Development of a Polymeric Gene Delivery Vector for Application in
Osteochondral Tissue Engineering

by

Clark Needham

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

Doctor of Philosophy

APPROVED, THESIS COMMITTEE

Antonios G. Mikos, Professor, (Chair)
Bioengineering

Cindy Farach-Carson, Professor
Biochemistry

Jane Grande-Allen, Professor
Bioengineering

F. Kurtis Kasper, Faculty Fellow
Bioengineering

Joseph A. Ludwig, Assistant Professor,
UT MD Anderson

March 1, 2014
Houston, TX
Development of a Polymeric Gene Delivery Vector for Application in
Osteochondral Tissue Engineering

by

Clark Needham

COMMITTEE MEMBERS

Dr. Cindy Farach-Carson

Department of Biochemistry and Cell Biology

Dr. Jane Grande-Allen

Department of Bioengineering

Dr. F. Kurtis Kasper

Department of Bioengineering

Dr. Joseph Ludwig

M. D. Anderson Cancer Center

Dr. Antonios G. Mikos (Chair)

Department of Bioengineering, Rice University
Abstract

Development of bPEI-HA as a Polymeric Gene Delivery Vector for Application in Osteochondral Tissue Engineering

by

Clark Needham

In this work, the polymeric gene delivery vector poly(ethylenimine)-hyaluronic acid (bPEI-HA) was optimized for transfection efficiency, incorporated into microparticles for controlled release, and applied directly in an oligo[poly(ethylene glycol) fumarate] (OPF) composite scaffold for osteochondral tissue generation. First, the effect of bPEI-HA synthesis parameters, specifically primary amines concentration, ligand targeting, and overall charge on the effectiveness of the vectors were investigated by altering the type and amount of hyaluronic acid (HA) oligosaccharide in the polymer. It was found that the length of the HA oligosaccharide had the most significant effect on cytotoxicity and transfection efficiency with human mesenchymal stem cells, while molar incorporation of HA, as opposed to the saccharide length and HA mass incorporation, had the greatest effect on zeta potential, but a minor effect on both cytotoxicity and transfection efficiency. Next, bPEI-HA/DNA complexes were incorporated into poly(DL-lactic-co-glycolic acid) (PLGA) microparticles and compared to microparticles containing bPEI/DNA complexes at several incorporation concentrations. It was found that the addition of HA to the bPEI vector allowed for increased loading concentration within these systems and significantly altered release kinetics without changing the morphology of the particles. Furthermore, the incorporation of HA onto the bPEI backbone significantly increased the transfection efficiency of the complexes released from the corresponding microparticle formulation. Finally, bPEI-HA was complexed with DNA encoding for the transcription factors RUNX2 and the SOX trio and incorporated into a composite hydrogel scaffold which was implanted into a rat osteochondral defect for 6 weeks. The in vitro release of this system was
characterized and found to have a significant burst release over the first week of exposure to water. The
*in vivo* analysis showed that the incorporation of DNA encoding for RUNX2 in the bone layer of these
scaffolds significantly increased bone growth. The results also indicate that a spatially loaded
combination of RUNX2 and SOX trio DNA loading leads to significantly better healing than empty
hydrogels or either factor alone. Finally, the results of this study suggest that subchondral bone is
necessary for correct cartilage healing. This research demonstrates the potential for gene delivery, and
specifically bPEI-HA combined with transcription factor DNA, to be applied to *in vivo* osteochondral
situations and result in improved tissue growth and quality.
Acknowledgements

This thesis work is dedicated to my parents, Jim and Alicia Needham who have been the best role models on the planet and showed me how to never give up.

I acknowledge support from the National Institutes of Health (R21 AR56076 and R01 AR57083). I extend my appreciation to my thesis committee members, Dr. Cindy Farach-Carson, Dr. Jane Grande-Allan, Dr. Kurt Kasper, Dr. Joseph Ludwig, and Dr. Antonios Mikos, for their support and advice.

I thank Dr. Joel Moake for the use of his flow cytometer and Dr. Michael Diehl for the use of his FPLC. I would also like to thank Dr. Rebecca Richards-Kortum for the use of the spectrophotometer and Dr. Michael Wong for the use of his zetasizer. Finally, I would like to thank Dr. Anita Saraf and Mr. Ryan Schweller for their guidance on the project.

This work would not have been possible without the support of my family and friends. I would especially like to thank my fiancé, Allison Heather, and my family, Jim, Alicia, Lauren, and Kent Needham for helping me power through all of the tough times with their support and encouragement. I also thank my friends at Rice for giving me an outlet and support when things were not going as planned, especially Robert, Joe, Allan, Rich, Matt, Brian, and Erica. I appreciate all of the help that I received from the administration and from the many other grad students who were always there to help me out when I needed it the most. Without all of your support I would have never made it - Thank you.
# Table of Contents

Abstract .................................................................................................................................................. 2

Acknowledgements ............................................................................................................................... 4

List of Figures ......................................................................................................................................... 10

List of Tables .......................................................................................................................................... 13

List of Abbreviations ............................................................................................................................ 14

Chapter 1 - Objective and Specific Aims ............................................................................................. 16

Chapter 2 - Non-viral Gene Delivery for Osteochondral Tissue Engineering ...................................... 17

  Motivation ........................................................................................................................................... 17

  Current Treatment Options .................................................................................................................. 18

  Native Cartilage Tissue ......................................................................................................................... 19

    Cartilage Extracellular Matrix .......................................................................................................... 21

    Cartilage Cell Types .......................................................................................................................... 22

  Non-Viral Gene Delivery ....................................................................................................................... 23

    The bPEI-HA Delivery Vector ............................................................................................................ 26

  Hyaluronic Acid .................................................................................................................................... 27

    Oligosaccharides of HA ....................................................................................................................... 28

    CD44 .................................................................................................................................................. 30

  Bone and Cartilage Development ........................................................................................................ 32

    Development of Articular Cartilage and Subchondral Bone .............................................................. 33

  Transcription Factors .......................................................................................................................... 34
Chapter 3 - Engineering a Polymeric Gene Delivery Vector Based on Poly(ethylenimine) and Hyaluronic Acid

ABSTRACT

INTRODUCTION

EXPERIMENTAL PROCEDURES

Materials

Degradation, Separation, and Characterization of HA Oligosaccharides

UV Spectroscopy

Synthesis of bPEI-HA

Assembly of Polymer/DNA Complexes

Zeta Potential and Dynamic Light Scattering

Cytotoxicity Studies

Transfection Efficiency

Statistics

RESULTS AND DISCUSSION

HA Degradation and Characterization
Chapter 4 - Modulation of Polyplex Release from Biodegradable Microparticles through Poly(ethylenimine) Modification and Varying Loading Concentration ................................................................. 69

ABSTRACT ......................................................................................... 69

INTRODUCTION .................................................................................. 70

EXPERIMENTAL PROCEDURES .............................................................. 72

Materials .................................................................................................. 72

Synthesis of bPEI-HA ............................................................................ 72

Assembly of Polymer/DNA Complexes .................................................. 73

Microparticle Preparation .................................................................. 74

Microparticle Characterization .............................................................. 75

Entrapment Efficiency ........................................................................ 75

Polyplex Dissociation and Detection .................................................... 75

In Vitro Release .................................................................................... 76

Transfection Efficiency ........................................................................ 77

Statistics ................................................................................................. 77

RESULTS .................................................................................................. 79

DISCUSSION ............................................................................................ 86
List of Figures

Figure 1: Schematic of the three layers of cartilage and their matrix fiber orientation ² .......................... 21
Figure 2: CD44 interaction with HA ........................................................................................................ 31
Figure 3. Graph demonstrating the concentration dependent HA peak shift. ...................................... 53
Figure 4. Representative ¹H NMR spectra of bPEI-HA ........................................................................ 56
Figure 5. Confirmation of actual synthesized bPEI-HA ratios ................................................................. 57
Figure 6. Cytotoxicity values for each bPEI-HA conjugate. ................................................................. 59
Figure 7. Zeta potential for each test group of bPEI-HA ...................................................................... 63
Figure 8. Transfection efficiency of each group of bPEI-HA, bPEI, or pDNA only on hMSCs. ............ 66
Figure 9: Representative SEM images illustrating morphology of particles ........................................ 80
Figure 10: DNA release curves from A) DNA only incorporating groups, B) bPEI/DNA complex
incorporating groups, and C) bPEI-HA/DNA complex incorporating groups; and D) bPEI release curves
from bPEI/DNA complex incorporating groups, and E) bPEI-HA release curves from bPEI-HA/DNA
complex incorporating groups. .............................................................................................................. 84
Figure 11: Transfection efficiency for each of the polymer containing groups .................................... 86
Figure 12: (a) Cumulative bPEI-HA and (b) DNA release in vitro. All groups were loaded with the same
amount of bPEI-HA and DNA. 3KCMC represents hydrogels fabricated with CMC and OPF synthesized
with 3 kDa PEG. 10KCMC represents hydrogels fabricated with CMC and OPF synthesized with 10 kDa
PEG. 10K represents hydrogels fabricated only with OPF synthesized with 10 kDa PEG. Region"***
denotes the time in which all groups' release are significantly different. Region"**" denotes times in
which 3KCMC and 10KCMC are significantly different from the 10K group, but not from each other.
Region "***" denotes times in which only the 10KCMC and 10K groups are significantly different from
each other .................................................................................................................................................. 103
Figure 13: Percent bone generation identified through micro-CT. Empty represents the group which had
no bPEI-HA/DNA complexes. RUNX2 represents the group which had only bPEI-HA and DNA
encoding for RUNX2 in the bottom layer. SOX trio represents the group which had only bPEI-HA and DNA encoding for the SOX trio in the top layer. Combination represents the group which had only bPEI-HA and DNA encoding for the SOX trio in the top layer and only bPEI-HA and DNA encoding for RUNX2 in the bottom layer.

Figure 14: Overall defect histological analysis where (a) corresponds to percent filling with new tissue score and (b) corresponds to percent implant degradation. Empty represents the group which had no bPEI-HA/DNA complexes. RUNX2 represents the group which had only bPEI-HA and DNA encoding for RUNX2 in the bottom layer. SOX trio represents the group which had only bPEI-HA and DNA encoding for the SOX trio in the top layer. Combination represents the group which had only bPEI-HA and DNA encoding for the SOX trio in the top layer and only bPEI-HA and DNA encoding for RUNX2 in the bottom layer. 0, 1, 2, and 3 correspond to histology scores described in Table 2. Groups connected by lines are significantly different from each other.

Figure 15: Bone tissue histological analysis where (a) corresponds to filling with new bone, (b) corresponds to subchondral bone morphology, and (c) corresponds to bone bonding with surrounding tissue. Empty represents the group which had no bPEI-HA/DNA complexes. RUNX2 represents the group which had only bPEI-HA and DNA encoding for RUNX2 in the bottom layer. SOX trio represents the group which had only bPEI-HA and DNA encoding for the SOX trio in the top layer. Combination represents the group which had only bPEI-HA and DNA encoding for the SOX trio in the top layer and only bPEI-HA and DNA encoding for RUNX2 in the bottom layer. 0, 1, 2, and 3 correspond to histology scores described in Table 2. Groups connected by lines are significantly different from each other.

Figure 16: Cartilage tissue histological analysis where (a) corresponds to the morphology of the new surface tissue, (b) is the morphology of the generated cartilage, (c) is the thickness of new cartilage, (d) is the joint surface regularity, (e) is the chondrocyte distribution, (f) is the chondrocyte cellularity, (g) is the safranin O staining, and (h) is the quality of the surrounding cartilage. Empty represents the group which had no bPEI-HA/DNA complexes. RUNX2 represents the group which had only bPEI-HA and DNA encoding for RUNX2 in the bottom layer. SOX trio represents the group which had only bPEI-HA
and DNA encoding for the SOX trio in the top layer. Combination represents the group which had only bPEI-HA and DNA encoding for the SOX trio in the top layer and only bPEI-HA and DNA encoding for RUNX2 in the bottom layer. 0, 1, 2, and 3 correspond to histology scores described in Table 2. Groups connected by lines are significantly different from each other.

Figure 17: Representative histological sections of osteochondral tissue formation after 6 weeks of implantation of OPF hydrogels in the Combined treatment group which had bPEI-HA and DNA encoding for the SOX trio in the top layer and RUNX2 in the bottom layer. Sections were stained with (a) Safranin-O/Fast Green and (b) hematoxylin and eosin. Scale bars = 500 µm.

Figure 18: Representative histological sections of osteochondral tissue formation after 6 weeks of implantation of OPF hydrogels in the RUNX2 treatment group which had bPEI-HA and DNA encoding for RUNX2 in the bottom layer. Sections were stained with (a) Safranin-O/Fast Green and (b) hematoxylin and eosin. Scale bars = 500 µm.

Figure 19: Representative histological sections of osteochondral tissue formation after 6 weeks of implantation of OPF hydrogels in the SOX Trio treatment group which had bPEI-HA and DNA encoding for the SOX trio in the top layer. Sections were stained with (a) Safranin-O/Fast Green and (b) hematoxylin and eosin. Scale bars = 500 µm.

Figure 20: Representative histological sections of osteochondral tissue formation after 6 weeks of implantation of OPF hydrogels in the Empty hydrogel treatment group which had no incorporated bPEI-HA/DNA complexes. Sections were stained with (a) Safranin-O/Fast Green and (b) hematoxylin and eosin. Scale bars = 500 µm.
List of Tables

Table 1. Description of the bPEI-HA synthesis groups with respect to HA oligosaccharide type, molar incorporation, and NMR integration ratio.................................................................54
Table 2. Complex size as measured by DLS for each test group of bPEI-HA. ........................................62
Table 3: Size and entrapment efficiency of DNA and polymer for all microparticle test groups..............79
Table 4: DNA release rates presented as percent of encapsulated material released per day..................82
Table 5: Polymer release rates presented as percent released per day. ....................................................82
Table 6: In Vivo Experimental Groups ................................................................................................94
Table 7: Evaluation Parameters for Osteochondral Defects68-71 ..........................................................99
# List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>bPEI</td>
<td>Branched Poly(ethyleneimine)</td>
</tr>
<tr>
<td>HA</td>
<td>Hyaluronic Acid</td>
</tr>
<tr>
<td>bPEI-HA</td>
<td>Branched poly(ethyleneimine)-hyaluronic acid conjugate</td>
</tr>
<tr>
<td>hMSCs</td>
<td>Human mesenchymal stem cells</td>
</tr>
<tr>
<td>DHB</td>
<td>2,5 - dihydroxybenzoic acid</td>
</tr>
<tr>
<td>MWCO</td>
<td>Molecular Weight Cutoff</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>pDNA</td>
<td>Plasmid DNA</td>
</tr>
<tr>
<td>eGFP</td>
<td>Enhanced green fluorescent protein</td>
</tr>
<tr>
<td>CMV</td>
<td>cytomegalovirus</td>
</tr>
<tr>
<td>MW</td>
<td>molecular weight</td>
</tr>
<tr>
<td>KU</td>
<td>Kilounits</td>
</tr>
<tr>
<td>FPLC</td>
<td>Fast protein liquid chromatography</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>DLS</td>
<td>Dynamic light scattering</td>
</tr>
<tr>
<td>PLGA</td>
<td>Poly(DL-lactic-co-glycolic acid)</td>
</tr>
<tr>
<td>rBPEI-HA</td>
<td>Rhodamine tagged bPEI-HA</td>
</tr>
<tr>
<td>PEG</td>
<td>Poly(ethylene glycol)</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>-----------</td>
<td>------------------------------</td>
</tr>
<tr>
<td>OPF</td>
<td>Oligo[ poly(ethylene glycol) fumarate]</td>
</tr>
</tbody>
</table>
Chapter 1 - Objective and Specific Aims

The objective of this work is to evaluate the use of bPEI-HA as a non-viral gene delivery vector and to incorporate this vector into a controlled release strategy to promote osteochondral tissue healing. Finally, this work sought to develop a potential therapy option utilizing this vector through its application with plasmid DNA encoding for transcription factors in an *in vivo* setting to investigate the ability to promote tissue healing in an osteochondral defect. To achieve the goals outlined above, work encompassing 3 specific aims was performed and resulted in the work described in depth below.

*Specific Aim 1: Engineering a Polymeric Gene Delivery Vector Based on Poly(ethylenimine) and Hyaluronic Acid*

This work uses a full factorial design to investigate the design aspects of the bPEI-HA delivery vector. The results of this work led to a deeper understanding of the gene delivery system and the identification of a specific delivery vector which has relatively superior performance.

*Specific Aim 2: Modulation of Polyplex Release from Biodegradable Microparticles through Poly(ethylenimine) Modification and Varying Loading Concentration*

This work resulted in the development and testing of a controlled release strategy for complexed gene delivery vector and DNA using PLGA. This study resulted in the identification of the influences of design parameters within the system on loading efficiency and release kinetics of the system.

*Specific Aim 3: Osteochondral Tissue Regeneration through Polymeric Delivery of DNA encoding for Transcription Factors In Vivo*

The final study described in this thesis investigated the effectiveness of complexed DNA and bPEI-HA when spatially confined within a OPF hydrogel and applied into a rat osteochondral defect. This work shows the potential of the gene delivery vector, but also the limitations associated with its application *in vivo*. 
Chapter 2 - Non-viral Gene Delivery for Osteochondral Tissue Engineering

Motivation

In 2005, 27 million Americans were reported to have osteoarthritis\(^1\). Cartilage degeneration is a debilitating end result of a number of diseases which have treatments that are often ineffective or have a limited lifespan. These treatments often involve surgical intervention and over 200,000 bone grafts and 500,000 surgical cartilage repairs are performed each year\(^2\). Because of the inadequacy in these current treatments, tissue engineering and gene delivery are very promising alternative treatment methods that are currently being developed for these disorders. Through the combination of bioactive scaffolds capable of promoting osteogenesis and chondrogenesis with infiltrated progenitor cells capable of differentiation, new, more appropriate cartilage generation could be achieved to provide a new and more ideal alternative treatment. Gene therapy is especially promising in these medical applications because it offers the opportunity to deliver a relevant dosage of otherwise unusable, attractive bioactive factors to a target site for an extended time through inducing differentiation in local cells to help regenerate tissue. Additionally, many therapeutic growth factors have a very limited lifespan in the body due to fast metabolic breakdown outside of the protective environment of the cell. Gene therapy can avoid this breakdown by manufacturing the factors at the target site inside of local cells. This work will describe the development and investigation of a polymeric gene delivery vector and its application towards in vivo regeneration of osteochondral tissue through the use of DNA encoding for appropriate osteogenic and chondrogenic transcription factors.
Current Treatment Options

Articular cartilage degeneration presents itself clinically as a problem that is associated with a number of disorders and has a variety underlying causes. Regardless of which type of articular cartilage disorder is present, cartilage is a tissue which has a very limited ability for self-repair. Because of a limited blood supply, an injury which does not penetrate to the subchondral bone will not result in a clot formation, an inflammatory response, or the infiltration of regenerative cells\textsuperscript{3, 4}. While the chondrocytes themselves will respond to the injury, this response is often inadequate and will result in degeneration of the tissue over time. An injury which is deep enough to affect the subchondral bone, on the other hand, has a better chance of repair than a superficial injury. This is because once the bone is penetrated the healing cascade can begin with clot formation. Blood and mesenchymal stem cells enter the wound and attempt to regenerate the tissues, but replace the injured collagen II with collagen I, resulting in a fibrous tissue, called fibrocartilage, which has significantly less ideal biomechanical properties. This inadequate and incomplete healing results in chronic pain for and reduction in mobility of the joint, and often warrants a medical intervention.

Many options currently exist for the treatment of cartilage injuries, including auto- and allografts, surgical debridement of the wound, penetration of the subchondral bone, and full prosthesis implantation\textsuperscript{4}. While these treatments can result in favorable outcomes, these favorable results often revert over time back into a painful and debilitating condition requiring further intervention. These limitations in current treatment options leave an open door to new promising alternatives that are being made available through tissue engineering and gene therapy research.
Native Cartilage Tissue

Articular cartilage is the functional surface for the synovial joints of the body and is responsible for maintaining low friction within the joint and padding impacts sustained in the joint’s normal usage. The loss of this surface, if significant enough, results in severe and often debilitating pain. This loss can be a result of disease, injury, or degeneration due to over-use and age. Regardless of the cause, treatment of joint surface loss is an emerging area of research, especially in regenerative medicine because of the body’s limited ability to heal this tissue and the inadequacy of current treatments.

The synovial joints in the body include nearly all of the joints of the limbs which are used for locomotion. Typically, joints consist of a joint capsule, which envelops and seals the joint and contains synovial fluid, and articular cartilage, which lines the load bearing or articulating surfaces of the joint. While the synovial lining of the joint has a readily available blood supply, the articular cartilage does not, making it a very unique avascular tissue. This avascular nature makes this tissue a viable candidate for current polymeric gene therapy and tissue engineering treatments due to the ability of delivered agents to remain relatively localized at the implantation site. Furthermore, this avascular nature allows this tissue to be immunoprivileged and mitigates the current tissue engineering hurdles associated with generating effective, functional vasculature because the tissue already receives its nutrients through diffusion.

Articular cartilage is made up of three distinct layers which differ in both purpose and physiology. The uppermost layer is called the surface zone and is characterized by flattened cells and higher proteoglycan extracellular matrix content. This top zone acts as the lubrication point for the joint and consists of a type II collagen web with type IX collagen dispersed throughout to help with shear force resistance. Further, this zone acts as a barrier to keep
molecules from penetrating into the cartilage from the synovial fluid. To correctly handle the shear forces at the surface, the collagen fibers and extracellular matrix are generally oriented horizontally in the tissue and relatively more tightly packed than in the lower two levels.

The middle layer is known as the mid zone and has rounded cells found in columns with high levels of aggregan and collagen type II present. The columns are formed as progenitor cells proliferate when the cartilage is forming during development. This zone is responsible for resisting both the shear forces from the surface and the compressive forces that are present deeper in the tissues. Because this layer is required to effectively integrate the upper and lower levels while resisting both shear and compressive force, the extracellular fibers are oriented in a range of directions between vertical and horizontal.

The calcified, or deep zone, cartilage is characterized by type X cartilage and is the lowest level which serves to integrate the articular cartilage into the underlying bone. This zone is the primary source of compression resistance in the articular cartilage. As such, this zone has vertically oriented fibers of ECM which allow for effective compression resistance. A general schematic of the fibers within these three layers can be seen below in Figure 1.
Figure 1: Schematic of the three layers of cartilage and their matrix fiber orientation

Cartilage Extracellular Matrix

Because the work described below seeks to regenerate cartilage and bone tissues and up to 95% of cartilage is made of ECM, it is important to have a clear understanding of what correctly regenerated tissue would consist of. In the case of cartilage, the extracellular matrix is often used as an indication of correct regeneration because it is this ECM which gives cartilage its superior load-bearing and wear resistance qualities. Further, incorrect ECM generation is what distinguishes fibrocartilage from correctly generated tissues.

One main extracellular component that is found ubiquitously in the body is collagen. Collagen II is found throughout the articular cartilage, and collagens V, VI, IX, X, XI, XII, and XIV are found in smaller quantities in specific regions of the cartilage column based on the differentiation state of the chondrocytes present. For example, only prehypertrophic and hypertrophic chondrocytes produce collagen X. Additionally, a complex series of glycosaminoglycans and proteoglycans are found throughout the cartilage column and add a
great degree of functionality and protection to the cartilage. Up to 12% of the cartilage ECM is made of chondroitin sulfate and keratin sulfate. These sulfated glycosaminoglycans are negatively charged and help to regulate the viscoelastic behavior of cartilage by retaining water\(^4\). Aggrecan is also present in articular cartilage and aids in the retention of water. Finally, hyaluronic acid (HA) is also present within cartilage in small but significant amounts and can bind aggrecan, thus playing a role in stabilizing the ECM\(^7, 8\).

**Cartilage Cell Types**

This work sought to generate the correct cell type within osteochondral defects through differentiation of infiltrated cells, thus it is important to understand genotype and phenotype of the native cartilage cells to provide a target for regeneration. The cells within the cartilage are known as chondrocytes and are responsible for creating and maintaining the correct ECM composition required to keep the cartilage functional\(^6\). These cells operate without a readily available blood supply and without many cell to cell contacts. Most of their interactions are either through hydrostatic pressures from joint loading, soluble molecule signaling, or contact with the ECM. One specific cell-matrix interaction mechanism worth noting for this work is the CD44-HA interaction. CD44 is present on chondrocytes and is the primary means of binding HA glycosaminoglycan chains\(^8\). This receptor is responsible for connecting the chondrocytes to the HA-Aggrecan aggregates in the extracellular matrix and often endocytosis of HA for metabolic purposes\(^8\).

In the *in vivo* portion of this work, infiltrated cells were the target of released polymer/DNA complexes. The cells infiltrating into the defect for regeneration will include mesenchymal stem cells (MSCs). These cells are found in many locations throughout the body and are perivascular in multiple organ types, including the bone marrow\(^9\). These cells were
considered a target for this work because they are multipotent progenitor cells that are capable of proliferating in clonal cell populations. Further, these cells are capable of differentiation into many cell types given the correct set of signals, including the bone and cartilage cells that are important for this work.  

Non-Viral Gene Delivery

Gene delivery is best summarized as the practice of altering the expression of biological targets of interest by altering or adding genetic information within a target cell. Delivery of genetic material is an attractive alternative to the direct delivery of drugs because of the possibility to alter the cells' phenotype and have localized, extended delivery of a therapeutic agent, especially one that might be otherwise impossible to administer. In the case of non-viral gene delivery, a gene delivery vector is often used to help in the delivery of the target DNA into the cell and is often necessitated because of the relatively inefficient uptake of DNA at the cell surface coupled with the natural degradation of intercellular DNA by serum proteases. Vectors provide the needed protection while improving the efficiency of introduction of DNA into the cell. While there are numerous examples of these vectors, the scope of this discussion will be limited to several examples of polymeric delivery of DNA.

Cartilage treatment is an area within gene therapy which could see immediate therapeutic benefits because the tissue's limited interaction with blood and immune cells allows the delivery vectors and genetic material to remain effective and in close proximity to target cells for longer periods. Towards this end, there are two potential approaches to regeneration of cartilage tissue using gene delivery, in vivo and ex vivo. Ex vivo approaches are currently the most investigated approach to genetic engineering because of the highly reduced risk to the patient. Here, cells from the patient are expanded and transfected outside of the body to help mitigate the potential
for immunological reactions. *In vivo* approaches are attractive because it simplifies the process of implantation and scaffold preparation. Additionally, this approach overcomes the regulatory hurdles associated with the sterility required when implanting processed cells into a human. This proposal will utilize an *in vivo* approach to genetic engineering through transfecting cells which migrate into the injury site and come into contact with the implanted scaffold.

**Branched PEI**

The work presented here is based on the polymeric gene delivery vector branched poly(ethyleneimine) (bPEI). PEI is a highly cationic polymer that can either be linear or branched and is capable of delivering DNA from the outside of the cell into the cytoplasm. It achieves this through association with the cell surface, endocytosis into the cell, and endosomal release through its ability to act as a proton sponge\(^{13-15}\). In the proton sponge hypothesis, an endosome containing the bPEI/DNA complex is created after endocytosis. As the endosome develops into a lysosome, the bPEI acts as a buffer and osmotically swells the endosome until it ruptures. At this point, the DNA is released from the complex and taken to the nucleus by intracellular mechanisms\(^{15, 16}\). Once in the nucleus, the DNA is expressed through the activation of a promoter region on the DNA strand. This promoter is generally a viral promoter which is activated by conditions within the cell\(^{17}\). Expression of the DNA will gradually decrease over time as this promoter region is recognized by the cell as foreign, methylated, and inactivated\(^{18}\).

Generally, it is believed that the ideal molecular weight (MW) for transfection with bPEI is between 5 and 25 kDa. The work proposed below will utilize 25 kDa bPEI which is attractive for these applications because of its relatively smaller complex formation compared to smaller MW bPEI\(^{13}\). Results have been shown that bPEI with larger molecular weights have a higher toxicity due to aggregation on the exterior of the cell, but also have higher transfection
efficiencies than those with lower molecular weights\textsuperscript{13, 19}. Many cationic gene delivery vectors, including bPEI, utilize their amine groups to complex and deliver DNA. The theoretical primary: secondary: tertiary ratio of amines in the bPEI that is assumed throughout this work is 1:2:1. There is some suggestion in the literature, which is worth taking into consideration, that the actual ratio is closer to 1:1:1 and more highly branched than the theoretical 1:2:1 ratio\textsuperscript{13}. This ratio is important because different amines serve different purposes within the vector and complexes.

The positive charge of the amine groups in these particles can is based on their acid dissociation constant, or pKa. In bPEI, the pKa is highest for primary amines and decreases to the lowest value for tertiary amines\textsuperscript{20}. The result of this is that as the pH drops from physiological levels to a pH of around 5.0 found in endosomes, bPEI protonation increases from \~20\% to \~45\%\textsuperscript{20, 21}. This high change shows that bPEI is a potent proton sponge capable of endosomal release through the buffering capacity of its tertiary and secondary amines. On the other hand, the initial charge, mainly from primary and secondary amines, is important in complexation because it allows the polymer to interact with the phosphodiesters on the DNA backbone and gives the bPEI an overall positive charge at neutral pH. Efficient complexation and attraction to the negatively charged moieties on the cell surface is a key component of the success of the bPEI gene delivery vector.

Another important consideration in gene delivery with a polycationic vector like bPEI is the ratio of nitrogen atoms in the vector to phosphate atoms in the DNA, or N:P ratio. This gives a general measurement of the overall charge of the system, as well as an idea about how tightly the DNA will be packed with the vector. For this proposed work, the N:P ratio will be held constant at 7.5:1, as this value has been shown to be the most effective in previous studies\textsuperscript{22}. 
As stated before, when bPEI and DNA are added into solution together, electrostatic interaction causes them to bind together and form complexes. Interestingly, even after the DNA has been added to bPEI, a large fraction of the bPEI remains in solution. This finding has led to some research into purification of the complexes to reduce cytotoxicity, but this results in a decrease in transfection efficiency 13, 23. The work proposed below does not incorporate any purification step during complex formation because of the premium placed on transfection efficiency and the decrease in cytotoxicity because of the incorporation of hyaluronic acid.

One important aspect of the work presented here is the incorporation of a ligand to target cell receptors and to mitigate the high charge density and ζ potential of bPEI. There are other similar examples in the literature which also take this approach. Specifically, bPEI has been conjugated to transferrin and PEGylated after complexation with DNA to decrease interaction with blood proteins, aggregate formation, and ζ potential 24. Additionally, multiple ligands have been conjugated to bPEI and used to deliver DNA to desired cell types and receptors including the sugars mannose 25, glucose 26, and galactose 22, 27-29, folate 30, 31, the RGD peptide 32, uronic acids 33, and HA 34-36. Finally, a number of diverse approaches to modify the ζ potential and intracellular release mechanisms of bPEI complexes have been investigated 32, 36-40. The overriding result from all of these investigations is the finding that a lower ζ potential will result in lower cytotoxicity of the complexes and that targeting of the vectors with a ligand is possible and can result in a specific transfection pattern according to the presence of the targeted receptor.

The bPEI-HA Delivery Vector

The current limitations in polymeric gene therapy generally include low transfection efficiencies, high toxicity, and biological barriers which hinder the vectors’ actions and access to the target cell 13. While bPEI suffers from many of these limitations, the addition of hyaluronic
acid (HA) to the system can mitigate many of these issues. First, bPEI is highly cytotoxic due to its high density of positive charges, due to the amines, which leads to bPEI build-up on the surface of a cell and eventually cell death. HA is negatively charged, giving it the ability to limit the overall positive charge density of bPEI-HA. Also, it will be shown in chapter 3 below that HA can be found at the surface of the complexes, lending a negative zeta potential at the surface of the complex. Furthermore, through utilizing the different cellular functions of HA oligosaccharides, the transfection efficiency is increased through targeting ligands on the cell surface, which results in increased internalization. Finally, by incorporation into a scaffold for delivery, bPEI-HA can be directly administered to the target cell population, overcoming many of the biological barriers to delivery that exist in other situations.

Other work has been completed that analyzes the post-condensation modification of bPEI/DNA complexes with HA to mitigate the ζ potential problems at the surface of the complexes. This work was successful, but does not directly link the HA to the vector. By linking chemically, it is hypothesized that the complex uptake through receptors specific for HA will be increased. Also, previous work has used large HA molecules, whereas this work will use smaller oligosaccharides which will specifically target ligands on the cell surface. Finally, through direct conjugation of HA to bPEI, a vector which can be encapsulated and released from a scaffold to complex with DNA is created, rather than vector which necessitates further processing after complex formation.

**Hyaluronic Acid**

Hyaluronic acid (HA), or hyaluronan, is a linear non-sulfated glycosaminoglycan with a relatively short *in vivo* half life, that is found in every tissue and body fluid in humans. This polysaccharide is a long linear string of the disaccharide β-1,4-glucaronic acid-β-1,3-N-acetyl-D-
glucosamine. HA is usually found as a very high molecular weight polymer associated with everything from protecting cells from viral infection to lubrication, due to its hydrophilicity and location in the glycocaylx. It is introduced into the glycocaylx by cells through synthesis at the cell surface and immediate extrusion into the extracellular space as a linear chain. Within the body, most HA is found in the skin with lesser amounts found in the joints, bones, and muscles. Finally, HA is an ideal lubricant for joints and is extruded by cells along the synovial lining, and thus is found in relatively high concentrations in the synovial fluid of the joint capsules; the focal tissue for this work.

HA carries a significant negative charge at physiological pH because of the carboxyl groups on the polysaccharide chain. This charge is important for this work because it helps mitigate the positive charges of the amine groups within the bPEI polymer that are responsible for its high cytotoxicity. This negative charge is also the source of HA’s ability to create zones of steric exclusion around cells. Through incorporation of HA into the gene delivery vector, it is hypothesized that the vector can more easily penetrate the glycocaylx, resulting in higher transfection. Also significant to this work is the fact that many cells have hyaladherins, receptors designed to bind to HA. The hMSCs that were used in the in vitro studies in this work specifically have CD44, a hyaladherin, on the cell surface. Additionally, CD44 is present on the cells of the developing articular joints during cavitation, suggesting that as cells in this work differentiate towards the chondrogenic phenotype, they may maintain CD44 on the cell surface.

Oligosaccharides of HA

This project utilizes relatively small oligosaccharides of HA attached to bPEI to achieve superior gene delivery of transcription factors relative to raw bPEI. When designing such a gene...
delivery vector, special care must be given to choosing the correct length of HA oligosaccharide for incorporation. Oligosaccharides from HA are present naturally in the body from degradation, as well as in osteoarthritic joints, and have a variety of positive and negative effects on the cells they come into contact with \(^8\). Chapter 3 of this work will explore in depth the usefulness of each oligosaccharide when combined with bPEI for transfection.

For chondrocytes, the minimum HA oligosaccharide size that is needed for association with the cell surface receptor, CD44 has been shown to be a hexasaccharide \(^8,^{44}\). Further, the turnover rate of CD44 in chondrocytes (48 hours), suggests that the receptors are endocytosed if there is no significant connection to the extracellular matrix. Finally, work has shown that in the presence of interleukin-1 (IL-1) chondrocytes accelerate endocytosis of CD44 receptors into lysosomes for degradation \(^8\).

It has also been found that the minimum size of HA fragment needed for association and binding with aggrecan is 10-12 saccharides \(^8,^{45}\). Also, the minimum size for attachment to the cell receptors responsible for matrix interactions is also 10-12 saccharides, and the affinity increases as the chain length increases. This is relevant to this work because the uptake of CD44 by endocytosis is inhibited by the attachment of aggrecan to the HA chain. This research would suggest that the optimal HA fragment for cellular uptake should be between 6 and 10 saccharides long, a finding confirmed in chapter 3.

Another novel effect of HA oligosaccharides on cells is the ability of the four saccharide sugar of HA to suppress cell death through the up-regulation of HSP72 \(^{45}\). Furthermore, low MW HA in general is a cause of inflammation and is an angiogenic factor. This is noticeable especially because its presence at the site of injury and role in cancer tumor development. On
the other hand, the four sugar oligosaccharide also suppresses proteoglycan synthesis, which is a negative outcome for this work. The positive potential for this small oligosaccharide was enough to warrant its investigation when incorporating into the gene delivery vector.

With many options for oligosaccharides to incorporate and positive and negative effects for each, an investigation into the optimal size for incorporation into the vector is necessitated and will be discussed later in Chapter 3. A summary of each of the oligosaccharides and their effects are listed below in Table 1, adapted from the work of Asari.

<table>
<thead>
<tr>
<th>Number of HA Sugars</th>
<th>Protein Interactions</th>
<th>Functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>HABPI</td>
<td>Suppression of Cell Death, Suppression of proteoglycan synthesis, Induction of MMPs and cytokines</td>
</tr>
<tr>
<td>6</td>
<td>Chondrocyte CD44, Smooth Muscle CD44</td>
<td>Chemotaxis, Suppression of HA cable formation, Activation of NF-kappa B in chondrocytes</td>
</tr>
<tr>
<td>10</td>
<td>Aggrecan, Versican, Link Protein</td>
<td>Up regulation of PTEN in tumor cells</td>
</tr>
<tr>
<td>12</td>
<td></td>
<td>Suppression of smooth muscle cell proliferation</td>
</tr>
<tr>
<td>15</td>
<td></td>
<td>Up regulation of tumor cell migration, CD44 cleavage</td>
</tr>
<tr>
<td>34</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1: Functions of HA Oligosaccharides

CD44

An understanding of the mechanisms and distribution of CD44 is important to this work because it is the primary HA receptor in the MSC population that is being targeted by the polymeric gene therapy vector in this work. RHAMM and many other hyaladherins are present throughout the body, but were not considered for various reasons. Specifically for RHAMM, it
is found in the intercellular space and interacts with smaller HA fragments than CD44. This fact, along with the fact that it only couples with CD44 and does not induce uptake itself, lead to CD44 being the rate limiting receptor in many cases. CD44 is a single pass membrane protein that is approximately 85 kDa and has 4 functional domains. As shown in Figure 2, the smallest HA fragment capable of interaction with CD44 is a hexasaccharide. Error! Reference source not found.

**Figure 2**: Representation of CD44 interaction with HA. As shown, the smallest HA fragment capable of binding the extracellular domain is a hexasaccharide.

Important to this work is the role which CD44 plays in the internalization and degradation of HA. HA is mainly cleared through the lymph or local catabolism through internalization and degradation in lysosomes. It is this internalization by CD44 of HA fragments which this work hopes to harness to internalize the bPEI-HA gene delivery vector.
The work presented in the next chapter summarizes the findings on this point and suggests that this cellular machinery can in fact be harnessed.

**Bone and Cartilage Development**

In order to fully understand the goals of this project, a short discussion is necessary on the role and purpose of transcription factors in cell biology. Transcription factors are the proteins responsible for correctly positioning RNA polymerase at a promoter sequence on the DNA strand, aiding in pulling apart the DNA to initiate transcription, and releasing the polymerase from the promoter region once transcription has begun. Thus, the transcription factors can decide which and how much and which DNA will be transcribed to RNA for protein production by deciding when and where transcription will begin.

With this information in mind, it is easy to see that transcription factors are very high upstream in the protein expression chain and can induce the production of a number of diverse downstream proteins for the length of the transcription factor’s life within the nucleus. The advantage of transcription factors is that a relatively small amount is capable of inducing expression of a number of downstream molecules in physiologically relevant amounts. In our case, developmental transcription factors will induce the transcription of a number of different downstream proteins found in the final chondrocyte and osteoblast phenotypes. The correct expression of these molecules without the use of transcription factors would normally necessitate an extended regimen of relatively higher dosing with a number of other growth factors. This research will focus on the delivery of the genes encoding the SOX trio (SOX5, SOX6, and SOX9) to achieve chondrogenesis and RUNX2 to achieve osteogenesis.
Development of Articular Cartilage and Subchondral Bone

To conceptualize the role of the developmental transcription factors in this research, an understanding of how bones and articular cartilage develop is needed. Development of the limbs is a complex process initiated by the budding of mesenchymal cells in the embryo which occurs after the mesoderm becomes the lateral plate and somatic layers and after dorsal and ventral orientation has been decided\textsuperscript{46}. At this point, the different layers of the mesenchyme have already committed to a lineage. Most importantly, the lateral plate layer becomes the cartilage and bone of the new limb. Development continues, and the first macro-event that can be observed is the condensation of mesenchymal cells that will eventually form cartilaginous rods which take the shape and placement of the bones\textsuperscript{6, 46}. One interesting point is that these cartilage cells will not develop into the bone. Rather, they undergo differentiation and apoptosis to allow for bone ingrowth. During development, invading progenitor cells which are destined to become bone will actually differentiate into cartilage in the absence of capillary networks and blood flow, suggesting that these cells look to their environment for developmental cues\textsuperscript{6, 46}.

Normal bone development is regulated by a number of factors including bone morphogenetic proteins (BMPs), fibroblast growth factors (FGFs), Wnts, hedgehog proteins, insulin-like growth factors IGFs), and retinoids. All of these factors are integral in the proper development of a functional and symmetrical skeletal system in the developing body. Along with a number of hormones acting as paracrine factors, these endocrine elements develop mesenchyme into bone and cartilage. The first step in the development of cartilage is the condensation of the prechondrogenic cells. Next, cell-cell interactions within the condensation lead to chondroblast differentiation. This is characterized by changes in cell morphology from fibroblast-like cells to cells more spherical in shape, a significant increase in volume of up to 10
times, and the secretion of aggrecan and collagen type II\textsuperscript{6, 47, 48}. This process is driven by transcriptional factors including the SOX trio, an integral part of this work. This developing region grows through the proliferation of chondrocytes and through their excretion of extracellular matrix. For this work, this is the developmental stage which would be ideal to recreate, as it would allow for the repopulation and extracellular regeneration of a defect site. Eventually, the cells at the innermost portion of this condensation stop dividing and become hypertrophic. These cells secrete type X collagen and become the site of bone growth and regulation. It is these cells which are the developmental source of bone because they signal for the ingrowth of vessels, the formation of a bone collar, and the mineralization of the extracellular matrix around them. Once this scaffold for the formation of bone has been created, these cells undergo apoptosis which allows for the ingrowth of bone cells and the finalization of the area to the bone phenotype.

**Transcription Factors**

**SOX Trio (SOX5, SOX6, and SOX9)**

The SOX trio has been shown to be able to induce chondrogenesis in multiple cell types\textsuperscript{49}. This has been definitively proven through the analysis of the addition or subtraction of the many factors considered necessary for chondrogenesis\textsuperscript{49}. Specifically, these factors include SOX5, SOX6, SOX9, IGF-1, FGF-2, Ihh, BMP-2, TGFβ, and Wnt. A recent study has shown that no single factor was capable of inducing the chondrogenic phenotype, but that when the SOX trio was cultured with chondrogenic media containing BMP-2 and TGFβ, the best results were achieved\textsuperscript{49}. Also, of the SOX trio, only SOX9 is capable of inducing the production of the rest of the SOX trio, proving its dominant role\textsuperscript{49}.  

34
SOX9

SOX9 is arguably the most important of the SOX trio, with SOX5 and SOX6 playing mainly supporting roles, because it is key to initiating prechondrogenic condensation in embryos and the differentiation of MSCs down the chondrogenic pathway\textsuperscript{6}. SOX9 is present at the initial differentiation of MSCs into prechondrocytes and remains expressed until the chondrocytes differentiate into hypertrophic chondrocytes\textsuperscript{6}. This information is important because it suggests that SOX9 is responsible for maintaining the prehypertrophic chondrocyte differentiation state. One significant piece of evidence which points towards the importance of SOX9 in chondrogenesis is the condition known as campomelic dysplasia in which a deficiency in SOX9 expression in an embryo results in impaired bone development\textsuperscript{6, 50, 51}.

Evidence in the literature suggests that SOX9 is a master regulator and is needed for correct expression of other important transcription factors during development, including SOX5, SOX6, and RUNX2\textsuperscript{50}. One interesting point that must be included with this statement is the fact that SOX9 should be removed after mesenchymal condensations have occurred for RUNX2 and osteoblast differentiation to occur correctly\textsuperscript{50}. This detail can be used to speculate that SOX9 could be used to create osteochondroprogenitor cells during the mesenchymal condensations. Studies have shown that during development, if SOX9 is removed, many other patterning factors remain present\textsuperscript{50}. This demonstrates that SOX9 is mainly a differentiation factor rather than a developmental patterning factor. Additionally, work has shown that SOX9 is also responsible for controlling *noggin* and *chordin* which protect cells from apoptosis during digit development\textsuperscript{50}.

SOX9 is an important cartilage transcription factor necessary to maintain the correct cartilage phenotype throughout the life of an individual. It has been shown that levels of SOX9
generally decrease with age and degeneration\textsuperscript{52}. It is possible to speculate that the loss of SOX9 over time is at least partially responsible for the degeneration and loss of the chondrocyte phenotype found in the cartilage of older patients. This constant availability of SOX9 needed to maintain cartilage is one of the main therapeutic advantages of a system capable of extended expression of SOX9.

\textit{SOX5 and SOX6}

SOX5, SOX6, and SOX9 have been found to work better when used together rather than as individual treatments. SOX5 and SOX6 are present at the same point in differentiation as SOX9 and can help amplify its chondrogenic effects. Though these two transcription factors are responsible for promoting SOX9 activity, they do not have any effect on the expression levels of the factor. As mentioned before, when SOX5 and SOX6 are removed, SOX9 is still present at normal levels. However, when SOX9 is removed, SOX5 and SOX6 are no longer found\textsuperscript{6, 51}. In development, these two transcription factors are not needed to create mesenchymal condensations, but rather work with SOX9 to drive chondrogenesis as well as collagen II production in native development\textsuperscript{6}. In summary, these two factors are needed with SOX9 to promote the appropriate production of extracellular matrix proteins and tissue, which is ultimately the goal of this project. Work has shown that these three transcription factors are sufficient to make non-chondrogenic cells express chondrogenic markers\textsuperscript{6}.

\textbf{RUNX2 (Cbfa1)}

RUNX2, also known as Cbfa1, is another transcription factor important in the development of the skeletal system. This factor is a member of the Runt-domain family of transcription factors and specifically drives the differentiation of osteoblasts\textsuperscript{51}. In the absence of RUNX2, there is no development of endochondral or intramembranous bone formation while an
increase in its dosage level will lead to faster bone development and a decrease in RUNX2 dose will lead to slower development. This shows the direct impact of this transcription factor on osteogenesis in a developmental setting. This role in osteogenic differentiation is why this transcription factor was selected for application towards creating an effective bilayered scaffold.

**Controlled Release**

One aspect of polymeric gene therapy which often can be detrimental in a therapeutic application is the transient response seen with the use of the non-viral DNA complexes, due to the DNA’s non-incorporation into the host cell genome\(^{53}\). There are many situations in which a more extended presence of the therapeutic gene and resulting protein is desirable and necessary. For such cases requiring a more sustained expression, controlled release of polymer/DNA complexes that allows for continual transfection with DNA complexes over an extended period of time is one potential solution.

Controlled delivery of bioactive molecules is an important aspect of any tissue engineering project and applies to the work presented below. This work evolved from an initial iteration of controlled release from coaxial electrospun meshes, to release from PLGA microparticles, to the final study which releases material directly incorporated into OPF hydrogels. With this evolution in mind, the discussion of controlled release in this work will be limited to the methods used in this work - the release of genetic material from PLGA microparticles and from OPF hydrogels.
Release from PLGA Microparticles

Poly(DL-lactic-co-glycolic acid) (PLGA) is a biocompatible, biodegradable polymer widely used to create controlled release systems, especially microparticle based systems. PLGA degradation occurs as bulk degradation through hydrolysis into lactic acid and glycolic acid which are natural byproducts of the body's metabolism and can be cleared from the body. In the case of a PLGA microparticle system, during particle synthesis a number of different therapeutic agents and molecules can be entrapped within the PLGA particles and stored until the PLGA degrades sufficiently to release the molecules entrapped inside.

PLGA microparticle and nanoparticle systems similar to the one described in this work have been utilized in a number of applications involving genetic material, including vaccination and siRNA delivery for therapeutic goals. More pertinent to this work, several studies have examined the release of bPEI/nucleotide complexes from PLGA microparticles. These studies have shown that all sizes and types of nucleotides, ranging from siRNA to plasmid DNA, are capable of successful incorporation and release from PLGA systems. The novelty of this work lies not only in the potential application of these microparticles towards osteochondral regeneration, but also in the examination of how modifications to the bPEI affect its release and the overall characteristics of the system.

OPF Hydrogels and Their Release of Genetic Material

Oligo(polyethylene glycol fumarate) (OPF) was selected for the in vivo portion of this work due to its existing characterization with respect to gene delivery and osteochondral repair. OPF is formed through a condensation polymerization between PEG and fumaryl chloride resulting in a linear polyester. The resulting polymer is a polyethylene glycol (PEG) based hydrogel which has favorable biological properties, including being biocompatible, bioinert, and
non-immunogenic, as well as having inert degradation products\textsuperscript{69}. Unlike strictly PEG hydrogels, the addition of the fumarate ester groups allows OPF to degrade in an aqueous environment\textsuperscript{70}. Further, the mesh size of the hydrogels can be highly controlled by the molecular weight of the PEG chains incorporated into the polymer\textsuperscript{71}. Finally, relatively higher concentrations of fumarate ester groups within the polymer will lead to accelerated degradation of the hydrogel mesh in water. This polymer is ideal for the applications described in this work as it has been shown in previous work to be capable of supporting bone\textsuperscript{72} and cartilage\textsuperscript{73} tissue growth.

Several studies have explored the release of DNA from OPF hydrogels and generally characterized the system\textsuperscript{66-68}. These studies have explored release of plasmid DNA from OPF hydrogels both \textit{in vitro} and \textit{in vivo}, as well as directly from the hydrogel or from an incorporated carrier. OPF is attractive as a delivery vehicle for a number of reasons. First, it is a synthetic rather than natural polymer and therefore offers tunable properties such as mesh size, degradation rate, and mechanical properties. Additionally, it is attractive for use with plasmid DNA and other nucleotides because it can be crosslinked under physiological conditions without harsh processing that could degrade or injure the genetic material\textsuperscript{67}. The inherent hydrophilicity of the system and hydrogel fabrication process results in an ability to entrap large concentrations of polymer/DNA complexes at relatively high loading efficiencies\textsuperscript{66-68}. When incorporated directly into the hydrogel during fabrication, release of plasmid/polymer complexes was found to have a favorable profile lasting several weeks\textsuperscript{68}. Finally, OPF implants utilized \textit{in vivo} are generally encapsulated with fibrous tissue by the body rather than infiltrated with native cells\textsuperscript{69}. This has led to the incorporation of cells directly into the OPF hydrogel during fabrication in many regenerative strategies. The work presented here uses an alternative approach in which a
porogen is used to impart channels and voids which can be filled by the body to effectively integrate the implant with the native cells infiltrating the defect.
Chapter 3 - Engineering a Polymeric Gene Delivery Vector Based on Poly(ethylenimine) and Hyaluronic Acid

* This Chapter was published as: CJ Needham, AK Williams, SA Chew, FK Kasper, AG Mikos.

Engineering a polymeric gene delivery vector based on poly (ethylenimine) and hyaluronic acid.

Biomacromolecules 2012; 13 (5), 1429-1437

ABSTRACT

In this work, the effects of primary amines, ligand targeting, and overall charge on the effectiveness of branched poly(ethylenimine)-hyaluronic acid conjugate (bPEI-HA) zwitterionic gene delivery vectors are investigated. To elucidate the relative importance of each of these parameters, the zeta potential, cytotoxicity, and transfection efficiency were explored for a variety of formulations of bPEI-HA. It was found that the length of the hyaluronic acid (HA) oligosaccharide had the most significant effect on cytotoxicity and transfection efficiency with human mesenchymal stem cells. Test groups of bPEI incorporating HA with a length of 10 saccharides had significantly higher transfection efficiency (14.6±2.0%) and lower cytotoxicity than other formulations tested, with the cytotoxicity of the group containing the greatest mass of 10 saccharide showing similar results as the positive controls at the highest polymer concentration (100 μg/ml). Additionally, molar incorporation of HA, as opposed to the saccharide length and HA mass incorporation, had the greatest effect on zeta potential, but a minor effect on both cytotoxicity and transfection efficiency. This work demonstrates the relative importance of each of these tunable design criteria when creating a zwitterionic
polymeric gene delivery vector and provides useful specific information regarding the design of bPEI-HA gene delivery vectors.

INTRODUCTION

Therapeutic gene delivery is a developing field that offers an attractive alternative to the direct delivery of therapeutic proteins. Gene delivery opens the possibility of altering cellular protein expression to achieve a therapeutic or otherwise desired response, such as differentiation. The use of non-viral gene delivery vectors mitigates some issues generally associated with viral gene therapy, including limited gene insertion size, immune response, mutagenesis, and large scale production limitations.\textsuperscript{74, 75}

Branched poly(ethylenimine) (bPEI) has been shown to be a promising polymeric non-viral gene delivery vector capable of efficient transfection in a number of cell types and situations.\textsuperscript{14, 16} A positive aspect of bPEI with relation to the present work is the high density of amines and the resulting ease with which chemical alteration can be performed, providing the ability to tailor the polymer for specific applications. For example, in the specific application of tissue engineering, controlled release of gene delivery agents for tissue regeneration can be achieved by loading the gene delivery complexes into implanted carrier scaffolds. To be successful in controlled release, gene delivery systems used in these applications must be able to protect DNA, such that it remains viable through a number of diverse situations that could arise during the construction and loading of the therapeutic carrier scaffolds. Branched PEI systems have shown the capability to withstand multiple preparation methods and retain the viability of DNA for this and other applications.\textsuperscript{57, 76}

Although there are several general concerns with the use of bPEI, such as cytotoxicity, previous studies have shown that modification of bPEI can address some of these concerns while
improving the transfection efficiency of the vector. The incorporation of another molecule, especially a ligand capable of cellular interaction, into the bPEI delivery vector system can be one of the most effective methods of improving the performance of the vector. Several examples of incorporated ligands to allow the targeting of cellular receptors or processes include mannose and other sugars, folate, RGD peptides, uronic acids, and hyaluronic acid (HA). These previous efforts were designed so that the incorporated ligand would harness the native cellular machinery and increase the cellular uptake of the polymer/DNA complexes, thus increasing transfection efficiency.

Of the many modifications to the PEI system, HA has many promising characteristics that warrant its further use and investigation. HA is a linear non-sulfated glycosaminoglycan with a relatively short in vivo half life that is found in essentially every tissue and body fluid in humans. This polysaccharide is a long linear polymer consisting of repeats of the disaccharide β-1,4-glucaronic acid-β-1,3-N-acetyl-D-glucosamine and carries a significant negative charge at physiologic pH because of the carboxyl groups on the polysaccharide chain. Given its ubiquitous distribution, many cell types within the body, including the hMSCs used in this study, have hyaladherins including CD44, CD54, and CD168 designed to bind to HA on the cell surface. It is because of these favorable characteristics that the system investigated in this work utilizes the direct conjugation of HA to the bPEI polymer chain for gene delivery.

Previous work involving the modification of PEI with HA has yielded important information regarding the feasibility of creating a zwitterionic polymer capable of transfection. A wide range of MW of HA can be obtained, due to the linear nature of the polymer and the availability of degradation enzymes, and both large and small MW varieties have been investigated for use in a bPEI-HA conjugate system. Generally, the previous approached can be
broken down into two groups. One approach used to create these conjugates focuses on higher MW HA. 77, 78, 82, 83 This approach to conjugation involves activating the carboxyl groups on the HA chain and utilizing these groups to conjugate the amines of bPEI to the large MW HA via an amide bond. In this work involving larger MW HA, the range of bPEI:HA mass ratios explored involved more bPEI than HA within the system. The other main approach to creating bPEI-HA conjugates utilized much smaller HA and a synthesis method based on connecting the primary amines of the bPEI to one of the terminal ends of the linear HA chain via a reductive amination process. 22, 76 This work is the inverse of the large MW HA approach, in that it utilized more HA than bPEI as well as attached the HA to a bPEI backbone rather than bPEI to a HA backbone. The work presented in this manuscript will continue the development of the latter approach and fully define and test the design parameters which affect the polymers’ efficacy as a transfection agent. By expanding the range of tested HA:bPEI synthesis ratios the work presented in this manuscript will lend greater understanding to both synthesis approached and will explore the various design parameters involved in the efficacy of these polymers with respect to cytotoxicity, transfection efficiency, and zeta potential.

Studies have shown that the negative charges and native nature of HA mitigate the high toxicity commonly associated with the high density of positive charges of bPEI, regardless of HA MW. The present work focuses on the use of relatively low MW HA, which will allow for a broad examination of the polymer conjugate system due to the ability to induce greater variation in amine concentration with less variation in HA weight incorporation. Additionally, HA association with bPEI/DNA complexes can positively alter the packing efficiency and transfection efficiency of the complexes. 35, 36 By combining HA and bPEI, the advantages of each can be used to generate a more efficient vehicle for cellular uptake and gene delivery. 22
However, the relative importance of each of the parameters in this system with regards to cytotoxicity, zeta potential, and transfection efficiency of the complexes are unclear.

The studies presented here focus on the design aspects of engineering a more effective gene delivery vector utilizing bPEI directly conjugated with HA. Specifically, the objective of the present work is to investigate the effects of HA oligosaccharide length and concentration within the bPEI-HA vector on its zeta potential, cytotoxicity, and its ability to transfect human mesenchymal stem cells (hMSCs) \textit{in vitro}. Additionally, by investigating the effects of the experimental parameters explored in this example system, the general mechanisms found in other systems could potentially be elucidated and better understood.

**EXPERIMENTAL PROCEDURES**

**Materials**

Chemicals used for the synthesis and purification of HA oligosaccharides, specifically sodium borate, sodium chloride, sodium cyanoborohydrate, sodium acetate, and ammonium acetate were purchased from Sigma-Aldrich (St. Louis, MO). Hyaluronidase was purchased from Worthington Labs (Lakewood, NJ). HA was purchased from LifeCore Biomedical (Chaska, MN). Branched PEI (MW=25,000) and polyacrylamide bead P2 desalting columns were purchased from Sigma-Aldrich (St. Louis, MO). Ultrapure 2,5 - dihydroxybenzoic acid (DHB) MALDI matrix was purchased from Protea Biosciences (Morgantown, WV). Human MSCs were obtained from the Texas A&M University Cell Distribution Center at the Texas A&M University HSC COM in Temple, Texas. The calcein assay kit was obtained from Molecular Probes (Carlsbad, CA). VivaSpin centrifuge dialysis membranes of 30,000 molecular weight cutoff (MWCO) were obtained from the Sartorius Corporation (Edgewood, NY). Anion exchange columns were obtained from GE Lifesciences (Piscataway, NJ). Cell culture materials
included α-MEM, glutamine, trypsin, and phosphate buffered saline (PBS) and were obtained from Gibco (Carlsbad, CA). Plasmid DNA (pDNA) encoding for enhanced green fluorescent protein (eGFP) with the cytomegalovirus (CMV) promoter (pCMV-eGFP, 4.7 kb, cat no. 6085-1) was obtained from Clontech (Palo Alto, CA).

**Degradation, Separation, and Characterization of HA Oligosaccharides**

HA oligosaccharides were obtained through enzymatic degradation of relatively high molecular weight (MW) HA using hyaluronidase through a previously described process. Briefly, a 100 ml solution of 100 mM NaAc and 150 mM NaCl was prepared. 1 g of HA was then added and allowed to dissolve overnight while stirring at 37°C. Once fully dissolved, 100 kilounits (KU) of hyaluronidase (Worthington Labs, Lakewood, NJ) were added to the solution and the reaction was allowed to incubate either to completion (overnight) or for 90 min, depending on the size of oligosaccharides desired. At the completion of the degradation time period, the solution was boiled for 10 min, centrifuged, and the supernatant collected to deactivate and remove the enzyme.

The resulting oligosaccharides in the supernatant were then separated using an anion exchange column (Mono Q 5/50 GL) in a fast protein liquid chromatography (FPLC) instrument. This separation was performed using a simple gradient that ran from 0% to 30% of a 1 M solution of NaCl over 90 min at a flow rate of 1 ml/min with fractions taken every 2 min. After collection, fractions at the same position from multiple runs were concentrated through lyophilization and direct combination of the resulting powder.

Characterization and confirmation of the HA oligosaccharide length was achieved utilizing \(^1\)H NMR and MALDI-TOF. \(^1\)H NMR spectra were obtained using a 400 MHz spectrophotometer (Bruker Avance 400 – Zurich, Switzerland) with deuterated water as a solvent.
and internal reference (δ=4.79). All spectra were recorded at room temperature and processed using MestRe-C software (MestReLab Research – S.L., Spain). MALDI-TOF experiments were performed using a MS Autoflex MALDI TOF-TOF instrument (Bruker Daltonics – Fremont, CA) with 2,5-dihydroxybenzoic acid (DHB) as a matrix. For mass spectrometry, each fraction was desalted using D-Salt Polyacrylamide 1800 Desalting Columns from Thermo Scientific (Oxford, IL) to reduce adducts in the system.

**UV Spectroscopy**

UV spectroscopy was used to determine the concentration of the HA fragments after separation. A concentration dependent peak absorbance shift was observed in the 200-250 nm wavelength range for HA. The relation between concentration and peak position was tested using 6.4 kDa HA and confirmed using 0.975 MDa and 1.59 MDa HA over a variety of HA concentrations, salt concentrations, and pH values in a Cary 5000 UV-Vis-NIR spectrophotometer (Agilent Technologies – Santa Clara, CA). Once established, this relation was used to directly determine the HA concentration in the fractions collected from the above mentioned HA purification process. HA oligomers with a length of 2 saccharides were observed to deviate from the established optical relationship and thus were desalted, lyophilized, and directly massed on an ultrasensitive scale (Mettler Toledo AX105, Columbus, OH) to ensure that the correct amounts were used in subsequent steps.

**Synthesis of bPEI-HA**

Synthesis of bPEI-HA was achieved utilizing a previously described reductive amination reaction. Briefly, HA and bPEI were added in the desired ratios to a 0.1 M sodium borate buffer in the presence of an excess of the reducing agent sodium cyanoborohydride. This mixture was held at 42°C for 120 hours to allow the reaction to reach the maximum possible
attachment of the HA chains to the primary amines of the bPEI. The resulting product was
dialyzed and resuspended with deionized water three times according to the manufacturer’s
protocol in a VivaSpin centrifuge dialysis tube with a 30,000 MWCO to remove salts and all
unreacted products. The recovered bPEI-HA was lyophilized, weighed, and used to complete the
ensuing studies described below. To verify the presence and ratios of bPEI and HA in the
resulting product, $^1$H NMR was performed at room temperature in the 400 MHz
spectrophotometer with deuterated water as a solvent and internal reference ($\delta=4.79$). All
spectra were recorded at room temperature and processed using MestRe-C software. By
integrating and comparing the peaks associated with either HA or bPEI within the polymers, the
ratio of bPEI to HA in each polymer was ascertained.

**Assembly of Polymer/DNA Complexes**

To form the polymer/DNA complexes, the procedures outlined previously were
followed.$^{22}$ Briefly, each of the bPEI-HA groups was dissolved in PBS at a concentration of 1
mg/ml and filtered through a 0.2 µm filter for sterilization. This solution was then brought to a
temperature of 37 °C and a pH of 7.4 and allowed to sit overnight. The following day, DNA
encoding for eGFP was separated into 50 µl aliquots for each experimental group, with each
aliquot containing the same amount of DNA. Each prepared bPEI or bPEI-HA polymer solution
was then added dropwise to the prepared DNA solution such that the N:P ratio was maintained at
7.5:1 for each case. Once the addition was complete, the samples were immediately vortexed,
centrifuged, and incubated for 2 hours at room temperature to allow for complete complexation.

**Zeta Potential and Dynamic Light Scattering**

The zeta potential of polymer/pDNA polyplexes was determined for each group at a
constant N:P ratio of 7.5:1, which has been determined to be optimal for bPEI-HA in previous
work. Size and zeta potential for each group was tested according to previously established procedures. Briefly, a Zen 3600 Zetasizer from Malvern Instruments (Worcestershire, U.K.) was used to measure the dynamic light scattering and electrophoretic mobility of the polyplexes at 25°C. Size was recorded and the zeta potential was calculated using the Smoluchowski equation and measured values. Zeta potential measurements were performed in 10 rounds of 10 samples, while DLS measurements were performed in 10 runs of 10 samples.

**Cytotoxicity Studies**

Human mesenchymal stem cells (hMSCs) were expanded and cultured according to the procedures established by Prockop et al and with the approval of the Rice IRB. All cells were passage 4 or lower when utilized in this work. To determine the cytotoxicity of each distinct group of bPEI-HA relative to each other and to unmodified bPEI, hMSCs were plated onto 96 well plates at a density of 40,000 cells/cm² and allowed to attach overnight. The cells were then exposed to bPEI or bPEI-HA in solution at one of three concentration levels of 10, 50, or 100 µg/ml for 24 hours. Polymer solutions were prepared by dissolution in α-MEM at the appropriate concentrations and filtration through a 0.2 µm filter for sterilization. After 24 hours of exposure, the solution was removed and the cells were rinsed twice with PBS and were analyzed for viability using a calcein assay for mammalian cells from Molecular Probes (Carlsbad, CA). Calcein was selected over other testing methods because of its similar reliability coupled with the ability to visualize the cytotoxicity through fluorescence. To complete the cytotoxicity assay, cells were incubated with 2 µM Calcein-AM for 30 min. For these studies, untreated hMSCs served as live controls, while hMSCs exposed to 90% ethanol for 15 min served as negative controls. Fluorescence of the Calcein-AM was measured for each group using a fluorescence microplate reader (FLx800 Bio-TEK Instruments) equipped with a 485/582
filter (excitation/emission) to measure calcein fluorescence. Live cell fraction was determined in the manner described by Temenoff et al.\textsuperscript{92} and six samples were tested for each group.

**Transfection Efficiency**

For transfection efficiency, hMSCs were expanded and plated onto 6 well plates at a density of 5,000 cells/cm\textsuperscript{2}. These cells were allowed to attach overnight in the presence of complete medium (500ml α-MEM, 100ml Fetal Bovine Serum (FBS), 6ml L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin). After attachment, the cell cycles were synchronized through incubation in α-MEM medium without the presence of serum for 30 hours; the doubling time of hMSCs.\textsuperscript{93} Though it was not anticipated that cell synchronization would have an effect on transfection efficiency, cell cycles were synchronized to mitigate potentially confounding factors. Once synchronized, the cell cycles were restarted by incubation in complete medium for 6 hours.

Transfection was then performed on the prepared cells with the above prepared complexes as previously described.\textsuperscript{22} Briefly, complexes were added dropwise to each test well to achieve a final DNA concentration of 50 µg/ml. The controls consisted of untreated cells and cells treated with DNA only. Each group was cultured for 72 hours after exposure to the complexes and then analyzed for transfection efficiency through flow cytometry. Six samples were tested for each group.

To prepare the samples for flow cytometry, cells were washed three times with PBS to remove any dead cells or debris. The remaining live cells were then trypsinized and collected. After collection, the cells were fixed through exposure to a chilled 4% formaldehyde solution for 1 hour. After fixation, the cells were again washed with PBS before being analyzed with a flow cytometer (Becton Dickenson FACS Scan) under high flow rate using the CellQuest Pro
software from BD Biosciences (San Jose, CA). PBS containing untreated cells was run through the cytometer to set the gates used to identify the cells on the output graph. The cytometer was set to identify green fluorescence and the total number of cells flowing through it. A limit of 5,000 cells was set for each group, and, in the cases of two of the bPEI only samples, where toxicity was too high, readings were terminated after three min with no new data points. Finally, to account for the population of native cells with rightward shift, markers were placed at 1% of the control samples.

**Statistics**

Statistical analysis was performed on the data collected for zeta potential, DLS, transfection efficiency, and cytotoxicity using ANOVA with a p value < 0.05. Post hoc analysis was performed via Tukey-Kramer HSD to identify statistical significance between each of the groups.

**RESULTS AND DISCUSSION**

**HA Degradation and Characterization**

HA degradation, characterization, and quantification were successfully performed according to the procedures outlined above. Degradation of larger HA chains by hyaluronidase occurs through hydrolysis of the endo-N-acetylhexosaminic bonds between the HA base saccharides and results in HA oligomers. Complete degradation (overnight) of HA results in 2 saccharide HA, while incomplete degradation results in a mixture of oligosaccharides of varying lengths. With incomplete degradation, a longer degradation time results in a broad distribution of HA oligomers, which has a relatively lower average mass. Previous work has shown that a 90 min degradation time resulted in the best distribution of HA oligosaccharides of differing lengths.
The oligosaccharide mixture generated by degradation was separated utilizing anion exchange chromatography, resulting in the purification of samples with average lengths of 10 and 16 saccharides, as determined by mass spectrometry. Each of these fractions was then successfully quantified for use in further studies by utilizing the relationship between peak position and HA mass shown in Figure 3. The identification of this relationship between peak absorbance wavelength and concentration allowed for the rapid identification of concentration without further processing the HA oligosaccharides, and associated sample loss. HA is extremely hydrophilic and the identification of this relationship could potentially provide another method to quantify HA that has not been completely dried.
This curve shows an exponential relation between the concentration and peak absorption wavelength.

Figure 3. Graph demonstrating the concentration dependent HA peak shift.

bPEI-HA Synthesis

<table>
<thead>
<tr>
<th>Group Name</th>
<th>Oligosaccharide Length (# of Saccharides)</th>
<th>Moles of HA per Mole of bPEI</th>
<th>Measured NMR Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low2</td>
<td>2</td>
<td>$4.4 \times 10^{-4}$</td>
<td>0.0285</td>
</tr>
<tr>
<td>Med2</td>
<td>2</td>
<td>$8.8 \times 10^{-4}$</td>
<td>0.0640</td>
</tr>
<tr>
<td>High2</td>
<td>2</td>
<td>$1.3 \times 10^{-3}$</td>
<td>0.1049</td>
</tr>
<tr>
<td>Low10</td>
<td>10</td>
<td>$4.4 \times 10^{-4}$</td>
<td>0.1661</td>
</tr>
<tr>
<td>Med10</td>
<td>10</td>
<td>$8.8 \times 10^{-4}$</td>
<td>0.3831</td>
</tr>
<tr>
<td>High10</td>
<td>10</td>
<td>$1.3 \times 10^{-3}$</td>
<td>0.6493</td>
</tr>
<tr>
<td>Low16</td>
<td>16</td>
<td>$4.4 \times 10^{-4}$</td>
<td>0.2666</td>
</tr>
<tr>
<td>Med16</td>
<td>16</td>
<td>$8.8 \times 10^{-4}$</td>
<td>0.6402</td>
</tr>
<tr>
<td>High16</td>
<td>16</td>
<td>$1.3 \times 10^{-3}$</td>
<td>1.0202</td>
</tr>
<tr>
<td>LowMix</td>
<td>2, 10, and 16 in a 1:1:1 Molar</td>
<td>$4.4 \times 10^{-4}$</td>
<td>0.2074</td>
</tr>
</tbody>
</table>
Table 2. Description of the bPEI-HA synthesis groups with respect to HA oligosaccharide type, molar incorporation, and NMR integration ratio.

<table>
<thead>
<tr>
<th>Mixture</th>
<th>HA oligosaccharide type</th>
<th>Molar Incorporation</th>
<th>NMR Integration Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>MedMix</td>
<td>2, 10, and 16 in a 1:1:1 Molar Mixture</td>
<td>8.8 x 10^{-4}</td>
<td>0.3472</td>
</tr>
<tr>
<td>HighMix</td>
<td>2, 10, and 16 in a 1:1:1 Molar Mixture</td>
<td>1.3 x 10^{-3}</td>
<td>0.5785</td>
</tr>
</tbody>
</table>

In this work, 12 distinct formulations of bPEI-HA conjugates, shown in Table 2, were synthesized and investigated. Through proper experimental design, these 12 groups allow for the relative importance of primary amines, overall net charge within the polymer, and ligand targeting through HA oligosaccharide length to be identified with respect to designing a cationic gene delivery vector. It is important to note that the secondary and tertiary amines of the bPEI are unchanged by the reaction mechanism employed and thus were not considered as a design characteristic in the present study. Four populations of HA oligosaccharides were tested; 2, 10, and 16 saccharides, in addition to a 1:1:1 molar mixture of the three sizes. The 10 saccharide population was chosen for its ability to interact with the ligands on the cell surface. The 2 saccharide population was selected because it represents complete degradation of the HA with hyaluronidase and is incapable of hyaladherin interaction. 16 saccharide HA was chosen as the largest population because of its relative ease of procurement compared to other larger oligosaccharides coupled with the fact that in the groups containing a mixture of HA, the average molecular weight of the mixture was very similar to the average molecular weight of the 10 saccharide groups. This similarity allows for these groups to be compared and conclusions to be drawn about the relative importance of the oligosaccharide length. For each of these oligosaccharide lengths, three molar levels of incorporation were examined: a low (4.4 x10^{-4}), medium (8.8 x10^{-4}), and high (13.2 x10^{-4}) number of moles of HA per mole of bPEI. These
incorporation values were chosen based on multiples of the amount used in previous work that established this vector.\textsuperscript{22} By utilizing these HA oligosaccharide populations and amounts, the relative importance of each design characteristic with respect to zeta potential, cytotoxicity, and transfection efficiency was effectively explored for these experimental groups. After synthesis and purification, each of the designed polymers was chemically verified using 1H NMR. Several representative spectra are shown in Figure 4, and as expected, when the overall amount of HA incorporated into the polymer increases, the peaks representing HA increase in intensity. For each NMR result, regions of the peaks representing the HA (\(\delta=4.0-3.1\)) in the polymer were integrated and compared to the integrated peaks representing the bPEI (\(\delta=3.1-2.5\)). This ratio was then plotted against the theoretical mass ratio for each polymer in order to verify each individual synthesis by comparing between synthesis groups. The resulting relationship, shown in Figure 5, correlated well between the groups with an \(R^2\) value of 0.9928, though it is important to note that the relationship between the theoretical mass ratio (HA/bPEI) used for synthesis and the measured NMR integration ratio (HA/bPEI) would be expected to differ slightly for a given group, considering small variation is the efficiency of conjugation. However, the relationship between the two ratios verified that each of the polymers used for further testing was synthesized and provided an easy check for the composition of the polymers.
These spectra were used to verify the relative ratios of bPEI and HA in each synthesized polymer.

**Figure 4. Representative $^1$H NMR spectra of bPEI-HA**
Ratios confirmed through comparing theoretical synthesis ratios with the measured 1H NMR ratios found from integration of the HA and bPEI peaks after synthesis.

**Figure 5. Confirmation of actual synthesized bPEI-HA ratios**

**Cytotoxicity**

One of the greatest hurdles generally encountered in the application of bPEI and other highly cationic polymers as gene delivery vectors is their high toxicity. It has been shown that by incorporating negatively charged molecules in a gene delivery system, the overall cytotoxicity can be decreased. In the case of bPEI, a relatively high concentration of positive charges associated with the nitrogen along the polymer backbone is thought to be responsible for the cytotoxicity of the vector. By conjugating a negatively charged polymer to
this backbone, some of the positive charge associated with these nitrogen groups can be effectively neutralized.

Cytotoxicity studies were performed on each of the polymer groups to identify the roles of the design parameters on the overall toxicity to hMSCs. Results from each group are presented as a ratio relative to the live controls run with each concentration, such that a value of 1 represents the live control. Although it is anticipated that the cytotoxicity of the complexes will differ from the cytotoxicity associated with the polymers alone, the polymers alone were explored in this case to provide a conservative measure of the cytotoxicity of the system that would be applicable regardless of the nucleotide type or size included in the system. By identifying the most important parameters with relation to cell survival, a gene delivery vector that maximizes transfection efficiency while minimizing cytotoxicity could be developed. The results of this cytotoxicity study are presented in Figure 6. As expected, pure bPEI was especially toxic to cells at all concentrations with a live fraction of only 0.058 ± 0.010 at the lowest tested concentration. By contrast, at the highest concentration, the group containing high amounts of 10 saccharide HA was not different from the live controls. Interestingly, the High10 group presented a live fraction value of greater than 1, which was unexpected but may reflect the propagation of error inherent in the methodology used to calculate the ratios presented. Collectively, these results clearly indicate that incorporation of HA into bPEI has the potential to significantly decrease cytotoxicity.
Cells were exposed to each group of bPEI-HA with concentrations of (A) 10 μg/ml, (B) 50 μg/ml, and (C) 100 μg/ml for 24 hours and tested using a Calcein assay. Error bars represent 1 standard deviation, and groups that share letters are not significantly different (p<0.05).

Figure 6. Cytotoxicity values for each bPEI-HA conjugate.
By examining how each of the groups performed at the lowest concentration (10 µg/ml), interesting conclusions can be drawn regarding the role of primary amines, HA oligosaccharide length, and overall charge in the cytotoxicity of the conjugates towards hMSCs. At 10 µg/ml, the Low2 and High2 groups do not possess significantly different cytotoxicity from pure bPEI. This result is expected, as only a small number of moles of HA are incorporated into the polymer backbone. Also, at 10 µg/ml, group Med2 is significantly different from raw bPEI and has similar cytotoxicity to the Low2, High2, and Low16 groups. Though important, the differences between bPEI and the Low2, Med2, and High2 groups are minor and reflect the fact that the Low2, Med2, and High2 groups only have small HA mass incorporation. When considered globally within this study, these results suggest that HA mass and negative charge incorporation have a greater impact than primary amine concentration in relation to cytotoxicity. As further evidence for this assertion, the cytotoxicities of the Med16 and HighMix groups are not statistically different. Though these groups have a relatively similar HA mass incorporation, they have very different concentrations of primary amines. These observations establish that the overall negative charge incorporation is more important than primary amine concentration within the polymers. This is expected as the cytotoxicity of bPEI is due to the positive charges interacting with and disrupting the cell membrane.53, 75

To elucidate the relative importance of the length of HA oligosaccharide with respect to primary amine incorporation and overall charge, numerous groups can be examined. Groups Med10 versus MedMix and High10 versus HighMix have essentially the same mass incorporation of HA into the polymer conjugates, but groups containing 10 saccharide HA have significantly lower cytotoxicity when compared to their counterparts containing a distribution of HA oligosaccharides. The highest overall viability was achieved with the High10 group, a high
level of 10 saccharide HA incorporation, but not the highest overall HA mass incorporation. This result displays the fact that the incorporation of HA in a 10 oligomer size range significantly decreases cytotoxicity and is more important with relation to cytotoxicity than HA mass incorporation, which may indicate that the polymers containing 10 saccharide HA are interacting directly with the hyaladherins on the cell surface instead of the cell membrane. Interaction with native cellular machinery could result in less cytotoxicity than disruption of the cell surface with charged molecules. Considering that the 16 saccharide HA can also interact with the hyaladherins, intramolecular interactions within the polymers due to the various lengths of the linear HA chain could be influencing the cytotoxicity of the polymers. These results demonstrate that the length of HA, or potentially any negatively charged polymer or ligand, is essential to decreasing cytotoxicity and should be taken into consideration when designing and synthesizing zwitterionic polymer conjugates as gene delivery vectors.

Zeta Potential and Dynamic Light Scattering (DLS)

In this work, an N:P ratio of 7.5:1 was selected and has been shown to be effective at achieving transfection. By maintaining a constant N:P ratio, a potentially confounding factor was removed in the present study, although future work could investigate the effect of this specific parameter on transfection efficiency and zeta potential. Complexes were assembled in 150 mM NaCl. While a higher salt concentration generally yields higher transfection percentages, an isotonic 150 mM NaCl solution was used in this work to mimic the conditions potentially found within the body. Each of the synthesized polymers was tested for zeta potential and DLS to determine their charge and size in solution. The results of this can be seen in Figure 7.
While some of the results contained high standard deviations, this could be the result of uncomplexed DNA and polymer within the solutions, as well as complex aggregation affecting the distribution of the data. The DLS data generally mirrored the trends found in the transfection efficiency and zeta potential, but did not present statistical significance. Specifically, the Low2, Med2, and High2 groups had the largest size and zeta potentials closest to 0 within the test groups, potentially suggesting ineffective complexation. The sizes of the remaining groups generally followed the trend within each HA type; specifically, that a lower zeta potential resulted in a larger complex size. One unexpected result within the DLS data was the large size observed for the bPEI only complexes, as it was expected that the complex size should be smaller than the bPEI-HA polymer complexes. The larger size observed with DLS for the bPEI complexes could reflect potential aggregation within the solution prior to testing, although aggregation was not directly assessed. By contrast, the zeta potential results were helpful in gaining a greater understanding of the system.

<table>
<thead>
<tr>
<th>Test Group</th>
<th>Average Complex Size (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low2</td>
<td>380.7 ± 84.2</td>
</tr>
<tr>
<td>Med2</td>
<td>696.6 ± 325.4</td>
</tr>
<tr>
<td>High2</td>
<td>341.7 ± 62.4</td>
</tr>
<tr>
<td>Low10</td>
<td>231.6 ± 78.4</td>
</tr>
<tr>
<td>Med10</td>
<td>340.8 ± 35.0</td>
</tr>
<tr>
<td>High10</td>
<td>283.2 ± 325.3</td>
</tr>
<tr>
<td>Low16</td>
<td>185.2 ± 2.6</td>
</tr>
<tr>
<td>Med16</td>
<td>195.6 ± 35.9</td>
</tr>
<tr>
<td>High16</td>
<td>172.2 ± 63.0</td>
</tr>
<tr>
<td>LowMix</td>
<td>234.1 ± 146.7</td>
</tr>
<tr>
<td>MedMix</td>
<td>337.3 ± 202.3</td>
</tr>
<tr>
<td>HighMix</td>
<td>143.0 ± 24.4</td>
</tr>
<tr>
<td>bPEI</td>
<td>911.2 ± 313.1</td>
</tr>
</tbody>
</table>

Results are presented as mean ± standard deviation.

Table 3. Complex size as measured by DLS for each test group of bPEI-HA.
Error bars represent 1 standard deviation with n=10, and groups that share letters are not statistically different (p<0.05).

Figure 7. Zeta potential for each test group of bPEI-HA.

As expected, the overall charge of bPEI/DNA complexes was positive due to the excessive positive charge relative to the negative charges along the DNA backbone. The data demonstrate that there is a point of HA mass incorporation for these bPEI-HA conjugates at which the net overall charge of the polymer/DNA complexes in solution becomes negative. The negative zeta potential in solution suggests that, when complexed, HA is present at the surface of the complexes. This is most likely due to the high hydrophilicity of the HA at the temperatures tested relative to bPEI and the fact that the open primary amines will associate with the negative
charges present in the complexes and the HA. Also, interestingly, the differences in zeta potential between the Low10, Med10, and High10 groups and the LowMix, MedMix, and HighMix groups are not significant among mass matched sets. This suggests that the complexes have a similar net charge in solution regardless of the HA oligosaccharide length that was conjugated to the bPEI backbone. This lends credence to the theory that ligands on the cell surface are being targeted and are responsible for the increased transfection efficiency and decreased cytotoxicity. In addition to this effect, an HA incorporation dependent charge difference can be seen among groups incorporating the same length of HA oligosaccharide. The data suggest that the medium level of HA incorporation results in the greatest net negative charge of polymer/DNA complexes. This is further confirmed by examining the High10, Med16, and HighMix groups. All three of these groups have a similar mass incorporation, but the Med16 group, which has fewer overall moles of HA present has a significantly more negative zeta potential. It is hypothesized that this effect is due to two competing forces within the polymers, namely primary amine concentration and overall charge. In the case of the lowest molar ratio of incorporation, the primary amine concentration is relatively high while the overall incorporation of negative charges (HA) is relatively low. This leads to a relatively more neutral zeta potential because the DNA is efficiently packed and the low negative charges are present on the surface. In the case of the medium level of HA, the packing efficiency is lower, but the interactions are still strong enough to be effective due to a significant amount of primary amines that remain available for interaction with the DNA backbone. The HA in these polymers is forced to the surface due to this packing combined with the native hydrophilicity of HA. In the case of high incorporation, interactions between the DNA and polymer are weaker due to
decreased amine availability, resulting in interactions within the zwitterionic polymers that decrease the negative charges present at the interface of the molecule and water.

**Transfection Efficiency**

Testing the overall transfection efficiency of each of these polymer groups allows the effects of each design parameter to be elucidated and a cohesive picture to form with respect to the potential effectiveness of these polymers. Each of the polymers was tested and the results are presented in Figure 8. The most effective transfection was achieved with the Low10 (12.15 ±1.46%), Med10 (13.38 ± 5.69%), High10 (14.58 ± 2.01%), Med16 (11.86 ± 0.77%), and MedMix groups (14.43 ± 0.85%).

Error bars represent 1 standard deviation with n=6, and groups that share letters are not significantly different (p<0.05).
HA oligosaccharide length is the major design factor that influences transfection efficiency. The results for the Low10 and High10 groups, when compared to the LowMix and HighMix groups, clearly indicate that the groups incorporating 10 saccharide HA yield significantly higher transfection than groups incorporating other lengths of HA. Though this could be an effect of selecting an HA polymer of a length that allows for the optimal interaction with the DNA or bPEI, this could be also be due to the potential for the 10 saccharide HA to interact with hyaladherin ligands on the cell surface. By harnessing the cell machinery already designed for the uptake of HA from the extracellular environment, cellular uptake could be improved with these polymers and transfection increased. It is important to note that the 16 saccharide HA should also be available to interact with the HA cell surface receptors, and this can be seen in the results of the Med16 group. The relatively low transfection efficiency observed for the High16 group could be due to the high amount of HA incorporation for this group, which could cause instability within the polymer/DNA complexes. The previous results from cytotoxicity and zeta potential could suggest that there is a ligand-mediated effect, but it is probable that the improved transfection efficiency is due to a combination of length and hyaladherin targeting. However, elucidation of the mechanism of interactions associated with the transfection observed with each group was beyond the focused aims on the present study.

In addition to these potential ligand interactions, there is a range of molar incorporation of HA conjugate that should be included into the system to generate more effective transfection. The groups incorporating a medium number of moles of HA into the bPEI backbone resulted in
significantly higher transfection in each group of HA investigated, with the exception of 10 saccharide HA. This shows that an appropriate balance must be realized within the molecules to allow for efficient cell association, complex uptake, and endosomal release. The zeta potential roughly mirrors the transfection efficiency and the rationale behind the results is similar. These results suggest that some of the HA is present on the surface of the complexes and is contributing to both the increased uptake and negative zeta potential. The medium HA incorporation ratio balances the primary amines in the system such that packing occurs but is not too tight to limit efficient unpacking and dissociation, and thus transfection.

Least important to transfection is the overall HA charge incorporation. The data from the Low2, Med2, and High2 group show that a low incorporation of HA mass results in significantly decreased transfection efficiency. On the other hand, once a threshold incorporation of HA mass is reached, further incorporation does not have a major effect on transfection. This effect is reflected in data from the High10, Med16, and HighMix groups. Specifically, while each of these groups has a similar mass incorporation, the transfection efficiency is far from uniform, with the HighMix group being significantly lower. It is hypothesized that this is because the conjugate selection is most important for association of the complexes with the cell surface and amine concentration is most important in endosomal buffering and escape, as has been indicated in the literature\textsuperscript{20}. These two major factors mask any effects that charge would have on the transfection efficiency.

It is important to note that the above conclusions and considerations were formed using plasmid DNA encoding for eGFP. It is hypothesized that in cases utilizing other types or sizes of nucleic acids, the results would be very similar. Because the vector was tested for cytotoxicity in the absence of nucleic acid, the trends and conclusions here should be conserved with the
application of this vector towards other lengths and types of nucleic acids. The identification of ligand effects in these results suggests that the polymers themselves, rather than the polymer/DNA complexes, are significantly different from each other and native bPEI with respect to their effects on cells. With this in mind, one could assume that the differences observed with these polymers would extend to other types and lengths of nucleotides. If cells lacking hyaladherins on their surface were to be targeted with these polymers, the results might not be as significant in the group that contained 10 saccharide HA. In these cases, the other conclusions that were drawn regarding primary amine concentration and overall charge incorporation should remain valid.

CONCLUSION

The results presented here demonstrate that the selection of HA oligosaccharide for incorporation into bPEI-HA polymers has the greatest effect on the transfection efficiency and cytotoxicity of bPEI-HA polymers. Specifically, use of 10 saccharide HA with these polymers significantly increases the transfection efficiency and decreases cytotoxicity when used with hMSCs. Also important in the system is the primary amine concentration of the polymer. A medium level of primary amines incorporated into the polymers yields higher transfection efficiency, while greater HA incorporation, and thus overall negative charge, lowers cytotoxicity. These results confirm that use of bPEI-HA yields improved gene delivery vector characteristics over bPEI alone and further illuminates the relative importance of primary amine concentration, overall charge incorporation, and HA oligosaccharide selection when designing these polymers.
Chapter 4 - Modulation of Polyplex Release from Biodegradable Microparticles through Poly(ethylenimine) Modification and Varying Loading Concentration

* This Chapter was published as: CJ Needham, SR Shah, PM Mountziaris, FK Kasper, AG Mikos. Modulation of Polyplex Release from Biodegradable Microparticles through Poly (ethylenimine) Modification and Varying Loading Concentration. Pharmaceutical research 2014; 31 (1), 77-85

ABSTRACT

This work investigates the effects of hyaluronic acid (HA) conjugated onto branched poly(ethylenimine) (bPEI) and varying loading concentrations of these polymers complexed with DNA on their release from poly(DL-lactic-co-glycolic acid) (PLGA) microparticles and the transfection of target cells. To examine the effect of alteration of the gene delivery polymer on the system, we observed the morphology, size, loading efficiency, polymer and DNA release, and the transfection efficiency for the microparticles formed with three internal phase loading concentrations during microparticle formation. Addition of HA to this vector allowed for increased loading concentration within these systems and significantly altered release kinetics without changing the morphology of the particles. The incorporation of HA onto the bPEI backbone significantly increased the transfection efficiency of the complexes released from the corresponding microparticle formulation. The results show that the modification of bPEI with HA and the concentration of loaded polymer/DNA complexes can significantly alter the entrapment and release profiles from PLGA microparticles. This is significant in that it offers
insight into the effects of modification of gene delivery vectors on a controlled release system designed to achieve a sustained therapeutic response.

INTRODUCTION

Polymeric gene delivery is a growing and promising area of biomedical research because it allows the direct alteration of protein expression within native or introduced cells to achieve a desired therapeutic response\(^{53}\). By inserting nucleic acids into the cell directly, protein and other nucleic acid targets which might otherwise be impossible to utilize can be used as therapeutic agents or vaccines\(^{17, 60, 61}\). While there are many benefits to utilizing this approach, one aspect that could be considered a drawback is the transient response seen with the application of the non-viral DNA complexes, due to the DNA’s non-incorporation into the host cell genome\(^{53}\).

There are many applications in which a more prolonged expression is desirable and necessary, such as the prolonged exposure to a transcription factor to more effectively influence progenitor cell differentiation. For such cases requiring a more sustained expression, controlled release that allows for sustained transfection with DNA complexes over an extended period of time is one potential solution.

One polymeric gene delivery agent which has been thoroughly investigated is branched poly(ethylenimine) (bPEI). While this polymer has been shown to be a relatively effective polymer for gene delivery, it also possesses certain negative characteristics which limit its potential use, especially cytotoxicity\(^{14, 16, 75}\). Alterations to this polymer by pairing hyaluronic acid (HA) with bPEI, especially by forming bPEI-HA through covalently linking the two, have been shown to decrease the cytotoxicity of the resulting polymer while significantly increasing the transfection efficiency\(^{22, 77, 78, 82, 83, 96}\). In this conjugate polymer, the negative charges within
the HA mitigate the positive charges associated with bPEI to decrease cytotoxicity. Further, incorporation of specific oligosaccharides of HA can increase transfection efficiency due to intramolecular interactions within the complexes, as well as by potentially allowing association with the hyaladherins on the cell surface\textsuperscript{96}.

While extensive work has been vested in utilizing poly(DL-lactic-co-glycolic acid) (PLGA)/cationic polymers as a complexation agent for vaccination and siRNA delivery\textsuperscript{54, 55}, this work will focus on the use of PLGA microparticles as a controlled release source for polymer/plasmid DNA complexes. Numerous studies have explored the release of bPEI/nucleotide complexes from PLGA microparticles\textsuperscript{57, 58, 60-64, 97-99}. These studies have investigated the effect of bPEI’s inclusion in systems containing nucleotides ranging from siRNA\textsuperscript{56-58} to plasmid DNA\textsuperscript{59-64}. However, limited studies have examined the effect of alterations to the bPEI structure itself on the system other than studying the differences between branched and linear PEI and copolymerization with the polymers of the microparticles\textsuperscript{59}. This work was motivated by the need to understand how alterations within polymers used for gene delivery affect their release from microparticles, which will facilitate more effective control over the design of these polymers for efficient loading and delivery to target cells. This angle of research is especially important when polymers designed for cell targeting are being evaluated.

The studies presented in this manuscript seek to understand how alteration of bPEI with HA oligosaccharides affects the characteristics of PLGA microparticle systems encapsulating these polymers complexed with plasmid DNA. Specifically, this work seeks to compare the morphology, entrapment efficiency, release, and transfection efficiency of PLGA microparticles containing different concentrations of DNA only, bPEI/DNA complexes, and bPEI-HA/DNA complexes. By understanding how the incorporation of HA into bPEI affects controlled release,
general conclusions on the use of other alterations to the bPEI system in PLGA microparticles can be elucidated and better understood.

**EXPERIMENTAL PROCEDURES**

**Materials**

Sodium borate, sodium chloride, and sodium cyanoborohydrate used for the synthesis and purification of bPEI-HA were purchased from Sigma-Aldrich (St. Louis, MO). HA was purchased from LifeCore Biomedical (Chaska, MN). VivaSpin centrifuge dialysis membranes of 30,000 molecular weight cutoff (MWCO) were purchased from the Sartorius Corporation (Edgewood, NY) and anion exchange columns were bought from GE Lifesciences (Piscataway, NJ). Poly(DL-lactic-co-glycolic acid) of 50-50 copolymer ratio was purchased from Lakeshore Biomaterials (Birmingham, AL). Branched PEI (M<sub>w</sub>=25,000) was obtained from Sigma-Aldrich (St. Louis, MO). All cell culture was performed with CRL-1764 (ATCC, Manassas, VA) rat fibroblast cells. The cell culture materials: α-MEM, glutamine, trypsin, and phosphate buffered saline (PBS), were obtained from Gibco (Carlsbad, CA). Plasmid DNA (pDNA) encoding for enhanced green fluorescent protein (eGFP) with the cytomegalovirus (CMV) promoter (pCMV-eGFP, 4.7 kb, cat no. 6085-1) was obtained from Clontech (Palo Alto, CA).

**Synthesis of bPEI-HA**

Synthesis of bPEI-HA was achieved utilizing a previously described reductive amination reaction<sup>22, 96</sup>. Briefly, HA and bPEI were added in a 2:1 w:w ratio to a 0.1 M sodium borate buffer in the presence of an excess of the reducing agent sodium cyanoborohydrate. This mixture was held at a temperature of 42°C for 120 hours to allow the reaction to fully complete.
The resulting product was dialyzed with deionized water three times in a VivaSpin centrifuge
dialysis tube with a 30,000 MWCO, according to the manufacturer’s protocol, to remove salts
and all unreacted products. The recovered bPEI-HA was lyophilized, weighed, and used to
complete the ensuing studies described below. To verify the presence and ratios of bPEI and HA
in the reaction product, $^1$H NMR was performed at room temperature in a Bruker 400 MHz
NMR with deuterated water as a solvent and internal reference ($\delta=4.79$). All NMR spectra were
processed using MestRe-C software according to a previously established protocol$^{96}$.

Assembly of Polymer/DNA Complexes

In order to correctly track the release of bPEI and bPEI-HA, each was separately tagged
with a rhodamine tagging kit (Thermo Scientific, Rockford, IL) according to the manufacturer’s
instructions to allow fluorescent detection upon release as previously reported$^{57}$. The product
was then dialyzed against an excess of ultrapure (type 1) water (Super-Q Water Purification
System, EMD Millipore, Billerica, MA) water for 72 hours, lyophilized, and its mass was
quantified on a scale. To form the polymer/DNA complexes, procedures outlined previously
were followed$^{22, 96}$. Briefly, rhodamine tagged bPEI-HA or bPEI (r-bPEI-HA and r-bPEI
respectively) was dissolved in PBS at a concentration of 5 mg/ml and 2 mg/ml respectively and
filtered through a 0.2 µm filter for sterilization. This solution was then brought to a temperature
of 37 °C and allowed to sit overnight to dissolve. Once dissolved, each polymer was frozen until
use in complex formation. Throughout this and subsequent processes, precautions were taken to
avoid rhodamine quenching by light.

To form the internal aqueous phase for microparticle formation, the DNA needed for
each synthesis was added to a water solution large enough such that the final volume of the
internal phase after addition of the polymer would be a constant 60 µl in every group. Then,
tagged bPEI or bPEI-HA polymer solution at room temperature was added dropwise to the prepared DNA solution such that an N:P ratio was maintained at 7.5:1 for each group. Once mixed, the samples were immediately vortexed, briefly centrifuged, and incubated for 30 min at room temperature to allow for complete complexation. The final solution was then used for microparticle loading or release analysis.

**Microparticle Preparation**

Microparticles were prepared using a 5\% w/w blend of poly(ethylene glycol) (PEG) in PLGA. This blend was dissolved in dichloromethane at a concentration of 250 mg/ml and microparticles were created using a water-in-oil-in-water double emulsion technique as previously described \(^{100, 101}\). Briefly, the bPEI-HA/DNA complexes were formed as described above for loading into the microparticles. For all groups the internal phase was kept to a constant 60 \(\mu\)l. After incubation at room temperature for 30 min to allow for complete complexation, this 60 \(\mu\)l internal phase was added to the dissolved PLGA/PEG blend and vigorously vortexed for 1 min to form an emulsion. 2.5 ml of 0.3\% PVA solution was then added to the emulsion and vortexed for 1 min. Once complete, the resulting solution was added to 200 ml of 1\% isopropyl alcohol, 0.15\% PVA solution stirring at 800 rpm. The microparticles were allowed to stir for 4 hours to allow for complete removal of the methylene chloride from the particles.

After the 4 hour solvent evaporation period was complete, the microparticles were removed from the stir plate and strained through a 300 \(\mu\)m mesh to remove larger particles. Once strained, the particles were centrifuged and rinsed 3 times with ultrapure (type 1) water (Super-Q Water Purification System, EMD Millipore, Billerica, MA) to remove any remaining components of the external phase. Once washed, the microparticles were lyophilized for at least
24 hours to completely dry them. Upon removal from the lyophilizer, all samples were purged with nitrogen and stored in a freezer at -20°C until further use.

**Microparticle Characterization**

Microparticle morphology was observed utilizing scanning electron microscopy (SEM) and size was determined through the use of a Multisizer3 Coulter Counter (Beckman Coulter, Brea, CA) with 3 samples of 2500 particles each. For SEM analysis all samples were coated with 20 nm of gold on a Denton Desk V Sputter system (Denton Vacuum, Mooresville, NJ) and examined under a 30 kV beam on a FEI Quanta 400 ESEM FEG (FEI, Hillsboro, OR).

**Entrapment Efficiency**

To discover the relative entrapment of bPEI, bPEI-HA, and DNA at each loading concentration, each component was examined individually within the same batch of microparticles. Briefly, 15 mg from each microparticle preparation group was dissolved in 0.5 ml of dichloromethane for 30 min. Once dissolved, the entrapped polymer and DNA were extracted with 1 ml of nuclease free TE buffer as previously described through vortexing for 1 min and centrifuging at 11,000 X g for 1 min to completely separate the oil and water layers. Once the extraction was complete, r-bPEI and r-bPEI-HA were fluorescently detected using a plate reader while DNA was detected as described below.

**Polyplex Dissociation and Detection**

Solution containing polyplexes of polymer and DNA were analyzed utilizing an adaptation of a previously described method of complex dissociation for DNA detection. Briefly, aliquots of sample were thawed if necessary and vortexed before use in the assay. Once agitated, 80 µl of sample was added to the wells of an opaque 96 well plate. Each sample was run in triplicate and fresh standards consisting of freshly prepared DNA and polymer
(corresponding to the polymer used for each sample’s release) were prepared at an N:P ratio of 7.5:1 to be used as a control for each plate. Once prepared, samples were analyzed for rhodamine concentration in a plate reader with excitation/emission wavelengths of 530/575. Once the rhodamine, and thus polymer concentration, was recorded, 160 µl of TE buffer at a pH of 12 and containing 0.5 v/v% PicoGreen dye were added to each sample well. The plate was incubated for 5 min on a shaker table at 60 rpm and then immediately analyzed in a plate reader with excitation/emission wavelengths of 485/530. This analysis was used because it enabled direct correlation between polymer detected and DNA detected for each sample and was used in all cases, except for DNA only release. For groups incorporating DNA only, a PicoGreen assay was performed according to the manufacturer’s protocol as polyplex dissociation was not necessary.

**In Vitro Release**

*In vitro* release was performed in nuclease free PBS. Three samples of 20 mg of microparticles from each group were measured and placed in 1.5 ml nuclease free centrifuge tubes. 0.5 ml of nuclease free PBS were then added to the centrifuge tubes and they were placed on a shaker table at 90 rpm in a warm room set at 37°C. At 6, 12, and 24 hours and 2, 3, 4, 7, 11, 14, 18, 21, 25, and 28 days, the particles were centrifuged at 2,000 X g for 2 min and the supernatant was collected. Following collection, new PBS was added, the microparticles were resuspended, and the samples were returned to the shaker table. Release samples were then stored at -20°C until the release solutions were analyzed as described in the polyplex dissociation and detection section. Release results were broken into 4 distinct phases for analysis and interpretation: 0-24 hours, 1-3 days, 3-18 days, and 18-28 days.
Transfection Efficiency

The released material from each test group was assessed for its capability to achieve effective transfection. To prepare solutions for analysis, dry microparticles were suspended in PBS at a concentration of 50 mg/ml. These samples were placed on a shaker table at 90 rpm in a warm room held at 37°C. After 1, 2, 3, and 4 weeks, the supernatant was removed from each group and immediately used for transfection. All release samples were handled such that sterility of the samples was maintained.

CRL1764 rat fibroblast cells were seeded onto 12 well plates at a concentration of 20,000 cells per well. After 12 hours for attachment, 0.2 ml of release supernatant (corresponding to the release from 10 mg/ml of microparticles) were added to each well with 0.3 ml of serum free media. After 12 hours of exposure, 0.5 ml of complete medium were added to each well. 72 hours after initial supernatant addition, cells were lifted with 0.05% trypsin and fixed in formalin. The fixed cells were then run through a flow cytometer (Becton Dickinson FACS Scan, Franklin Lakes, NJ) under high flow rate using the CellQuest Pro software from BD Biosciences to assess transfection efficiency. Controls corresponding to fluorescence of 1% of cells treated with supernatant from blank PLGA microparticles were first run through the cytometer to set the criteria to determine transfection efficiency. Finally, a detection limit of 2000 cells was set for each group in the cytometer.

Statistics

Statistical analysis was performed on the data collected for microparticle size, encapsulation efficiency, release characteristics, and transfection efficiency using two-way ANOVA with a p value < 0.05. Post hoc analysis was performed via Tukey-Kramer HSD to
identify statistical significance (p<0.05) between each of the groups. All data are presented as mean ± standard deviation and number of replicates is noted in each case.
RESULTS

Each of the microparticle formulations had a diameter between 12.8 and 18.3 μm and no statistical significance between any of the groups was identified. Particle morphology was investigated with SEM and no significant differences were observed between each particle formulation. Representative SEM images for the medium loading concentration for each case are shown in Figure 9. Entrapment efficiency was quantified for each of the experimental groups and is shown in Table 4. The highest DNA entrapment was found in the DNA only low group with an entrapment of 65.6 ± 7.1%, while the lowest entrapment was in the bPEI High loading group, with a value of 2.5 ± 4.4%. This same group also had the lowest polymer entrapment, with 2.7 ± 1.0%, while the bPEI-HA Low group had the highest entrapment with 63.7 ± 2.1%.

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>Size (μm)</th>
<th>DNA Entrapment Efficiency (% of Loaded)</th>
<th>Transfection Polymer Entrapment Efficiency (% of Loaded)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA Low</td>
<td>15.8 ± 11.1</td>
<td>65.5 ± 7.1&lt;sup&gt;A&lt;/sup&gt;</td>
<td>-</td>
</tr>
<tr>
<td>DNA Middle</td>
<td>16.6 ± 10.3</td>
<td>58.0 ± 2.4&lt;sup&gt;A,B&lt;/sup&gt;</td>
<td>-</td>
</tr>
<tr>
<td>DNA High</td>
<td>15.0 ± 9.6</td>
<td>43.3 ± 9.1&lt;sup&gt;B,C&lt;/sup&gt;</td>
<td>-</td>
</tr>
<tr>
<td>bPEI Low</td>
<td>18.3 ± 12.9</td>
<td>25.5 ± 6.7&lt;sup&gt;C&lt;/sup&gt;</td>
<td>45.2 ± 8.7&lt;sup&gt;E&lt;/sup&gt;</td>
</tr>
<tr>
<td>bPEI Middle</td>
<td>12.8 ± 9.8</td>
<td>28.9 ± 2.5&lt;sup&gt;C&lt;/sup&gt;</td>
<td>24.6 ± 8.2&lt;sup&gt;G&lt;/sup&gt;</td>
</tr>
<tr>
<td>bPEI High</td>
<td>17.9 ± 14.6</td>
<td>2.5 ± 4.4&lt;sup&gt;D&lt;/sup&gt;</td>
<td>2.7 ± 1.0&lt;sup&gt;H&lt;/sup&gt;</td>
</tr>
<tr>
<td>bPEI-HA Low</td>
<td>13.8 ± 9.4</td>
<td>61.8 ± 11.6&lt;sup&gt;A,B&lt;/sup&gt;</td>
<td>63.7 ± 2.1&lt;sup&gt;E&lt;/sup&gt;</td>
</tr>
<tr>
<td>bPEI-HA Middle</td>
<td>13.9 ± 9.3</td>
<td>61.8 ± 0.7&lt;sup&gt;A,B&lt;/sup&gt;</td>
<td>61.4 ± 11.2&lt;sup&gt;E,F&lt;/sup&gt;</td>
</tr>
<tr>
<td>bPEI-HA High</td>
<td>14.5 ± 9.7</td>
<td>50.7 ± 10.2&lt;sup&gt;A,H&lt;/sup&gt;</td>
<td>43.2 ± 3.4&lt;sup&gt;F&lt;/sup&gt;</td>
</tr>
<tr>
<td>Blank</td>
<td>15.8 ± 9.1</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Microparticles prepared by drying at room temperature at 800 rpm in a 400 ml beaker for 4 hours. Groups containing the same letter are not significantly different from each other and all data are presented as average ± standard deviation for n=3.

Table 4: Size and entrapment efficiency of DNA and polymer for all microparticle test groups.
A) DNA Middle, B) bPEI Middle, and C) bPEI-HA Middle test formulations. Scale bar in lower right corner of (a) indicates 100 μm and applies to all panels.

**Figure 9:** Representative SEM images illustrating morphology of particles.
Some statistical differences were observed within the 9 groups in terms of entrapment efficiency. The DNA and polymer entrapment can each separately be broken into four groups, marked A-D in Table 4, within which test formulations in each group were not statistically significant from each other. Specifically, as shown in Table 4, the DNA entrapment of groups with unmodified bPEI (bPEI Low, bPEI Middle, bPEI High) was significantly lower than all other groups. There was no statistical significance between the DNA entrapment of any of the bPEI-HA groups (bPEI-HA Low, bPEI-HA Middle, bPEI-HA High), but these values were all significantly higher than the loading of the bPEI complex groups (bPEI Low, bPEI Middle, bPEI High).

In terms of polymer loading, the bPEI Middle and High loading groups were significantly different from all other groups, including each other. Both of these groups had much lower polymer loading than all other groups, with the polymer loading in the bPEI High groups being the lowest statistically. This extremely low incorporation was likely due to macroscopically observable aggregation of the polymer/DNA complexes to the point that they could not be incorporated into the particles. This led to the exclusion of the bPEI High group from the release and transfection efficiency experiments. Finally, the bPEI-HA high and low loading groups had higher polymer entrapment efficiencies, with a statistically significant difference between bPEI-HA High and Low.

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>Phase 1 (0-24 Hours) (% of total encapsulated per day)</th>
<th>Phase 2 (1-3 Days) (% of total encapsulated per day)</th>
<th>Phase 3 (3-18 Days) (% of total encapsulated per day)</th>
<th>Phase 4 (18-28 Days) (% of total encapsulated per day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA Low</td>
<td>14.6±2.4%&lt;sup&gt;A&lt;/sup&gt;±&lt;sup&gt;B&lt;/sup&gt;</td>
<td>1.8±0.0%&lt;sup&gt;A&lt;/sup&gt;</td>
<td>0.6±0.1%&lt;sup&gt;A&lt;/sup&gt;</td>
<td>0.3±0.1%&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td>DNA Middle</td>
<td>14.3±0.6%&lt;sup&gt;A&lt;/sup&gt;±&lt;sup&gt;B&lt;/sup&gt;</td>
<td>1.7±0.1%&lt;sup&gt;A&lt;/sup&gt;</td>
<td>0.7±0.0%&lt;sup&gt;A&lt;/sup&gt;</td>
<td>0.1±0.0%&lt;sup&gt;B&lt;/sup&gt;</td>
</tr>
<tr>
<td>DNA High</td>
<td>19.8±7.7%&lt;sup&gt;A&lt;/sup&gt;</td>
<td>1.8±0.7%&lt;sup&gt;A&lt;/sup&gt;</td>
<td>0.9±0.2%&lt;sup&gt;A&lt;/sup&gt;</td>
<td>0.2±0.0%&lt;sup&gt;H&lt;/sup&gt;</td>
</tr>
<tr>
<td>bPEI Low</td>
<td>6.9±1.8%&lt;sup&gt;A&lt;/sup&gt;±&lt;sup&gt;H&lt;/sup&gt;</td>
<td>1.9±0.5%&lt;sup&gt;A&lt;/sup&gt;</td>
<td>0.7±0.2%&lt;sup&gt;A&lt;/sup&gt;</td>
<td>0.7±0.1%&lt;sup&gt;C&lt;/sup&gt;</td>
</tr>
<tr>
<td>bPEI Middle</td>
<td>2.3±0.7%&lt;sup&gt;C&lt;/sup&gt;</td>
<td>0.6±0.1%&lt;sup&gt;B&lt;/sup&gt;</td>
<td>0.2±0.0%&lt;sup&gt;B&lt;/sup&gt;</td>
<td>0.2±0.0%&lt;sup&gt;H&lt;/sup&gt;</td>
</tr>
<tr>
<td>Experimental Group</td>
<td>Phase 1 (0-24 Hours) (% of total encapsulated per day)</td>
<td>Phase 2 (1-3 Days) (% of total encapsulated per day)</td>
<td>Phase 3 (3-18 Days) (% of total encapsulated per day)</td>
<td>Phase 4 (18-28 Days) (% of total encapsulated per day)</td>
</tr>
<tr>
<td>--------------------</td>
<td>------------------------------------------------------</td>
<td>------------------------------------------------------</td>
<td>------------------------------------------------------</td>
<td>------------------------------------------------------</td>
</tr>
<tr>
<td>bPEI Low</td>
<td>15.3±3.1%&lt;sup&gt;B&lt;/sup&gt;</td>
<td>4.9±1.4%&lt;sup&gt;A&lt;/sup&gt;,&lt;sup&gt;H&lt;/sup&gt;</td>
<td>1.8±0.2%&lt;sup&gt;A&lt;/sup&gt;</td>
<td>1.6±0.2%&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td>bPEI Middle</td>
<td>9.4±5.4%&lt;sup&gt;A&lt;/sup&gt;</td>
<td>2.7±1.1%&lt;sup&gt;B&lt;/sup&gt;</td>
<td>1.6±0.3%&lt;sup&gt;A&lt;/sup&gt;</td>
<td>1.4±0.4%&lt;sup&gt;A&lt;/sup&gt;,&lt;sup&gt;B&lt;/sup&gt;</td>
</tr>
<tr>
<td>bPEI-HA Low</td>
<td>43.7±9.4%&lt;sup&gt;B&lt;/sup&gt;</td>
<td>8.2±3.8%&lt;sup&gt;A&lt;/sup&gt;</td>
<td>0.9±0.2%&lt;sup&gt;B&lt;/sup&gt;</td>
<td>0.7±0.4%&lt;sup&gt;B&lt;/sup&gt;</td>
</tr>
<tr>
<td>bPEI-HA Middle</td>
<td>20.8±4.2%&lt;sup&gt;A&lt;/sup&gt;</td>
<td>5.9±1.1%&lt;sup&gt;A&lt;/sup&gt;,&lt;sup&gt;H&lt;/sup&gt;</td>
<td>0.9±0.1%&lt;sup&gt;B&lt;/sup&gt;</td>
<td>0.7±0.1%&lt;sup&gt;B&lt;/sup&gt;</td>
</tr>
<tr>
<td>bPEI-HA High</td>
<td>14.1±4.8%&lt;sup&gt;A&lt;/sup&gt;</td>
<td>3.7±0.7%&lt;sup&gt;A&lt;/sup&gt;,&lt;sup&gt;H&lt;/sup&gt;</td>
<td>0.9±0.3%&lt;sup&gt;B&lt;/sup&gt;</td>
<td>0.7±0.2%&lt;sup&gt;B&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Groups with the same letter are not significantly different within each phase. Each data point is presented as average ± standard deviation for n=3.

**Table 5: DNA release rates presented as percent of encapsulated material released per day.**

Table 5 and Table 6 demonstrate that significant differences in release rate were more frequent between categories (DNA, bPEI, bPEI-HA) of loading with less variation within each category. This trend is also apparent in the overall cumulative release curves presented in Figure 10.
Region "#" denotes the time in which the bPEI Low and bPEI Middle release are significantly different. Region "*" designates the time in which each of the three polymer release curves were significantly different from each other. Region "***" designates the time in which the bPEI-HA Low release is significantly different from the bPEI-HA High release, but not the bPEI-HA Middle release which is not significantly different from either the bPEI-HA High or bPEI-HA Low groups. Release was measured from 20 mg of microparticles in PBS at 37°C for each group. Each data point is presented as average ± standard deviation for n=3.

Figure 10: DNA release curves from A) DNA only incorporating groups, B) bPEI/DNA complex incorporating groups, and C) bPEI-HA/DNA complex incorporating groups; and D) bPEI release curves from bPEI/DNA complex incorporating groups, and E) bPEI-HA release curves from bPEI-HA/DNA complex incorporating groups.

Groups only incorporating DNA were characterized by the highest relative DNA burst release with the DNA High group demonstrating significantly higher burst (19.8±7.7% in phase 1 for DNA High) when compared to all other polymer containing groups. This was followed by a relatively high phase 2 (1-3 days) release when compared to other groups with the exception of bPEI Low release (1.9±0.5%). Finally release rates from these DNA groups over the third and fourth phase decreased gradually.

For groups incorporating bPEI/DNA complexes, profuse aggregation during polymer/DNA complex formation resulted in the exclusion of the bPEI High group from investigation. DNA release from these groups was characterized by a significantly lower burst release when compared to the DNA only group followed by a gradual slowing of the release rate through phases 2, 3, and 4. Polymer release from these groups was characterized by a relatively
small burst release followed by a linear release over time with the phase 3 and phase 4 release rates being significantly higher than the rates seen in bPEI-HA incorporating groups in most cases.

Finally, bPEI-HA incorporating groups had a DNA release profile that was distinct compared to the other groups. The burst release from these groups was in the middle of release rates. The phase one release was relatively low, but a significantly accelerated release for each of the bPEI-HA containing groups within phase 3 was observed. This was in significant contrast to each of the other groups. The polymer release from the bPEI-HA containing groups, on the other hand, was characterized by a large phase 1 and 2 release followed by a reduced phase 3 and 4 release.

Transfection was observed with each of the polymer containing groups and is presented in Figure 11. The most effective transfection was observed in the group containing the highest concentration of bPEI-HA/DNA complexes (bPEI-HA High group) with a value of 31.1 ± 17.6% resulting from cell exposure to material released in the second week. For all groups, transfection was characterized by a small but significant initial transfection sustained over 2 weeks with a gradual decline to negligible transfection with week 4 release material.
The black dotted line corresponds to 1% used as a control value. Cells were exposed to material released over 1 week time periods and tested for GFP expression. Group with "A" is significantly different from all other groups within the same phase.

**Figure 11: Transfection efficiency for each of the polymer containing groups.**

**DISCUSSION**

The objective of the studies presented here was to determine the effect of HA conjugation to bPEI on a PLGA microparticle system loaded with polymer/DNA complexes in terms of DNA and microparticle morphology, polymer release, and transfection efficiency. To completely explore the differences in this system between DNA only, bPEI/DNA, and bPEI-HA/DNA complex containing microparticles, three concentrations of internal loading phase DNA were studied for each group; 0.25, 0.75, and 1.25 mg/ml. In groups containing polymers, a constant N:P ratio of 7.5:1 was maintained. This resulted in 9 study groups (DNA Low, DNA Middle, DNA High, bPEI Low, bPEI Middle, bPEI High, bPEI-HA Low, bPEI-HA Middle, and bPEI-HA High) that allowed for complete analysis of the effects of loading amount and type on entrapment efficiency, release, and particle morphology to be examined.
Entrapment efficiency has been linked to a number of parameters within these water in oil in water microparticle-based drug delivery systems, including hydrophilicity of the polymers, surfactant concentrations, internal phase volume, and salt concentrations in the external phase. This work suggests that internal phase concentration is a key factor when using a DNA complexation agent in microparticle encapsulations. For the loading agent to be fully incorporated, it must be evenly dispersed and not aggregated inside of the internal phase. This information explains the lower loading of the all of the bPEI groups relative to bPEI-HA groups. It has been shown that as the concentration of bPEI increases, so does the propensity for aggregation, while bPEI-HA complexes are less likely to aggregate due to the inclusion of the HA which acts to stabilize the polymer. During microparticle preparation, bPEI/DNA complexes in the highest concentration could visually be seen aggregating, while no aggregations were noted in the bPEI-HA groups at any concentration. It is hypothesized that these aggregations in the bEPI complex groups were then not effectively incorporated into the particles, leading to the decreased loading efficiency. In fact, the group containing the highest concentration of bPEI (bPEI High) showed negligible encapsulation due to this aggregation effect and was excluded from further investigation. When this group was explored, it was found that the DNA was entrapped in the particles larger than 300 μm which were strained out prior to release studies (data not shown). In other words, the very large aggregates were coated with PLGA. By contrast, the incorporation of HA in the bPEI-HA groups decreased the aggregation of the complexes during loading, resulting in a higher loading efficiency.

The most important factor influencing the release of the entrapped DNA was the polymer type in the polymer-DNA complexes. The incorporation of bPEI-HA into the system significantly accelerated the phase 3 release. This is most probably due to the presence of HA
within the system causing accelerated PLGA degradation while also increasing the hydrophilicity of the system. These are two characteristics that have been shown in previous work to accelerate release of plasmid DNA from PLGA microparticles\textsuperscript{60, 63, 97}. This accelerated release was observed to a greater degree in the DNA kinetics and not as significantly in the polymer release. While the polymer release kinetics differed between the bPEI and bPEI-HA groups, they maintained the same general release curves with a quick phase 1 and 2 release followed by a slow phase 3 and 4 release. The differences between nucleic acid and bPEI release kinetics have been previously examined using Dynamic Light Scattering, which indicated that the vector and nucleic acid were loaded as complexes, they were released separately and then formed complexes immediately\textsuperscript{57, 104}.

The overall trend in transfection and release kinetics can be explained by examining the degradation rate of the PLGA polymer in each group. Previous work has shown that the DNA not involved with burst release is released according to the PLGA degradation rate found in each of the test groups\textsuperscript{63}. With this assumption, we can see that the incorporation of an acid, HA, into the system would result in the accelerated degradation of the PLGA system causing an accelerated release of the contained DNA in groups incorporating bPEI-HA.

The transfection efficiency experiments illustrated the potential for released complexes to initiate transfection and result in effective gene expression. The transfection peaked after 2 weeks and then dropped off in each of the groups. The highest transfection was observed in the high concentration of bPEI-HA/DNA complexes after 2 weeks. When taken in context of the DNA release curves and entrapment efficiency, the transfection closely followed the observed trends discussed above. Specifically, as the rate of DNA release dropped precipitously after 2 weeks of incubation, so did the transfection efficiency in all groups. Further, the highest
transfection efficiency was observed in the bPEI-HA High group, which had high second week release and high total encapsulation amounts. This suggests that when more DNA and polymer are encapsulated and released in a certain time period, enhanced transfection from bPEI-HA/DNA PLGA microparticles can be achieved.

**CONCLUSIONS**

The experiments above illustrate the potential to engineer the release kinetics of polymer/DNA complexes from a PLGA microparticle system while achieving significant transfection efficiency on target cells. Aqueous loading phase concentration is of upmost importance to the loading efficiency. For release characteristics and transfection efficiency, aqueous loading phase concentration does not seem to be as important as the polymer loading type. The addition of HA to the system significantly alters the release by accelerating the sustained release of DNA from the degrading microparticles. These results demonstrate that utilizing a bPEI-HA gene delivery vector in a PLGA microparticle system is a viable and effective method for initiating extended transfection on target cells.
ABSTRACT

Native osteochondral repair is often inadequate due to the inherent properties of the tissue, and current clinical repair strategies can result in healing with a limited lifespan and donor site morbidity. This work investigates the use of polymeric gene therapy to address this problem by delivering DNA encoding for transcription factors complexed with the branched poly(ethylenimine)-hyaluronic acid (bPEI-HA) delivery vector via a porous oligo[poly(ethylene glycol) fumarate] (OPF) hydrogel scaffold. To evaluate the potential of this approach, a bilayered scaffold mimicking native osteochondral tissue organization was loaded with DNA/bPEI-HA complexes and the release kinetics of the system determined. Next, bilayered implants either unloaded or loaded in a spatial fashion with bPEI-HA and DNA encoding for either Runt-related transcription factor 2 (RUNX2) or SRY (sex determining region Y)-box 5, 6, and 9 (the SOX trio), to generate bone and cartilage tissues respectively, were fabricated and implanted in a rat osteochondral defect. At 6 weeks post-implantation, micro-computed tomography (micro-CT) analysis and histological scoring were performed on the explants to evaluate the quality and quantity of tissue repair in each group. Additionally, the incorporation of DNA encoding for RUNX2 in the bone layer of these scaffolds significantly increased bone growth. The results also indicate that a spatially loaded combination of RUNX2 and SOX trio DNA loading significantly improves healing relative to empty hydrogels or either factor alone. Finally, the results of this study suggest that subchondral bone is necessary for correct cartilage healing.
KEYWORDS

Polymeric gene delivery, OPF, bPEI-HA, SOX trio, RUNX-2

INTRODUCTION

Osteochondral injuries are an area of in depth research due to the inadequacy of native healing and the limited lifespan and quality of conventional treatments. Cartilage tissue is often limited in its ability to regrow due to its heavy dependence on appropriate extracellular matrix generation and lack of blood flow. Tissue engineering is one area of research that could address these current inadequacies and provide an improved therapy for osteochondral injuries.

In particular, gene delivery is one developing and promising option for osteochondral injury repair in the context of tissue engineering. Gene delivery has the ability to utilize often otherwise unusable therapeutic proteins by directly inducing their expression within the cells of a target tissue through delivery of nucleotides into those cells. Osteochondral and chondral tissues are especially promising for polymeric gene delivery approaches because of the limited blood flow to the region, which can cause problems in DNA polymer complex delivery, and the potential for the delivered genes to induce differentiation of infiltrated mesenchymal stem cells (MSCs). By using transcription factors that regulate many downstream proteins, gene delivery could be capable of inducing more physiologically correct differentiation in target cells. This work utilized the transcription factors Runt-related transcription factor 2 (RUNX2), or CBFA1, which has been shown to induce osteogenic differentiation, and SRY (sex determining region Y)-box 5, 6, and 9 (the SOX trio), which has been shown to induce chondrogenic differentiation.
To deliver these transcription factors into the cell, a gene delivery vector is needed. This work utilizes branched poly(ethylenimine) (bPEI), a commonly used polymer for gene delivery, modified with hyaluronic acid (HA). The resulting product, bPEI-HA has been shown to mitigate many of the negative effects associated with bPEI, such as its high cytotoxicity, while improving transfection efficiency and potentially providing cellular targeting through hyaladherins on the cells' surface. 22, 35, 36, 96

Studies have examined the role of the SOX trio and RUNX2 in chondrogenesis and osteogenesis both separately and in combination with each other and other bioactive factors. 105, 106 It has been shown that the SOX trio is capable of inducing chondrogenic differentiation in target cells both in vitro and in vivo107 and that this combination of transcription factors has the potential to do so in vitro without the use of inductive factors. 49 Further, when cells transduced with the SOX trio were implanted into a rat osteochondral defect for 8 weeks, they were found to promote defect healing. 108 In other studies, PLGA scaffolds bPEI and bPEI based vectors complexed with DNA encoding for the SOX trio have been shown capable of inducing cartilage growth in vivo and in vitro. 109, 110

RUNX2 has also been shown to be an effective driver of differentiation. 111 When DNA encoding for RUNX2 is transduced into stem cells which are seeded onto scaffolds and implanted into animals, substantially more bone can be grown than in control situations. 112-114 Additionally, when adenoviral RUNX2 is immobilized on a scaffold, it is still capable of increasing osteogenic differentiation. 115 One important consideration with relation to this study is that it has been shown that these two groups, the SOX trio and RUNX2, have inhibitory effects on each other when applied to the same cell population in vitro and in vivo. 116, 117 In fact, it has been shown that SOX9 is dominant and inhibitory to RUNX2 in vivo. 118 This work investigates
the hypothesis that by spatially loading these two complexes to mimic native tissues, distinct zones of osteogenesis and chondrogenesis can be achieved without negative interactions.

Oligo[poly(ethylene glycol) fumarate] (OPF) is a promising hydrogel for use in tissue engineering, including for applications involving bone\textsuperscript{72} and cartilage\textsuperscript{73} tissue growth, and combined osteochondral repair\textsuperscript{65-68}. This hydrogel is formed by condensing poly(ethylene glycol) (PEG) and fumaryl chloride to form a linear polyester. The mesh size can be controlled by varying the molecular weight of the incorporated PEG chains, and higher concentrations of fumarate ester groups inside the polymer result in higher crosslinking and more points for degradation\textsuperscript{70, 71}. OPF has numerous favorable biological properties including being biocompatible, bioinert, and non-immunogenic, in addition to having inert degradation products\textsuperscript{69}.

OPF is attractive as a delivery vehicle for a number of reasons. First, it is a synthetic rather than natural polymer and therefore offers tunable properties such as mesh size, degradation rate, and mechanical properties to allow for modification for a specific application. Moreover, it is attractive for use with plasmid DNA and other nucleotides because it can be crosslinked under physiologic conditions without harsh processing that could degrade or injure the genetic material\textsuperscript{67}. The inherent hydrophilicity of the system and hydrogel fabrication process results in an ability to entrap large concentrations of polymer/DNA complexes at relatively high loading efficiencies\textsuperscript{66-68}. Furthermore, OPF has been used to deliver DNA and bPEI in previous work by either direct loading of complexes or by loading complex laden gelatin particles\textsuperscript{66-68}. These studies have explored release of plasmid DNA from OPF hydrogels both \textit{in vitro} and \textit{in vivo}, as well as directly from the hydrogel or from an incorporated carrier. When
incorporated directly into the hydrogel during fabrication, release of plasmid/polymer complexes was found to have a favorable profile lasting several weeks.\textsuperscript{68}

In the present study, we hypothesize that the regeneration of bone and cartilage tissue will be enhanced by the delivery of bPEI-HA and DNA encoding for transcription factors in a porous OPF hydrogel scaffold. The inclusion of carboxymethyl cellulose (CMC) as a porogen in the OPF scaffold is also expected to increase tissue distribution inside of the scaffolds relative to previous studies involving solid OPF scaffolds by generating voids inside the scaffold which cells can migrate into. Additionally, the dual delivery of RUNX2 and the SOX trio is expected to improve the quality and amount of bone and cartilage generation within the defects. To examine these hypotheses, release from constructs was analyzed \textit{in vitro} and the constructs were implanted in a rat osteochondral defect for 6 weeks and analyzed using micro-CT and histology.

**MATERIALS AND METHODS**

\textit{In Vivo} Experimental Design

Composite scaffolds consisting of OPF, CMC, and bPEI-HA/DNA complexes were examined \textit{in vivo} for their ability to generate tissue in a rat knee osteochondral defect model. Groups for this study were designed to examine the interactions and efficacy of the use of DNA encoding for the transcription factors SOX 5, SOX 6, and SOX 9 (the SOX trio), and RUNX2 delivered with bPEI-HA. The groups examined here are summarized below in Table 7 and include a material control, RUNX2 DNA only, and SOX trio DNA only in order to identify the effects of each component individually as well as a combination group used to identify combinatory effects of RUNX2 and the SOX trio.

\textit{Table 7: In vivo experimental groups for rat osteochondral defect implantation.}
<table>
<thead>
<tr>
<th>Group</th>
<th>Top (Chondrogenic) Layer</th>
<th>Bottom (Osteogenic) Layer</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Empty</td>
<td>OPF only</td>
<td>OPF only</td>
<td>Scaffold control</td>
</tr>
<tr>
<td>SOX Trio</td>
<td>bPEI-HA/SOX trio complexes</td>
<td>OPF only</td>
<td>Elucidate the effects of the SOX trio alone</td>
</tr>
<tr>
<td>RUNX2</td>
<td>OPF only</td>
<td>bPEI-HA/RUNX2 complexes</td>
<td>Elucidate the effects of RUNX2 alone</td>
</tr>
<tr>
<td>Combined</td>
<td>bPEI-HA/SOX trio complexes</td>
<td>bPEI-HA/RUNX2 complexes</td>
<td>Show potential interactions between SOX trio and RUNX2</td>
</tr>
</tbody>
</table>

**Assembly of bPEI-HA/DNA Complexes**

Branched PEI-HA was synthesized as previously described,\textsuperscript{22, 96} using a reductive amination reaction to directly conjugate the hyaluronic acid fragments (6.4kDa) (LifeCore Biomedical, Chaska, MN) to the primary amines of the bPEI (Sigma-Aldrich, St. Louis, MO). The structure was verified with $^1$H NMR to ensure correct conjugation as has been described previously.\textsuperscript{22, 96}

Plasmid DNA encoding for RUNX2, SOX5, SOX6, and SOX9 (Origene, Rockville, MD) was expanded using DNA expansion kits according to the manufacturer's instructions (Qiagen, Venlo, Netherlands), collected, and used directly. For loading into hydrogels, bPEI-HA and DNA were combined drop wise in a constant 7.5:1 Nitrogen:Phosphate (N:P) ratio and allowed to complex in ultrapure (type 1) water (Super-Q Water Purification System, EMD Millipore, Billerica, MA) at room temperature for 30 mins before use. After complexation, complexes were lyophilized for 48 hrs in preparation for use in hydrogel loading.
OPF Synthesis and Characterization

Synthesis of OPF was performed as previously described. Briefly, anhydrous dichloromethane (EMD, Billerica, MA) was obtained through refluxing in the presence of calcium hydride (Sigma Aldrich, St. Louis, MO) followed by distillation. Anhydrous PEG ($M_n = 9.31 \pm 0.06 \text{kDa}, M_w = 13.11 \pm 0.05 \text{kDa}$) (Sigma Aldrich, St. Louis, MO) was generated through distillation in toluene (Fisher Scientific, Waltham, MA) and then added to the anhydrous dichloromethane. Triethylamine (Sigma Aldrich, St. Louis, MO) and fumaryl chloride (Acros, Geel, Belgium) were added to this PEG solution drop wise and the reaction was incubated for 2 days. Purification was then performed and characterization of the product was performed through analysis with gel permeation chromatography using PEG standards and $^1$H NMR to verify correct structure and fumarate PEG ratios as previously described. The OPF used in this work has a $M_n$ of approximately 19.8 ± 0.3 kDa and a $M_w$ of 89.9 ± 3.9 kDa.

Composite Scaffold Fabrication

Scaffolds for use in the studies described below were composites consisting of an OPF hydrogel crosslinked around CMC particles up to 100 µm in diameter. For fabrication of the composites, OPF and poly(ethylene glycol)-diacrylate (PEGDA) ($M_n = 10,000 \text{Da}$) were first dissolved in PBS [2:1 OPF:PEG-DA weight ratio, 38% w/v gel in water]. Immediately before OPF crosslinking was initiated, dried DNA/bPEI-HA complexes were suspended in the OPF/PEGDA solution. For each layer, a DNA concentration of 6.2 mg/ml (after swelling) was maintained, which corresponds to levels previously shown to elicit a response in vivo. Once dispersed, ammonium persulfate (APS) and $N,N,N',N'$-tetramethylethane-1,2-diamine (TEMED) were added to the solution [3.6 mM] and it was vigorously stirred for 30 s. At the end of these 30 s, CMC particles were added to the mixture and it was stirred for an additional 45 s before
being added into custom poly(tetrafluoroethylene) (PTFE) molds. For bilayered scaffolds, the bottom osteogenic layer was allowed to crosslink for 3 min in a PTFE mold before addition of another mold directly on top was filled with crosslinking chondrogenic layer hydrogel. Once the two layers were stacked, they were allowed to finish crosslinking for 10 min prior to use.

Scaffolds for use in in vitro release and in vivo implantation were fabricated as 8 mm diameter by 1.2 mm thickness discs which were then punched with a custom punch such that the final scaffold punch dimensions were 1.2 mm in diameter by 1.2 mm thickness with each layer having a 0.6 mm thickness. These dimensions correspond to a 1.5mm diameter by 1.5 mm thickness implant after swelling.

*In Vitro Release Study*

Release of eGFP DNA (pCMV-eGFP, 4.7 kb, Clonotech cat no. 6085-1, Palo Alto, CA) from OPF/CMC/bPEI-HA/DNA composite scaffolds was studied *in vitro* over 28 days at 37º C and 90 rpm on a shaker table. Plasmid eGFP DNA was selected as a representative DNA strand because of its similar overall size. For each group, 3 samples of 4 scaffolds of the size implanted into the animals (1.5mm diameter x 1.5mm thickness swollen) were suspended in 0.5 ml of PBS, and the supernatant was removed and tested at 0.5, 1, 2, 3, 7, 14, 21, and 28 days. The groups studied included OPF synthesized with 10K PEG with and without incorporating CMC as a porogen.

DNA was detected by dissociating the bPEI-HA/DNA complexes in a high pH solution combined with the use of a PicoGreen detection kit (Thermo Fisher, Grand Island, NY) as previously described.57,96 Specifically, 80 µl of release sample was added to each well of an opaque 96 well plate. Fresh bPEI-HA/DNA complexes at an N:P ratio of 7.5:1 were prepared and used as controls in all cases, and all samples were run in triplicate. Once the plates were
prepared, 160 µl of pH 12 tris-EDTA (TE) buffer with 0.5% v/v PicoGreen dye was added to each well. The plate was then incubated at 60 rpm for 5 min before being read on the plate reader with excitation/emission wavelengths of 485/530 nm.

Surgical Procedure

32 Lewis rats (Charles River Laboratories, Wilmington, MA) 12 weeks of age for skeletal maturity were used in this study. All manipulations described followed protocols approved by the Institutional Animal Care and Use Committee at Rice University and published NIH guidelines for the care and use of laboratory animals have been observed. All implants were fabricated directly before implantation. Each construct contained CMC and OPF in an 1:1 weight ratio during fabrication as well as 8.4 µg DNA per scaffold in the combined scaffolds (4.2 µg DNA in either the RUNX2 or SOX trio only scaffolds). All animals survived the surgery and post-operative period with no signs of distress or infection for the duration of the study.

To complete the surgery, previously established procedures were followed to create defects in the right femoral condyle of the rat. The surgical site was steriley prepared and a lateral approach was used to expose the knee joint through blunt dissection. A lateral parapatellar incision was made and the patella dislocated medially to access the knee. The joint capsule was then opened and the defect was established along the midline and midway up the trochlear groove from the knee with a 0.9 mm drill bit and was created with a 1.5 mm drill bit. The defect was then flushed with saline and filled with the implant prepared as described above. The patella was then physically relocated and the wound closed in three layers. Animals were closely monitored for any signs of discomfort.
**In Vivo Tissue Preparation**

Six weeks after implantation, animals were anesthetized with 4-5% isofluorane and euthanized with carbon dioxide. A subsequent bilateral thoracotomy was performed to ensure death before the treated knees were extracted. The knees were then placed in 10% neutral buffered formalin for 1 week for fixation. Micro-CT scans were then performed on all samples before they were decalcified in 5% formic acid for 14 days and dried in an ethanol gradient from 50-100% (50, 70, 80, 90, 95, 100%). *In vivo* animal samples were analyzed for cartilage and bone generation through micro-CT and histology according to guidelines previously established for use in a bilayered osteochondral defect in a rabbit model.\(^{125-128}\)

**Histology**

For histological analysis, the dried samples were embedded in paraffin and sectioned in 6 µm slices. Sections from two locations within the defect (center and medial) were obtained and stained with hematoxylin and eosin (H&E) and Safranin O/Fast Green. Each of these sections was independently analyzed by three evaluators according to procedures described previously.\(^{125,127}\) Briefly, the region of the defect corresponding to the native cartilage thickness was analyzed for cartilage tissue generation and the lower portion corresponding to native bone was analyzed for bone regeneration. Osteochondral repair was evaluated using 13 predetermined measures of regeneration which are listed in Table 8 and cover the quantity and quality of the regeneration of tissues, scaffold degradation, and integration of new tissues with surrounding native tissues.

<table>
<thead>
<tr>
<th>Table 8: Evaluation parameters for osteochondral defects(^{125-128})</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Overall Defect Evaluation</strong></td>
</tr>
<tr>
<td><strong>Percent Filling with New Tissue</strong></td>
</tr>
<tr>
<td>Percent</td>
</tr>
<tr>
<td>90-100%</td>
</tr>
<tr>
<td>50-90%</td>
</tr>
<tr>
<td>10-50%</td>
</tr>
<tr>
<td>Percent Implant Degradation</td>
</tr>
<tr>
<td>-----------------------------</td>
</tr>
<tr>
<td>0-10%</td>
</tr>
<tr>
<td>90-100%</td>
</tr>
<tr>
<td>50-90%</td>
</tr>
<tr>
<td>10-50%</td>
</tr>
<tr>
<td>0-10%</td>
</tr>
</tbody>
</table>

**Subchondral Tissue Evaluation**

<table>
<thead>
<tr>
<th>Percent Filling with New Bone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percent</td>
</tr>
<tr>
<td>90-100%</td>
</tr>
<tr>
<td>50-90%</td>
</tr>
<tr>
<td>10-50%</td>
</tr>
<tr>
<td>0-10%</td>
</tr>
</tbody>
</table>

**Subchondral Tissue Morphology**

| Mostly trabecular bone | 4 |
| Mostly compact bone | 3 |
| Mostly cartilage | 2 |
| Mostly fibrous tissue | 1 |
| Only fibrous tissue or no tissue | 0 |

**Extent of New Bone Bonding with Adjacent Bone**

| Complete on both edges | 3 |
| Complete on one edge | 2 |
| Partial on both edges | 1 |
| No continuity on either edge | 0 |

**Cartilage Evaluation**

(Cartilage regions identified by scoring team considering scaffold design and adjacent native tissues)

<table>
<thead>
<tr>
<th>Morphology of New Surface Tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exclusively AC</td>
</tr>
<tr>
<td>Mainly Hyaline Cartilage</td>
</tr>
<tr>
<td>Fibrocartilage (spherical morphology in &gt;75% of cells)</td>
</tr>
<tr>
<td>Mostly Fibrous Tissue (spherical morphology in &lt;75% cells)</td>
</tr>
<tr>
<td>No tissue</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Morphology of New Cartilage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exclusively AC</td>
</tr>
<tr>
<td>Mainly Hyaline Cartilage</td>
</tr>
<tr>
<td>Mainly Fibrocartilage</td>
</tr>
<tr>
<td>Only Fibrous Tissue/ No Tissue</td>
</tr>
</tbody>
</table>

**Thickness of New Cartilage**

| Similar to surrounding cartilage | 3 |
| Greater than surrounding cartilage | 2 |
| Less than surrounding cartilage | 1 |
| No cartilage | 0 |

**Joint Surface Regularity**

| Smooth, intact surface | 3 |
| Surface fissures (<25% new surface thickness) | 2 |
| Deep fissures (25-99% new surface thickness) | 1 |
| Complete disruption of the new surface | 0 |

**Chondrocyte Distribution**

| Columnar | 3 |
| Mixed Columnar-clusters | 2 |
| Clusters | 1 |
| Individual or disorganized cells | 0 |

**Chondrocyte Cellularity**

| Similar number of chondrocytes | 3 |
| More chondrocytes | 2 |
| Fewer chondrocytes | 1 |
| No Chondrocytes | 0 |

**Safranin O Staining**

| Similar staining intensity | 4 |
| Stronger Staining intensity | 3 |
| Moderate staining intensity | 2 |
| Poor staining intensity | 1 |
| Little or no staining intensity | 0 |

**Chondrocyte and GAG Content of Adjacent Cartilage**

| Normal cellularity with normal GAG content | 3 |
| Normal cellularity with moderate GAG content | 2 |
| Clearly less cells with poor GAG content | 1 |
| Few cells with little or no GAGs or no cartilage | 0 |
**Micro-CT Imaging and Analysis**

Before decalcification for histology was performed, micro-CT analysis was used to determine the mineralization as previously described. All images were taken with a high resolution SkyScan-1172 micro-CT imaging system. Samples were oriented so that the axis of the defect was vertical in the micro-CT chamber. The scanner resolution was set to 10.2 μm/pixel with a voltage of 100 KV and a current of 100 μA. The resolution of the camera was set to high (1280 x 1024 raw images). The defect was then reconstructed by backprojecting the raw images in a NRecon CT Reconstruction software package.

1.5 mm diameter, 1.5 mm height volumes of interest were then selected within the reconstructed images at the site of the defect for each sample. To calculate the percent bone formation within these volumes of interest, a lower binarization threshold of 45 and upper binarization threshold of 255 were set to identify bone morphology within the samples. With these values, percent bone formation within the samples was defined as the bone volume within the volume of interest divided by the total volume of interest expressed as a percentage.

**Statistics**

Statistical analysis was performed on the data collected for *in vitro* release and micro-CT analysis using two-way ANOVA with a priori value of 0.05. Post hoc analysis was performed via Tukey-Kramer HSD to identify statistical significance (p<0.05) between each of the groups. Each of the *in vivo* histological scoring parameters was analyzed using the Kruskal Wallis test of variance and the Wilcoxon rank sum test for difference of groups. All data are presented as means ± standard deviation and *in vitro* studies had an n of 4 while *in vivo* studies had a n of 8.
RESULTS

In Vitro Release and Scaffold Fabrication

Scaffolds for implantation in vivo and in vitro analysis were successfully fabricated utilizing the bilayered 2-step process described above to embed bPEI-HA/DNA complexes in a spatially loaded fashion. In vitro release was studied over 28 days and is shown in Figure 12. OPF with and without CMC as a porogen was found to have a large burst release of both bPEI-HA and DNA. The OPF 10K group without CMC had a significantly smaller burst release after the first 24 hours of DNA (16.3±0.3 µg) relative to the CMC containing group. Each curve was characterized by a large burst release rate over the first three days, followed by a very slow and decreasing release rate over the remainder of the study period. CMC significantly increased the total DNA released from the composite scaffolds at all time points.

Figure 12: Cumulative DNA release in vitro. All groups were loaded with the same amount of bPEI-HA and DNA. 10KCMC represents hydrogels fabricated with CMC and OPF synthesized with 10 kDa PEG. 10K represents hydrogels fabricated only with OPF synthesized with 10 kDa PEG. Region“*” denotes the time in which all groups’ release are significantly different.
**Gross Observation**

During the tissue harvest, no signs of gross infection or any other adverse tissue responses were observed and all animals were ambulatory for the duration of the study.

**Micro-CT**

Micro-CT images were taken for each sample and analyzed to determine the percentage of bone present within the defect volume. There was no significant difference in percent regenerated bone volume between groups, as shown in Figure 13, but the analysis demonstrated measureable bone growth in all in vivo study groups with a high percentage formation of 29.5±8.0% found in the combination treatment group.

![Figure 13: Percent bone generation identified through micro-CT.](image)

Error bars represent the standard deviation of each group and for all groups n=8.
Histology

Histological analysis was performed on the collected sections from the medial edge and the center of each defect as described above to determine the regeneration and tissue response within the created defects. The results for each parameter outlined in Table 8 are displayed in Figure 14, Figure 15, and Figure 16 and numerous statistical differences were observed. Representative histological results from the center of each group are included as in Figure 17, Figure 18, Figure 19, and Figure 20.
Figure 14: Overall defect histological analysis where (a) corresponds to percent filling with new tissue score and (b) corresponds to percent implant degradation. 0, 1, 2, and 3 correspond to histology scores described in Table 2. Groups connected by lines are significantly different from each other.
Figure 15: Bone tissue histological analysis where (a) corresponds to filling with new bone, (b) corresponds to subchondral bone morphology, and (c) corresponds to bone bonding with surrounding tissue. 0, 1, 2, 3, and 4 correspond to histology scores described in Table 2. Groups connected by lines are significantly different from each other.
e  Chondrocyte Distribution

Percent Obtaining Each Score

Combined  Runx2  Sox Trio  Empty

f  Chondrocyte Cellularity

Percent Obtaining Each Score

Combined  Runx2  Sox Trio  Empty
Figure 16: Cartilage tissue histological analysis where (a) corresponds to the morphology of the new surface tissue, (b) is the morphology of the generated cartilage, (c) is the thickness of new cartilage, (d) is the joint surface regularity, (e) is the chondrocyte distribution, (f) is the chondrocyte cellularity, (g) is the safranin O staining, and (h) is the quality of the surrounding cartilage. 0, 1, 2, 3, and 4 correspond to histology scores described in Table 2. Groups connected by lines are significantly different from each other.
Figure 17: Representative histological sections of osteochondral tissue formation after 6 weeks of implantation of composite scaffolds in the Combined treatment group which had bPEI-HA and DNA encoding for the SOX trio in the top layer and RUNX2 in the bottom layer. Sections were stained with (a) Safranin-O/Fast Green and (b) hematoxylin and eosin (H&E). Scale bars = 500 μm. Magnified images indicate (c) a thick layer with a mixture of fibrocartilage (FC) and mostly hyaline cartilage (C) and (d) underlying regenerated subchondral cortical bone (CB) and near complete degradation of the composite scaffold. Boxed regions are shown in higher magnification with scale bars = 250 μm.
Figure 18: Representative histological sections of osteochondral tissue formation after 6 weeks of implantation of composite scaffolds in the RUNX2 treatment group which had bPEI-HA and DNA encoding for RUNX2 in the bottom layer. Sections were stained with (a) Safranin-O/Fast Green and (b) hematoxylin and eosin (H&E). Scale bars = 500 µm. Images with higher magnification indicate (b) regions of fibrocartilage (FC) with little safranin-o staining and (c) regions with remaining hydrogel (H) indicated by voids in contact with newly formed cortical bone tissue (CB).
Figure 19: Representative histological sections of osteochondral tissue formation after 6 weeks of implantation of composite scaffolds in the SOX Trio treatment group which had bPEI-HA and DNA encoding for the SOX trio in the top layer. Sections were stained with (a) Safranin-O/Fast Green and (b) hematoxylin and eosin (H&E). Scale bars = 500 µm. Images with higher magnification indicate (c) regions with very thin surface (TS), fibrous tissue (FT) and some little fibrocartilage with minor safranin-o staining (FC) and (d) subchondral regions with significant hydrogel remaining (H) and poor bone regeneration with fibrous tissue present (FT).
Figure 20: Representative histological sections of osteochondral tissue formation after 6 weeks of implantation of composite scaffolds in the Empty hydrogel treatment group which had no incorporated bPEI-HA/DNA complexes. Sections were stained with (a) Safranin-O/Fast Green and (b) hematoxylin and eosin (H&E). Scale bars = 500 µm. Images with higher magnification indicate regions with (c) a thin chondral layer (TS) made of fibrous tissue (FT) and some fibrocartilage (FC) and (d) regions within the subchondral bone layer with significant hydrogel remaining (H) with ingrowing fibrous tissue (FT).

In all of the groups a portion of the composite scaffold remained in the defect region.

The groups with either an empty hydrogel or with only the SOX trio delivered had significantly more hydrogel present and significantly less new tissue growth into the defect as seen in Figs 3, 8, and 9. The combined treatment group had significantly higher tissue filling and less hydrogel present than all other groups as shown in Figs 3 and 6. Finally, the RUNX2 treatment group statistically fell between the best case combined treatment group and the more negative Empty and SOX trio only groups for tissue filling and implant degradation as shown in Fig 3.
For bone growth and quality, the SOX trio only group scored significantly lower in many cases. For percent filling with bone the SOX trio treatment group grew significantly less bone than all other test groups as demonstrated in Figure 15 and 8. For subchondral tissue morphology, the SOX trio scored significantly lower than all groups except for the blank hydrogels, signifying a poorer quality of the generated tissue. These results can be seen in the bone excerpts from the representative histological sections, Figures 6-9. While RUNX2 outperformed the SOX trio in bone quality and quantity, it was not significantly different from the empty gels and scored significantly worse than the combined group which was significantly better than all other test groups as seen in Figs 4a, 4b, and 6. These differences also are reflected in the example histological sections. Finally as seen in Fig 4c, there were fewer statistical differences in the bone bonding scoring, but the SOX trio did score significantly lower than the combined treatment group.

In the cartilage scoring portion, significant differences were found. For joint surface regularity and the thickness of the new cartilage shown in Fig 5c and 5d, the combined treatment group significantly outperformed the SOX trio group, signifying an improvement in the surface healing of the defect which is clearly seen in the representative histological sections in figs 6 and 9. Conversely, for chondrocyte distribution the SOX trio group was found to have a significantly better distribution than the combined treatment group, shown in Fig 5e. Similar trends as were found for the bone histological scoring were present in the cartilage scoring categories which were listed in this paragraph, but did not achieve levels of significance.

**DISCUSSION**

Composite scaffolds were designed and fabricated to facilitate cellular infiltration and tissue ingrowth. OPF was selected due to its established ability to support regrowth of cartilage
and bone while remaining non-toxic to target tissues immediately after implantation and during and after degradation. Additionally, by using a hydrogel, loading of high amounts of DNA in a spatially bilayered approach was possible while retaining the ability to release the genetic material and polymer delivery vector simultaneously. Because previous work has shown that implanting porous hydrogels into a defect can result in improved tissue ingrowth, this work utilized CMC as a porogen.\textsuperscript{130,131} It was shown that in all groups a significant level of tissue ingrowth within the scaffold was achieved.

The primary aim of this study was to determine if DNA encoding for transcription factors delivered by bPEI-HA to a rat osteochondral defect via an OPF/CMC composite scaffold is capable of improving tissue generation in an \textit{in vivo} situation. To achieve this goal, first, bilayered OPF/CMC hydrogels loaded with DNA encoding for eGFP complexed with bPEI-HA as representative complexes were fabricated and their release kinetics studied \textit{in vitro} to characterize the system. GFP was selected as a representative gene for use in \textit{in vitro} studies due to its similar size to the plasmids used in this study (4.7 kb vs. 4.7-5.8 kb for Runx2 and the Sox trio) and due to its use as an analog in the literature.\textsuperscript{88,96} Once release was characterized, implants were fabricated incorporating bPEI-HA and DNA encoding for the SOX trio, DNA encoding for RUNX2, or a spatially controlled combination of both and implanted into a rat osteochondral defect for 6 weeks.

A 6 week time point was selected in this work because of the release kinetics of the system observed \textit{in vitro}. Release of both DNA and bPEI-HA in this study was characterized by a strong burst and was almost wholly completed in the first week of exposure to release media. The reason for this rapid release as opposed to a delayed release situation is likely due to the incorporation of CMC as a porogen. As the CMC dissolves out of the scaffolds, channels for
polymer/DNA complex escape and release were created. It is also probable that the unconfined nature of the release study effects the dissolution rate of the CMC and thus the release. A 6 week time period was selected because of its previous use as an endpoint with osteochondral defects\textsuperscript{132-134} and rapid release coupled with the transient nature of polymeric gene delivery led to our speculation that a longer time point would not show as drastic of differences between groups.

The results presented above indicate that in this approach, the RUNX2 treatment group are more capable than the SOX trio treatment group at achieving the desired tissue growth of either bone or cartilage respectively. While the micro-CT analysis was unable to identify significant differences between groups, RUNX2 containing groups did significantly better in bone and general histological scoring than groups without RUNX2. This disparity between tests is interesting, but the data produced by the histological sections is superior due to its direct visualization of the cells and tissue morphology rather than detection of mineralization.

The SOX trio only group proved incapable of generating significant cartilage growth within the cartilage layer. It did, however score significantly better with respect to the distribution of the chondrocytes that did form, as seen in Fig 5e. This could indicate that the SOX trio is having an effect on the target cells. The most probable reason for this disparity in appropriate tissue generation is the inherent burst release associated with this system. Previous work has shown that subchondral bone growth is necessary to support cartilage growth.\textsuperscript{135} If it is assumed that the bioactive factors in this system are released quickly, the DNA encoding for the SOX trio attempted to grow cartilage in this situation without the mechanical support of subchondral bone. It is possible that in future work, if release of the cartilage treatment factors was delayed, that more complete cartilage healing could be observed.
It was found that combination therapy consisting of RUNX2 and SOX trio resulted in greater tissue filling, implant degradation, and bone generation and quality. Several previous studies have found that the combination of RUNX2 and the SOX trio results in negative bone and cartilage regrowth, and it has been shown that they can counteract each other's action.\textsuperscript{116, 117} In fact, if they were transfecting the same cells, it would be expected from previous work that the SOX trio would be dominant.\textsuperscript{118} As for the lack of interaction, we hypothesize the spatial aspect of the loading excluded the infiltrated cells from being transfected with both transcription factors. Because the bPEI-HA/DNA complexes were entrapped in the OPF mesh network at the time of composite scaffold fabrication and implantation, the two DNA treatments were kept separate. Once implanted, the natural confines of the defect could limit diffusion of the complexes and the ability of RUNX2 and the SOX Trio to interact.

One potential explanation for the faster implant degradation and tissue ingrowth in the combination treatment group could be the presence and amount of bPEI-HA in the defect. In combination treatment groups, more bPEI-HA was present because of the bilayered loading. OPF degrades via hydrolysis and the incorporation of an acid into the system accelerates the rate of this hydrolysis.\textsuperscript{136} The faster degradation of the hydrogel would allow more space for cellular infiltration and allow more cells to receive treatment with RUNX2 before the complexes were released and cleared from the defect.

The primary limitation of the current study is its scope and additional studies could be considered to attempt to refine the general approach presented here. The most obvious approach would involve creating a different composite scaffold capable of delaying release from the chondrogenic layer until appropriate subchondral bone has been formed. With that said, the research presented here has shown that DNA encoding transcription factors delivered with bPEI-
HA is capable of improving tissue healing \textit{in vivo}. Additionally, spatial loading of two different treatments in a hydrogel scaffold is superior in this system to either treatment alone and improves tissue ingrowth and implant degradation.

\textbf{CONCLUSIONS}

The experiments described above show the potential of a bilayered approach in a rat osteochondral defect. Spatial design and loading of the described implants resulted in measurable improvements in overall tissue generation and quality, especially in the bone layer. This improvement in healing is especially true when a combination of the SOX trio and RUNX2 are used, though the inclusion of DNA encoding for RUNX2 also had a significant effect on tissue growth. The results here demonstrate the ability to apply polymeric gene therapy and transcription factors \textit{in vivo} directly without implanting transfected or transduced cells and in spatial combination with each other in an osteochondral defect.
Chapter 6 - Conclusion

In this work, hyaluronic acid was conjugated to bPEI and the system explored to evaluate the effects of alterations on characteristics which are important to effective polymeric gene delivery. BPEI-HA was then incorporated into biodegradable microspheres and the system characterized for release and effectiveness. Finally, bPEI-HA was combined with DNA encoding for developmental transcription factors and applied with in a biodegradable, composite hydrogel scaffold in an *in vivo* osteochondral defect.

In the first specific aim, the effects of primary amines, overall charge, and HA length within the bPEI-HA system were investigated by testing various formulations of the polymer. We found that the type of HA saccharide used was the most important design characteristic of the system, while moles of HA incorporated was also important for zeta potential, transfection efficiency, and cytotoxicity. This study demonstrated that incorporation of HA into the delivery vector has the potential to significantly increase transfection efficiency while decreasing cytotoxicity.

In the second specific aim, bPEI-HA/DNA and bPEI/DNA complexes or raw DNA was loaded into a PLGA microparticle system at different concentrations to determine the attributes of the system. We found that the incorporation of bPEI-HA into the system increased the loading and transfection efficiency without altering the morphology or size of the produced particles. Further, the incorporation of HA resulted in significantly altered and accelerated release profiles of both DNA and the polymer in this system. This study indicated that the use of bPEI-HA in a PLGA microparticle system is feasible and has many advantages towards generating a particle capable of controlled release and transfection of nearby cells.

In the final specific aim, bPEI-HA/DNA complexes were loaded into an OPF composite scaffold to develop a bilayered osteochondral tissue engineering system capable of regenerating bone and cartilage *in vivo*. We found that the incorporation of DNA encoding for the Sox trio and Runx2 combined was...
capable of inducing superior tissue growth and quality, especially of bone. Further we found that the incorporation of only Runx2 in the system has positive effects on the bone growth and quality when compared to groups without Runx2 DNA. Additionally, the incorporation of bPEI-HA in this system likely accelerated the degradation of the OPF hydrogel implant resulting in superior tissue ingrowth. This work suggests that polymeric gene delivery is a viable option for osteochondral tissue generation in vivo.

The gene delivery vector explored in this work, bPEI-HA, is a promising vector for non-viral gene therapy and the findings presented in this work have gone a long way towards developing options for its application. When developing a controlled release system involving this, or any, gene delivery vector, it is important to consider the overall loading potential of the system. While the PLGA system used in specific aim 2 has a preferable release profile for in vivo application, the loading of this system is insufficient to elicit a therapeutic response in the target tissues.

We have demonstrated that bilayered application of bPEI-HA/DNA encoding for the SOX trio and RUNX2 has an effect on tissue regeneration in vivo. To our knowledge, this is the first time that unconstrained application of these transcription factors in vivo has been performed. This work has developed the bPEI-HA gene delivery vector to a point in which the synthesis system is understood and superiority over bPEI has been shown for many cases.
References


42. Toole, B. P., Seminars in Cell and Developmental Biology 2001, 12, 79-87.
49. Toshiyuki Ikeda; Satoru Kamekura; Akihiko Mabuchi; Ikuyo Kou; Shoji Seki; Tsuyoshi Takato; Kozo Nakamura; Hiroshi Kawaguchi; Shiro Ikegawa; Ung-il Chung, Arthritis & Rheumatism 2004, 50 (11), 3561-3573.