in a higher number of CD206$^+$ anti-inflammatory M2 macrophages in the wound area compared to the controls. The reduction in the macrophage count of the control wounds can be related to the decrement of the total cellular influx into the wound area resulting in minimal neotissue mass and poor healing. Results of the above immunostaining experiments have further strengthened the notion that IL-4/SLac treatment enhances diabetic wound healing. Abundance of macrophages displaying the M2 pro-healing phenotype in IL-4/SLac treated wounds complements the accelerated cell proliferation and tissue formation response detected in H&E and Masson’s trichrome staining of the same wounds. According to the normal wound healing timeline, by 14 days, a normal non-diabetic
wound would have entered the proliferation stage of healing characterized by the presence of pro-resolution M2 macrophages and absence of pro-inflammatory M1 macrophages (Figure 4.1).¹⁸ Although a diabetic wound typically heals more slowly due to chronic inflammation, the above results suggest the possibility of overcoming such long periods of inflammation. IL-4/SLac treatment seems to mitigate the inflammatory response by sustained localized delivery of the cytokine, leading to acceleration in the healing process. The relatively higher M2 and minimal M1 macrophage numbers in these wounds indicate resolution of inflammation by day 14. This is accompanied by the development of proper granulation tissue (as seen in Section 4.3) marking the beginning of the proliferation phase.

Figure 4.15. Quantification of macrophage populations. Cell counts in the IL-4/SLac treated wounds, determined by ImageJ software, showed a much larger population of CD206⁺ M2 macrophages relative to the M1 population. Different Greek letters indicate significant differences (p < 0.001).
4.5. Angiogenesis and Wound Contraction

Wound tissue sections were further analyzed by another set of immunofluorescent staining experiments focused on evaluating angiogenesis and wound contraction, which occur during the proliferation and remodeling phases of healing, respectively. The formation of new blood vessels within the wound bed is important for sustaining the newly formed granulation tissue. It requires a complex interplay between GFs stimulating angiogenesis, appropriate ECM and the migration and mitogenic stimulation of endothelial cells.\textsuperscript{37, 42} Wound contraction refers to the commencement of connective-tissue compaction and the contraction of the wound, orchestrated mainly by myofibroblasts.\textsuperscript{37} Myofibroblasts are differentiated from fibroblasts and show smooth muscle cell-like properties due to the presence of large bundles of alpha smooth muscle actin (α-SMA)-containing microfilaments in their cytoplasm.\textsuperscript{43} Myofibroblasts facilitate the wound repair process by gripping the wound edges and decreasing the size of the wound in a contractile fashion.\textsuperscript{44} In evaluating angiogenesis and wound contraction, the marker used for immunostaining was α-SMA, as it is expressed by both myofibroblasts and smooth muscle cells perivascular lining of blood vessels. However, myofibroblast organization is different to the smooth muscle cell arrangement around a blood vessel, allowing the identification of both using one marker (Figure 4.16). During wound contraction, myofibroblasts display stress fibers and form adhesion complexes with the ECM, which generate contractile force.\textsuperscript{44} Thus, alignment of rows of myofibroblasts along newly formed fibronectin and collagen fibrils gives the appearance of fibrous layers in immunostained sections.\textsuperscript{45} Contrastingly, smooth muscle-lined blood vessels often appear as circular structures in cross-sections of wound tissue.\textsuperscript{39, 46}
Figure 4.16. Evaluation of angiogenic response and presence of myofibroblasts at day 14. A higher number of α-SMA⁺ structures were observed in IL-4/SLac treated wounds compared to the controls. Circular structures were identified as blood vessels and the fibrous layers as myofibroblasts. Immunostain panel consists of a smooth muscle cell/myofibroblast marker (α-SMA⁺; red) counterstained with a nuclear stain (DAPI⁺; blue). Scale bar = 200 μm.
Figure 4.17. Quantification of vessel density at day 14 post injury. The number of red circular capillary structures (identified by α-SMA$^+$ staining) per unit area was counted using Image J software to determine vessel density in the wound bed. IL-4/SLac treated wounds (especially 20 ng IL-4 in SLac) showed significantly higher vessel density compared to controls. Different Greek letters indicate significant differences (p < 0.001).

Qualitative and quantitative assessment of immunofluorescent images revealed a significantly higher number of α-SMA$^+$ circular structures, identified as cross-sections of blood vessels, in the three groups of IL-4 /SLac treated wounds, compared to control groups (Figure 4.16-4.17). Moreover, the presence of α-SMA$^+$ myofibroblast layers in IL-4 /SLac treated wounds was also higher. It was noted that the second type of α-SMA staining is found in areas separate from the capillary structures, indicating the non-endothelial cell origin of this staining and suggesting the presence of myofibroblasts. Additionally, myofibroblast α-SMA staining was abundantly expressed throughout the granulation tissue in most wounds treated with IL-4/SLac. Conversely, SLac only and IL-4 in HBSS groups showed minimal α-SMA$^+$ structures. This is in congruence with the
literature that suggest IL-4 can induce fibroblasts to differentiate into myofibroblast-like cells containing α-SMA, whereas IL-1, TNF-α, IL-6 and bFGF fail to do so.\textsuperscript{47, 48}

It is evident from these findings that sustained delivery of IL-4 from the SLac scaffold leads to enhanced healing of diabetic wounds as observed by: i) efficient myofibroblast-driven wound contraction and, ii) the formation of a highly vascularized granulation tissue.

#### 4.6. Discussion and Conclusions

One of the main causes of pain, suffering and morbidity in approximately 370 million diabetic patients worldwide is the diabetic foot ulcer (DFU).\textsuperscript{25} A series of multiple mechanisms (including decreased cell/GF response, disruption in production/activity of proteases and low blood supply) can contribute to the lack of healing in DFUs, and ultimately lead to leg amputation in 15% of the patient population.\textsuperscript{6}

As a result of their incomplete, uncoordinated healing process and particularly the impaired ability to fight infection, DFU patients display inadequate, often pathological inflammatory responses.\textsuperscript{3} To address this issue of delayed healing in diabetic wounds, we have attempted to advance the inflammatory response by sustained localized delivery of an anti-inflammatory cytokine IL-4. As a carrier matrix for the delivery of IL-4, a previously published biocompatible, degradable and injectable MDP hydrogel termed SLac was utilized.\textsuperscript{13, 49}

Gross morphology wound measurements at day 14 indicate that application of a one-time dose of 2 – 200 ng of IL-4 per wound via SLac matrix encapsulation had a positive effect on closing of diabetic wounds. These measurements were supplemented by further evaluation using H&E staining, Masson’s trichrome staining and
immunofluorescent staining. Analysis of various indicators of healing such as re-epithelialization, granulation tissue formation, macrophage response, angiogenesis and myofibroblast organization has demonstrated that the IL-4 loaded SLac hydrogel positively affects the inflammation, proliferation and remodeling stages of the healing process, leading to accelerated healing of diabetic wounds. Comparatively, the controls tested showed minimal healing. Application of either unloaded SLac gel alone or IL-4 in a buffer solution did not result in significantly enhanced wound closure, potent re-epithelialization, and the formation of a highly cellularized, vascularized, and contractile granulation tissue that was readily observed in the IL-4/SLac treated wounds. Therefore it is important to note that both the pro-healing tendencies of the cytokine itself (such as polarization of M2 macrophages and stimulation of ECM macromolecule production), and the desirable physico-chemical properties of the MDP hydrogel scaffold play central roles in facilitating the healing process.

Prior investigations have focused on achieving faster healing of diabetic wounds by use of a variety of GFs, cytokines and ECM macromolecules. Greenhalgh et al. have shown that administration of recombinant PDGF-BB (10 μg per wound daily for 5 days) or administration of recombinant FGF (1 μg per wound daily for 5 days) via a PEG-PBS vehicle, on full-thickness dermal wounds in db/db mice resulted in ~80-90% wound closure by 21 days. Also, moderately thick granulation tissue, minimal-to moderate epithelial migration and extensive neovascularization was seen. Comparatively, the PEG-PBS vehicle showed only ~48% wound closure. In a later study by the same group, it was shown that a combination of PDGF (10 μg) with TGF-α (1 μg) leads to ~95-100% wound closure by 21 days in the same experimental set up. Although these
findings indicated stimulation in diabetic wound healing, the dosages, dosing frequency and healing times were higher than that used in the current study (2-200 ng IL-4 given once).

In a more recent study, Johnson and colleagues have shown utility of a heparin-binding form of EGF (HB-EGF) in diabetic wound healing. They developed a coacervate delivery system to provide sustained, localized release of HB-EGF in polygenic type 2 diabetic NON-cNZO10/LtJ mice. Their design was based on polyethylene argininylaspartate diglyceride (PEAD) that self-assembles with heparin to form a complex coacervate capable of encapsulating HB-EGF. The coacervate containing 1 μg of HB-EGF was applied once to 5-mm full-thickness dermal wounds. Results demonstrated ~85% re-epithelialization at day 7, in wounds treated with the HB-EGF-coacervate, relative to ~55% in controls saline and free HB-EGF. They also show higher basal keratinocyte proliferation (at day 3), collagen deposition (at day 14) and wound contraction (at day 14) in the HB-EGF-coacervate cases. Comparatively, the work in this chapter showed similar success in healing of larger (8-mm diameter) wounds on a monogenic strain of diabetic mice (C57BLKS/J-m/Leprdb db/db) with lower concentrations of cytokine (2-200 ng).

Kawanabe et al. explored the ability of sphingosine-1-phosphate (S1P) to accelerate diabetic wound healing. S1P is lysophospholipid capable of increasing migration and proliferation of endothelial cells in endothelial injury, inflammation, angiogenesis, and wound healing. Researchers injected S1P (10 μM or 100 μM in 100 μL PBS, which is about 380 ng or 3800 ng S1P per wound) once daily for 13 days, to full-thickness 9-mm diameter wounds. They found a significant decrease in wound size
and higher vessel density in granulation tissue, compared to the BSA/PBS control, in the wounds treated with the higher concentration of S1P, with 100% wound closure at day 13.\textsuperscript{52} The dosages and dosing frequency in this case are again much higher when compared to the current study, albeit a slightly larger wound size.

There have not been very many studies investigating the application of exogenous IL-4 on wound healing. Of note are those reported by Kucukcelebi \textit{et al.}\textsuperscript{54} and Salmon-Ehr \textit{et al.}\textsuperscript{20} In the first study, Sprague-Dawley rats were induced with diabetes by intramuscular injections of streptozotocin, and 6-cm full-thickness incisional wounds were created on the dorsal aspect of the rats and immediately closed with nylon sutures. This animal model differs significantly to the genetically diabetic mouse model used in the current study, and requires constant monitoring of diabetic hyperglycemia. IL-4 was then injected to the wound edge at a concentration of 0.1 μg, 1 μg or 10 μg. Results indicate that 1 μg IL-4 treatment increases the breaking strength of incisional wounds in diabetic rats, and at day 14 this treatment group showed significantly stronger wounds than controls. The authors attribute this to higher synthesis of ECM proteins within the wound upon IL-4 treatment.

Salmon-Ehr and colleagues, however, does not use a diabetic animal model.\textsuperscript{20} Instead, 8-mm full thickness biopsy punch wounds were created on the mid-dorsum of healthy male Swiss mice. IL-4 was applied topically to wounds at a dose of 250 ng in carboxymethyl cellulose, daily for 4 days. Researchers report a rapid increase in wound closure rates (~50% of wound area remaining at the end of 4 days, compared to 90% in the control PBS) and in the formation of granulation tissue. The study described in this chapter utilized the same model of wounding, but in genetically diabetic mice that show
impaired healing. Furthermore, IL-4/SLac was administered only once throughout the study in considerably lower doses.

In summary, when examined in parallel to other studies related to diabetic wound healing, it is evident that the work detailed herein stands competitive and shows great promise as a potential therapeutic agent. The IL-4/SLac treatment tested here combines a vital bioactive factor with a successful MDP scaffold that displays all the right criteria for an optimal wound dressing: biocompatibility, ease of application, ability to conform to wound surface, provide a moist environment, protect against dehydration, allow gas exchange, and slowly release bioactive agents. Additionally, the inclusion of IL-4 allows for its use in slow-healing wounds with impaired healing mechanisms.

Future work may be directed towards dual bimodal release of IL-4 and another GF playing a central role in wound healing such as EGF, FGF or VEGF. The second GF can be encapsulated in liposomes for delayed release and made available for impact in the later stages of wound healing i.e. proliferation and maturation.

4.7. Materials and Methods

4.7.1. Synthesis of SLac peptide

MDP with the sequence K(SL)₃RG(SL)₃KGRGDS (SLac), was synthesized on a low loading Rink Amide MBHA resin at a 0.15 mM scale using a Focus XC automated solid phase peptide synthesizer (Aapptec, Louisville, KY) by using an optimized protocol reported previously. Peptide was cleaved from the resin using a mixture of TFA, triisopropylsilane, water, ethanedithiol and anisole in a 36:1:1:1:1 ratio. Resulting peptide had neutralized termini due to the acetylated N-terminus and amidated C-terminus.
Cleaved peptide was roto-evaporated, precipitated in cold ether and dried overnight. Crude peptide was dissolved in Milli-Q water to form a 1% by weight solution and pH adjusted to 7.4 prior to dialysis (MWCO 500-1000 Da) for 3 days with buffer changes twice daily. Post-dialysis, the peptide solution was frozen and lyophilized. Synthesis of the correct peptide was confirmed by matrix-assisted laser desorption/ionization time-of-flight (Bruker Daltonics, Billerica, MA) mass spectroscopy (MALDI-TOF).

4.7.2. Preparation of loaded MDP gels and controls

Lyophilized SLac MDP was dissolved at 20 mg/mL in Milli-Q water with 298 mM sucrose, and the pH was adjusted to 7.4. Above peptide solution was mixed with recombinant murine MCP-1 or IL-4 (PeproTech) to form loaded gels containing 1 wt% peptide. MCP-1 was loaded at two concentrations in SLac gels:

1. 20 ng MCP-1 per wound – 20 mg/mL SLac mixed with 0.4 μg/mL MCP-1 (dissolved in HBSS), in 1:1 ratio.

2. 100 ng MCP-1 per wound – 20 mg/mL SLac mixed with 2 μg/mL MCP-1 (dissolved in HBSS), in 1:1 ratio.

IL-4 was loaded at three concentrations in SLac gels:

1. 2 ng IL-4 per wound – 20 mg/mL SLac mixed with 40 ng/mL IL-4 (dissolved in HBSS), in 1:1 ratio.

2. 20 ng IL-4 per wound – 20 mg/mL SLac mixed with 0.4 μg/mL IL-4 (dissolved in HBSS), in 1:1 ratio.

3. 200 ng IL-4 per wound – 20 mg/mL SLac mixed with 4 μg/mL IL-4 (dissolved in HBSS), in 1:1 ratio.
The controls used included SLac peptide gel alone (unloaded) and IL-4 in a buffer solution:

1. SLac alone - 20 mg/mL SLac mixed with HBSS, in 1:1 ratio.
2. IL-4 in HBSS (20 ng per wound) - 0.4 μg/mL IL-4 was mixed with HBSS, in 1:1 ratio.

Each wound received 100 μL of gel (or solution in the case of IL-4 in HBSS).

4.7.3. Diabetic mouse wound healing model

All experiments were approved by the Rice University Institutional Animal Care and Use Committee. C57BLKS/J-m/Lepr\textsuperscript{db} (db/db) female mice (Jackson Labs, Bar Harbor, ME) were 10 weeks old and weighed 40-45 g at the start of the experiments. Mice were anesthetized using isofluorane (2% for induction and 1% for maintenance) and their backs depilated under sterile conditions. Mice were given Meloxicam (100 μL of 0.5 mg/mL) to relieve pain 30 min prior to surgery and both 24 h and 48 h post-surgery. Four full-thickness punch-biopsy wounds (8-mm in diameter) were created on the dorsal aspect of each mouse. Directly after, wounds were irrigated with HBSS and treated with IL-4/SLac gels, MCP-1/SLac gels or controls (listed above). Treatments were applied topically using a 21 gauge needle, 100 μL per wound. To avoid drying of the wounds, they were covered with transparent Tegaderm\textsuperscript{TM} dressings (Nexcare Products, Maplewood, MN) that were changed at day 7. At each time point, wounds were imaged with a scale and wound area analyzed using ImageJ software. Wound area remaining at day 3, 7 and 14 were calculated as a percentage of the initial wound area at day 0. After 14 days, animals were sacrificed using an overdose of isoflurane, CO\textsubscript{2} asphyxiation, and bilateral thoracic puncture. The dorsal skin containing the wound tissue was excised,
washed with PBS, and fixed in neutral buffered formalin for 24 hours prior to processing for histological analysis.

4.7.4. **Histology and immunofluorescence staining**

Tissue was processed to paraffin blocks, sectioned at 7 µm, deparaffinized and stained for cellular infiltrate and re-epithelialization using H&E. Masson’s trichrome staining was carried out according to manufacturer’s instructions (Sigma-Aldrich, St. Louis, MO) to visualize granulation tissue formation and collagen deposition. Macrophage polarization at Day 14 was determined using immunostaining for M1, M2 and naïve macrophages: goat anti-mouse CCR7\(^+\) (Novus, Littleton, CO); rabbit anti-mouse CD206\(^+\) conjugated to biotin (Bioss, Woburn, MA); rat anti-mouse F4/80\(^+\) (eBioscience, San Diego, CA); and nucleic DAPI counterstain.

Angiogenic and myofibroblast evaluation was performed by immunostaining for smooth muscle actin: rabbit anti-mouse α-SMA\(^+\) (GeneTex, Irvine, CA), and nucleic DAPI counterstain. Secondary antibodies used were donkey anti-goat FITC 488 (GeneTex), Streptavidin AF 647 (ThermoFisher, Waltham, MA), donkey anti-rat Dylight 550 (ThermoFisher) and donkey anti-rabbit AF 647 (ThermoFisher).

Immunofluorescent sections were imaged using a Nikon A1 confocal microscope (Nikon, Tokyo, Japan). Wound closure, as seen in H&E stained sections, was analyzed in terms of the length of epithelial tongue over the longitudinal diameter of wound (given as a percentage). Macrophage numbers were quantified by counting cells stained as M1, M2 or naïve macrophages, using ImageJ software (cells per mm\(^2\), n=2 separate sections, n=8 samples).
4.7.5. Statistical analysis

Data is represented as mean ± S.D. One way ANOVA was conducted for multiple comparisons of parametric data, with Tukey post-hoc analysis for all pairwise comparisons of the mean responses to the different treatment groups. All samples were tested in 8 replicates (n=8). Values of p < 0.05 were considered statistically significant.
References


Chapter Five
Conclusion

In an effort to create an optimal ECM mimic, our lab has successfully developed a new generation of self-assembling biomaterials that combine favorable and tunable physico-chemical properties with biocompatibility, tissue integration, bioactivity and controlled release.\textsuperscript{1-12} These self-assembling multidomain peptides developed over the past decade have received much attention given their ability to self-assemble into β-sheets, and form robust hydrogels as a function of pH, temperature, ionic strength and other conditions.\textsuperscript{2,3,5,7,8} MDPs present a highly versatile platform that can be customized to accomplish a variety of goals, including absorption and release of bioactive factors, regeneration of dental pulp tissue and surgical hemostasis.\textsuperscript{4, 10, 11, 13, 14} This thesis discussed the work I have carried out over the last 5 years to investigate the potential use of MDP scaffolds in tissue regeneration, by controlled release of growth factors and cytokines.

In the first two years of my doctoral research, I demonstrated how orthogonal self-assembly concepts were utilized to design composite hydrogels containing the supramolecular entities: MDPs and liposomes.\textsuperscript{12} These composites were rationally designed, with the intent of developing a system that can mimic the level of complexity seen in sophisticated natural systems. The so-formed two-component constructs allow for controlled temporal release of bioactive factors. The individual components of the self-assembled gel, and the MDP-liposome composites (MLCs) were characterized by TEM, SEM and rheometry, demonstrating that peptide nanofibers and lipid vesicles both retain their structural integrity in the composite gel.\textsuperscript{12} In vitro release studies from the
composite gels loaded with MCP-1, EGF and PlGF-1, showed delayed release by liposomes within the MLCs. This was in comparison to a relatively rapid release from hydrogel matrices. Thus, a bimodal release system was established with MLCs.\textsuperscript{12}

In the third and fourth years of my study, I further built upon the functionality these release systems by demonstrating robust in vivo responses to MLCs. Specifically; I have shown that PlGF-1 can temporally modulate VEGF-receptor activation in endothelial cells, and robust vessel formation in rats, when released from liposomes within MLCs.\textsuperscript{15} Histological assessment of rat subcutaneous implants of PlGF-1 loaded MLCs demonstrated high cellular infiltration and neomatrix development. Immunohistochemical analysis indicated that blood vessel development begins after 2 days and reaches a peak at 5 days.\textsuperscript{15} Immature vessels were shown to resorb while mostly robust mature vessels remained at the end of 10 days. As such, controlled in vivo angiogenesis was observed with the use of PlGF-1 loaded MLCs.\textsuperscript{15}

These MLCs induce an unprecedented level of growth factor-mediated neovascular maturity. The stability and maturity of the MLC-induced vascular network is accentuated when compared with similar angiogenesis techniques that have yielded neoplasticity, much less mature vasculature, or involve cell based approaches that have been challenged with minimal uptake/ success.\textsuperscript{16-18} At present, the literature reports few polymer-based or GF-based material delivery systems that offer such robust vessel growth as seen in these MLCs.\textsuperscript{10,15}

In my fifth year, I explored the possibility of using cytokine-laden MDP scaffolds for stimulating healing of chronic diabetic wounds.\textsuperscript{19-21} I tested the effect of topically
applying IL-4 loaded SLac MDP hydrogels on full-thickness dermal wounds created on the backs of genetically diabetic mice. A two week study involving this animal model showed accelerated healing of wounds treated with the IL-4 loaded MDP gels, compared to the unloaded SLac control and IL-4 delivered via a buffer solution instead of a gel. Wound closure measurements as well as histological analysis (H&E and Masson’s trichrome staining) and immunofluorescent staining indicate higher re-epithelialization, granulation tissue formation, wound contraction and neovascularization in IL-4/SLac treated wounds relative to the controls. Furthermore, I was able to demonstrate a higher level of macrophage polarization to the pro-healing M2 phenotype, by treating these diabetic wounds with sustained and localized delivery of anti-inflammatory IL-4 from the SLac scaffold. The above results indicate the efficacy of an MDP scaffold-mediated delivery for a topically-acting cytokine, and suggest that future incorporation of multiple, synergetic GFs and cytokines into MDP gels may have utility in chronic wound therapy.

The in vivo success of these composite MDP hydrogels indicates their potential to exhibit comparable functionality to the natural extracellular matrix in terms of chemical communication by signaling molecules. Overall, composite MDP gels laden with bioactive factors can serve as dynamic matrices for tissue regeneration to treat a milieu of pathologies including peripheral vascular disease, diabetic ulcers, tissue infarcts and myocardial regeneration.
References


Appendix I

VEGF Encapsulation and Release

The first growth factor tested for encapsulation in liposomes was VEGF. Encapsulation was carried out in situ during hydration of dry lipid films. Unencapsulated VEGF was removed by passing the liposome solution through a Sephadex G-75 column. Since purification by a Sephadex column is based on the size exclusion principle, G-75 Sephadex beads with a fractionation range of 30-80 kDa were used particularly to allow retention of unencapsulated VEGF molecules (38.2 kDa in size) in the column. Encapsulation efficiency was determined by quantifying both entrapped and unentrapped VEGF via enzyme-linked immunosorbent assay (ELISA).\(^1\) It was found to be 2.64% ± 0.31 (App. Figure 1). Due to this low efficiency of encapsulation, a 100 ng/ml VEGF solution was subjected to the liposome preparation process without actual lipids in an attempt to evaluate the stability of VEGF throughout all the processing steps including sonication, freeze-thawing and extrusion. It was determined that only 9.1% ± 0.9 of the original VEGF concentration could be detected after all processing steps. Thus it seemed that the relatively harsh conditions prevailing during liposome preparation are not well tolerated by VEGF. Based on the above results which indicated a high degree of protein fragility pertaining to VEGF, it was decided that MCP-1, EGF and FGF-2 would be tested for endurance in the same manner as above. ELISA analysis of GF/cytokine fractions after each step in the liposome preparation protocol showed that 68.0% ± 3.2 of MCP-1, 82.4% ± 1.4 of EGF and 15.7% ± 0.7 of FGF-basic remained at the end. Therefore, MCP-1 and EGF were chosen for encapsulation in liposomes.
App. Figure 1. Determining encapsulation efficiency of VEGF using Sephadex columns and ELISA. The concentration of VEGF in the original solution was 100 ng/mL.

Efficiency of encapsulating MCP-1 was determined by in the same manner as above, employing ELISA, and it was found to be 48.7% ± 2.6. Similarly, encapsulation efficiency for EGF was 37.3% ± 4.5. However, during MCP-1 and EGF release experiments, the ELISA method of release detection demonstrated many complications. Data showed inexplicable, abnormal trends and could not be reproduced. Therefore, a shift to another analytical method was made: detection via a conjugated fluorophore. Chapter Two details this analytical method.

VEGF release from peptide gels without liposomes was attempted as well (App. Figure 2). Release of complete VEGF load could be observed by the end of 6 days. Analysis of VEGF remaining in the gels after disintegration correlated well with release profiles. Again, preliminary release studies employed ELISA as the detection method,
which was later changed to fluorescence detection with MCP-1-CFDA and EGF-FITC (Figure 2.9).

**App. Figure 2.** VEGF release profiles. VEGF was released from the matrix of K(SL)₃RG(SL)₃KGRGDS peptide gel without liposomes. Analysis of the remaining VEGF after breaking down the gel indicated that the VEGF load was released almost completely by 6 days. This correlated well with the release profile showing ~100% release after the same time period.

*Determining release via ELISA:*

GF/cytokine release from liposomes in gel matrix was assayed in a transwell setup as shown in Figure 2.11. Gels were incubated at 37°C and 5% CO₂ for up to 3 weeks. 100 µL of the release media was removed from the inner well and replenished at a series of time points (hours 2, 4, 8, 12 and days 1, 2, 3, 5, 7, 9, 12, 15, 18, 21, and 24). Samples were frozen at -80°C until analysis. ELISA was performed on release media samples to determine GF concentration using ELISA kits for VEGF, MCP-1 and EGF purchased from R&D Systems.¹ Standard series were prepared in duplicate using both kit standards and GF stock from Sigma-Aldrich. Samples were diluted with PBS (supplemented with 0.1% BSA) to ensure that concentration will be within the detection range of the kit and
tested in triplicate. In the first step, 200 µL volumes of samples and standards were added to the well plate pre-coated with primary antibody for capture of protein. Plate was incubated for 2 hours at room temperature and then washed three times with 400µL volumes of wash buffer supplied in the kit. Next, 200 µL volumes of horseradish peroxidase-conjugated secondary antibody were added to the plate and incubated for 1-2 hours. Wash procedure was repeated, 200 µL volumes of tetramethylbenzidine (TMB) substrate solution provided in the kit were added to the plate, and plate was again incubated for 20-30 minutes covered in aluminum foil (protected from light). Finally, 50 µL volumes of a stop solution were added to halt color development and the resultant yellow solutions were measured for absorbance at 450 nm. Concentrations corresponding to the absorbance values were determined by plotting a five parameter logistics (5-PL) curve (App. Figure 3).

**App. Figure 3.** Sample standard 5-PL curve plotted for determining EGF concentration via ELISA.
Appendix II

Differently-Sized Liposomes

Liposomes having diameters of 100 nm, 200 nm and 400 nm were synthesized to explore differences in release kinetics. 100-200 nm liposomes are known as small unilamellar vesicles (SUV) while anything larger is termed a large unilamellar vesicle (LUV).  

100 nm and 200 nm liposomes were made by the hydration of dry lipid films and extrusion through a 100 nm or 200 nm pore size membrane (App. Figure 4). LUVs were made through the ether injection method.

**App. Figure 4.** Schematic showing steps in synthesis of liposomes by hydration of dry lipid films. Lipids were mixed, solvent evaporated by passing nitrogen, and container kept on high vacuum to allow complete drying. Dry lipid films were hydrated with PBS and mixture was incubated for 1 hour with intermittent agitation. Then it was subjected to 5 freeze-thaw cycles (rapidly frozen in a dry ice-butanol bath and thawed in a water bath at 41°C). Finally, the liposome suspension was extruded through a 100 nm membrane using a Mini-Extruder.
In the ether injection method, the lipid mixture is normally prepared in diethyl ether. Thus, DPPC, DPPG and cholesterol were mixed in diethylether while the EGF solution was made in 1xPBS supplemented with 0.1% BSA. Lipids in ether were then injected into the GF solution very slowly at 42°C while the ether evaporated (App. Figure 5). Large particles/flocculates were centrifuged out at the end. Purification was done by dialysis. Liposomes were sized by dynamic light scattering experiments (App. Figures 6-7) and release studies conducted with EGF-FITC as described in Chapter Two. Fluorescence was used to measure GF concentrations in release aliquots. Release profiles indicated that LUVs were less stable than SUVs and more likely to leak out material earlier (App. Figure 8). Higher release percentages were observed at days 1, 3 and 7 in LUV-containing gels.

**App. Figure 5.** Experimental set-up for the ether injection method of preparing LUVs.
**App. Figure 6.** Size distributions of differently-sized liposomes after 1 day. a) 100 nm SUVs with a peak mean of 114 nm, b) 200 nm SUVs with a peak mean of 182 nm and c) LUVs with a peak mean of 398 nm.

**App. Figure 7.** Stability of LUVs. After 3 days, LUVs seem to break down to form smaller vesicles, possibly releasing most of their load in the process.
App. Figure 8. EGF-FITC release from SUVs and LUVs in gels.
Appendix III

Modeling Release Data

The Korsmeyer-Peppas equation describes diffusion-related drug release from hydrophilic polymeric matrices. The Weibull function is typically employed to describe triphasic or sigmoidal release curves. Previous studies involving biodegradable microspheres have indicated suitability of Weibull function for release from delivery systems that show an erosion-dominated process coupled with minimal diffusive release rates. ‘β’ or the shape factor in the Weibull equation characterizes the shape of the curve as exponential (β=1), sigmoid or S-shaped with upward curvature followed by a turning point (β>1) or parabolic, with a higher initial slope and after that consistent with exponential (β<1).

<table>
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<th>Release from hydrogel only</th>
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<tr>
<td><strong>Single release profiles</strong></td>
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App. Table 1. R^2 values and constants in curve fits (burst release model and sigmoidal release model) of each GF release profile (single release).
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<th>EGF-PIGF-1 bimodal release curves</th>
<th>Korsmeyer-Peppas equation for burst release: ( \frac{M_t}{M_\infty} = k(t^n) )</th>
<th>Weibull equation for sigmoidal (delayed) release: ( \frac{M_t}{M_\infty} = 1 - e^{-\alpha(t^\beta)} )</th>
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<td>Release from hydrogel only</td>
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**App. Table 2.** \( R^2 \) values and constants in curve fits (burst release model and sigmoidal release model) of the EGF-FITC/PIGF-1-TAMRA bimodal release profiles.
Appendix IV

SDF-1α Release from Gels In Vitro and In Vivo

Stromal cell-derived factor-1α (SDF-1α), a mesenchymal stem cell homing chemokine, was used for release studies after conjugation to a fluorophore. SDF-1α purchased from PeproTech Inc., (Rocky Hill, NJ) was conjugated to 5(6)-carboxyfluorescein diacetate succinimidyl ester (CFDA-SE) using a standard labeling protocol. 9 mM CFDA-SE in sterile anhydrous DMSO was mixed with 0.25 mM SDF-1α in PBS in a molar ratio of 20:1. The mixture (total volume = 25 μL) was incubated overnight in the dark at 4°C with continuous gentle agitation. The unconjugated dye was removed from the conjugated protein by using SpinOut™ GT-600 0.1mL columns (G-Biosciences, St. Louis, MO) and the conjugated protein was eluted out with PBS. The conjugated SDF-1α-CFDA was mixed with 20 mg/mL SLac to form a composite gel. Release of SDF-1α-CFDA from the matrix of the gel was studied (App. Figure 9).

![App. Figure 9](image_url)

App. Figure 9. Release profile for SDF-1α-CFDA, showing release from gel matrix along with the burst release model curve fit (n=3 for each sample).
In vitro cell migration assays were conducted to analyze human mesenchymal stem cell (hMSC) migration in response to SDF-1α release from the hydrogel. Since SDF-1α is a mesenchymal stem cell homing chemokine, its release from a SLac hydrogel can stimulate hMSC to migrate across a membrane, towards the chemical signal.\textsuperscript{8, 10} Cells were seeded in transwell inserts (18000 cells/well in 200 μL media) with SDF-1α releasing SLac gels at the bottom of the outer well (App. Figure 10a). Gels were topped with 1 mL of hMSC media. Control cases contained only hMSC media with SDF-1α. The set-up was incubated at 37°C for 24 h, after which migrated cells were stained with crystal violet and counted under the optical microscope (App. Figure 10b).

**App. Figure 10.** hMSC migration study. a) Transwell set-up with cells seeded in the insert and composite gel plated at the bottom of the outer well.\textsuperscript{*}  
b) Cells migrated to the underside of the insert membrane were stained with crystal violet. c) Migration assay results showing the number of migrated cells in response to SDF-1α released from the gel or from media. Different Greek letters indicate significant differences. (n=3, p <0.05) After 24 h, the hMSC migration response to 100 ng SDF-1α in gel is similar to 50 ng in media. This corresponds to the SDF-1α-CFDA release profile, d), that indicates 50% release by day 1.

\textsuperscript{*} Image taken from webpage: http://www.springerimages.com/Images/RSS/1-10.1007_978-1-59745-401-8_27-0
Results of the migration assay demonstrated that after 24 h, the number of cells migrated correlates linearly to the mass of SDF-1α in the media of the outer well, up to 100 ng. With regard to release of SDF-1α from the gel: when 100 ng of the chemokine was loaded in the 200μL gel, the migration response after 24 h was similar to that of 50 ng SDF-1α in media. This suggests that possibly 50% of the load in the gel has been released after 24 h, and it corresponds to the SDF-1α-CFDA release profile which shows the same: approximately 50% release by day 1 (App. Figure 10c-d).

In vivo studies involving SDF-1α were conducted subcutaneously in rats (discussed in detail in Chapter Three). Specifically we hypothesized that controlled release of SDF-1α can tailor cellular recruitment. The chemokine was integrated into the MDP matrix (MDP(SDF-1α)) to elicit a rapid release accelerating stem cell recruitment acutely. MDP(SDF-1α) gels were injected into the dorsal aspect of Wistar rats. Harvested tissue at Day 2, 5 and 10 was immunostained to determine cellular infiltrate. Identification of the implant was facilitated by cellular density and hydrogel morphology of fixed, embedded sections. Immunofluorescent CD68+ macrophages were seen in MDP(SDF-1α) implants and control implants with only MDP, at all time points. Thy-1+ mesenchymal stem cells (MSC) were identified at Day 2 and 5, with an expected dependence on SDF-1α loading (App. Figure 11-12). In qualitative comparison, control MDP gels showed a lower presence of Thy-1+ MSC. Of note, the most pronounced MSC recruitment was observed in Day 5 explants, wherein SDF-1α was loaded in the matrix. Thus it appeared that SDF-1α has an effect on recruiting MSCs into hydrogels. However, the effect was not as distinct and pronounced as expected in some implants and hence these studies were not pursued further.
App. Figure 11. Immunostaining for monocytes/macrophages (CD68⁺; red), mesenchymal stem cells (Thy-1⁺; green), and nuclei (DAPI; blue) within the control MDP and MDP(SDF-1α) implants at Day 2, 5 and 10. High macrophage infiltration is observed in all cases. MSC recruitment can be seen in MDP(SDF-1α) implants at Day 2 and 5. Scale bar 200 μm.
**App. Figure 12.** Component images of MDP(SDF-1α) implant at Day 5, in which SDF-1α is released from the gel matrix. Macrophage infiltration as well as MSC recruitment can be seen. Tissue sections were immunostained for macrophages (CD68⁺; red), mesenchymal stem cells (Thy-1⁺; green), and nuclei (DAPI; blue). Scale bar 100 μm.
References


Appendix V

List of Publications


(9) Wickremasinghe, N. C.; Kumar, V. A.; Shi, S.; Carrejo, N.; Hartgerink, J. D., “Enhancing diabetic wound healing with IL-4 loaded peptide gels” (In preparation)