LOCALIZED AND SUSTAINED RELEASE OF PLASMID DNA OR siRNA FROM BIOMATERIAL SCAFFOLDS TO PROMOTE OSTEOGENESIS

by

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Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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(date) May 25, 2010

*We also certify that written approval has been obtained for any proprietary material contained therein.
To my loving husband, Nick
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>2D</td>
<td>Two dimensional</td>
</tr>
<tr>
<td>3D</td>
<td>Three dimensional</td>
</tr>
<tr>
<td>α–MEM</td>
<td>Alpha-modified Eagle’s medium</td>
</tr>
<tr>
<td>μg</td>
<td>Micrograms</td>
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<tr>
<td>μl</td>
<td>Microliters</td>
</tr>
<tr>
<td>μm</td>
<td>Micrometers</td>
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<tr>
<td>μM</td>
<td>Micromolar</td>
</tr>
<tr>
<td>°C</td>
<td>Degrees Celsius</td>
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<tr>
<td>ADM</td>
<td>Accell® delivery medium</td>
</tr>
<tr>
<td>ALP</td>
<td>Alkaline phosphatase</td>
</tr>
<tr>
<td>AR-S</td>
<td>Alizarin red S</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenetic protein</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>Calcium chloride</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CaP</td>
<td>Calcium phosphate</td>
</tr>
<tr>
<td>Cbfa1</td>
<td>Core binding factor α 1</td>
</tr>
<tr>
<td>cm</td>
<td>Centimeter</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>CT</td>
<td>Computed tomography</td>
</tr>
<tr>
<td>deGFP</td>
<td>Destabilized GFP</td>
</tr>
<tr>
<td>Dex</td>
<td>Dexamethasone</td>
</tr>
<tr>
<td>diH₂O</td>
<td>Deionized water</td>
</tr>
<tr>
<td>dl</td>
<td>Deciliter</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle medium</td>
</tr>
<tr>
<td>DMEM-LG</td>
<td>Dulbecco’s modified eagle medium – low glucose</td>
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<tr>
<td>DMEM-HG</td>
<td>Dulbecco’s modified eagle medium – high glucose</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dsDNA</td>
<td>Double stranded DNA</td>
</tr>
<tr>
<td>dsRNA</td>
<td>Double stranded RNA</td>
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<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>eGFP</td>
<td>Enhanced GFP</td>
</tr>
<tr>
<td>ES</td>
<td>Embryonic stem (cell)</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>N/P</td>
<td>Nitrogen to phosphate ratio</td>
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<tr>
<td>NP</td>
<td>Nanoparticle</td>
</tr>
<tr>
<td>OP-1</td>
<td>Osteogenic protein – 1</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>pDNA</td>
<td>plasmid DNA</td>
</tr>
<tr>
<td>PEG</td>
<td>Poly(ethylene glycol)</td>
</tr>
<tr>
<td>PEI</td>
<td>Poly(ethyeneimine)</td>
</tr>
<tr>
<td>PEO</td>
<td>Poly(ethylene oxide)</td>
</tr>
<tr>
<td>pg</td>
<td>Picogram</td>
</tr>
<tr>
<td>PGA</td>
<td>Poly(glycolic acid)</td>
</tr>
<tr>
<td>PLLA</td>
<td>Poly(L-lactic acid)</td>
</tr>
<tr>
<td>PLGA</td>
<td>Poly(lactic-co-glycolic acid)</td>
</tr>
<tr>
<td>psi</td>
<td>Pounds per square inch</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>RFP</td>
<td>Red fluorescent protein</td>
</tr>
<tr>
<td>RGD</td>
<td>Arginine – glycine – aspartic acid peptide</td>
</tr>
<tr>
<td>RISC</td>
<td>RNA-induced silencing complex</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>RNase</td>
<td>Ribonuclease</td>
</tr>
<tr>
<td>rpm</td>
<td>Rotations per minute</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>SCID</td>
<td>Severe combined immunodeficient</td>
</tr>
<tr>
<td>sec</td>
<td>Second</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>shRNA</td>
<td>Short hairpin RNA</td>
</tr>
<tr>
<td>siRNA</td>
<td>Short interfering RNA</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>w/v</td>
<td>Weight to volume ratio</td>
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Biomaterial Scaffolds for Non-Viral Gene Delivery: Localized and Sustained Release of Plasmid DNA or siRNA to Promote Osteogenesis

Abstract

by

MELISSA DIANE KREBS

There is a critical need for bone replacement tissue. Each year in the United States, roughly 0.5-1 million patients experience problematic healing of a bone fracture. Tissue engineering and regenerative medicine seek to engineer lost or damaged tissue, often with a combination of biomaterial scaffolds, bioactive factors, and transplanted cells. This thesis aimed to develop gene delivery systems that promote osteogenesis, by investigating the hypothesis that the sustained and localized delivery from a biomaterial scaffold of DNA encoding for osteogenic factors or siRNA for knockdown of osteogenic-inhibitory genes will lead to improved osteogenesis. The release of plasmid DNA and DNA complexed with poly(ethyleneimine) (PEI-DNA) from gas-foamed poly(lactic-co-glycolic acid) (PLGA) scaffolds was tailored by the incorporation of varying amounts of alginate polysaccharide, and prolonged release of PEI-DNA nanoparticles from the PLGA scaffolds was attained for at least 35 days. The use of injectable biomaterial scaffolds was also examined, as these can be placed into a defect in a minimally invasive manner. PLGA can be dissolved in an FDA-approved solvent, tetroglycol, and when injected into an aqueous environment, it will solidify. Porous injectable PLGA scaffolds
were fabricated by the addition of porogens and water into the PLGA solution. These porous scaffolds were thoroughly characterized and were shown to support the growth of cells throughout the bulk of the scaffold. Furthermore, DNA was incorporated into the scaffolds, and the release rate tailored by incorporation of the DNA into microspheres. Additionally, alginate hydrogels were examined for the release of DNA and DNA incorporated into calcium phosphate nanoparticles. These injectable hydrogels containing DNA-calcium phosphate nanoparticles and preosteoblastic cells were shown to promote heterotopic osteogenesis in vivo. In addition to the delivery of DNA for upregulation of osteogenic factors, another powerful option for the promotion of osteogenesis through genetic regulation could be to deliver siRNA to downregulate factors that inhibit bone formation. siRNA incorporated into calcium-crosslinked or photocrosslinked alginate hydrogels or collagen hydrogels was released in a sustained manner for at least 1-2 weeks. The siRNA remained bioactive, capable of silencing gene expression in cells. The treatment of human mesenchymal stem cells with siRNA against osteogenic inhibitory genes was examined to determine if they could be promoted to differentiate down the osteogenic lineage via this approach. However, the silencing of five different genes identified as potential targets from the literature (GNAS, P2RY11, adenosine kinase, noggin, and chordin) failed to have a significant effect on their differentiation, indicating the complexity of stem cell fate decisions. Overall, this thesis demonstrates the utility of biomaterial scaffolds for providing sustained and localized gene delivery. The released genetic material can influence cellular behavior, and these systems offer great promise for tissue regeneration.
CHAPTER 1. Introduction

1.1 Problem Statement

There is a critical need for replacement tissues due to organ failure and tissue loss. In the United States in 2007, there were approximately 97,000 people on waiting lists for organ transplantation, yet only ~27,500 organ transplants were performed that year.\(^1\) This persistent lack of available organ donations is a large part of the motivation in advancing the field of tissue engineering, with the goal of using both biology and engineering principles to create new tissue by encouraging the growth of cells under the proper conditions.\(^2\) In general, the field of tissue engineering seeks to regenerate diseased or damaged tissues by providing the necessary physical, biochemical, and cellular cues that promote tissue regeneration.

Every year in the United States, musculoskeletal conditions cost $254 billion due to health care costs and lost productivity, and are the top reasons for patient visits to their doctors each year.\(^3\) Roughly 30% of Americans currently have some form of arthritis, 50% of women and 12% of men over the age of 50 will suffer from a fracture related to osteoporosis during their life, and each year millions of people are disabled with musculoskeletal injuries in road accidents.\(^3\) There is a critical need for bone tissue replacement in particular. In the United States, there are 8 million bone fractures every year with 5-10\% of these exhibiting problematic healing.\(^4\) Bone grafts are second only to blood in being the most commonly transplanted tissue, with more than 500,000 bone graft procedures performed in the U.S. each year.\(^5\)
For large bone defects, the gold standard in treatment is the use of autografts, where bone is harvested elsewhere from the patient (typically the iliac crest) and then placed into the defect. However, the disadvantages include donor site morbidity and lack of sufficient amount of bone that can be harvested. Allografts can be used as an alternative to autografts, where the tissue is harvested from a donor. These are used less frequently due to concerns over host rejection and disease transmission. To help mitigate these concerns, allografts can be decellularized and sterilized, but this additional processing negatively impacts both the mechanical strength and osteoinductivity of the grafts. The use of tissue engineering to regenerate the affected bone is an attractive approach as it does not contain the inherent disadvantages associated with bone grafts.

1.2 Hypothesis

Tissue engineering approaches often use a biomaterial scaffold with bioactive factors to help induce the formation of the desired tissue within a defect site. Also, cells can be incorporated into the biomaterial system in order to help repopulate the defect with the appropriate cell type. For bone regeneration, bone morphogenetic proteins (BMPs) are frequently used as the bioactive factors to help stimulate osteoblastic differentiation and matrix secretion. In particular, one of the BMPs shown to have a strong osteoinductive effect is BMP-2. This growth factor is involved in osteoinductive signaling pathways and promotes the differentiation of mesenchymal stem cells into osteoblasts. The delivery of BMP-2 for bone regeneration holds much promise but still suffers from the need to deliver large amounts of protein to elicit an effect due to protein degradation in vivo and poor retention locally at the site of injury.
An attractive alternative is localized gene delivery to allow sustained expression of specific osteoinductive growth factors by host and/or transplanted cells near the defect site. An important consideration in using gene delivery \textit{in vivo} is the retention of the genetic material at the site of the injury to promote a strong therapeutic effect. Although many groups have performed direct injections of DNA for increased gene expression to promote osteogenesis, the duration of expression is difficult to control and often limited, and the timing of the injection is extremely important for therapeutic benefit.\textsuperscript{14} Biodegradable materials can be of great use in allowing sustained delivery of DNA at the desired location.\textsuperscript{15}

The delivery of DNA to cells serves to upregulate the expression of a desired protein. However, it is also possible to deliver short interfering RNA (siRNA) to downregulate gene expression. The use of siRNA in tissue engineering applications is an intriguing option, as it may be beneficial to silence the expression of genes that hinder a regenerative process.\textsuperscript{16} Additionally, it may be useful in guiding the differentiation of progenitor cells (either present in the defect or transplanted there) down a desired pathway. For instance, recent work has demonstrated that there are several genes in mesenchymal stem cells (MSCs) that when silenced, promote the cells’ differentiation down the osteoblastic lineage.\textsuperscript{17}

The central hypothesis for this thesis is that the sustained, localized delivery from a biomaterial scaffold of DNA encoding for osteogenic factors or siRNA for knockdown of an osteogenic-inhibitory gene, combined with the transplantation of cells of osteogenic potential such as osteoblasts or mesenchymal stem cells, will promote osteogenesis. A thorough investigation into the use of different biomaterials
that will provide sustained and localized release of DNA or siRNA to both transplanted cells as well as surrounding host cells will impact the fields of gene delivery and bone tissue engineering.

1.3 Specific Aims and Strategy

The following specific aims will address the hypothesis of this work:

**Aim 1:** To engineer and characterize systems for sustained, localized delivery of DNA from poly(lactide-co-glycolide) (PLGA) and alginate biopolymer matrices, and to demonstrate that delivery of DNA encoding for osteogenic factors can enhance osteogenesis. The strategy to accomplish this aim is as follows:

1. Engineer stable DNA-nanoparticles, characterize them, and examine their ability to transfect cells and thereby achieve gene upregulation.
2. Achieve sustained release of bioactive DNA and DNA-nanoparticles from gas-foamed PLGA scaffolds, injectable PLGA scaffolds, and alginate hydrogels by varying scaffold properties such as material, molecular weight, net charge, and crosslinking.
3. Transplant osteogenic cells within a DNA-containing alginate hydrogel subcutaneously and demonstrate enhanced osteogenesis *in vivo.*

**Aim 2:** To demonstrate that knockdown of an osteogenic-inhibitory gene will induce MSC differentiation down an osteogenic pathway, and further, that this knockdown can occur in a sustained manner within a three-dimensional alginate or collagen hydrogel system which exhibits prolonged, localized delivery of siRNA. Also, to demonstrate that
MSCs and the siRNA delivered together within an alginate or collagen hydrogel system will promote osteogenesis \textit{in vivo}. The strategy to accomplish this aim is as follows:

1. Achieve sustained release of siRNA from alginate and collagen hydrogels over time by varying scaffold properties such as molecular weight, net charge, and crosslinking. Verify bioactivity of released siRNA for gene knockdown using a reporter gene, GFP, constitutively expressed in a cell line.

2. Apply siRNA against osteogenic-inhibitor gene to MSCs in monolayer and demonstrate that knockdown of this gene will promote differentiation into the osteogenic phenotype \textit{in vitro} as evidenced by the expression of osteogenic markers.

3. Treat MSCs with siRNA against BMP antagonists, BMP-2, or both together to examine the effects each has on osteogenesis and to determine if the combined effects are additive or synergistic.

\textbf{1.4 Significance}

These studies will have an impact on both the fields of gene delivery as well as bone tissue engineering. Although others have investigated the use of biomaterials for the delivery of genetic material, many challenges remain. The fabrication of scaffolds that will incorporate DNA or siRNA must involve gentle enough processing so as to allow these molecules to maintain their bioactivity and not be denatured or degraded. Furthermore, for many applications it is beneficial for the genetic material to be released over a sustained period of time from the biomaterial scaffold in order for it to have a prolonged effect. Finally, cells must be able to uptake the genetic material that is
released, and as they do not readily uptake naked DNA or siRNA, these molecules often need to be complexed with carrier polymers or chemically modified to promote penetration of the cell membrane.

In addition to these challenges, the transfection efficiency must be considered. Viral methods for gene delivery are highly efficient but also have significant disadvantages, including the risk for immune reaction, genetic mutations based on recombination events, and expression that is prolonged beyond the time required for defect healing. Although significantly safer, non-viral gene delivery methods tend to be much less efficient. As such, it is important to ensure that a non-viral gene delivery system from a biomaterial scaffold is able to provide sufficient cell transfection to have a therapeutic effect.

Due to all of these challenges, further research in this field is necessary to help advance the state of the art in biomaterial gene delivery. This thesis aims to contribute significantly to this field by examining several different biomaterial systems. The release of DNA from gas-foamed poly(lactide-co-glycolide) (PLGA) scaffolds was tailored by the addition of varying amounts of alginate to the system in order to improve the use of these scaffolds for gene delivery by providing more sustained release. Porous, injectable PLGA scaffolds were engineered and thoroughly characterized, and the release of DNA from these scaffolds was prolonged by its incorporation into PLGA microspheres prior to adding it to the injectable PLGA solution. Calcium crosslinked alginate hydrogels were examined for the delivery of naked DNA and DNA encapsulated in calcium phosphate nanoparticles, and this system was shown to promote osteogenesis in vivo. Additionally, the release of siRNA from calcium crosslinked and photocrosslinked alginate and
collagen hydrogels was examined for the first demonstration of localized and sustained delivery of siRNA from macroscopic biopolymer scaffolds. Although the systems developed herein can be applied to many situations in which localized and sustained gene delivery is desired, this thesis will focus on the promotion of osteogenesis to help repair bone defects.

1.5 References


CHAPTER 2. Tissue Engineering Approaches for Bone Regeneration

2.1 Introduction to Bone Fractures and Healing

When bone is fractured, there is a natural series of complex events that occur at the defect site. The blood vessels in the area are ruptured, and there follows hematoma formation and coagulation to stop the bleeding. During this process, inflammatory cells release cytokines that recruit progenitor cells to the area; these cells contribute to the formation of new extracellular matrix, and within about one week a soft tissue callus has formed which fills the defect space but which is not load bearing. Mesenchymal stem cells that have been recruited to the area will differentiate into osteoblasts or chondrocytes, and these cells will contribute to the formation of woven bone and cartilage, with the cartilage then being ossified during the process of endochondral ossification. At this point, weight bearing loads placed on the defect will lead to strengthening of the tissue at the load bearing sites and resorption of the callus tissue at the non-load bearing sites. The callus will ultimately be remodeled to fully restore the shape, size, intricate architecture, and function of the original bone.

However, fracture healing can be disrupted due to various factors, and the bone defect may not be able to heal naturally. As stated in the previous chapter, out of 8 million bone fractures every year in the U.S., 5-10% of these exhibiting problematic healing. Fractures which contain splintered bone often have difficulty healing properly as the dead fragments of splintered bone must be resorbed, delaying the healing process and often leading to overly enlarged callus formation that never properly heals. If the defect site is not immobilized properly, the strain on the callus will prevent proper
healing and ossification. Additionally, nonunion of the bone defect is a serious problem that results when the defect is too large to bridge itself naturally or when the host cells are incapable of natural repair, there are physiologically low levels of the necessary vitamins or minerals to aid in healing, or in patients with infections, diabetes, or an inability to vascularize new tissue. Thus, the treatment of bone defects caused by trauma or surgical resection that exhibit problematic healing remains a significant challenge in orthopedics.

Currently, defects are often treated in the clinic by autologous or allogenic grafts. Autologous grafts are the gold standard for treatment of bone defects. However, there is limited tissue availability for autologous grafts and they have the significant disadvantage of donor site morbidity. Allogenic grafts may provide a greater amount of tissue that can be used, but have the disadvantages of the possibilities for disease transmission or host rejection.

Tissue engineering of bone seeks to provide an alternate therapeutic approach to healing bony defects. There are many chemical, cellular, and mechanical cues within a bone defect, and tissue engineering approaches often seek to use or recreate these signals to craft a local environment that is conducive to bone regeneration. Other tissue engineering approaches may try to recreate the signaling environment that is found during fetal development to grow new bone. Often biomaterial scaffolds are used to provide a space-filling three dimensional matrix in which cells can migrate and proliferate. Ideally the scaffold material will be biodegradable so that as new tissue is formed by the cells at the site, the scaffold can degrade away over time, leaving only fully regenerated natural tissue at the site. Soluble factors such as growth factors or DNA are also frequently incorporated in order to recruit cells to the area and direct their
proliferation and differentiation, or to influence the behavior of a population of transplanted cells. It can be desirable to include a population of transplanted cells in the tissue engineering strategy, especially if there is an insufficient number of cells surrounding the defect to repopulate it, or if waiting for host cells to migrate to the area would result in a too long of a healing time or incomplete healing. Many scaffolds are formed *ex vivo* and then implanted. In contrast, injectable biomaterials offer a minimally invasive alternative for administration directly to the site of interest.

2.2 Biomaterial Scaffolds

The choice of the material used to make a scaffold for tissue engineering is an important consideration. It must be biocompatible, and ideally will degrade over time as new tissue is formed. The mechanical properties may be important as well. Furthermore, the surface chemistry of the scaffold must be able to support cell adhesion and proliferation.

2.2.1 Nondegradable vs. degradable materials

There are a variety of materials available that can be used to construct tissue engineering scaffolds. These materials can be nondegradable or degradable. Examples of nondegradable materials that are used in bone tissue engineering include metals such as titanium, some ceramics, and some synthetic polymers such as polytetrafluoroethylene (PTFE), polymethylmethacrylate (PMMA), PMMA/ polyhydroxyethylmethacrylate (PHEMA), and high density polyethylene.\textsuperscript{4-7} These materials have been met with good success when used as bone substitutes, but one of the major long-term difficulties with
their use is that they have higher mechanical properties than native bone tissue. As such, the phenomenon of stress shielding is a major concern over time, wherein the stronger implant will absorb the mechanical stress in the area, so that the native bone surrounding the implant is not subjected to this stress. This mechanical environment in a load-bearing area results in the bone being resorbed at a higher rate than it is deposited, and can thus cause the implant to loosen over time, causing patient discomfort and the need for additional surgeries to correct the problem.

Degradable biomaterials are of great interest as they circumvent the problems associated with the nondegradable implants. The choice and design of the degradable material is critical to the tissue regeneration process, as a material that degrades too rapidly will not provide sufficient mechanical support over time and may not allow enough time for tissue regeneration throughout the defect. Conversely, a very slowly degrading material will offer disadvantages similar to a nondegradable material. The optimal choice would be a biomaterial scaffold that degrades at the same rate as new tissue is formed, thus preserving the mechanical stability and bridging of the defect. The degradation rate of a polymer scaffold has been demonstrated to impact both the quantity and quality of bone tissue formation.

Degradation rates are influenced by the polymer structural and chemical properties and by the environment to which it is exposed, such as mechanical loading, pH, ionic strength, enzymatic activity, and temperature. There is a wide range of degradable materials that have been examined for bone tissue engineering. These materials include synthetic polymers such as the FDA-approved poly(glycolic acid) (PGA), poly(lactic acid) (PLA), and their copolymers poly(lactic-co-glycolic acid)
(PLGA). These α-hydroxy esters undergo bulk degradation by hydrolysis into lactic acid and glycolic acid which can be excreted from the body, although unfortunately they can also serve to decrease the pH of the local environment before they are removed.\textsuperscript{10} Other examples of synthetic classes of polymers that have been investigated are polyanhydrides, which degrade by surface erosion, and poly(propylene fumarate) which degrades by hydrolysis into fumaric acid and propylene glycol.\textsuperscript{10} There are also many natural biodegradable polymers such as collagen, hyaluronic acid, and alginate which have been used for bone regeneration applications. Collagen and hyaluronic acid can be enzymatically degraded, while alginate hydrogels dissociate over time as their divalent cationic crosslinkers leach out. These and other materials have also been chemically modified with methacrylates to allow crosslinking by application of UV light, and some of these materials can degrade by hydrolysis.\textsuperscript{11}

2.2.2 Natural vs. synthetic materials

2.2.2.1 Synthetic materials

Synthetic polymers offer a major advantage in that they can be customized to achieve a wide range of chemical and mechanical properties. Furthermore, they can be manufactured in large quantities, and there are no concerns with these materials being immunogenic, although biocompatibility for a given application must still be closely examined.\textsuperscript{12} These polymers will often be subjected to fibrous encapsulation, as proteins adsorb to their surface and a tissue capsule forms around the implant.\textsuperscript{7} This can be problematic especially if the implant is supposed to release bioactive factors to the surrounding tissue. Thus, much research has been focused on the surface modification of
these polymer scaffolds to encourage migration, adhesion, and proliferation of the desired cell populations; this topic will be discussed in more detail below. However, the advantages of being able to tailor the mechanical properties, degradation, and chemical features provide sufficient advantage that synthetic polymers are heavily researched for tissue engineering scaffolds.

There are also many synthetic ceramic materials that have been examined; these seem very promising due to their high compressive strength and chemical composition that is similar to native bone. However, they are very brittle and thus their use is often restricted to non-load-bearing sites. Two widely researched ceramics are tricalcium phosphate, which is chemically similar to amorphous bone precursor, and hydroxyapatite, which is chemically similar to bone mineral. Hydroxyapatite does not degrade appreciably over time, while tricalcium phosphate can be resorbed. Osteoid can be produced on the surface of the ceramics when they are bonded directly to healthy bone. Injectable calcium phosphate cement has also been widely examined and appears to be very promising for use as an osteoconductive scaffold. The implant has an initial compressive strength similar to bone and undergoes remodeling such that it can eventually be fully replaced by new bony tissue.

2.2.2.2 Natural polymers

Natural polymers have also gained attention due to their many beneficial properties such as low immunogenicity, non-toxicity, and similarity to the extracellular matrix. Many natural polymers are extremely hydrophilic and are often used in the form of a hydrogel. Collagen is frequently used as a scaffold for bone regeneration, as
this molecule is the primary protein component of bony tissue. The disadvantages to the use of collagen include its weak mechanical properties and its potential for eliciting an immune response. Another interesting natural material is demineralized bone matrix, which is bone that has been decalcified but which retains collagen and other proteins including growth factors. This material has been used clinically, and appears to have strong osteoinductive potential, likely due to the growth factors that remain in the matrix.\textsuperscript{17} However, concerns remain as this is an allogenic graft and thus poses risks for disease transmission. Also, some researchers have examined the use of coralline ceramics, or coral made by marine invertebrates. The natural porosity of these ceramics appears to be in the ideal range for bony tissue ingrowth and they offer promise for use as osteoconductive scaffolds.\textsuperscript{15, 18}

\textbf{2.2.2.1 Polysaccharides}

Polysaccharides in particular are of great interest as natural materials because they have many properties that lend them well to use in biological systems, including their hydrophilic nature and water solubility, high oxygen permeability, unique mechanical properties due to high water-uptake and retention in aqueous environments, biocompatibility, and biodegradation. Additionally, the native extracellular matrix is largely made of glycosaminoglycans (GAGs) which are long, unbranched chains of repeating disaccharides. Since cells are surrounded by polysaccharides in the extracellular matrix (ECM), polysaccharide scaffolds placed into a tissue defect may provide the cells an environment that is similar to their native environment.\textsuperscript{16} The unique intrinsic properties of polysaccharides will be discussed in greater detail below.
Cell Adhesivity

Some polysaccharides can promote cell adhesion, while others are non-adhesive to cells. Hyaluronic acid is a ubiquitous mammalian-derived polysaccharide that is a native extracellular matrix molecule. Cells are able to naturally adhere to this material through the CD44 receptor on their surface. As such, it has found use in promoting cellular adhesion to various biomaterial systems. For instance, recently, hyaluronic acid has been added to electrospun PLLA and to electrospun PCL scaffolds to promote cell adhesion and infiltration into the scaffolds, and has been used to coat polysulfone membranes for increased cell attachment. Another material that has been utilized in its native form for increasing cell adhesion is chitosan. Interestingly though, chitosan itself, a positively charged polysaccharide, is not cell adhesive. However, when incubated in the presence of proteins, such as those found in serum-containing cell culture medium, the proteins will adsorb to the material due to electrostatic interactions. Cells will then adhere to these adsorbed proteins on the surface. This makes chitosan unique as most polysaccharides do not readily adsorb proteins, but the positive charge of chitosan does allow this to happen. This has been utilized in various systems, including a micropatterned co-culture system, blended into scaffold formulations with PGA, PLGA, or poly(butylene succinate), in chitosan-sugar nanoscaffolds, and in textile-based chitosan scaffolds.

There are several polysaccharides that are naturally non-adhesive to cells which are also utilized in tissue engineering. Agarose, derived from algae, is one such material. Micromolded agarose has been used to provide a non-adhesive surface for self-assembly of microtissue structures, and also self-assembly of chondrocytes into cartilage tissue.
Alginate, also derived from algae, does not allow for cell adhesion either. However, one group did find that by crosslinking the alginate with iron instead of calcium ions, it was possible to achieve serum protein adsorption and therefore increased cell attachment without additional modification of the material.\textsuperscript{32} The use of non-adhesive substrates such as alginate could also have great potential for non-fouling surfaces that would, for example, prevent the adhesion of bacteria.\textsuperscript{33}

\textit{In vivo}, cells will bind to the ECM via interaction of integrins on the cell membrane with ligands found in the ECM. There are many different types of ligands found in the ECM, such as collagen, laminin, fibronectin, and vitronectin. However, only small peptide fragments of these large molecules are actually recognized by the cell; for example, the sequence of arginine – glycine – aspartic acid (RGD) is a cell adhesive peptide fragment in fibronectin and other ECM proteins.\textsuperscript{34} By chemically modifying a material to contain these short cell adhesion peptides, cells can adhere to materials that may have been otherwise nonadhesive. This has been widely used in the field of tissue engineering, as it allows the use of materials that may have other advantages but that are naturally lacking cell adhesiveness to now be used for the adhesion and further culturing of cells on or in the material. There are several recent examples of these modifications in the literature, including the modification of chitosan with a laminin peptide module for cell adhesion and neurite outgrowth,\textsuperscript{35} with cell adhesive peptides for neural tissue engineering,\textsuperscript{36} and with GRGDS for cell adhesion.\textsuperscript{37} Another recent report demonstrates the modification of hyaluronic acid with RGD peptides for brain tissue engineering,\textsuperscript{38} although this chemical modification was shown much earlier by Glass, et al.\textsuperscript{39} Alginate
has also been modified with the addition of an RGD peptide sequence,\textsuperscript{40} and this system has been extensively used in the literature since then.

\textit{Mechanical Properties}

Another interesting feature of polysaccharides is their mechanical properties. Due to their hydrophilicity, they are able to uptake and retain a significant amount of water; this provides them a great ability to bear compressive loads, as the attraction for water molecules creates a resistance against water flow out of the material even under strong compressive forces.\textsuperscript{41} Polysaccharides display viscoelastic mechanical properties; their elastic modulus will vary greatly depending on the rate of loading.\textsuperscript{42} The unique mechanical properties of polysaccharides are often exploited in tissue engineering. There are several recent reports of this in various systems, especially with the use of chitosan. Chitosan has been shown to increase the mechanical properties of calcium phosphate cement for bone regeneration applications.\textsuperscript{43, 44} Wang, et al. hypothesized that this is due to the ability of the chitosan to bind the calcium phosphate particles together more tightly as the cement set.\textsuperscript{45} Chitosan and chitin have been mixed into poly(ethylene oxide) (PEO) scaffolds to increase the modulus of elasticity.\textsuperscript{46} Branched chitosan was found to provide higher mechanical properties than linear chitosan with no effect on cell adhesion; the authors hypothesized that this was due to increased hydrogen bonding in the branched structure compared to the linear structure.\textsuperscript{47}
Hydrophilicity

The hydrophilic nature of polysaccharides also offers additional benefits for use in tissue engineering. Since polysaccharides are soluble in aqueous solutions, typically at neutral pH (with some exceptions), this allows the formation of scaffolds or micro- or nano-particles under physiologic conditions. The implication of this is that cells or bioactive factors such as DNA or growth factors can be easily incorporated into these materials without concerns about toxicity or denaturation. A recent report demonstrates the successful use of alginate microspheres to encapsulate cells to protect them during calcium phosphate cement mixing and setting. Encapsulated cells have been shown to maintain their bioactivity, as shown by the encapsulation of bone marrow stromal cells in alginate microspheres, which were shown to retain their capacity to undergo osteogenic differentiation while encapsulated. However, another study highlighted the importance of construct design when encapsulating chondrocytes in alginate microspheres, as they showed that different cell densities and alginate concentrations gave different chondrogenic responses.

Additionally, polysaccharides have been utilized to increase cell retention within scaffolds. For instance, cells have been mixed within an alginate hydrogel, which was then dripped onto scaffolds made of chitosan and alginate, and subsequently crosslinked with calcium chloride solution to immobilize the cells in the scaffold. This was shown to retain more cells within the fibrous scaffolds compared to the conditions without the added alginate. Also, PLLA scaffolds have been filled with chondrocytes encapsulated in agar to increase the retention of the cells within these scaffolds.
Protein Affinity

The native ECM acts as a reservoir for a number of growth factors. Various polysaccharides have an affinity for protein, and are therefore often used for modulating release of protein (typically growth factors) from a hydrogel, or even for retention of native growth factors that diffuse into the hydrogel in an in vivo environment. The major polysaccharide that has been utilized in this realm is heparin, a highly sulfated glycosaminoglycan. There have been numerous recent reports on the addition of heparin to biomaterial systems for the retention of growth factors within the system. Woodruff, et al. also demonstrated that delivering heparin by release from a fibrin matrix to a bone defect decreased the wound healing time, which they hypothesized was due to increased local growth factor concentration (without the delivery of growth factors) due to the heparin affinity interactions with native proteins. Alginate sulfate has been shown to have a very similar effect as heparin; when mixed within alginate scaffolds, alginate sulfate provides affinity binding of heparin-binding proteins. Also, hyaluronic acid has been shown to trap bone morphogenetic protein 2 (BMP-2) into a PLLA - hyaluronic acid layered scaffold by affinity interactions. On the other hand, it may also be possible to increase protein release from a system by incorporating a polysaccharide component that does not have a natural affinity for protein, and which serves to increase the hydrophilicity and therefore decrease the protein affinity of the system. One group has shown that the addition of chondroitan sulfate to collagen scaffolds increases the hydrophilicity of the scaffolds and also provides increased protein release.
DNA Affinity

As mentioned previously, chitosan is a positively-charged polysaccharide. This positive charge allows it to form complexes with DNA via electrostatic interactions. This has been used extensively for gene delivery applications. Chitosan mixed with DNA can form nanoparticles for delivery of the DNA to cells; these complexes retain an overall positive charge and are thus able to directly interact with the negatively charged cell membrane. These complexes are then uptaken by cells via endocytosis. There is a plethora of literature examining the use of chitosan-DNA nanoparticles for gene delivery; for an extensive review of this topic, see Dang, et al.\textsuperscript{64} Two recent examples of the use of this technology include the addition of chitosan-DNA nanoparticles to an electrospun scaffold for gene delivery to promote bone regeneration,\textsuperscript{65} and the synthesis of thiolated chitosan – DNA nanoparticles which showed enhanced and sustained gene transfection.\textsuperscript{66} Chitosan has also been used in scaffold-format, with DNA subsequently adsorbed to the surface. For instance, chitosan and collagen scaffolds have been fabricated, and plasmid DNA or adenovirus were subsequently adsorbed to the surface.\textsuperscript{67, 68} Similarly, scaffolds made of only chitosan have been used for delivery of adenovirus to periodontal ligament cells.\textsuperscript{69} A more complex formulation involved the fabrication of a gene activated matrix that was comprised of chitosan and collagen scaffolds, which were loaded with chitosan-DNA nanoparticles.\textsuperscript{70} Another system that has been demonstrated is the creation of chitosan – alginate polyelectrolyte draw fibers that are used for gene delivery.\textsuperscript{71}
**Inherent Signaling Properties of Hyaluronic Acid**

One of the interesting features about using polysaccharides in tissue engineering applications is that many have intrinsic features which are naturally bioactive. For instance, hyaluronic acid has been shown to have intrinsic bioactivity in many tissue engineering applications. This polysaccharide has been implicated as a stem cell niche.\(^7_2\) It has been found to act as a signaling molecule for hMSCs.\(^7_3\) Turner, et al. demonstrated the ability to maintain a hepatic progenitor cell phenotype even over the course of 4 weeks of culturing these cells in hyaluronic acid hydrogels.\(^7_4\) Sulfated hyaluronan has been shown to suppress the proliferation of keratinocytes and increase their differentiation into mature keratinocytes.\(^7_5\) A commercial product called Hyalomatrix (Addmedica, Paris, France) has been shown to dramatically improve wound healing; this product consists of a bilayer scaffold of hyaluronan and silicone membrane.\(^7_6\) The silicone membrane acts as a barrier against microorganisms in the external environment, while the hyaluronan environment seems to provide a cultivating environment for keratinocyte stem cells to proliferate and differentiate, thus repopulating the dermis and allowing for increased healing.\(^7_6\)

**Blood compatibility**

Heparin is a polysaccharide with anticoagulant properties. It is able to bind thrombin, catalyzing the subsequent binding of antithrombin with thrombin, which inactivates the thrombin molecule. This prevents thrombin from catalyzing the change of soluble fibrinogen into an insoluble fibrin clot. The heparin itself can then dissociate from the bound thrombin / antithrombin complex, and can be reused to bind another free
thrombin molecule. This ability of heparin to be recycled and still maintain its bioactivity has found much interest in the tissue engineering field as a mechanism by which to increase the blood compatibility of various biomaterial implants. Any material which is implanted in vivo will initiate the clotting cascade, and this could lead to the eventual deposition of a collagen wall around the implant, as the body is trying to isolate the unknown object if it is unable to otherwise get rid of it. This could drastically decrease the utility and effect of the implant, so in many cases it is desirable to disable the coagulation cascade in that area. The use of heparin for this purpose is reported extensively throughout the literature. It has been released from scaffolds that are implanted at a defect site, and has been used in biomaterial surface modifications (see Olsson, et al. for a review on this topic).

Mimicking the Native ECM

As the native ECM is largely comprised of polysaccharides, it has been examined if the inclusion of some of these polysaccharides into biomaterial systems may indeed serve to mimic the native ECM and thereby enhance the cellular response to the biomaterial. This has been widely explored for chondrogenesis as hyaluronic acid and chondroitin sulfate are major cartilage ECM components, but may apply to other tissues as well. For example, the addition of chondroitan sulfate and hyaluronic acid to collagen hydrogels for culturing chondrocytes was found to increase cartilage formation compared to collagen alone. Chung and Burdick showed that hyaluronic acid hydrogels showed increased chondrogenesis of mesenchymal stem cells as compared to inert PEG hydrogels. Wei, et al. demonstrated that the addition of chondroitan sulfate to fibrin
matrices increased the chondrogenic differentiation of adipose-derived stem cells as compared to fibrin alone. Also, Varghese, et al. showed that the addition of chondrotian sulfate to PEG hydrogels increased the chondrogenic differentiation of mesenchymal stem cells. It is likely that this increase in chondrogenesis shown in these various systems may be due to culturing the chondrocytic cells in an environment that is more like the cartilage ECM found in vivo.

In summary, although polysaccharides are certainly not the only option for natural materials, they have many unique properties that are beneficial for use as tissue engineering scaffolds.

2.2.3 Scaffold design

In addition to the particular biomaterial chosen, the structural design of the scaffold can also play an important role in encouraging tissue regeneration. Ideally, the scaffold should fill the dimensions and shape of the defect, and contain an architecture that allows for capillary ingrowth, cell migration, and sufficient nutrient diffusion throughout.

Appropriate micron-scale porosity is important for the design of biomaterial scaffolds in tissue engineering applications, as it can permit increased cell adhesion, migration, proliferation, and new extracellular matrix production. Biomaterial scaffold porosity is especially critical when the regeneration strategy implemented relies only on the recruitment of host cells to the defect site, as is the case for techniques which do not include cell transplantation. In this situation, the migration of the host cells
into the scaffold and their subsequent proliferation and secretion of extracellular matrix is required to obtain tissue repair in the defect. Therefore the scaffold must permit, and ideally promote, cellular infiltration throughout. Nanoscale porosity has been shown to insufficient for cell migration, growth, and new extracellular matrix deposition.\textsuperscript{89} For osteogenesis, porosity has been shown to be important in stimulating new bone growth. For instance, porous and nonporous hydroxyapatite particles capable of delivering BMP-2 were compared, and it was found that no bone formation occurred with the solid particles while osteogenesis was achieved with the porous particles.\textsuperscript{90} Furthermore, this group found that pores on the order of 300-400 µm promoted the greatest extent of osteogenesis when implanted subcutaneously \textit{in vivo}.\textsuperscript{90} However, it has also been shown that pores of different sizes in the range of 350-800 mm have no effect on bone formation subcutaneously \textit{in vivo}.\textsuperscript{91} Hulbert, et al. first defined the minimum pore size necessary to achieve osteogenesis \textit{in vivo} as being greater than 100 µm.\textsuperscript{92} Since then, there has been extensive research in further examining the importance of porosity on osteogenesis; this topic has been reviewed extensively by Karageorgiou and Kaplan.\textsuperscript{86}

The mechanical properties of a scaffold are also important. Scaffolds that are too weak will not be able to bear loads on their own and scaffolds that are too strong can cause stress shielding, as mentioned earlier. Even beyond this general principle though, the stiffness of materials has been shown in many studies to directly impact the osteogenic differentiation and response of cells. Engler, et al. demonstrated that the stiffness of polyacrylamide substrates influenced the differentiation of mesenchymal stem cells.\textsuperscript{93} Without exogeneous growth factors or other osteogenic supplements, MSCs were shown to differentiate into osteoblasts on the stiffest gels examined, 25-40 kPa.
osteoblast cells cultured in 3D hydrogel scaffolds were shown to exhibit increased osteogenesis with increasing scaffold stiffness up to 300 kPa.\textsuperscript{94} These pre-osteoblast cells were also examined on 2D substrates of increasing stiffness and found to exhibit increased osteogenesis on the stiffest substrates up to 424 kPa.\textsuperscript{95}

Surface modifications can also be made to scaffolds which influence cell behavior. For example, mesenchymal stem cells were shown to differentiate down the osteogenic lineage when cultured on polyacrylamide hydrogels that had collagen I covalently bound to the surface, but not on hydrogels that had collagen IV, laminin, or fibronectin instead.\textsuperscript{96} The adhesion of cells to biomaterial scaffolds is very important for matrix deposition and proliferation, and there has been significant work in the surface modification of biomaterials to increase cellular adhesion. The use of various techniques for enhancing the adhesion of osteoblasts and mesenchymal stem cells to biomaterials has been reviewed.\textsuperscript{97} For example, the covalent coupling of a cell adhesion peptide containing the arginine-glycine-aspartic acid (RGD) sequence to PEG hydrogels has been shown to influence osteoblast behavior; higher concentrations of RGD peptide led to increased mineral deposition.\textsuperscript{98}

2.3 Cell Sources

Another important scaffold design parameter for bone regeneration is the ability to incorporate cells within the material for cell transplantation approaches. The cellular component of auto- and allografts has been shown to play a critical role in bone regeneration.\textsuperscript{99} It is thus likely that tissue engineering approaches that incorporate cellular transplantation may be more promising for bone regeneration. There are various
cell populations that can be considered for osteogenesis. The cells may either be fully differentiated cells or they may be stem cells, capable of differentiating into a variety of mature cell types.

Osteoblasts are fully differentiated cells that would be applicable for bone regeneration, as they are the cells responsible for bone formation and mineralization. Although at first glance this may seem the most obvious choice of a cell population to use, there are several disadvantages to using fully differentiated cells. One of the largest disadvantages is that these cells do not have a high proliferation potential. This makes it extremely difficult to obtain a sufficient number of cells to treat a defect. Furthermore, the source of cells is a concern; autogenic cells harvested from the donor will lead to an additional injury site where the tissue is harvested from. Also, it is difficult to get enough cells to have an effect as there is a limited availability of cells in harvested tissue. Allogenic cells carry the risks of disease transmission and host rejection.

For these reasons, most research in cell transplantation techniques focuses on the use of stem cells, as they have much greater proliferation capacity and can generally be harvested more easily. Mesenchymal stem cells (MSCs) in particular are a promising cell population for regenerative medicine applications. They are capable of differentiating into a wide variety of connective tissue cells,\textsuperscript{100} they can be easily harvested from the bone marrow,\textsuperscript{101} expanded rapidly \textit{in vitro},\textsuperscript{102} and they migrate towards injured tissue sites.\textsuperscript{100} The differentiation of MSCs down the osteogenic pathway can be promoted by various exogenous growth factors or transcriptional regulation within the cell. The transcriptional regulation can be either positive (promoting differentiation) or negative (inhibiting differentiation).\textsuperscript{103} Adipose-derived mesenchymal stem cells have also been
shown to be capable of differentiation into osteoblasts and offer promise as another source of MSCs. Additionally, it may be possible to use embryonic stem (ES) cells and induce their differentiation down the osteogenic lineage; however, significant concerns surround the use of these cells such as tumorgenicity, immunorejection, homogeneity of cells after differentiation, and ethical concerns. Another interesting prospect for the future is the use of induced pluripotent stem (iPS) cells, which are adult fibroblast cells transformed into embryonic-like stem cells. These cells would not carry the ethical concerns of ES cells, yet the other challenges associated with the use of ES cells will still remain. These cells have yet to be examined for their ability to differentiate into osteoblasts.

2.4 Bioactive Factor Delivery

In addition to the biomaterial scaffold and a population of transplanted cells, it is also very common to deliver bioactive factors to help encourage the osteogenic process within a bone defect. There are many bioactive factors that can be used, with growth factors and DNA encoding for growth factors being the most commonly employed.

2.4.1 Growth Factor Delivery

Many growth factors that promote the bone healing response have been identified. The BMPs, which belong to the TGF-β family, are widely used as these proteins have been shown to strongly induce bone formation. One study compared fourteen of the BMPs and found that all of them had strong stimulatory effects on osteogenesis of osteoblastic cells, but BMP-2, -6, and -9 had the strongest osteoinductive effects on
murine MSCs. This group also found that upregulation of BMP-2, -6, -7, and -9 expression by adenoviral transfection promoted bone formation in vivo. BMPs bind to BMP receptors (type I or type II) on the cell surface, which then activates intracellular transcription factors smad-1, -5, and -8. These activated factors then bind with smad-4 and the newly formed complexes are transported into the nucleus where they regulate gene transcription. Additionally, BMPs activate other smad-independent molecules such as MAP kinase, pI3 kinase, and JNK.

There are also several BMP inhibitors that can be expressed. These include noggin, chordin, gremlin, sclerostin, and others. These inhibitors bind to the various BMP molecules with different affinities. For instance, noggin binds to BMP-2 and -4 with high affinity but has low affinity for BMP-6 and -7.

Another molecular pathway that can influence osteogenesis is the Wnt pathway. Wnt binds to a receptor, Frizzled, and co-receptor, LRP-5/6, and activates downstream signaling through a glycogen synthase kinase complex. This process then protects β-catenin from degradation, and β-catenin is translocated into the nucleus where it can activate gene transcription. Wnt signaling stimulates the expression of Runx2, a major osteogenic transcription factor. This process is blocked by sFRP-1.

From the BMP family, BMP-2 has been used in many tissue engineering strategies as it has been found to have a strong osteoinductive effect. It is involved in signaling pathways for osteoinduction and can stimulate mesenchymal stem cells to differentiate into osteoblasts. The injection of BMP-2 into bone defects in rats has been shown to accelerate fracture healing. A controlled, randomized study of 450 tibial fracture patients was conducted which showed that those receiving BMP-2 in a
collagen sponge had improved wound healing compared to the control group.\textsuperscript{114} Human MSCs, however, may not be as responsive as rodent cells to the induction of osteogenesis in the presence of BMP-2.\textsuperscript{115}

BMP-7, also known as osteogenic protein-1 (OP-1), is another widely used BMP molecule for bone regeneration. OP-1 was shown to accelerate fracture healing when injected into a bony defect in goats, although by 4 weeks the OP-1 treated groups and the control groups showed no statistical differences in any of the examined parameters.\textsuperscript{116} A clinical trial demonstrated that OP-1 embedded in a collagen sponge produced a healing response comparable to that of autografts.\textsuperscript{117}

Although the BMPs are widely used, there are other molecules that have been shown to enhance or support bone regeneration. These include insulin-like growth factor (IGF),\textsuperscript{118, 119} fibroblast growth factor (FGF),\textsuperscript{120, 121} platelet-derived growth factor (PDGF),\textsuperscript{122} and vascular endothelial growth factor (VEGF).\textsuperscript{123}

Although the delivery of growth factors for bone regeneration holds much promise, there is still a need to deliver large amounts of protein to elicit an effect, due to protein degradation \textit{in vivo} and poor retention locally at the site of injury.\textsuperscript{124}

\section*{2.4.2 Introduction to Gene Delivery}

An alternative to the delivery of growth factors is gene delivery. In this approach, cells are exposed to genetic material which encodes for these growth factors. When the cells uptake DNA, they will then express the encoded growth factor, which provides local exposure to these factors for extended periods of time. Alternatively, if cells uptake short
interfering RNA (siRNA) their expression of a specific protein will be silenced, and this can influence the behavior of these cells and neighboring cells as well. This approach will be discussed in detail in the next chapter.

2.5 References


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CHAPTER 3. Gene Delivery for Controlling Cell Behavior

3.1 Delivery of DNA by Viral and Non-viral Methods

Gene therapy involves the delivery of DNA or RNA to a population of target cells in order to treat a disease or stimulate tissue growth in a defect by altering the gene expression profile of the select cells. Depending on the desired therapeutic approach, genes can either be upregulated or downregulated. Gene delivery can be achieved by several methods, both viral and non-viral.

3.1.1 Viral Gene Delivery

Viral gene delivery offers the major advantage of increased transduction of cells due to its high efficiency. There are many types of viruses that can be used to deliver genetic material. Retroviruses offer the advantage of integrating the delivered gene directly into the host genome for permanent expression, thus providing a high level of expression of the desired protein over time. These viruses contain single stranded RNA which is reverse transcribed into double stranded DNA upon infection of a cell.\(^1\) This double stranded DNA is then inserted into the cell’s genomic DNA. Retroviruses are able to efficiently transfect many cell types and allow for sustained gene expression, but one of the major safety concerns associated with these viruses is the risk of mutagenesis based on random insertion into the genome.\(^2\) Furthermore, the gene size that can be delivered with these viruses is limited to less than 8 kb, and retroviruses can only infect dividing cells.\(^2\) Also, the continual expression of a gene is probably not desirable in many cases once the tissue has been regenerated or the disease state has been corrected.
Retroviruses have been studied for osteogenesis. Skeletal myoblasts transduced with retroviruses encoding for either BMP-2 or Runx2 were found to have increased osteogenic differentiation both in vitro and in vivo. Rat bone marrow stromal cells transduced with a BMP-4 retrovirus and embedded in a gelatin matrix were able to heal a critical-sized cranial defect in rats. Murine stromal cells transduced with a retrovirus for expression of BMP-2 and seeded onto PLGA matrices induced heterotopic bone formation in the quadriceps muscles of mice. Rabbit periosteal cells were isolated and transduced with a retrovirus encoding for BMP-7 and were found to express BMP-7 at both the RNA and protein level.

Lentiviruses are another class of virus that can be used for gene therapy; these are a class of complex retroviruses. The human immunodeficiency virus (HIV) is a lentivirus. These viruses also offer the advantage of stable integration into the host genome leading to high levels of continual protein expression, but unlike simple retroviruses they are able to transduce both dividing and non-dividing cells. However, they have the same drawbacks as the simple retroviruses as mentioned above. The use of lentiviruses for increasing osteogenesis has been examined by several groups. Rat bone marrow stromal cells transduced with lentivirus encoding for BMP-2 were shown to produce higher levels of alkaline phosphatase activity in vitro, and when implanted in a collagen sponge were able to provide spinal fusion in vivo. Heterotopic bone formation in the hind limb muscles of mice was observed upon implantation of rat bone marrow stromal cells that had been transduced with a lentivirus encoding for BMP-2.

Another type of viruses that are used for gene transfer are adenoviruses. These are double-stranded DNA viruses, and are able to incorporate genes up to 30 kb. These
vectors are widely used as they can be produced in high virus titers, they are able to transfect both dividing and non-dividing cells, and because they do not integrate into the host genome, thus avoiding concerns over mutagenicity and prolonged expression. Of course the transient expression offered by these vectors may be a drawback in certain applications, and another difficulty with the use of adenoviruses is that the host has a high immune response to them. Replication-deficient adenoviruses have also been constructed, and these are supposed to be safer and less immunogenic than the original adenoviruses used; however, many groups have found that the animal will produce antibodies against the encoded protein, which can lead to significant autoimmune problems. Many groups have examined the use of adenoviruses in promoting osteogenesis, especially for the upregulation of BMP-2. The upregulation of BMP-2 expression in rat MSCs by transduction with retrovirus or adenovirus or transfection with cationic lipids was compared. Although the adenovirus-transfected cells exhibited increased osteogenesis in vitro, all three types of genetic upregulation produced similar levels of osteogenesis in vivo with the adenovirus group only slightly outperforming the other two.

Adeno-associated viruses are another type of virus used for gene delivery; these are DNA viruses that can hold up to 4.7 kb in gene size and that are naturally replication deficient. These viruses offer several major advantages, including their ability to transduce both dividing and non-dividing cells, their tendency to integrate to a specific site in the human chromosome 19, their stability, and their low immunogenicity. However, they are more difficult to manufacture, they require a helper virus to replicate, and they still have the potential for insertional mutagenesis. Transplantation of MSCs
with adeno-associated viruses encoding for BMP-2 and VEGF resulted in improved tibial defect healing in mice. Another group demonstrated that coating biomaterial scaffolds with adeno-associated virus encoding for BMP-2 induced bone formation in rat muscles. A tetracycline-sensitive promoter was used to create an adeno-associated virus for BMP-2 so that the expression of the BMP-2 could be regulated. Only mice that received an analogue of tetracycline in their drinking water were able to produce ectopic bone formation.

3.1.2 Non-viral Gene Delivery

Although viral vectors are highly efficient delivery vehicles, as mentioned above there are major concerns with immunogenicity, toxicity, the risk of recombination, and possible extended expression of the protein beyond the timeframe required for the healing of the defect. As a result, much effort has been invested into the development of efficient non-viral methods, which include the creation of nanoparticles with encapsulated DNA using liposomes, polycations, or inorganic compounds. These methods provide only transient gene expression, which for some disease states may be a drawback. However, it has been demonstrated that a short-term expression of BMP-2 in a cell population was sufficient to induce bone formation by mouse MSCs, suggesting that stably modifying cells genetically for bone regeneration may not be necessary. With that said, it is important to ensure that large populations of cells that are already at the defect site or that are recruited there are transfected in order for the gene delivery to provide a therapeutic effect. As mentioned in Chapter 1, the duration of expression resulting from a single injection of DNA is limited and hard to control, and
timing the injection can be difficult in order to provide a therapeutic benefit.\textsuperscript{32} To this end, much research has been conducted in using biodegradable material scaffolds that have DNA incorporated into them in order to provide a sustained delivery of DNA at the desired location.\textsuperscript{33} Thus the expression of the protein at the defect site, although transient due to the non-viral gene delivery, will be strong enough to promote tissue healing.

There has been much work on gene delivery from biomaterial scaffolds, yet many challenges remain. The incorporation of genetic material into a biomaterial while maintaining its bioactivity can be difficult, as many materials are formed into scaffolds using harsh conditions such as high temperatures or organic solvents. For instance, the polymer poly(lactic-co-glycolic acid) (PLGA) is only soluble in organic solvents. However, it was found that if the polymer is ground into fine particulates that are then mixed with a porogen, pressed into a pellet and gas-foamed under high CO\textsubscript{2} pressure, the PLGA particulates will fuse to each other around the porogen particles to form a porous scaffold.\textsuperscript{34} Additionally DNA can be lyophilized and incorporated into the matrix while still maintaining its bioactivity.\textsuperscript{35} Although this system is promising, it has some major disadvantages: naked DNA is released almost immediately from the scaffold with little further release, and DNA complexed with PEI adsorbs to the surface of the PLGA scaffold and does not release into the surrounding tissue.\textsuperscript{35} Although some success was seen using this scaffold in a critical-sized bone defect as the final healing response was improved compared to not having a scaffold or a scaffold with DNA incorporated, the defect was still not completely healed.\textsuperscript{36} Thus a more robust response is required for this technique to have ultimate clinical utility. Another more recent approach was taken to chemically modify gas-foamed PLGA scaffolds to contain lysine residues; DNA could
then be added to bind to the lysine.\textsuperscript{37} The release of DNA from these scaffolds is shown to be sustained for up to 18 days and is still bioactive as evidenced by the successful transfection of cells cultured on the scaffolds. However, this technology needs further validation and \textit{in vivo} characterization to determine its ultimate potential for gene delivery.

An alternative to using hydrophobic biomaterial scaffolds is the use of hydrogel scaffolds. Here, DNA can be incorporated into the water soluble polymer prior to crosslinking to form a three dimensional hydrogel. Alternatively, depending on the porous network it may be possible in some cases to soak a lyophilized crosslinked hydrogel in a nucleotide-containing solution and have the nucleotides absorbed into the matrix. There have been several reports on the use of hydrogels for gene delivery. Collagen with and without reinforcement from PLGA fibers was examined for the release of plasmid DNA; however all formulations released the DNA in four or fewer days.\textsuperscript{38} Another hydrogel that has been examined for DNA delivery is oligo(poly(ethylene glycol) fumarate) (OPF), which showed sustained release ranging from 30-60 days.\textsuperscript{39} However, when implanted into a cranial defect in rats, this hydrogel formulation loaded with plasmid DNA encoding for BMP-2 showed minimal bone formation and minimal hydrogel degradation after 30 days.\textsuperscript{40} A silk-elastinlike hydrogel was used for sustained release of plasmid DNA over the course of at least one month.\textsuperscript{41} This system has been examined by this group in several reports, but they have yet to demonstrate \textit{in vivo} utility of the non-viral delivery system.\textsuperscript{42} PEG-hyaluronic acid hydrogels were also examined for release of plasmid DNA.\textsuperscript{43} The release of naked DNA from these hydrogels was relatively rapid (less than 1 week) while DNA complexed with PEI did not release at very
high amounts from the hydrogels. Also, alginate hydrogels with PEI-DNA nanoparticles incorporated have been studied for application in therapeutic angiogenesis and were found to slightly increase blood vessel formation.\textsuperscript{44} Overall, although there has been much work in the delivery of DNA from biopolymer scaffolds, each system requires further development and poses difficulties that must be surmounted. There still exists a need for the development of a scaffold system that can provide sustained release of bioactive DNA and provide an environment that is both conductive and inductive for osteogenesis in order to achieve substantial bone regeneration in a defect. This thesis will examine the use of three different biomaterial scaffold systems for DNA delivery in an attempt to help advance this field and aid in the discovery of promising gene delivery systems.

3.2 Localized and Sustained Delivery of siRNA

In addition to the delivery of plasmid DNA to promote the increased expression of a desired protein, there is also much work being done in trying to design delivery systems for short interfering RNA (siRNA) to decrease protein expression. Gene expression can be silenced by the targeted destruction of specific mRNA molecules in a highly conserved process known as RNA interference (RNAi).\textsuperscript{45,46} RNAi has the potential to treat diseases that can be corrected by the decreased expression of specific proteins, for example in cancer therapeutics\textsuperscript{47} or in tissue regeneration.\textsuperscript{48-50} Despite the enormous potential, the use of siRNA has not yet been successful clinically, largely due to difficulties providing effective delivery of the siRNA to the desired cells \textit{in vivo}.\textsuperscript{51,52} Similar to any RNA molecule, siRNA is highly prone to degradation by ribonucleases.
that are found throughout the body and in the environment.\textsuperscript{52-55} Another difficulty is that in rapidly dividing cells, the silencing effect that the siRNA provides may only last a few days.\textsuperscript{46} Furthermore, the localized delivery of the siRNA to a specific site \textit{in vivo} is highly challenging.\textsuperscript{56} In fact, it has been said that “the three biggest problems with RNAi therapeutics remain ‘delivery, delivery, and delivery.’”\textsuperscript{57}

siRNA functions directly in the cytoplasm, where it is assembled into an RNA-induced silencing complex (RISC) that is comprised of several proteins and the siRNA. This complex becomes activated by ATP, the double stranded siRNA becomes single stranded, and the complex begins to seek out mRNA to which it is complementary.\textsuperscript{58} When a complementary mRNA sequence is found, the mRNA is destroyed by the nuclease activity of the RISC.\textsuperscript{59} In lieu of delivering siRNA into a cell, it is also possible to deliver long double stranded RNA or a plasmid or virus that encodes for short hairpin RNA (shRNA). The longer dsRNA or shRNA molecules are cleaved by an enzyme called Dicer into the short siRNA sequences that are part of the RISC complex.\textsuperscript{60,61} The difficulty with the use of these methods is that the longer double stranded RNA molecules can induce a cellular immune response via the interferon system.\textsuperscript{62}

There are disease states in which a systemic effect of RNAi may be desirable; some examples include non-localized cancers, HIV, neurodegenerative diseases, respiratory viruses, heart and vascular disease, and many others. However, there are other instances in which the systemic delivery of siRNA would be undesirable, as only a localized area needs treatment. Examples of this include tissue regeneration at a defect site and localized tumors or tumor resection sites. Here, the localized delivery of siRNA to a target site will be the focus of the following discussion.
3.2.1 Delivery of Naked siRNA

One method that some groups have utilized for the delivery of siRNA to a particular site is direct injection of a solution of siRNA in saline or an excipient solution. As the siRNA is not complexed with anything, it is termed “naked” siRNA. Generally, naked siRNA injected into the body has a very short half life, on the order of minutes, limiting its usefulness. However, it has been found that some tissues are able to uptake naked siRNA to a much higher degree than other tissues, for instance the eye, central nervous system, and lung, making localized delivery of siRNA to these tissues a possibility. One of the largest complications leading to loss of sight is uncontrolled retinal neovascularization in patients that have ischemic retinal disorders. It was demonstrated that inhibition of VEGF by antibodies could reduce ocular vascularization by about 50%, although further reduction could not be achieved due to difficulties in delivering the antibodies throughout the retinal tissue. Thus, other groups have examined the direct delivery to the eye of siRNA targeting VEGF to reduce this neovascularization using RNA interference. Reich, et al. decreased ocular neovascularization by direct injection of naked siRNA against VEGF into the retina of mice. Shen, et al. demonstrated that the delivery of siRNA targeting VEGF receptor 1 (VEGFR1) resulted in decreased ocular vascularization with siRNA remaining present in these cells for at least 5 days. There are two clinical trials currently underway exploring the delivery of siRNA against VEGF or VEGFR1 to treat age-related macular degeneration.
There have been several reports on the local delivery of siRNA to the nervous system. Dorn, et al. demonstrated that intrathecal injection of naked siRNA complementary to a pain-related ion channel showed diminished pain response. The silencing of agouti-related peptide by direct injection of siRNA into the hypothalamus of mice led to decreased body weight and increased metabolism, which could impact obesity. The intracerebroventricular injection of naked siRNA has also been shown to provide gene silencing that is confined to the brain. Using this method, dopamine and serotonin transporters have been silenced, affecting the locomotive behaviors of mice.

Studies have also demonstrated the ability to deliver naked siRNA to the lungs, typically by an intranasal route. siRNA targeting heme oxygenase-1 was delivered intranasally, and the silencing of this gene was specific to the lung. Intranasal delivery of naked siRNA, delivered either before or after infection with the severe acute respiratory syndrome (SARS) virus, was found to decrease SARS virus levels in the lungs of rhesus macaques and substantially reduce the symptoms of the disease. Two other viruses that infect the respiratory system and for which vaccines or other antiviral treatments are currently not available include respiratory syncytial virus (RSV) and parainfluenza virus. These RNA viruses encode in part for RNA polymerases to aid in the replication of the viral genome, and Bitko, et al. demonstrated that the intranasal delivery of naked siRNA targeted to a subunit of the viral polymerases was able to prevent and treat both of these viruses in mice. There is currently a clinical trial underway to examine the efficacy of this treatment in humans suffering from RSV infection. Intratracheal instillation, where a material is introduced directly into the lungs via a catheter or needle placed in the trachea, has also been used to deliver
unmodified siRNA to the lungs to silence the expression of macrophage inflammatory protein 2, which helped to reduce the migration of neutrophils to the lung after acute lung injury.\textsuperscript{77}

### 3.2.2 Chemical Conjugation of siRNA

Another avenue that has been explored to increase the activity of siRNA \textit{in vivo} is to conjugate the siRNA molecule with a chemical entity that will help increase the cellular uptake of the siRNA and/or help improve its pharmacokinetics. The various chemical modifications of siRNA have been reviewed extensively by Damha’s group.\textsuperscript{78,79} Frequently, lipophilic moieties such as cholesterol are conjugated to the siRNA as they have been shown to provide greater \textit{in vivo} stability by associating with serum proteins in the blood, and also to provide increased cell uptake due to increased cell membrane permeability.\textsuperscript{80,81} Cholesterol has been conjugated to siRNA complementary to the huntingtin gene, found in patients with Huntington’s disease, and when injected directly into the striatum of mice was found to locally silence gene expression and decrease the pathology of Huntington’s disease in these mice.\textsuperscript{82} Wu, et al. demonstrated that delivery of cholesterol-conjugated siRNA targeted to herpes viral proteins was able to protect mice from herpes simplex virus 2 when the siRNA was applied directly to the vaginal mucosa.\textsuperscript{83} Chen, et al. infused cholesterol-conjugated siRNA molecules directly into the central nervous system in rats and demonstrated that oligodendrocytes, a cell type that is difficult to transfect, were able to uptake the siRNA.\textsuperscript{84}
3.2.3 siRNA-Laden Polymeric Particles

Nano- or micro-particles with siRNA encapsulated in them can be fabricated from various biopolymers. The use of siRNA-laden particles has been widely researched, as the siRNA can be protected from environmental factors which may degrade it and is typically uptaken by cells with a higher efficiency.\textsuperscript{85} Again, there is much work in the systemic delivery of siRNA particles, but here the technologies that have demonstrated the ability to deliver siRNA locally will be focused on.

3.2.3.1 siRNA Incorporated Into Liposomes

To increase the half-life of siRNA \textit{in vivo}, it can be encapsulated within liposomes or complexed with cationic lipids to form siRNA-laden nanoparticles. Liposomes typically consist of a phospholipid bilayer that surrounds an aqueous core, and it is within this core that siRNA (or other nucleotides or proteins or drugs) is contained. A more popular alternative to liposomes is the use of lipoplexes, which are cationic lipids mixed with the anionic siRNA to form complexes based on electrostatic interactions; however, these complexes tend to be more unstable in solution and may aggregate over time.\textsuperscript{86} Additionally, they can be cytotoxic or induce an inflammatory response.\textsuperscript{87} Cationic lipid – siRNA complexation was recently demonstrated to silence the expression of red fluorescent protein (RFP) when injected locally into a tumor expressing RFP that was formed subcutaneously.\textsuperscript{88} Another group demonstrated the uptake of anti-VEGF siRNA in a subcutaneous tumor when siRNA complexed with a cholesterol derivative was injected locally into the tumor.\textsuperscript{89} siRNA incorporated into lipoplexes has also been found to be uptaken at a high efficiency in vaginal mucosa when
directly applied to the area. This has been shown to effectively silence the expression of herpes viral proteins to protect mice from herpes simplex virus 2.\textsuperscript{90} siRNA against lamin A/C was incorporated into lipoplexes with localized silencing found in the vaginal mucosa.\textsuperscript{91} Additionally, this group demonstrated local delivery of siRNA lipoplexes to the colon, another mucosal surface. By silencing the expression of TNF-\(\alpha\) in the colon, inflammation was reduced in mice with inflammatory bowel disease.\textsuperscript{91} A recent interesting approach to localized delivery of siRNA involved the synthesis of oleic acid-based liposomes that had a magnetic core comprised of a colloidal suspension of magnetic nanocrystals. siRNA was able to interact with these liposomes by electrostatic interaction of the siRNA with the oleic acid, thus essentially forming lipoplexes. When these magnetic liposomal particles carrying siRNA targeted to epidermal growth factor receptor were injected intravenously and allowed to travel systemically in mice, they were found to accumulate in high amounts locally in a tumor to which an external magnetic field was focused.\textsuperscript{92} However, there were still particles that made their way into other tissues, including predominantly the lung, liver, and spleen.

### 3.2.3.2 Polymeric Nanoparticles Containing siRNA

Frequently, cationic polymers are used to form nanoscale siRNA complexes based on electrostatic interaction of the negatively charged siRNA with the positively charged polymer. Poly(ethyleneimine) (PEI) is one such polymer. PEI is a synthetic polymer that can be either linear or branched, and with a high percentage of free amine groups which are positively charged. PEI complexed with siRNA targeting VEGF has been injected directly into subcutaneous tumors, and the silencing of VEGF and
subsequent diminishment of tumor growth was confirmed.\textsuperscript{93} PEI-siRNA was also delivered intratumorally with siRNA complementary to STAT3 to promote the apoptosis of the tumor cells.\textsuperscript{94} To increase the circulation time of PEI-RNA particles, the PEI can be modified with PEG, as this hydrophilic molecule does not adsorb serum proteins. The drawback of adding PEG to PEI is that it will reduce the positive charge of the polymer, thus lessening its interaction with cell membranes which leads to decreased uptake of the PEI particles. siRNA complexed with PEG-PEI was successfully delivered by tracheal intubation to the lungs, with the majority of the particles ending up in the lungs for gene silencing, although some siRNA complexes were found in other tissues.\textsuperscript{95} Chitosan is another polycation that is used for siRNA complexation; it is a polysaccharide derived from the shells of crustaceans. Chitosan-siRNA nanocomplexes with siRNA targeted to GFP have been delivered to the lungs of GFP transgenic mice by nasal administration and were shown to silence GFP expression by approximately 40\%.\textsuperscript{96} Chitosan complexed with siRNA against red fluorescent protein (RFP) has been delivered intratumorally into a subcutaneous tumor expressing RFP, and was shown to reduce RFP expression by approximately 83\%.\textsuperscript{97}

Nanoparticles can also be modified with a targeting ligand to increase uptake by only the desired cell population, even if the siRNA formulation is delivered systemically. For instance, siRNA-laden nanogels composed primarily of N-isopropylmethacrylamide were conjugated with a peptide that targeted the EphA2 receptor.\textsuperscript{98} Although these have not yet been tested \textit{in vivo}, the silencing of EGFR was seen in a cell line expressing EphA2 receptor, with no silencing found in a cell line that does not express this receptor. Poly(propyleneimine) (PPI) dendrimers have also been used to create siRNA-containing
nanoparticles; these particles were modified with a peptide of luteinizing hormone-releasing hormone (LHRH) for increased uptake by cancer cells.\textsuperscript{99} This group demonstrated that the siRNA particles were indeed uptaken to a much higher degree \textit{in vivo} by the tumor cells.

Alternatively, the nanoparticles can be designed such that they do not release their payload until they receive a physical signal to do so. One example of this is the use of gold nanospheres to which thiolated siRNA complementary to NF-κB is bound.\textsuperscript{100} Only particles in cells exposed to near-infrared light, which can penetrate deep into tissues, will release the siRNA into the cytoplasm. This group demonstrated targeted knockdown of NF-κB in tumor tissue, which increased the susceptibility of the tumor to chemotherapeutic drugs.

Polymeric microparticles can also be used for the delivery of siRNA. For instance, Murata, et al. encapsulated anti-VEGF siRNA in PLGA microspheres that were about 40 µm in diameter, injected them next to a subcutaneous tumor, and found decreased tumor growth over time.\textsuperscript{101}

### 3.2.4 Macroscopic Biomaterial Scaffolds with siRNA Encapsulated

As described in the sections above, many of the current technologies being examined for siRNA delivery rely on the formation of nanoscale siRNA complexes and in some cases microscale complexes. The disadvantage of using these techniques for local delivery is that they can be rapidly dispersed from the delivery site due to their small size. Therefore, the challenge of getting a sufficient amount of siRNA to the desired location still remains. There is a paucity of work examining the use of
macroscopic biomaterial scaffolds for the local delivery of siRNA. Biomaterial scaffolds could be placed at a tissue defect site, with siRNA released from the scaffold over time to incorporated cells and the surrounding host cells. The siRNA could be released either by diffusion through the biopolymer pores or as the polymer degrades. In this way, the siRNA would act locally, and the scaffold itself could aid in tissue regeneration by providing a conductive or inductive environment for cellular growth within the defect. The work in our laboratory was the first to examine the use of such a system.102 Ionically crosslinked alginate, photocrosslinked alginate, and collagen hydrogels were exhibited to provide varying siRNA release profiles, and in all cases the siRNA released was bioactive and able to silence protein expression in cells. The use of collagen scaffolds to deliver siRNA-dendrimer nanoparticles has also recently been demonstrated, although it was found that ~60% of the siRNA was released in the first two days with little subsequent release.103 However, the nanoparticles delivered from these scaffolds were shown to reduce protein expression for up to 7 days when cells were cultured directly on the scaffolds. Singh, et al. incorporated DNA, siRNA and chemokines into PLGA microparticles which were then incorporated into hydrogels made either of dextran vinyl sulfone and poly(ethylene glycol) (PEG) or PEG alone.104 The chemokines are released from the hydrogels to promote the migration of antigen-presenting cells into the hydrogels, and these cells were then able to uptake the siRNA and DNA from the PLGA microparticles. Further developing these biomaterial scaffold systems and others that incorporate siRNA offers exciting potential for the delivery of siRNA to a specific site in vivo. The work in this thesis aims to help advance this exciting opportunity.
3.3 References


CHAPTER 4. DNA Delivery from Gas-Foamed PLGA Scaffolds

4.1 Introduction

To achieve a longer-lasting effect from gene delivery without repeated dosing, it is necessary to develop systems that can be placed in the physiological site of interest which will release the genetic material over time in a prolonged manner. In this way, the cells around this site are continuously exposed to the genetic material for a period of time. Biodegradable material scaffolds can be of great use in allowing a sustained delivery of DNA at the desired location.\(^1\) The choice of the scaffold material is a major consideration; it must be biocompatible and ideally will degrade over time as new tissue is formed. One such biomaterial that has been extensively used for various tissue engineering applications is poly(lactic-co-glycolic acid) (PLGA), a biodegradable polymer that has already been approved by the FDA for use in several clinical applications.\(^2,3\) The rate of degradation of the polymer can be altered by changing the ratio of lactic acid blocks to glycolic acid blocks or the molecular weight (MW) of the polymer.\(^4\)

PLGA can be formed into highly porous scaffolds without the need for toxic solvents by using gas-foaming technology.\(^5\) PLGA pellets are first ground and sieved to a specific size distribution, as is the porogen (usually sodium chloride or sucrose). The PLGA and porogen are then mixed together at ratios up to 95% porogen and 5% PLGA, and pressed in a mold. This forms a disk which is then placed into a pressure vessel and exposed to carbon dioxide at high pressures. After overnight equilibration, the CO\(_2\) is rapidly released, causing the glass transition temperature of PLGA to decrease and
thereby allow the PLGA particulates to fuse to one another throughout the scaffold.6 The porogen can then be leached out into an aqueous solution to leave behind a highly porous PLGA structure (Figure 4.1).

Due to the absence of harsh solvents or high temperatures during the fabrication, these scaffolds can be used for gene delivery by mixing in lyophilized plasmid DNA, either naked or complexed with a polycationic carrier such as polyethyleneimine (PEI), prior to pressing and foaming.7 These scaffolds loaded with PEI-DNA encoding for BMP-4 have been used for osteogenesis with some success, as they exhibited increased bony tissue in a defect compared to a blank scaffold, but the bone formed still did not span the defect.8 At least two major challenges associated with the use of these scaffolds for DNA release remain. These are: (1) a large burst release within 24 hours followed by minimal subsequent release from the scaffold when naked DNA is used, and (2) the minimal overall release observed when cationic PEI-DNA nanoparticles are used, due to electrostatic interactions with the PLGA scaffold surface.7

We have developed a modification to the system that permits controlled, sustained release of naked DNA and DNA complexed with PEI. The addition of alginate, a polysaccharide which can form a hydrogel upon crosslinking with divalent cations, to the scaffolds delays the release of naked DNA while increasing the release of PEI-DNA. In this work, the ability to modulate the release by varying the amount of alginate added is demonstrated, and its potential impact on gene delivery for tissue engineering is discussed.
4.2 Materials and Methods

Plasmid DNA encoding for short hairpin RNA (shRNA) to silence the expression of GFP was obtained from OriGene Technologies (Rockville, MD). PEI “Max” with molecular weight of 25 kD was obtained from Polysciences, Inc. (Warrington, PA). Poly(lactide-co-glycolide) (PLGA) 50:50 copolymer with molecular weight (MW) of 22 kD and inherent viscosity of 0.21 dl/g was obtained from Lakeshore Biomaterials (SurModics Pharmaceuticals, Birmingham, AL).

Sodium alginate powder (product name of 20/40, FMC Biopolymers, Princeton, NJ) with MW of 196,000 g/mol as determined by SEC-MALS was lyophilized until dry. It was then subjected to gamma irradiation at 5 MRad (Phoenix Lab, University of Michigan, Ann Arbor). The MW of the irradiated alginate was found to be 37,000 g/mol. The percentage of guluronic (G) and mannuronic (M) acid in the alginate was 66% G-content and 34% M-content as determined by NMR (FMC Biopolymers). A 1% alginate solution was purified by dialysis for 4 days (Spectra/Por 3500 MWCO, Spectrum Laboratories, Rancho Dominguez, CA), subjected to activated charcoal treatment by mixing with 0.5 g activated charcoal (50-200 mesh, Fisher Scientific, Fairlawn, NJ) per 100 ml of 1% alginate solution for 30 minutes, sterilized through a 0.22 µm filter, and lyophilized until dry.

PEI-DNA condensates were made by combining 50 µg DNA in 4 ml of 5 mM HEPES (pH 7.4) with 300 µl of 10 mM PEI in 3.7 ml of 5 mM HEPES to obtain a final N/P ratio of 20. After a 30 minute incubation at room temperature (RT), 0.2 ml or 2 ml of a 2% alginate solution in PBS was added and briefly vortexed. After an additional 30 minute incubation at RT, 160 µl of 50% w/v sucrose (Sigma Aldrich, St. Louis, MO) was
added to the mixtures, briefly vortexed and incubated another 30 minutes at RT. The condensates were then flash frozen in liquid nitrogen and lyophilized for 4 days. Condensates were made with and without PEI, and with and without the two amounts of alginate.

To fabricate the scaffolds, the lyophilized PEI-DNA-alginate mixtures were mixed with milled sucrose (250-425 µm) and PLGA (106-250 µm). Scaffolds with 90% porosity were prepared at 90:10 porogen:PLGA ratio by mixing the porogen and PLGA together and pressing in a 13 mm die (Pike Technologies, Madison, WI) at 3.5 metric tons for 1 min using a hydraulic press (Carver, Wabash, IN). When DNA or PEI-DNA with or without alginate was used in a scaffold, the lyophilized condensates were weighed, and this mass was considered as a portion of the porogen mass. The pressed disks were then exposed to 800 psi CO₂ for 20 hours in a high-pressure vessel (Parr, Moline, IL). The CO₂ was then rapidly released, and the foamed scaffolds were placed first in 5 ml of 100 mM CaCl₂ for 1 min to crosslink the alginate, and then in 10 ml DMEM to begin leaching the sucrose. After 4 hours, the DMEM was removed and 5 ml fresh DMEM was added to continue the porogen leaching process for an additional 20 hours.

For release studies, the scaffolds were kept in DMEM and sampled at various timepoints. The media was assayed for DNA content by using the dsDNA PicoGreen® kit with a standard curve consisting of known amounts of DNA in the same medium.

For scanning electron microscopy (SEM) analysis, scaffolds were frozen and then lyophilized after leaching. The scaffolds were carefully sectioned with a razor blade to expose the interior morphology. These scaffolds were then sputter coated with palladium
for 60 seconds (Desk II, Denton Vacuum, Moorestown, NJ). A Philips XL30 environmental scanning electron microscope was used to image the scaffolds under a 15 kV electron beam.

For the cell transfection studies, HEK293 (human embryonic kidney) cells stably transfected with destabilized GFP (deGFP) were a generous gift from Piruz Nahreini, Ph.D. (University of Colorado Health Sciences Center). The cells were cultured in DMEM-HG + 10% FBS, and were plated in 24-well plates at a density of 150,000 cells per well. After overnight incubation, the media was removed and replaced with enough spent media from the release studies to contain 1 µg plasmid DNA encoding for shRNA against eGFP, with serum-free DMEM added when necessary to achieve a total volume of 0.5 ml. 48 hours post-transfection, cells were harvested from plates and assayed by flow cytometry (EPICS XL-MCL, Beckman Coulter, Fullerton, CA) to determine the percentage of the cell population expressing GFP.

For determination of the relative amount of DNA bound to PEI with or without alginate, an equal volume of 400 ng/ml ethidium bromide (Fisher Scientific, Fairlawn, NJ) was added to a freshly prepared solution and the fluorescence (510 nm excitation, 590 nm emission) read with a plate reader (Tecan, Durham, NC).

4.3 Results and Discussion

PLGA scaffolds possessing 90% porosity were created by gas foaming. These scaffolds contained DNA or PEI-DNA, with or without alginate. Alginate, a negatively charged polysaccharide which can be crosslinked with calcium to form a hydrogel, was added to the system as a method for preventing the strong adhesion of the positively-
charged PEI-DNA complexes to the PLGA. As the alginate is negatively charged, it was mixed in with the PEI-DNA nanoparticles prior to lyophilization to surround the nanoparticles, so that when they were later mixed into the PLGA to form the scaffold, it would prevent the adhesion of the PEI-DNA to the PLGA. It was also added to retain naked DNA in the scaffold longer by providing a hydrogel component through which the DNA must diffuse before it could be released from the scaffold.

In order to ensure that the addition of the alginate would not alter the porous structure of the scaffolds, the interior morphologies of the scaffolds with varying amounts of alginate were examined by scanning electron microscopy (Philips XL30) (Figure 4.2). The alginate is apparent within the scaffolds, especially at the higher concentration, but a substantial porous structure is preserved, which would allow for cells to migrate and proliferate within the scaffolds.

Next, the rate of release of the DNA from the various scaffold formulations was examined. The burst release in the first 24 hours of naked DNA was decreased to less than 60% of the total amount incorporated with the addition of either 4 or 40 mg of alginate into the system, and sustained incremental release was achieved for up to 10 days (Figure 4.3A). This is an improvement compared to the release of naked DNA in the PLGA scaffolds without alginate, which show an immediate burst and no subsequent release. The addition of alginate into scaffolds containing PEI-DNA increased the release of the DNA from these scaffolds, with more alginate allowing a greater release of DNA; the scaffolds made with the lower amount of alginate showed sustained release for at least 35 days (Figure 4.3B).
These release curves demonstrate that this advancement to this gene delivery system holds much promise in allowing for a more sustained effect over time. Although it is possible to achieve gene transfection by delivering naked plasmid DNA to some tissues, the level of expression and the duration of that expression are low, and a high amount of DNA is required to obtain any discernible effect. Therefore, it is more likely that the scaffolds delivering the PEI-DNA nanoparticles would be of greater utility for gene delivery. I anticipate that by using one of the formulations containing alginate with the PEI-DNA nanoparticles, a more sustained expression of the desired protein would be obtained due to the gradual release of the particles from the scaffolds over time.

Next, the bioactivity of the released PEI-DNA was examined by supplying cells with spent media from the release studies and measuring the level of gene expression obtained. The plasmid DNA used encoded for shRNA to silence GFP expression, and thus the knockdown of GFP was measured in cells constitutively expressing GFP. For all conditions, no cell transfection was found, as there was no silencing of the GFP expression (data not shown). Although DNA was present in the spent media, and still in double stranded form (as it was measured with the dsDNA dye PicoGreen), it was not having an effect on the cells. This was expected with the conditions containing naked DNA, as plasmid DNA alone cannot transfec cells \textit{in vitro}. However, further investigation was required to determine why the conditions containing PEI-DNA were not providing increased gene transfection.

It was important to determine if the DNA present in the release media from the PEI-DNA scaffolds was still complexed with the PEI. If it had somehow become uncomplexed, this would explain the lack of \textit{in vitro} transfection. One technique that can
be used to determine the complexation of PEI with DNA is an ethidium bromide exclusion assay. In this assay, the ethidium bromide is able to intercalate with the DNA only if the DNA is not complexed to PEI. When PEI is added to DNA, it displaces the ethidium bromide, as evidenced by a lower fluorescent signal. Naked DNA and PEI-DNA were compared to PEI-DNA mixed with the low amount of alginate (4 mg). As shown in Table 4.1, the fluorescent signal was strongest with naked DNA and decreased substantially with the PEI-DNA complexes. However, the fluorescence increases upon the addition of alginate to the mixture, indicating that the negatively charged polysaccharide is partially decoupling the DNA from the PEI. This is likely due to competition between the negatively charged DNA and alginate for electrostatic interaction with the positively charged PEI. Another group has previously shown that the electrostatic interaction between DNA and polycations such as chitosan, polyethylenimine (PEI), or poly-L-lysine can be disrupted in the presence of other charged compounds. However, these ethidium bromide fluorescence values also indicate that not all of the PEI-DNA is uncomplexed, so perhaps simply delivering a higher total amount of DNA would allow for appreciable transfection efficiency.

<table>
<thead>
<tr>
<th>Sample</th>
<th>EtBr Fluorescence</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>1</td>
</tr>
<tr>
<td>PEI-DNA</td>
<td>0.05</td>
</tr>
<tr>
<td>PEI-DNA + alginate</td>
<td>0.66</td>
</tr>
</tbody>
</table>

Table 4.1 Ethidium bromide exclusion assay demonstrating quenched fluorescence of DNA by addition of PEI, and subsequent increase of fluorescence by adding alginate, indicating some PEI-DNA becomes uncomplexed.
These scaffolds are promising for gene delivery applications, but further work is necessary to characterize their efficacy. Furthermore, they may not be the best option for including a transplanted cell population, as seeding cells within these scaffolds can be challenging due to the hydrophobicity of the PLGA and difficulties achieving a uniform cell seeding distribution on a preformed scaffold. Therefore it is possible that these scaffolds hold much promise for local DNA delivery in situations where a transplanted cell population is not required. However, a cell transplantation approach may be a better avenue towards achieving increased osteogenesis. Furthermore, these preformed scaffolds can only be surgically implanted into a defect site, necessitating an additional surgical procedure for their use. In contrast, the use of injectable biomaterials would allow the scaffold to be placed at the site of interest in a minimally invasive manner. Therefore the pursuit of these scaffolds for gene delivery for bone regeneration was temporarily set aside in favor of pursuing injectable biomaterial systems for gene delivery.

However, the idea of incorporating a hydrogel component into a solid scaffold to aid in sustained gene delivery is novel, and worth further pursuit. Future studies will include the examination of transfection of cells seeded directly onto the scaffolds, as even if the released DNA is largely uncomplexed with PEI, it is possible that PEI-DNA nanoparticles still remain within the scaffold and would be able to affect cells that adhere to, migrate into, and proliferate within the scaffolds. Also, the released DNA would be available to affect surrounding cells if these scaffolds were implanted in vivo. Additionally, it would be interesting to pursue the addition of biomaterials other than alginate that can form a hydrogel. This may be especially interesting for materials such
as poly(ethylene glycol) (PEG) which are inert and therefore would not compete with the DNA for binding to the PEI. It will also be important to test the capability for these scaffolds to deliver DNA in vivo.

4.4 Conclusions

This technology shows much promise as a non-viral gene delivery system. Highly porous PLGA scaffolds containing varying amount of alginate were obtained by gas foaming and subsequent porogen leaching. By incorporating alginate into the system, the burst release of naked DNA was decreased and the release of PEI-DNA was increased. Additionally, all of these releases were found to be more prolonged over time. In fact, sustained release of PEI-DNA for at least 35 days was obtained in the scaffolds containing the low amount (4 mg) of alginate. However, it was also found that the alginate may be partially decoupling the PEI and the DNA. Future work will include examining the ability to keep the PEI-DNA complexed, for instance by finding a substitute for the alginate that would serve a similar purpose, or perhaps by examining the complexation of PEG to PEI which would serve to increase the stability of the PEI-DNA nanoparticles, as the PEG would help to shield the particles from interacting with the negatively charged alginate. Also, the efficacy of these scaffolds in vivo for transfection of either transplanted cells or host cells surrounding the implant will be determined.
Figure 4.1 Photograph of gas-foamed PLGA scaffold with 90% porosity, 5 mm in diameter.
Figure 4.2 SEM images of scaffolds. (A) PLGA only, (B) PLGA with PEI-DNA, (C) PLGA with PEI-DNA with low concentration alginate, (D) PLGA with PEI-DNA and high concentration of alginate. Scale bars = 200 μm.
Figure 4.3 Release from gas-foamed PLGA scaffolds of (A) naked DNA or (B) PEI-DNA either without alginate, with 4 mg alginate (low), or with 40 mg alginate (high) incorporated into the scaffold.
4.5 Acknowledgements

I would like to thank David Ciufo for assistance with some of the cell transfection studies.

4.6 References


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CHAPTER 5. Injectable Poly(lactic-co-glycolic) Acid Scaffolds with \textit{in situ} Pore Formation for Tissue Engineering and Gene Delivery

5.1 Introduction

Many solid biopolymer scaffolds with microscale porosity used for tissue engineering applications are first formed in the laboratory and then must be surgically implanted, such as the gas foamed PLGA scaffolds discussed in the previous chapter. Methods that are commonly used to produce such scaffolds include solvent casting,\textsuperscript{1,2} gas foaming methods,\textsuperscript{2,3} or solid free-form fabrication.\textsuperscript{4-6} For solvent casting and gas foaming approaches, a porogen is first mixed throughout a polymer, the polymer is fused together to form a continuous network, and then the porogen is subsequently leached out from the polymer to provide a porous, interconnected scaffold. The scaffolds made using the solvent casting technique are typically made from polymers that are not water-soluble and require toxic solvents for them to go into solution. The solvents must be thoroughly removed from the polymeric system prior to implantation in a patient, necessitating these scaffolds to be prefabricated on the bench, and eliminating the possibility of having an injectable system. Scaffolds formed by gas foaming and free-form fabrication techniques require the use of large, expensive equipment for scaffold manufacturing, which prevents \textit{in situ} formation at a tissue defect site.

In contrast, biomaterials that can be injected to form a scaffold \textit{in vivo} offer a minimally invasive alternative, which is valuable in a clinical setting because it
eliminates the requirement for surgical implantation, and thus reduces both patient risk and treatment expense. Many injectable scaffolds are hydrogel-based; these materials typically have inadequate mechanical properties for load-bearing sites. Furthermore, they often possess nanoscale porosity which is suboptimal for cell migration, growth, and new extracellular matrix deposition. Previous work by others has demonstrated the potential utility of injectable nonporous poly(lactic-co-glycolic acid) (PLGA) for the purpose of delivering drugs, proteins, and DNA to treat diseases such as chronic inflammatory disease, or for the delivery of bulking agents for the treatment of urinary incontinence, but these have not been engineered to possess micron-scale porosity for use in tissue engineering. Poly(propylene fumarate) (PPF) has been used to form porous injectable scaffolds with high mechanical properties which are quite promising for use in tissue regeneration strategies, but the use of foaming reactions to form this porosity can be uncontrolled and may cause local tissue damage. Additionally the elastic modulus of these scaffolds may be unsuitably high for certain applications.

Appropriate micron-scale scaffold porosity is an important biomaterial design criterion in tissue engineering applications as it can permit increased cell adhesion, migration, proliferation, and new extracellular matrix production. Biomaterial porosity is especially critical when the regeneration strategy implemented does not include cell transplantation, and therefore relies solely on the recruitment of host cells to the defect site. In this situation, if the host cells do not migrate into the scaffold, proliferate, and secrete extracellular matrix, there will be no tissue repair in the defect. Therefore the scaffold must permit, and ideally promote, cellular infiltration throughout.
In this chapter, the development of an injectable biomaterial scaffold that solidifies *in situ* with microporous, interconnected architecture on the surface and within the bulk is described. Furthermore, the ability to incorporate DNA into these scaffolds to allow for their use as a gene delivery vehicle is also demonstrated. The choice of the scaffold material is a major consideration; it must be biocompatible and ideally will degrade over time as new tissue is formed. The biomaterial used in this system is poly(lactic-co-glycolic acid) (PLGA), which is a biodegradable polymer that has been approved by the FDA for use in several clinical applications. The rate of degradation of the polymer can be altered by changing the ratio of lactic acid blocks to glycolic acid blocks or the molecular weight of the polymer. In the system described herein, the PLGA is dissolved in tetraglycol, also known as glycofurol, a water-miscible solvent which is used with some FDA-approved pharmaceuticals administered via injection; quantities of tetraglycol up to 0.07 ml per kg body weight per day are deemed safe. When a solution of PLGA in tetraglycol is injected into an aqueous environment, the tetraglycol diffuses out of the polymer into the surrounding water. As the water-insoluble PLGA comes into contact with the water, it precipitates by phase inversion into a nonporous scaffold. The injection of pure PLGA in tetraglycol into an aqueous solution will form a solid scaffold with minimal porosity. Advancing this technology for tissue engineering applications may require modifying this injectable PLGA system to allow for *in situ* pore formation to create solid scaffolds with interconnected porosity throughout the polymer surface and bulk. In the work presented here, such scaffolds are successfully created by the addition of porogens and a small amount of water to the PLGA solution which solidifies *in situ* following injection into an aqueous solution to
form a microporous, solid scaffold.\textsuperscript{27} This system is characterized \textit{in vitro} by examining the morphologic appearance of resultant scaffolds, measuring their porosity, quantifying their degradation rates and mechanical integrity over time, and evaluating their ability to support cellular growth and infiltration. It is also shown that these scaffolds can be formed with a highly porous structure when injected subcutaneously in the backs of mice. Finally, the ability to deliver bioactive DNA in a sustained manner from these injectable PLGA scaffolds is demonstrated.

\textbf{5.2 Materials and Methods}

\textit{Scaffold Fabrication}

75:25 7E (i.v. 0.76 dl/g) or 50:50 6A (i.v. 0.58 dl/g) PLGA (Lakeshore Biomaterials, Birmingham, AL) was dissolved in tetraglycol (Sigma, St. Louis, MO) at 10\% w/v by stirring overnight at 60\(^\circ\)C. To create porous scaffolds, 210 mg of sodium chloride, sodium bicarbonate, ammonium bicarbonate, or sucrose (Fisher Scientific, Fairlawn, NJ) sieved to fall within a size distribution of 106-250 µm was placed in one 3 mL syringe, and 350 µl of the polymer solution was placed into a second 3 ml syringe, followed by the addition of ultrapure, deionized water (diH\(_2\)O) to the tip of this syringe (35 µl of diH\(_2\)O was added to 75:25 PLGA scaffolds while only 15 µl of diH\(_2\)O was added to 50:50 scaffolds). All components of the scaffold were mixed by joining the syringes together with a Luer-Lok connector. The polymer solution was then injected into 10 mL of phosphate buffered saline (PBS, Hyclone, Logan, UT) in a 20 ml glass vial where it precipitated by phase inversion to form a solid scaffold as the tetraglycol
diffused into the PBS, and to allow the porogen to be leached from the scaffold to form a porous structure. These scaffolds were placed in a humidified environment with 5% CO$_2$ at 37 °C for 24 hours then frozen and subsequently lyophilized for 48 hours and vacuum-sealed for storage at -20°C. Other scaffolds were fabricated by leaving out either or both the porogen or diH$_2$O.

**Scaffold Characterization**

Scanning electron microscopy (SEM) was used to characterize the surface and bulk morphology of the scaffolds. Scaffolds were sputter coated with palladium for 60 seconds (Desk II, Denton Vacuum, Moorestown, NJ). A Philips XL30 environmental scanning electron microscope was used to image scaffolds under a 15 kV electron beam.

Microcomputed tomography (micro-CT) was performed on nonporous (50:50 PLGA) and porous scaffolds (50:50 PLGA formed with water and sucrose porogen) (N = 3 each condition) using a Scanco uCT40 desktop scanner (Scanco Medical, Bruttisellen, Switzerland). Scan settings were E = 55k Vp, I = 145 µA, and integration time = 250 ms, and all samples were scanned at an isotropic voxel size of 16 µm. Raw scan data was reconstructed at 16 µm resolution into grayscale 2-D slice tomograms, and these were segmented to produce 3-D binarized images via semi-automatic contouring and global thresholding. The appropriate segmentation values were determined using histogram analysis accompanied by visual comparison of thresholded and non-thresholded areas of the 2D tomograms, and values were kept constant for all analyses. The volume fraction of PLGA within the total volume of the scaffold was computed for each of the porous and nonporous scaffolds.
Scaffold degradation studies were carried out at 37°C for a period of 4-8 weeks. Conditions included scaffolds formed with 1) PLGA, 2) PLGA + porogen, 3) PLGA + porogen + diH₂O, and 4) PLGA + diH₂O. Scaffolds made with 75:25 PLGA and 50:50 PLGA were studied to compare degradation rates based on the ratio of lactic acid to glycolic acid in the polymer chain. Salt was used as the porogen for the 75:25 PLGA scaffolds while sucrose was the porogen used for 50:50 PLGA scaffolds. Scaffolds were prepared as described above and injected into 20 ml glass scintillation vials containing 10 ml of PBS. The PBS was refreshed after 24 hours and then once a week for every subsequent week of the study. At each sampling time point, scaffolds were lyophilized for 48 hours before weighing.

Assuming that the PLGA scaffolds behave like elastic solids, constant strain rate compression testing was conducted to evaluate the elastic moduli of the scaffolds. A flat section was cut from each scaffold at all degradation timepoints. The height of each section was measured using digital calipers, and the area was measured by taking a photograph of the scaffold section and then using ImageJ (NIH) to quantify the area. The scaffolds used for mechanical testing varied from 0.9 - 3.6 mm in height and from 8.9 - 37.0 mm² in area. The range in heights is due to sectioning of the scaffolds by a razor blade, and the range in area is a result of the scaffold forming process. Compression testing was performed by placing the scaffolds in between two parallel platens on a TestResources 100R250 (Shakopee, MN) and subjecting them to a constant compression rate of 0.5 mm/min after application of a 0.1 N pre-load. The elastic modulus of each sample was calculated by determining the slope of the first 5% strain of the stress-strain curve.
**In vitro Cell Adhesion and Proliferation on the Scaffolds**

Cells were seeded and cultured on the scaffolds *in vitro* to examine differences in cell adhesion and proliferation on different scaffold formulations. Scaffold conditions examined were PLGA, PLGA + sucrose, and PLGA + sucrose + diH₂O. 50:50 PLGA was used in all scaffold formulations for *in vitro* studies. Prior to cell seeding, scaffolds were pre-wet and sterilized in 70% ethanol for 30 minutes. Scaffolds were rinsed 3X with PBS for 1 hr each and once with serum-free α-MEM (HyClone, Logan, UT) for 3 hrs under gentle agitation. Scaffolds were then soaked in complete media consisting of α-MEM with 10% fetal bovine serum (FBS) (HyClone) for 24 hours at 37°C with gentle agitation. Scaffolds were placed in 24-well ultra-low-adhesion plates (Corning, Corning, NY) and attached to the bottom of the wells using autoclaved silicone grease. Scaffolds were seeded with 500,000 MC3T3-E1 subclone 4 cells (ATCC #CRL-2593, American Type Culture Collection, Manassas, VA) suspended in 30 µl of complete media, then incubated at 37°C in a 5% CO₂ humidified incubator for 3 hrs prior to adding 1 ml of complete media. Media was changed every other day during maintenance of the cultures.

After 6, 10, and 15 days of culture, cells were stained with Cell Tracker Green CMFDA (Invitrogen, Carlsbad, CA) as per the manufacturer’s protocol. Cells on the scaffold surface were imaged using a confocal microscope (LSM510, Zeiss, Thornwood, NY). Images were taken every 1 µm in the z-direction for 100 µm from the scaffold surface and compiled into a single 3-D projection.

Additionally, DNA content was quantified for the cell-laden scaffolds 1, 6, 10, and 15 days post-seeding (N = 4 each time point, each condition) to estimate the change in the number of cells on the scaffolds over time. The scaffolds were transferred into 20
ml HDPE scintillation vials, 3 ml of nuclease-free water (Ambion, Austin, TX) was added, and the constructs were frozen at -20°C. The constructs were thawed and homogenized (Polytron PT3000, Kinematica Inc., Bohemia, NY) on ice at 6000 rpm for 1 minute. Subsequently, two additional freeze-thaw cycles were conducted to lyse the cells. The DNA released into the water was then assayed using the PicoGreen dsDNA assay kit (Invitrogen) as per the manufacturer’s protocol. The number of cells on each scaffold was determined by using an approximation of 8 pg DNA per cell.²⁸

In Vivo Formation of Porous Scaffolds upon Injection of Polymer Solution

The morphology of scaffolds formed in an in vivo environment was examined. 100µl of 75:25 PLGA and porogen mixture in tetraglycol was injected subcutaneously in the backs of NOD-SCID mice using a 16G needle (1 injection per mouse). NIH guidelines for the care and use of laboratory animals (NIH Publication #85-23 Rev. 1985) were observed. The polymer formulations used were (1) PLGA, (2) PLGA + sucrose + diH₂O, (3) PLGA + ammonium bicarbonate + diH₂O, and (4) PLGA + sodium bicarbonate + diH₂O. 24 hours post-injection, mice were sacrificed by CO₂ exposure and the scaffolds were excised, fixed in 3.7% formaldehyde, rinsed in PBS, and then lyophilized. The scaffolds were vacuum sealed and stored at -20°C until the surface and bulk morphology of the excised scaffolds examined using SEM.

DNA Delivery and Cell Transfection

Preparation of PLGA microparticles encapsulating DNA
Plasmid DNA (pDNA) encapsulated PLGA microparticles were prepared using a double emulsion-solvent evaporation method. Briefly, 500 µl of LacZ pDNA (1 mg/ml, Sigma) in Tris-EDTA buffer was emulsified in 5 ml of PLGA (75/25, i.v. 0.76 dl/g) solution (10% w/v in methylene chloride) using sonication (Sonic Dismembrator 550, Fisher Scientific) at 20 W for 30 sec. The water-in-oil emulsion was further emulsified in 49.5 ml of a 3% w/v aqueous solution of polyvinyl alcohol (PVA, Mw 30,000~70,000, Sigma) using a magnetic stirrer (1000 rpm for 5 min) to form a water-in-oil-in-water multiple emulsion. The emulsion was further stirred overnight at room temperature to remove the methylene chloride. The PLGA microparticles were recovered by centrifugation (1000xg for 5 min at 4 °C), washed with nuclease free water three times to remove PVA and unentrapped agent, and then lyophilized until dry to obtain a dry powder.

**pDNA encapsulated PLGA microparticle characterization**

The morphological examination of the PLGA microparticles encapsulating LacZ pDNA was performed using SEM with a 20 kV electron beam. The pDNA encapsulated PLGA microparticles were mounted onto a metal stub and sputter coated with palladium for 60 seconds (Desk II, Denton Vacuum, Moorestown, NJ).

**Preparation of injectable delivery systems**

The PLGA 75/25 (i.v. 0.23 dl/g, 100 mg) was dissolved in 1 ml tetraglycol at 37°C for 12 hrs. 220 mg of pDNA-loaded PLGA microparticles (100 µg pDNA) were suspended in 1 ml of PLGA solution in tetraglycol. To create porous PLGA scaffolds, 600 mg NaCl (106-250µm of diameter) was added into the microparticle-PLGA-
tetraglycol solution. 100 µl aliquots of this solution were then injected into 1 ml of PBS. These scaffolds were placed in a humidified environment with 5% CO₂ at 37 °C for DNA release. As a comparative group, the PLGA solution without the porogen was also prepared. To prepare naked pDNA-loaded PLGA scaffold with or without porogen as control groups, the PLGA was dissolved in tetraglycol at 20 w/v % (100 mg PLGA in 0.5 ml tetraglycol) by stirring overnight at room temperature. The LacZ pDNA (100 µg) was dissolved in tetraglycol (0.5 ml). This solution was then mixed with PLGA/tetraglycol solution to make a pDNA-loaded PLGA solution at a final concentration of 100 µg pDNA/ml PLGA solution. The mixture was rotated gently at 4°C overnight to form a homogenous solution. To make naked pDNA-loaded porous PLGA scaffold, 600 mg NaCl (106-250µm of diameter) was added into pDNA-loaded PLGA solution.

**Release kinetics of pDNA from PLGA microparticles in vitro**

The kinetics of in vitro release of pDNA from injectable PLGA was determined in 1 ml of PBS, pH 7.4 at 37 °C under continuous agitation. At various time points, the supernatant was withdrawn and fresh buffer was replenished. The amount of pDNA in the supernatant was determined using the Picogreen dsDNA assay. The experiments were performed in triplicate.

**In vitro transfection**

Transfection studies were conducted using human embryonic kidney 293 (HEK293) cells in vitro. The released pDNA samples were collected at various time points and lyophilized. The lyophilized pDNA was reconstituted using 50 µl PBS and complexed with PEI25k (N/P=2.5/1). HEK293 cells were plated in 96-well plates at
1×10^4 cells/well in 100 µl α-MEM containing 10% FBS. After 24 hrs incubation, the medium was removed, the cells were washed with PBS three times, and 100 µl serum-free medium was added into each well. The solution of pDNA/PEI25k complex was added to each well (10 µl/well), and incubated for 4 hrs at 37°C. Then the medium was removed, and fresh medium containing 10% FBS was added to each well. After 3 days, HEK293 cells were fixed and stained with β-Gal Staining Kit (Invitrogen) according to the manufacturer’s instructions.

Statistical Analysis

All data is reported as average values ± standard deviation. Statistical analysis was performed using GraphPad InStat program, Version 3.06 (La Jolla, CA). For each comparison, an unpaired t-test with two-tail P-value was used, with statistical significance defined as P<0.05.

5.3 Results

PLGA can be used in an injectable format when dissolved in a biocompatible, FDA-approved solvent, such as tetraglycol. However, the scaffold formed from injection of this PLGA solution into an aqueous environment lacks porosity. The goal of the work presented here was to incorporate porosity into the design of these in situ forming nonporous scaffolds to form scaffolds that can be used in tissue engineering applications. Microporous solid scaffolds that are formed upon injection into an aqueous environment were created by mixing a solution of PLGA dissolved in tetraglycol with a porogen and a
small volume of diH₂O and injecting this mixture into phosphate buffered saline (PBS). Figure 5.1 shows the macroscopic appearance of the scaffolds created using this technique. When PLGA in tetraglycol is injected into PBS, a nonporous scaffold results (Figure 5.1A). When sucrose or sodium chloride that has been milled and sieved to 106-250 µm is mixed into the PLGA solution prior to injection into PBS, a scaffold that is larger in size forms (Figure 5.1B), but there is still no macroscopic evidence of surface porosity. Since the same amount of PLGA was used for all conditions, the increased size is due to either the presence of undissolved porogen trapped in the center of the scaffold or interior porosity that was a result of the porogen leaching from the scaffold during the hardening of the polymer. Although the evidence of possible interior porosity was promising, the lack of surface porosity presented a limitation, as host cells at a defect site would not be able to penetrate the bulk of the scaffold until the surface had sufficiently degraded by hydrolysis. It was hypothesized that this outer solid skin might be forming due to rapid diffusion of the tetraglycol out from the surface of the scaffold, which was causing the PLGA to harden before all of the salt was exposed to the aqueous environment and leached away. Therefore, to obtain a scaffold that possesses interconnected, micron-scale porosity throughout the surface and the bulk, it was hypothesized that the addition of a small amount of diH₂O to the mixture prior to injection into the aqueous environment would aid in the initial precipitation of PLGA throughout the bulk of the scaffold by creating an emulsion. The result of adding a small amount of diH₂O to the mixture did result in a scaffold with both surface and bulk porosity as observed by gross inspection (Figure 5.1C). To verify that the addition of the porogen was required to achieve this porosity distribution, a mixture of PLGA in
tetraglycol with diH$_2$O, but without porogen was examined. The scaffolds formed in this manner without porogen display minimal porous structure (Figure 5.1D), indicating the need for both porogen and diH$_2$O to be present in the system to form scaffolds with porosity throughout.

The interior and exterior microarchitecture of the scaffolds were observed in greater detail using SEM. Figure 5.2 shows representative photomicrographs of scaffolds made using 75:25 PLGA, which is comprised of 75% glycolic acid and 25% lactic acid, and sodium chloride as the porogen (when porogen was used). The nonporous PLGA scaffolds do not possess surface or interior porosity (Figure 5.2A,B). When sodium chloride is added to the PLGA-tetraglycol solution, there is still no surface porosity (Figure 5.2D), but there is some interior porosity (Figure 5.2C). However, when both diH$_2$O and sodium chloride are added, the scaffolds exhibit both interior and exterior porosity (Figure 5.2E,F). Furthermore, the size of the pores is on the order of hundreds of microns. When PLGA in tetraglycol is mixed with diH$_2$O only, the resultant scaffolds do not exhibit micron-scale porosity on the surface or in the interior (Figure 5.2G,H), indicating that the porogen is a necessary component to achieve these microarchitectural features in the system. The use of a different PLGA composition, 50:50 PLGA (50% glycolic acid and 50% lactic acid), and a different porogen, sucrose, produced similar results (Figure 5.3).

To quantify the porosity obtained in the scaffolds, the nonporous scaffolds composed of PLGA in tetraglycol and the porous scaffolds made by mixing water and porogen in the PLGA/tetraglycol solution were examined using micro-CT. Representative 3-D image reconstructions with exposed cross-sections show minimal
porosity in the nonporous scaffolds, but significant porosity throughout the porous scaffolds (Figure 5.4). The percent porosity was calculated by determining the free volume (total volume – PLGA volume) and dividing by total volume. The total porosity was 32.19% ± 11.4% and 72.24% ± 4.0% for the nonporous and porous scaffolds, respectively. Statistical analysis indicates that these values are significantly different, with P<0.005.

The mass loss of the scaffolds in an aqueous environment (i.e., PBS) over time was then examined to determine their rate of degradation. Except for scaffolds made with only PLGA + diH2O, all scaffolds formed with 75:25 PLGA, using sodium chloride as a porogen in the porous scaffolds, exhibited a statistically significant loss in mass (P<0.05) between 24 hours and 1 week (Figure 5.5A). For all 75:25 scaffolds, there was minimal subsequent degradation observed between 1 week and 4 weeks. Scaffolds formed with 50:50 PLGA, using sucrose as the porogen in the porous scaffolds, demonstrated significant mass loss over 8 weeks (Figure 5.5B). As with the 75:25 scaffolds, all conditions in this experiment except the PLGA + diH2O showed statistically significant mass loss (P<0.05) between 24 hours and 1 week. In addition, all of these scaffolds showed statistically significant mass loss from 24 hours to 8 weeks. At 8 weeks the nonporous scaffolds had a significantly smaller mass than any of the other types of scaffolds, which were not statistically different from one another at that time. Interestingly, the 50:50 scaffolds showed linear degradation profiles, with best fit lines for the PLGA + sucrose and PLGA + sucrose + diH2O scaffolds having R^2 values greater than 0.875, and for PLGA and PLGA + diH2O scaffolds having R^2 values greater than 0.985.
The compressive elastic moduli of these injectable scaffolds were then determined at varying times of degradation (Figure 5.6) to examine possible changes in mechanical integrity. For both the 75:25 PLGA scaffolds with sodium chloride (Figure 5.6A) and 50:50 PLGA scaffolds with sucrose porogen (Figure 5.6B), the modulus did not vary significantly throughout degradation, with the exception of the nonporous scaffolds which were found to have a significantly higher modulus at 4 weeks than at 24 hours (P<0.05), possibly due to residual tetruglycol leaching more slowly from these nonporous scaffolds because of their solid skin and lack of porosity.

The degraded 50:50 PLGA scaffolds were also imaged using SEM after 8 weeks to examine how the exterior and interior structural morphologies change over time in an aqueous environment (Figure 5.7). The nonporous PLGA was largely degraded by this time point, and the outer surface remained nonporous (Figure 5.7A,B). The PLGA + porogen still shows a nonporous surface (Figure 5.7C) with some porosity on the interior (Figure 5.7D). On the other hand, PLGA + porogen + diH₂O maintained significant porosity on both the interior and the exterior of the scaffold (Figure 5.7E,F).

The scaffolds made by mixing diH₂O into the PLGA-tetruglycol solution exhibit some porous structure (Figure 5.7G,H), although to a lesser extent than those made by mixing in both diH₂O and porogen.

MC3T3-E1 cells, a preosteoblastic cell line derived from mouse calvaria, were then seeded and cultured on the nonporous and porous scaffolds \textit{in vitro} to investigate their ability to adhere to and proliferate on these materials with different surface and bulk microarchitectures. Prior to cell seeding, the scaffolds were incubated in serum-
containing medium to allow proteins in the serum to adsorb to the surface of the scaffolds and thereby facilitate cell attachment via integrin receptors. The scaffolds were each seeded with 500,000 cells and then imaged and assayed for DNA content 6, 10, and 15 days later as a measure of proliferation. Using confocal microscopy, scaffolds were imaged to depths of 100 µm. Cells were easily imaged throughout the porous scaffolds, and were seen to proliferate from day 6 (Figure 5.8A) to day 15 (Figure 5.8B). In contrast, cells were only found on the peripheral surface of the nonporous scaffolds (Figure 5.8C), which were more difficult to image since they do not allow light to pass through due to their solid nature. 24 hours after seeding the cells on the scaffolds, the cell number was calculated based on the measurement of DNA content to determine a seeding efficiency for each scaffold type. On average, the seeding efficiencies were found to be 29.6% ± 20% for the nonporous PLGA scaffolds, 26.0% ± 19% for the PLGA + sucrose scaffolds, and 16.6% ± 3% for the PLGA + sucrose + diH₂O scaffolds. These seeding efficiencies, which are measures of initial cell adhesion to the scaffolds, were not found to be statistically different. The change in cell number over time was then determined for all three scaffold types. DNA content at days 6, 10, and 15 was normalized to the DNA content at day 1 for each scaffold condition to measure the fold increase in cell number over time (Figure 5.8D).

Cells exhibited no proliferation on the nonporous PLGA scaffolds, and there was minimal proliferation observed on the scaffolds formed with PLGA + sucrose as only the value at day 6 (and not day 10 or 15) was significantly higher than that at day 1. The porous scaffolds formed with PLGA + sucrose + diH₂O exhibited increased cell numbers at days 6, 10, and 15 compared to day 1. Additionally, at days 10 and 15, the increase in
cell number on the porous scaffolds was statistically greater than that on the nonporous scaffolds at the same timepoint.

It is important to verify that the formation of these porous scaffolds is not limited to only an *in vitro* aqueous environment, given that the ultimate goal is to form them upon injection *in vivo* for use in tissue engineering. Various polymer formulations were injected subcutaneously in the backs of NOD-SCID mice, and the scaffolds were harvested 24 hours later. They were then observed for both surface and interior porosity by scanning electron microscopy (Figure 5.9). Although not previously investigated *in vitro*, two additional porogens, sodium bicarbonate and ammonium bicarbonate, were tested to examine the effect of various porogens on scaffold formation *in vivo*. To minimize animal usage, only scaffolds formed by injection of PLGA alone or with the addition of both porogen and diH$_2$O were examined. Therefore, four conditions were used: PLGA alone (Figure 5.9A,B), PLGA + sucrose + diH$_2$O (Figure 5.9C,D), PLGA + ammonium bicarbonate + diH$_2$O (Figure 5.9E,F), and PLGA + sodium bicarbonate + diH$_2$O (Figure 5.9G,H). The PLGA-alone injections appear solid and exhibit no surface porosity, as expected. In contrast, all three other scaffold conditions formed with porogen + diH$_2$O show both surface and bulk porosity.

Finally, the ability to use these scaffolds for DNA delivery was demonstrated. By mixing lyophilized plasmid DNA into the PLGA / tetraglycol solution, the genetic material was distributed throughout the polymer prior to scaffold formation. Scaffolds were made with and without porogen. It was found that the DNA release did not vary significantly when comparing the two types of scaffolds, and that the release was complete after only about one week (Figure 5.10). Therefore, to modulate the release and
allow for more sustained delivery over time, the DNA was first incorporated into PLGA microspheres (Figure 5.11). These microspheres were then mixed into the PLGA/tetraglycol solution immediately prior to scaffold formation. The PLGA microspheres do indeed slow the release considerably, and allow for sustained DNA delivery for at least 70 days (Figure 5.10). The PLGA microspheres incorporated into the scaffolds with porogen shows increased release compared to the scaffolds without porogen. Furthermore, when compared to the DNA release from the microspheres alone, the release is delayed by incorporation into either type of scaffold.

To verify that the released DNA was still bioactive, the released media was pooled, lyophilized and resuspended in a small volume of water. This was mixed with PEI and applied to cells grown in monolayer in vitro. The plasmid DNA used in these studies encoded for lacZ, so that the transfection efficiency could easily be assayed by staining with X-Gal. DNA from the first week of release from the scaffolds without microspheres gave the highest transfection efficiency, as expected due to the high amounts of DNA released early in these conditions (Figure 5.12). A lower level of transfection was observed for cells treated with release media from the first week from the scaffold formulations with microspheres and also from the microspheres alone. After one week, negligible amounts of DNA were released from the scaffolds without microspheres and so these conditions were not tested for bioactivity at later timepoints. The release media from days 8-22 showed highest transfection from the microspheres in the porous scaffolds (Figure 5.13). Although at this time the microspheres alone had released more total DNA, much of the release occurred in the first week, so there was actually less DNA available for transfection of cells in the 2 and 3 week release media.
The DNA was being released more slowly from the PLGA microspheres in nonporous PLGA scaffolds and so the transfection from these was also less than from the porous scaffolds. Similarly, the release from microspheres in the porous scaffolds again gave the greatest transfection for the release media from days 23-42 (Figure 5.14). Even from days 43-70 the DNA released from all three conditions is still bioactive and able to transfect cells (Figure 5.15). At this later timepoint the microspheres alone give higher transfection efficiency, as they release a substantial amount of the loaded DNA during weeks 7-10.

5.4 Discussion

There are many techniques and materials for forming scaffolds for tissue engineering applications, however, to our knowledge, there has not yet been a demonstration of an injectable solid scaffold that forms in situ by phase inversion with a microporous exterior and interior. In this work, we present a novel biomaterial system with the capacity to form such scaffolds both in an aqueous in vitro environment as well as in vivo.

PLGA is a biocompatible, biodegradable polymer that is frequently used in tissue engineering research for the formation of porous scaffolds that can be placed within a defect to support cellular migration of either surrounding host cells or transplanted cells into the scaffold, followed by proliferation of the cells and tissue-specific extracellular matrix deposition which can lead to natural tissue formation and subsequent defect repair. This polymer is insoluble in aqueous solutions, and so is often prepared in a toxic organic
solvent which must be removed prior to implantation into the body. However, there are several water-miscible solvents that have been FDA approved at various dosages for use in patients which can be used to dissolve PLGA, such as tetraglycol, \(N\)-methyl-2-pyrrolidone (NMP), dimethyl sulfoxide (DMSO), and 2-pyrrolidone.\(^{33}\) When a solution of PLGA in tetraglycol is injected into an aqueous environment, the solvent will diffuse out into the surrounding water, and as it does the hydrophobic PLGA will precipitate out of solution and harden into a nonporous scaffold.

The ability to form a solid scaffold using an injectable technology is attractive in the field of tissue engineering. As mentioned earlier, one of the key parameters for scaffolds in these applications is sufficient micron-scale porosity to allow cells to infiltrate and proliferate throughout the bulk of the scaffold placed in a tissue defect so that new tissue can be formed. Porosity is often created in solid scaffolds by the inclusion of porogens that can be leached away upon placement in an aqueous environment. The inclusion of such porogens was investigated in this system, with the stipulation that the porogens selected should be insoluble in tetraglycol so that they could retain their crystalline state and size when mixed into the polymer solution. In this manner, upon injection into an aqueous environment, the PLGA could harden around the porogen crystals, which would then be leached away, leaving behind a porous PLGA structure. There are many possible choices for the specific porogen used, with sodium chloride sieved to a specific size range, usually on the order of hundreds of microns, being the most frequently used.\(^{34}\) In this work, sodium chloride and sucrose, both of which are soluble in water but insoluble in tetraglycol, were the primary porogens examined. To further examine the ability to form a porous structure with a diverse range
of porogens, and because sodium chloride is not an optimal choice for \textit{in vivo} leaching,\textsuperscript{35} two additional porogens were investigated \textit{in vivo}, sodium bicarbonate and ammonium bicarbonate.

Initially, these porogens, milled and sieved to a specific size range, were investigated for their ability to aid in the \textit{in situ} formation of porous PLGA scaffolds upon injection into an aqueous solution. The scaffolds formed from a mixture of PLGA in tetruglycol with porogen are larger in volume than their nonporous PLGA-only counterparts, which indicates the presence of internal porosity, or possibly undissolved porogen entrapped in the structure which could ultimately be dissolved to allow for porosity. However, these scaffolds have a solid skin surrounding them, which does not degrade even after a month in an aqueous solution. This is problematic for tissue engineering approaches, as a solid surface clearly will not allow for cellular access into the bulk of the scaffold.

Therefore, the system was further modified to ensure that both surface and bulk porosity would be formed upon injection into a defect. The inclusion of a small amount of diH\textsubscript{2}O in the mixture was sufficient to provide this desired porosity. Pores on the order of hundreds of microns can be observed on the surface and throughout the bulk of the scaffolds using SEM and micro-CT, and furthermore much of the porosity appears to be interconnected. This is desirable for permitting host cell infiltration into and throughout the scaffold. It is hypothesized that the inclusion of a small amount of diH\textsubscript{2}O, mixed into the PLGA solution with porogen between connected two syringes, forms an emulsion that is predominately composed of the hydrophobic polymer in tetruglycol solution, but with dispersed water droplets into which the solvent can begin to diffuse.
prior to injection into a bulk aqueous environment. This preliminary diffusion around the droplets throughout the polymer solution may help to lower the polymer solubility locally in these areas, and therefore promote the formation of a micro-porous structure and prevent the development of a solid polymer skin around the scaffold. Further analysis by micro-CT verified that the porous scaffolds with both surface and bulk porosity possessed a high level of porosity as compared to the nonporous scaffolds.

The degradation rate of PLGA can be varied by changing the molecular weight of the polymer or the ratio of lactic acid to glycolic acid due to the greater hydrophobicity of lactic acid as compared to glycolic acid. Interestingly, in the studies presented here it was found that the 50:50 PLGA scaffolds all exhibited linear decreases in mass over the course of 8 weeks. In future systems, it will be of interest to further tailor a specific system to achieve degradation profiles so that the scaffold degrades at a similar rate as new tissue formation. Furthermore, the porosity of the scaffolds achieved with this system was found to persist throughout the degradation process, which indicates that there would be space for cells to continue proliferating and forming new tissue throughout the bulk of the scaffold as it degrades.

During the degradation studies, the mechanical properties of the scaffolds were found to not vary significantly over time, with the exception of the nonporous scaffolds which showed an increase in elastic modulus between 24 hours and 4 weeks. We hypothesize that this increase could be due to the glycofurol slowly leaching out from these scaffolds because of their nonporous structure, and residual solvent still present at earlier timepoints may serve to plasticize the polymer. Others have shown that residual solvent can plasticize polymers which decrease their mechanical properties.36,37
Depending on the particular tissue regeneration application, it may be desirable to obtain higher mechanical properties than those displayed by these scaffolds. It is possible that the elastic modulus might be increased by changing variables such as the concentration of PLGA in tetraglycol, the ratio of lactic acid to glycolic acid, the molecular weight of the polymer, or the amount of porogen and diH₂O used to fabricate the scaffolds. Additionally, it may be beneficial to incorporate an additional biomaterial, such as calcium phosphate, to further increase the mechanical properties of the resultant scaffolds.

It is important for the scaffolds to support the attachment and growth of cells, and so the ability of cells to adhere to and proliferate within these scaffolds was investigated. PLGA is a hydrophobic polymer that will not typically support cell attachment until serum proteins adsorb to its surface, which then present specific ligands to which cell surface receptors can bind. Although this can occur readily in vivo, for in vitro use the scaffolds were incubated first in serum-containing media prior to cell seeding. It was found that MC3T3 cells were able to adhere to these surfaces, and could be monitored over time. In the porous scaffolds, cells were found throughout the scaffold. This was in direct contrast to cells seeded on the nonporous scaffolds, which even after two weeks were still found only on the outer peripheral surface. This result confirmed that without surface porosity cells are unable to infiltrate into the bulk of the construct. Furthermore, by measuring the number of cells on the scaffolds, it was determined that the cells on the nonporous scaffolds did not increase in number over the course of 15 days, whereas those on the porous scaffolds did show proliferation. This indicates that the porous scaffolds
have a greater potential than the nonporous scaffolds to support cellular proliferation throughout the scaffold.

An important verification of the potential utility of this novel technology for tissue engineering is its ability to form porous structures upon injection \textit{in vivo}. The capacity to form scaffolds with interconnected surface and interior porosity in an animal model was confirmed via subcutaneous injections in SCID mice. As this system relies on leaching of the porogen \textit{in vivo} to form a porous structure, various porogens were used in this study to identify several that could have potential for \textit{in vivo} use. Sodium chloride can cause local tissue damage, so sucrose, ammonium bicarbonate, and sodium bicarbonate were examined. All three types of porogens, when mixed with the PLGA solution and \textit{diH}_2\textit{O}, created scaffolds with both surface and interior porosity. Demonstration of microporous polymer scaffold formation in the \textit{in vivo} environment confirms that this porous, biodegradable, injectable system has great potential for future use in minimally invasive regenerative medicine applications.

To further examine the potential for these scaffolds, their ability to serve as a gene delivery vehicle was investigated. By incorporating DNA into the PLGA / tetraglycol solution, the scaffolds formed upon injection into the site of interest could also be used for inductive tissue engineering, as the encapsulated DNA could transfect cells and increase the expression of the desired bioactive factors. Here DNA encoding for \textit{lacZ} was used as an easy way to assess the transfection efficiency and verify the bioactivity of the DNA. When naked DNA was incorporated into scaffolds with or without porogen, the release was complete after only one week, and the release profiles of the DNA from the two types of scaffolds was not different. To further engineer this system to modulate
the release, the DNA was first incorporated into PLGA microspheres prior to incorporation into the scaffolds. This way, the DNA had to diffuse through the PLGA microspheres and then through the scaffold itself in order to be released into the surrounding medium. As anticipated, the release rate was decreased, and bioactive DNA was delivered for at least 70 days at which time the study was concluded. The release of DNA was faster from the scaffolds containing porogen than from the nonporous scaffolds, likely due to a shorter diffusion path to the surrounding medium. It was also seen that the release rate was greater from the microspheres alone than from the microspheres incorporated into the scaffolds, as the DNA did not have to diffuse through the scaffold material after it had been released from the microspheres. In all conditions, the released DNA was found to be bioactive. However, the naked DNA released from the scaffolds so rapidly that only during the first week was there sufficient genetic material available to transfect cells. In contrast, DNA was released from the microspheres alone or microspheres incorporated into the PLGA scaffolds for at least 70 days, and able to transfect cells throughout this time. This offers great promise for the use of these injectable, porous scaffolds as gene delivery vehicles.

Despite the potential of these scaffolds for gene delivery, they were not pursued further for bone regeneration for this dissertation work. Instead, the focus for the remainder of this thesis work turned to the development of a system capable of delivering a cell population in addition to the DNA to a bony defect, as increased cellularity within a defect site will likely lead to improved regeneration. Although the glycofurol used in these scaffolds is safe in small amounts when delivered to an organism as a whole, cells cannot be mixed directly into the PLGA / glycofurol mixture. As such, these scaffolds
will be promising in regenerative medicine applications where the migration and proliferation of the surrounding host cells into the scaffold is sufficient for regeneration to occur. For this reason, hydrogels were next examined as they are injectable and cells can be easily incorporated into them.

5.5 Conclusions

A novel system for the formation of a porous PLGA scaffold upon injection in vivo was developed. The PLGA is dissolved in a biocompatible solvent which is water miscible. When the polymer solution is injected into an aqueous environment, the solvent will diffuse into the surrounding aqueous environment, causing the PLGA to harden upon contact with water. A porogen and a small amount of diH$_2$O are mixed into the PLGA solution to allow for the formation of a porous structure throughout the resulting scaffold upon injection. This porous structure, which can be formed in vitro and in vivo, can support cellular growth, and is promising as a simple, minimally invasive approach to in vivo microporous scaffold fabrication using solid polymer materials for ultimate application in tissue engineering. Furthermore, these scaffolds can be used for localized and sustained gene delivery to the site of interest.

Future work will include examining the ability to tailor the porosity and structure of these scaffolds by altering the amount of porogen included, the size distribution of the porogen, the amount of diH$_2$O included, and the polymer solution concentration. Additionally, the incorporation of DNA encoding for specific growth factors may encourage tissue regeneration in vivo, as local cells could be stimulated to migrate into
the scaffold, proliferate, and differentiate. The release of plasmid DNA encoding for important bioactive factors released in a controlled, sustained manner may help to accelerate tissue repair, and will continue to be examined in future studies. Furthermore, the incorporation of growth factors and their subsequent release and bioactivity would be interesting to examine.
Figure 5.1 Photographs of the macroscopic appearance of scaffolds. (A) PLGA only; (B) PLGA + porogen; (C) PLGA + porogen + diH₂O; (D) PLGA + diH₂O.
Figure 5.2 SEM photo-micrographs of 75:25 PLGA scaffolds depicting interior and surface microarchitecture. (A and B) PLGA only; (C and D) PLGA + sodium chloride; (E and F) PLGA + sodium chloride + diH₂O; (G and H) PLGA + diH₂O. (A, C, E, and G) are of scaffold interiors; (B, D, F, and H) are of scaffold exteriors. Scale bars represent 200 µm.
Figure 5.3 SEM photo-micrographs of 50:50 PLGA scaffolds depicting interior and surface microarchitecture. (A and B) PLGA only; (C and D) PLGA + sucrose; (E and F) PLGA + sucrose + diH$_2$O; (G and H) PLGA + diH$_2$O. (A, C, E, and G) are of scaffold interiors; (B, D, F, and H) are of scaffold exteriors. Scale bars represent 200 µm.
Figure 5.4 Micro-CT images of a cross-section through (A) nonporous and (B) porous scaffolds made from 50:50 PLGA. Scale bars represent 1 mm.
Figure 5.5 Scaffold degradation over time in PBS. (A) 75:25 PLGA with sodium chloride as porogen. (B) 50:50 PLGA with sucrose as porogen.
Figure 5.6 Elastic moduli of injectable scaffolds. (A) 75:25 PLGA with sodium chloride as porogen. (B) 50:50 PLGA with sucrose as porogen.
Figure 5.7 SEM photo-micrographs of 50:50 PLGA scaffolds depicting interior and surface microarchitecture after 8 weeks. (A) and (B) PLGA only; (C) and (D) PLGA + sucrose; (E) and (F) PLGA + sucrose + diH₂O; (G) and (H) PLGA + diH₂O. (A, B, C, E, and G) are of the scaffold interiors; (D, F, and H) are of the scaffold exteriors. All scale bars represent 200 µm except in (B) where it is 1 mm.
Figure 5.8 Interaction of pre-osteoblast cell line with various scaffold types studied in vitro. Scaffolds seeded with MC3T3-E1 cells stained with a cytoplasmic stain CellTracker® were examined with confocal microscopy. Photomicrographs of fluorescently labeled cells on scaffolds made with PLGA + sucrose + diH₂O at (A) day 6 and (B) day 15 demonstrate extensive cellular infiltration throughout the bulk of the scaffold and proliferation between the two time points. (C) A photomicrograph of cells on nonporous PLGA scaffolds at day 15 shows cells populating only the peripheral surface of the scaffolds. Scale bars represent 200 µm. (D) Graph showing fold increase in cell number on scaffolds compared to the number of cells measured at 24 hours post-seeding. Sample values significantly greater (P<0.05) than those of the same scaffold type at 24 hours are indicated by *, and sample values significantly greater (P<0.05) than the nonporous scaffolds at the same timepoint are indicated by **.
Figure 5.9 SEM photo-micrographs of nonporous and microporous scaffolds formed in vivo. (A) and (B) PLGA only; (C) and (D) PLGA + sucrose + diH$_2$O; (E) and (F) PLGA + ammonium bicarbonate + diH$_2$O; (G) and (H) PLGA + sodium bicarbonate + diH$_2$O. Panels A, C, E, and G are from the interior of the scaffolds. Panels B, D, F, and H are from the exterior of the scaffolds. All scale bars represent 200 µm.
Figure 5.10 Release profiles of naked DNA or DNA incorporated into PLGA microspheres from injectable PLGA scaffolds with and without porogen, and from PLGA microspheres alone.
Figure 5.11 SEM photomicrograph of DNA-laden PLGA microspheres. Scale bar represents 50 µm. Courtesy of Dr. Oju Jeon.
**Figure 5.12** Transfection of cells with DNA encoding for lacZ in release media pooled from days 1-7. The blue cells in the photomicrographs are transfected. The bar graph represents the quantification of the total percent of cells that were positively transfected. *p>0.05; all other values are statistically significantly different from each other (p<0.05). Courtesy of Dr. Oju Jeon.
**Figure 5.13** Transfection of cells with DNA encoding for lacZ in release media pooled from days 8-22. The blue cells in the photomicrographs are transfected. The bar graph represents the quantification of the total percent of cells that were positively transfected. All values are statistically significantly different from each other (p<0.05). Courtesy of Dr. Oju Jeon.
Figure 5.14 Transfection of cells with DNA encoding for lacZ in release media pooled from days 23-42. The blue cells in the photomicrographs are transfected. The bar graph represents the quantification of the total percent of cells that were positively transfected. All values are statistically significantly different from each other (p<0.05). Courtesy of Dr. Oju Jeon.
Figure 5.15 Transfection of cells with DNA encoding for lacZ in release media pooled from days 43-70. The blue cells in the photomicrographs are transfected. The bar graph represents the quantification of the total percent of cells that were positively transfected. *p>0.05; all other values are statistically significantly different from each other (p<0.05). Courtesy of Dr. Oju Jeon.
5.6 Acknowledgements

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5.7 References


CHAPTER 6. Calcium Phosphate - DNA Nanoparticle Gene Delivery from Alginate Hydrogels Induces in vivo Osteogenesis

6.1 Introduction

Gene delivery can be achieved by several methods, both viral and non-viral. Although viral vectors are highly efficient delivery vehicles, there are major concerns with immunogenicity, toxicity, the risk of recombination, and possible extended expression of the protein beyond the timeframe required for the healing of the defect. As a result, much effort has been invested into the development of efficient non-viral methods, which include the creation of nanoparticles with encapsulated DNA using liposomes, polycations, or inorganic compounds. Of these, inorganic compounds, especially calcium phosphate (CaP), are attractive as they are widely available, inexpensive, and relatively easy to prepare. Calcium phosphate precipitation of plasmid DNA, a technology developed 35 years ago, is widely used to transfet cells in vitro.

An important consideration in using gene delivery in vivo is the retention of the DNA at the site of interest to promote a strong therapeutic effect. Direct injections of DNA into an area result in a duration of expression that is difficult to control and often limited. Biodegradable scaffolds can be of great use in allowing sustained delivery of DNA at the desired location. The choice of the material is a major consideration. It must be biocompatible, and ideally should degrade over time as new tissue is formed. Many scaffolds are formed ex vivo and then implanted. Hydrogels offer a minimally
invasive option, as they can typically be injected directly into the site of injury. Also, it is easy to incorporate osteogenic cells into the hydrogel for subsequent cell transplantation.

Rather than delivering DNA at the site of interest and relying on uptake by surrounding cells, some groups have reported on ex vivo gene therapy of cells with subsequent transplantation of the engineered cells into the injured area within a scaffold.\textsuperscript{14-19} A major difficulty with this technique is that the amount of BMP produced by these pre-transfected cells is dependent on their survival during implantation and within the defect. In contrast, DNA delivery from a scaffold can provide a sustained supply of DNA for surrounding host cells or transplanted cells to uptake and express. Enhancement of bone regeneration resulting from the release of DNA encoding for osteogenic factors from scaffolds with and without cell transplantation has been demonstrated.\textsuperscript{20-24} However, many of these studies utilized viral vectors, which have disadvantages previously described. In addition, many involved implantation of scaffolds, which requires invasive surgical procedures.

When considering delivery from a scaffold, it is important to ensure that the DNA maintains its ability to transfect cells over time, and that it can withstand any processing techniques used to incorporate it into the scaffold. DNA complexed with liposomes can be unstable with agitation or during freezing,\textsuperscript{25} and the electrostatic interaction between DNA and polycations such as chitosan, polyethylenimine (PEI), or poly-L-lysine can be disrupted in the presence of other charged compounds (for example, negatively charged polysaccharides, such as alginate).\textsuperscript{26} Furthermore, both liposomes and polycations can be cytotoxic.\textsuperscript{1,27} CaP crystals complexed with DNA do not have problems with cytotoxicity at the concentrations used for transfection, but they can aggregate or grow over time,
rendering the particles ineffective for transfection, as the optimal size for cellular uptake by endocytosis has been shown to be around 100 nm or less.\textsuperscript{28,29} Therefore, it is important to make DNA nanoparticles that remain stable over time when incorporated into a scaffold for transplanted or host cell uptake.

In this chapter, the ability to deliver osteogenic cells and DNA complexed with CaP from an injectable hydrogel for the application of bone regeneration is shown.\textsuperscript{30} Plasmid DNA (pDNA) encoding for lacZ as a measure of transfection efficiency is examined, and also pDNA encoding for BMP-2 is used to enhance bone regeneration \textit{in vivo}. As mentioned previously, the transfection efficiency of standard preparations of CaP-DNA can decrease over time as the inorganic crystals continue to grow and aggregate, becoming too large for endocytosis by the cells.\textsuperscript{31} Therefore, CaP-DNA nanoparticles (NPs) have been optimized to remain stable over time. These NPs are incorporated into an alginate hydrogel to allow for localized delivery of the complexes to both transplanted cells and host cells at a bone defect site (Figure 6.1). One recent study demonstrated that alginate can serve as an effective matrix for localized gene delivery.\textsuperscript{32} In this study, alginate modified with adhesion ligands containing the RGD peptide sequence was used as it has been shown to act as a synthetic ECM material\textsuperscript{33} which can promote cell attachment, proliferation, osteoblastic differentiation, and bone formation.\textsuperscript{34-36} It is possible to incorporate osteoblasts or osteoprogenitor cells within this hydrogel to provide a cell population capable of taking up the genetic material being delivered, and therefore further promote osteogenesis. This nanoparticle-cell-alginate mixture can be injected, and as such it is a minimally invasive therapeutic option. Sustained release of CaP-DNA from the alginate over time is demonstrated, along with the ability of the CaP-
DNA NPs to transfect preosteoblast cells *in vitro*, and the combination of CaP-DNA NPs with cells in alginate to promote bone formation *in vivo*.

6.2 Materials and Methods

**Materials**

Sodium alginate powders were a generous gift from FMC Biopolymers (Princeton, NJ). The plasmid pcDNA3.1/Hygro/lacZ and Quant-It PicoGreen dsDNA quantitation reagent were obtained from Invitrogen (Carlsbad, CA). The plasmid encoding for BMP-2 was a generous gift from Chris Evans, Harvard Medical School. MC3T3-E1 Subclone 4 (ATCC #CRL-2593) cells were obtained from American Type Culture Collection (Manassas, VA). Phosphate buffered saline (PBS) and α-Modified Eagle’s Medium were obtained from Hyclone (Logan, UT). All other chemicals were obtained from Fisher Scientific (Fairlawn, NJ).

**Alginate Preparation**

Two different sodium alginate powders, with product names of 10/60 and 20/40, were lyophilized until dry, and the 20/40 alginate was subjected to gamma irradiation at 5 MRad (Phoenix Lab, University of Michigan, Ann Arbor). The molecular weight (MW) was found to be 37,000 g/mol for irradiated 20/40 alginate, and 121,000 g/mol for non-irradiated 10/60 alginate as determined by SEC-MALS (FMC Biopolymers). The percentage of guluronic (G) and mannuronic (M) acid for each alginate was determined by NMR (FMC Biopolymers); 10/60 was found to have 68% G-content and 32% M-content, and 20/40 5 Mrad was found to have 66% G-content and 34% M-content. The
peptide glycine – arginine – glycine – aspartic acid – serine – proline (GRGDSP; Commonwealth Biotechnologies, Richmond, VA), which contains the RGD cell-binding domain, was covalently coupled to the irradiated alginate as previously described. Both types of alginate were then purified by dialysis for 4 days, subjected to activated charcoal treatment, and sterilized through a 0.22 µm filter.

**Preparation and Characterization of Calcium Phosphate DNA Nanoparticles**

Two types of calcium phosphate DNA nanoparticles were fabricated: calcium phosphate core with pDNA coating, and calcium phosphate – DNA core with BSA coating. The fabrication of the CaP core – DNA coated NPs was based on a method described by Sokolova, et al. Briefly, equal volumes of 18.7 mM CaCl₂ (pH 9) and 11.23 mM Na₂HPO₄ (pH 9) were added simultaneously to a tube with a magnetic stir bar. The solution was mixed for 30 seconds and 200 µg of pDNA was added to quench the crystallization by coating the crystals. CaP-DNA core – BSA coated NPs were created by first preparing calcium phosphate – DNA particles and subsequently adding a solution of bovine serum albumin (BSA) to coat the particles and prevent further crystal growth. Specifically, 120 µg pDNA was mixed with 100 µl 2 M CaCl₂ and the solution added dropwise to 1 ml of 2X HBS (pH 7) while stirring. Then, 780 µl of distilled water was immediately added, the mixture stirred at room temperature for 30 minutes, and 200 µg of BSA was added.

The size of the NPs was determined by transmission electron microscopy (TEM). NPs were freshly prepared (N=3 preparations for each type), and a sample of each was diluted 1:50 with distilled water, spotted onto a nickel formvar grid, and allowed to dry at
37°C. The remaining NPs were kept for two weeks at 4°C, and samples were taken at one and two weeks for imaging. Images were scanned into digital format, and from these images, particle diameters (N>200 NPs) were calculated using ImageJ software (NIH, Bethesda, MD).

The DNA incorporation efficiency of each particle type (N=3) was determined by centrifugation of the NPs at 17,000 rpm for 30 minutes, followed by spectrophotometric measurement of the pDNA in the supernatant using the PicoGreen dsDNA assay kit. Plasmid DNA at known concentrations was used to construct the standard curve.

*In vitro Cell Transfection Using Calcium Phosphate DNA Nanoparticles*

CaP core – DNA coated and CaP-DNA core – BSA coated NPs were freshly prepared using DNA encoding for lacZ. MC3T3-E1 preosteoblastic cells were seeded the day before transfection at a density of 1x10⁵ cells/well in a 24-well plate. For transfection, the cells were rinsed once with PBS, followed by the addition of serum-free media containing 10% v/v of NPs. The cells were incubated for 5 hours at 37°C, and then the media was removed and replaced with complete medium (α-MEM + 10% FBS). 48 hours post-transfection, the cells were rinsed with PBS, fixed with 0.2% glutaraldehyde, and stained with X-Gal to assay lacZ expression. 24 hours after staining, the cells were rinsed with PBS and examined under the microscope to observe transfection efficiency.
In vitro DNA Release from Alginate Hydrogels

CaP NPs were freshly prepared, and mixed at a concentration of 10% v/v with a 2% 10/60 alginate solution reconstituted in PBS. The alginate was then crosslinked with a slurry of calcium sulfate (210 mg/ml in distilled water) at a ratio of 25:1 and cast between two glass plates spaced 0.75 mm apart. After 20 minutes, 10 mm diameter disks (N=8) were cut out and transferred to PBS containing calcium and magnesium. In the same manner, alginate disks containing naked pDNA and alginate disks without pDNA or NPs were created. The disks were incubated at 37°C under gentle agitation. Release samples were taken periodically by removing the PBS and replacing with fresh PBS. The released DNA was measured using the PicoGreen dsDNA assay kit.

In vivo Bone Formation

CaP NPs were freshly prepared and mixed into a 2% 20/40 5 MRad GRGDSP-alginate solution, followed by the addition of MC3T3 cells at a final concentration of 24x10^6 cells/ml. Additionally, as a control, cells in the modified alginate without NPs or pDNA were examined to obtain a background level of bone formation due to the cells alone. The alginate was crosslinked as described above with calcium sulfate, and kept in a syringe on ice until injection. 200 µl of each experimental condition was injected subcutaneously through an 18-gauge needle into the backs of anesthetized 5-week-old male C.B-17 SCID mice (Harlan, Indianapolis, IN). NIH guidelines for the care and use of laboratory animals (NIH Publication #85-23 Rev. 1985) were observed. Implants were harvested, fixed, and processed histologically at 2.5 and 6 weeks post-injection. Slides were stained with hematoxylin and eosin (H&E) or Goldner’s Trichrome.
6.3 Results

The size of the CaP – DNA NPs was quantified to determine their stability over time. The size and morphology of both types of NPs were examined at 0, 1, and 2 weeks post-fabrication by transmission electron microscopy. Representative images are depicted in Figure 6.2. The NPs are relatively uniform in size with a round morphology. The particle sizes were determined, and as shown in Table 6.1, the NPs did not grow or aggregate over time. The average particle diameters remained close to or below 100 nm at all timepoints, which has been shown to be the optimal particle size range for cell uptake by endocytosis.\textsuperscript{28,29} Additionally, the DNA incorporation efficiency was determined to be 66.5% +/- 3.5% for CaP core – DNA coat NPs, and 79.5% +/- 16.2% for CaP-DNA core – BSA coat NPs.

<table>
<thead>
<tr>
<th></th>
<th>CaP core – DNA coat diameter (nm)</th>
<th>CaP-DNA core – BSA coat diameter (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Week 0</td>
<td>74.77 ± 73.1</td>
<td>160.75 ± 178.8</td>
</tr>
<tr>
<td>Week 1</td>
<td>34.25 ± 38.5</td>
<td>62.86 ± 53.5</td>
</tr>
<tr>
<td>Week 2</td>
<td>41.55 ± 37.3</td>
<td>56.40 ± 98.7</td>
</tr>
</tbody>
</table>

Table 6.1 Size stability of CaP-DNA NPs over 2 weeks as determined by analysis of transmission electron microscopy images (average diameter ± standard deviation).

The ability of these NPs to transfect preosteoblast cells \textit{in vitro} in monolayer culture was examined using pDNA encoding for lacZ (Figure 6.3). The MC3T3-E1 subclone 4 preosteoblast cell line from mouse calvaria was used, as they have exhibited high capacity for osteoblastic differentiation.\textsuperscript{38} The transfection efficiency was low;
approximately 1% of cells stained positively for lacZ expression. However, it was verified that both particle types had the ability to transfect the preosteoblastic cells, and these low transfection efficiencies are on the order of those reported in the literature for calcium-phosphate DNA nanoparticles.

The in vitro release of naked pDNA and both types of CaP-DNA NPs from alginate hydrogels over the course of 78 days was quantified. As seen in Figure 6.4, all conditions show sustained release of pDNA throughout the course of these 78 days. The CaP-DNA core – BSA coat NPs were released most rapidly, followed by naked pDNA, then by CaP core – DNA coat NPs.

The ability of this novel injectable gene delivery system to transfect preosteoblasts and promote bone formation in vivo was tested. For this in vivo study, low MW alginate with RGD modification was used. As shown previously, irradiated alginate degrades more rapidly in vivo,\(^\text{36}\) and the RGD amino acid sequence allows cells incorporated within the alginate to adhere to the hydrogel.\(^\text{34}\) Degradation of the hydrogel is important to allow space for the cells to proliferate and secrete extracellular matrix, and to ultimately form new tissue.\(^\text{36}\) The addition of the RGD amino acid sequence allows cells to adhere to the hydrogel, promotes their proliferation, and has also been shown to increase the rate of bone formation when combined with preosteoblast or osteoblast cells.\(^\text{34}\) CaP-DNA NPs and preosteoblast cells were mixed into irradiated alginate modified with the cellular adhesive peptide, GRGDSP. These constructs were crosslinked with calcium and injected subcutaneously in the backs of SCID mice. Although pDNA encoding for lacZ was used in the in vitro studies to visualize the transfection, the pDNA in the NPs used in vivo encoded for BMP-2 to promote bone
formation. The constructs were harvested at 2.5 and 6 weeks post-injection, and the histology was examined. In the samples with the CaP core – DNA coat NPs, bony tissue as shown in Figure 6.5 was found in some of the implants as early as 2.5 weeks post-injection, and also at 6 weeks post-injection. In the samples with cells only (i.e., no NPs or DNA), no bony tissue was seen at any time point. This was also the case for samples with CaP-DNA core – BSA coat NPs.

6.4 Discussion

In this study, CaP-DNA was delivered from alginate hydrogels to enhance bone formation of transplanted preosteoblasts. CaP-DNA NPs were created using two different methods, one with the pDNA serving as a coating to halt particle growth, and the other with BSA serving the same purpose. Both types of NPs were on the order of 100 nm in diameter, the optimal size for cellular endocytosis,\(^{28,29}\) and remained stable in size for at least two weeks. The pDNA incorporation efficiency was high, indicating that the majority of the pDNA was complexed to the CaP NPs, and therefore available for cellular uptake. Furthermore, both types of NPs were capable of transfecting cells \textit{in vitro}, although at a low efficiency as expected.\(^ {31}\) However, we theorized that it may only be necessary for a portion of transplanted cells to be transfected with the BMP-2 plasmid in this system to enhance bone formation, because the cells that are expressing this protein will secrete it and signal to other neighboring cells to differentiate into osteoblasts and begin laying down extracellular matrix that will become bony tissue. Therefore, it should be possible to have a large population of cells differentiate into mature
osteoblasts, even if only a select portion of them actually exhibit upregulated expression of BMP-2 as a result of transfection.

To determine the amount of pDNA that is released from the alginate hydrogels over time due to diffusion of the pDNA out of the hydrogels or degradation of the hydrogels, we measured the release of pDNA from alginate for over two months. We found that the pDNA is released very slowly from the alginate during this period. The CaP-DNA core – BSA coat NPs released faster than naked pDNA or CaP core – DNA coat NPs. The pore size of alginate (both low and high MW) has been reported to be on the order of 5 nm,\(^3\) which likely explains the slow release of the pDNA from the hydrogels, and suggests that the majority of the release that we measured was due to degradation of the hydrogels rather than diffusion through them. With this system, the large fraction of pDNA remaining in the hydrogels would be available to transfec incorporated cells, and the smaller fraction of pDNA released from the hydrogel would be capable of transfecting host cells locally surrounding the hydrogel.

Low MW alginate produced by irradiation has been shown to degrade more rapidly than high MW alginate \(\text{in vivo}\).\(^3\) We hypothesized that incorporating preosteoblast cells within degradable alginate containing the CaP-DNA NPs would allow for sufficient NP release from the hydrogels and uptake of the NPs by the transplanted preosteoblast cells or surrounding host cells to improve bone tissue formation. Therefore, we proceeded with an \(\text{in vivo}\) study using low MW, RGD-modified alginate, MC3T3-E1 cells, and CaP-DNA NPs with pDNA encoding for BMP-2. Evidence of bony tissue was found in some of the samples containing the CaP core – DNA coat NPs after as little as 2.5 and 6 weeks. No bony tissue was observed at these early time points in samples with
MC3T3s alone (without NPs or pDNA) or, surprisingly, given our ability to transfet *in vitro*, in samples containing the CaP-DNA core – BSA coat NPs. It is possible that the BSA is interfering with cellular uptake *in vivo*; another group has reported decreased transfection with CaP-DNA NPs coated with BSA as compared to CaP-DNA NPs without BSA. In the future, the choice of a different protein coating, for instance one that promotes cellular uptake, may show more promising results. Other groups have shown increased pDNA NP uptake when they are coated with ECM molecules such as collagen or fibronectin. Such NP modifications might enhance the bone formation that was observed in both H&E and Goldner’s Trichrome stained histology in the CaP core – DNA coat NPs condition.

This injectable gene delivery system shows promise for bone regeneration applications. It is injectable, and therefore minimally invasive, it allows the inclusion of cells, and the CaP NPs provide an additional source of calcium and phosphate which are required for the formation of hydroxyapatite. Furthermore, the use of alginate hydrogels as the biomaterial delivery platform allows for modification of the scaffold’s biochemical and physical properties, such as its cell adhesiveness, mechanical properties, and degradation profile. The CaP NPs may transfet both transplanted cells delivered within the alginate, as well as host cells that will surround the hydrogel. These transfected cells will produce and secrete BMP-2, which can cause surrounding cells to differentiate down the osteoblastic lineage. As the pDNA is delivered via a non-viral method, the expression of the protein will be transient, ceasing when the transfected cells later senesce and die. Future directions include improving cellular uptake of these CaP-DNA NPs to increase transfection efficiency. Additionally, the use of different cell types, such
as mesenchymal stem cells, preosteoblasts, or primary osteoblasts, may be studied to see which are more readily transfected in this system and provide for increased bone growth. It would also be informative to examine what effect varying the biochemical and physical properties of the alginate used in the system has on gene uptake by cells within the 3D hydrogels and subsequent bone formation.
Figure 6.1 Schematic of experimental system. The top left side shows the fabrication method of the CaP core – DNA coat NPs, while the top right side shows the fabrication method of the CaP-DNA core – BSA coat NPs. The NPs were mixed in alginate hydrogels. For the in vivo study, preosteoblast cells were also mixed in the alginate prior to crosslinking with calcium. The final mixture was injectable, as shown.
Figure 6.2 Transmission electron microscopy (TEM) photomicro-graphs of (A) CaP core – DNA coat NPs and (B) CaP-DNA core – BSA coat NPs at time = 0 weeks.
Figure 6.3  Photomicrograph of MC3T3-E1 cells transfected by CaP-DNA core – BSA coat NPs (DNA encoding for lacZ; transfection demonstrated by X-Gal staining). Arrows indicate transfected cells.
Figure 6.4 Release profiles of CaP-DNA NPs and naked DNA from alginate hydrogels over time. Symbols in graph are as follows: ♦ no DNA, ▲ CaP core – DNA coat NPs, ● naked pDNA, X CaP-DNA core – BSA coat NPs.
Figure 6.5 Histology of implants. (A) 2.5 weeks post-injection, alginate with MC3T3-E1 cells and CaP core – DNA coat NPs, H&E staining. (B) 6 weeks post-injection, alginate with MC3T3-E1 cells (no NPs), Goldner’s Trichrome staining. (C, D) 6 weeks post-injection, alginate with MC3T3-E1 cells and CaP core – DNA coat NPs. (C) H&E staining, (D) Goldner’s Trichrome staining. a = alginate, b = bone. Scale bar = 100 nm.
6.5 Acknowledgements

Thank you to Erin Salter, Eric Chen, and Kathleen Sutter for their assistance with the production and characterization of the calcium phosphate nanoparticles. I would also like to thank James Dennis, Ph.D. and Mr. Amad Adwallah for assistance with the histology, and Therese Andersen of FMC Biopolymers for the alginate characterization. The authors gratefully acknowledge funding support for this work from Case Western Reserve University (EA), the Ellison Medical Foundation (EA), and a National Science Foundation Graduate Research Fellowship (MDK).

6.6 References


CHAPTER 7. siRNA Delivery from Biopolymer Hydrogels

7.1 Introduction

To this point, the work in this thesis has been focused on the delivery of plasmid DNA to upregulate protein expression in cells. However, it is also possible to deliver genetic material to downregulate protein expression. RNA interference (RNAi) is a powerful gene silencing mechanism which inhibits gene expression at the translational level by the targeted destruction of specific mRNA molecules. RNAi has the potential to revolutionize disease treatment and aid in the functional repair of damaged tissue by decreasing the expression of specific proteins, for example in cancer therapeutics by inhibiting promoters of angiogenic processes or in regenerative medicine by inhibiting proteins that negatively impact the healing process or by altering stem cell differentiation pathways. However, effective delivery of short interfering RNA (siRNA) to target cells in vivo remains a significant challenge to realizing its full therapeutic potential because it is highly prone to degradation by ubiquitous RNAses, targeting and retention of the siRNA at a specific location is problematic, and the silencing effect often only lasts a few days in rapidly dividing cells. siRNA can be delivered to cells either directly or by plasmid DNA that encodes for the siRNA molecule of interest. Delivery of siRNA itself offers the advantage of not requiring transport to the nucleus for expression, as it functions directly in the cytoplasm. This highly conserved process in eukaryotic cells utilizes intrinsic cellular machinery to degrade mRNAs that are
complementary to short (<30 nucleotides) double-stranded silencing RNA (siRNA).\textsuperscript{12} Current approaches for delivery of siRNA \textit{in vivo} include the direct injection of siRNA in saline, incorporation into liposomes, chemical conjugation with molecular entities to aid in targeting or stabilization of the molecule, complexation with positively charged peptides or polymers to form nanoparticles, or encapsulation within polymeric nano- or microspheres.\textsuperscript{13} However, since most of these techniques form nanoscale complexes with the siRNA, they can be rapidly dispersed, and therefore must either rely on targeting for uptake only by the desired cell population or delivery of high concentrations to allow sufficient dosage to reach the site of interest during a brief therapeutic window. Here, a new paradigm is presented whereby three-dimensional macroscopic, biopolymer scaffolds are utilized to retain siRNA locally and release it in a sustained manner to prolong the effect directly at the site of interest. Three different biodegradable, injectable hydrogel systems are shown to be capable of localized, sustained delivery of siRNA: calcium-crosslinked alginate, photocrosslinked alginate, and collagen (Figure 7.1). The released siRNA remained bioactive and able to silence protein expression in cells surrounding the hydrogels. Cells incorporated into these hydrogels with the siRNA also exhibited sustained gene knockdown within this three-dimensional microenvironment. This promising new class of injectable biopolymer-based siRNA delivery systems capable of achieving localized and sustained gene silencing to both host and transplanted cells may have great utility in tissue engineering and therapeutic medicine. This was the first report of injectable, biodegradable biopolymer constructs for the controlled delivery of siRNA, permitting long term inhibition of gene expression at a specific site.\textsuperscript{14}
The use of siRNA in tissue engineering applications is an intriguing option, as it may be beneficial to silence the expression of genes that hinder a regenerative process. Additionally, it may be useful in guiding the differentiation of progenitor cells (either host or transplanted cells present in the defect) down a desired pathway by silencing the expression of genes that normally suppress this differentiation. For instance, recently, a high-throughput siRNA screen identified at least 12 genes in MSCs that when silenced, induce osteogenic differentiation of the cells. These genes encoded for molecules that were cell fate-specific, and also for other molecules, for example those involved in self-renewal. One of the genes identified in this screen, GNAS, has been found to cause skeletal abnormalities when mutated in humans and mice. In mesenchymal stem cells, it has been found that knockdown of GNAS expression leads to increased osteogenic differentiation. Core binding factor alpha 1 (Cbfa1) is a transcription factor that regulates differentiation of MSCs into osteoblasts by regulating the expression of osteocalcin. If Cbfa1 is deleted in mice, the resulting embryos exhibit a lack of calcification in the body and are much smaller than wild type embryos. Increased GNAS expression, which leads to increased adenyl cyclase and cAMP, has been shown to decrease Cbfa1 by means of proteosomal degradation. Therefore, silencing the expression of GNAS will increase Cbfa1, and thereby stimulate MSCs to differentiate into osteoblasts. Additionally, two other genes identified in this screen as osteogenic suppressors were adenosine kinase (ADK) and a purinergic receptor 2, G-protein coupled, 11 (P2RY11) which are both involved in cAMP production. As the silencing of these molecules has been shown to increase osteogenic differentiation, it would seem that decreasing intracellular cAMP levels promotes osteogenesis. Also, ATP can bind to
purinergic receptors expressed by osteoblastic cells.\textsuperscript{25} ATP has been shown to inhibit bone formation by osteoblasts, and to stimulate bone resorption by osteoclasts.\textsuperscript{26,27} Therefore, it is possible that by silencing purinergic receptor expression, less ATP is bound by the cells. This reduction in ATP may contribute towards increased osteogenic response of MSCs.

Other studies have indicated that BMP antagonists can inhibit bone formation. As discussed in earlier chapters, the administration of BMP-2 to promote osteogensis offers great promise that has yet to be fully realized. It has been shown that BMP-2 may not have a strong osteoinductive effect on human MSCs.\textsuperscript{28} Although it has been suggested that humans may require higher concentrations of BMP or improved delivery systems for the molecule to have a stronger osteogenic effect, it could also be possible that by silencing its inhibitors the effect of BMP could be strengthened. By engineering the cells on a biochemical level, it could be possible to make them more receptive to BMP-2. For instance, noggin, a secreted protein, has been shown to have a negative effect on osteogenesis, as it inhibits BMP activity and thereby reduces bone formation.\textsuperscript{29-31} It has been found that increasing BMP in an osteoblastic culture will lead to an increase in cellular expression of noggin, which presumably provides the cells with a regulatory method to avoid overexposure to BMP.\textsuperscript{32} By suppressing noggin, cultures of osteoblastic cells show increased osteogenic gene expression profiles and increased calcified matrix deposition.\textsuperscript{29} It is possible that by silencing noggin in a population of MSCs, their differentiation down the osteogenic lineage would be encouraged. Similarly, another group has found that silencing chordin, another BMP-antagonist, showed increased osteogenic response of human MSCs.\textsuperscript{33} In this chapter the silencing of several of these
genes are examined to determine their effect on the osteogenesis of human MSCs. Once a candidate target gene has been identified that increases the osteogenic response of hMSCs, siRNA complementary to this gene can be delivered from the biopolymer hydrogels to promote bone tissue formation over time in vivo. Furthermore, a population of osteogenic cells could be incorporated into the siRNA-laden hydrogel to provide an additional source of cells to the defect site to aid in the tissue regeneration.

7.2 Materials and Methods

Materials

Sodium alginate powder with product name of 20/40 and chitosan (Novafect O25) were generous gifts from FMC Biopolymers (Princeton, NJ). PureCol collagen (97% type I collagen) was obtained from Inamed Biomaterials (Fremont, CA). Polyethylenimine (PEI) “Max” was obtained from Polysciences, Inc. (Warrington, PA). The RiboGreen RNA quantitation reagent, Trizol, and the PCR primers were obtained from Invitrogen (Carlsbad, CA). The HEK293 cells stably transfected with destabilized GFP (deGFP) were a generous gift from Piruz Nahreini, Ph.D. (University of Colorado Health Sciences Center). Nuclease-free water was obtained from Ambion (Austin, TX). Phosphate buffered saline (PBS), fetal bovine serum (FBS), 0.05% trypsin-EDTA, and α-MEM were obtained from Hyclone (Logan, UT). The 5X siRNA buffer, Accell siRNA, and Accell delivery (serum-free) medium (ADM) were obtained from Thermo Scientific Dharmacon (Lafayette, CO). The sequences for the Accell siRNAs are listed in Table 7.1. The QuantiTect Reverse Transcription kit was obtained from Qiagen (Valencia, CA). SYBR-Green PCR Master Mix was from Applied Biosystems (Foster City, CA).
Human BMP-2 was obtained from GenScript (Piscataway, NJ). The transwell membranes were obtained from Corning (Corning, NY). The 2-aminoethyl methacrylate (AEMA), 2-morpholinoethane sulfonic acid (MES), N-hydroxy-succinimide (NHS), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC), Dulbecco’s Modified Eagle’s Medium with 4.5 g/L glucose (DMEM-HG), dexamethasone, and alkaline phosphatase yellow pNPP liquid substrate were obtained from Sigma (St. Louis, MO). All other chemicals and supplies were obtained from Fisher Scientific (Fairlawn, NJ).

<table>
<thead>
<tr>
<th>Gene</th>
<th>siRNA Target Sequence</th>
</tr>
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<tr>
<td>eGFP</td>
<td>GCCACAACGUCUAUAUAUAUCAU</td>
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<tr>
<td>Human GNAS (pool of 4)</td>
<td>CCAUAGGGCAUGAUUAAACA CCAUGCAUGUAAUGGCUUU GUGUUGUGCAGCAUUAAA GCAUGCACCUGCAGUAA</td>
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<td>Human ADK (pool of 4)</td>
<td>GCAUUGGGGAUGAUAAUAUU GUGCCAAAUUUAGUGUAA GAAGCAAGCAGCAUAUAUUAUGAUGGAGCAAAAGAUU</td>
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<tr>
<td>Human P2RY11 (pool of 4)</td>
<td>GUGGCCAGGAUAAAGACUUC CGACAAACUCAGUGGGUUG GCUGUAUCUUCGAGCAGCUG CCAUACUGUGGUUGUUGU</td>
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<tr>
<td>Human chordin</td>
<td>CUAUCAGGGCGAAGGUGUA</td>
</tr>
<tr>
<td>Human noggin (pool of 2)</td>
<td>CCAUCAAUUCCUGAGUGACA UGGUGGACCUCAuxGAAC</td>
</tr>
<tr>
<td>Mouse noggin</td>
<td>GUUAUAAGAGUUCAUGUAUA</td>
</tr>
</tbody>
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**Table 7.1** List of siRNA sequences.
**Alginate Preparation**

The 20/40 sodium alginate powder was lyophilized until dry, and a portion of it was then subjected to 5 MRad gamma irradiation (Phoenix Lab, University of Michigan, Ann Arbor). The molecular weight was 37,000 g/mol for irradiated 20/40 alginate, and 196,000 g/mol for non-irradiated 20/40 alginate as determined by SEC-MALS (FMC Biopolymers). The percentage of guluronic (G) and mannnuronic (M) acid was determined by NMR to be 66% G-content and 34% M-content (FMC Biopolymer). The peptide glycine – arginine – glycine – aspartic acid – serine – proline (GRGDSP; Commonwealth Biotechnologies, Richmond, VA), which contains the RGD cell-binding domain, was covalently coupled to the alginates as previously described.\textsuperscript{34} Both types of alginate were then purified by dialysis for 4 days, subjected to activated charcoal treatment, and sterilized through a 0.22 µm filter. Prior to crosslinking, this alginate was dissolved at 2% w/v in nuclease-free PBS.

The photocrosslinkable alginate was prepared from the 20/40 5 MRad alginate as previously described, at a theoretical methacrylation of 45%.\textsuperscript{35} Prior to crosslinking, this alginate was dissolved at 2% w/v in nuclease-free water containing 0.05% w/v photoinitiator (Irgacure D-2959, Sigma).

**Transfection of HEK293 cells with siRNA**

HEK293 cells stably transfected with deGFP, cultured in DMEM-HG + 10% FBS, were plated in 24-well plates at a density of 7.9 x 10\textsuperscript{4} cells per cm\textsuperscript{2}. After overnight incubation, the media was removed and replaced with ADM containing eGFP siRNA at concentrations of 0, 0.1, 0.5, and 1 µM. At various timepoints the cells were harvested
from plates by trypsinization, resuspended in PBS, and assayed by flow cytometry (EPICS XL-MCL, Beckman Coulter, Fullerton, CA) to determine the percentage of the cell population expressing GFP.

*Release and bioactivity of siRNA from hydrogels*

Hydrogels containing 13.3 µg (10 µl) of siRNA were fabricated in transwell membranes with 0.4 µm pore size. First, the siRNA was mixed into 90 µl of 2% alginate or 3 mg/ml collagen solutions. The collagen solution was kept on ice during this process. For alginate mixed with chitosan or PEI, 45 µl of the 2% alginate solution was mixed with 45 µl of 2 mg/ml chitosan or 10 mM PEI solution. These 100 µl mixtures were then pipetted onto transwell membranes. To crosslink, the ionically-crosslinked alginate was placed into a solution of 100 mM CaCl₂ for 5 minutes (minimal siRNA was lost during this process, as there was no measurable amount of RNA in the calcium crosslinking solution using the RiboGreen RNA quantitation reagent). The photocrosslinkable alginate solution was exposed to 365 nm UV light at ~8-20 mW/cm² for 10 minutes. The collagen solution was placed into a 37 °C incubator for 45 minutes to allow hydrogel formation. For release studies, the transwell membranes were placed into the wells of a 24-well plate containing PBS, the PBS was replaced at various time points, and the siRNA content in each sample was measured using the RiboGreen RNA quantitation reagent. For bioactivity studies, HEK293 cells were seeded in 24-well plates at 150,000 cells per well in DMEM-HG + 10% FBS. The next day, the media was changed to ADM, the hydrogels were fabricated and crosslinked as described above, and then transferred into the wells with plated HEK293 cells. The cells were harvested 3 or 6 days
later and assayed for GFP expression by flow cytometry (EPICS XL-MCL, Beckman Coulter). The medium in the day 6 samples was replaced at day 3 with ADM + 1% fetal bovine serum.

Transfection of HEK293 cells incorporated within hydrogels

HEK293 cells were mixed into the photocrosslinkable alginate or collagen solutions at a density of 20 x 10^6 cells/ml, and then crosslinked in transwell membranes as before. One third of the hydrogels were not exposed to siRNA, one third had exogenous exposure to 13.3 µg siRNA added with the ADM in the well of the 24-well plate, and one third had 13.3 µg siRNA mixed into the hydrogel solutions with the cells prior to crosslinking. The hydrogels on the transwell membranes were cultured in the presence of ADM which was replaced with ADM supplemented with 1% FBS on days 1, 3, and 5. The cells within the hydrogels were imaged using a confocal microscope (LSM510, Zeiss) on days 3 and 6. Images were taken every 2 µm in the z-direction for 104 µm from the transwell membrane surface and compiled into a single 3-D projection.

hMSC isolation, preparation, siRNA transfection, and culturing

Human bone marrow aspirates were harvested from the posterior iliac crest of a healthy human volunteer donor after informed consent under a protocol approved by the University Hospitals of Cleveland Institutional Review Board. hMSCs were isolated from the bone marrow aspirates and cultured as previously described. All hMSCs used in these experiments were passage 2 cells. The day before transfection, all cells were seeded in 24-well plates in DMEM-LG + 10% FBS at a density of 2.6 x 10^4
cells/cm². The cells were then treated with siRNA at a concentration of 500 nM the following day, and subsequently cultured in various media for each siRNA transfection experiment.

For the cells treated only one time with siRNA, the media for all wells was replaced with the Accell Delivery Media (serum-free) with or without the appropriate siRNA for 3 days. Following this 3 day incubation, the media was then changed to serum-containing media with or without osteogenic supplements. For these experiments, the basic control medium consisted of DMEM-LG + 10% FBS. Supplements were added as indicated, at the following concentrations: 120 µM ascorbic acid-2 phosphate, 2 mM b-glycerophosphate, 10⁻⁷ M dexamethasone, and 100 ng/ml BMP-2. The adipogenic media consisted of DMEM-LG + 10% FBS with 1 µM dexamethasone, 100 µM indomethacin, 500 µM 3-isobutyl-1-methylxanthine, and 10 µg/ml insulin. In all cases, the media was changed every third day.

For the cells treated multiple times with siRNA, they were cultured in DMEM-LG + 2% FBS with or without the various osteogenic supplements and with or without siRNA. The media was changed every third day.

Alkaline phosphatase activity assay

The activity of ALP was measured based on the conversion of p-nitrophenyl phosphate to p-nitrophenol. Cells cultured in monolayer were harvested by removing spent media, rinsing each well with 250 µl PBS, then detaching the cells from the plate by trypsinization, and subsequently resuspending the cells in 500 µl PBS. The cells were
subjected to three freeze-thaw cycles (-20°C until frozen, then room temperature until completely thawed). The lysed cells were then tested for alkaline phosphatase activity by mixing 100 µl of lysed cells with 100 µl of the alkaline phosphatase substrate in a 96-well plate and incubating at 37°C for 30 minutes. The reaction was stopped by the addition of 50 µl of 0.1 N NaOH, and the absorbance at 410 nm read using a plate reader (Tecan, Durham, NC).

Reverse transcription and quantitative Real Time Polymerase Chain Reaction (qRT-PCR)

Cells were first rinsed in PBS, and then lysed by the addition of 200 µl Trizol per well. The lysed cells were transferred to an RNase-free microcentrifuge tube and stored at -80°C for less than 30 days prior to isolating the RNA. The manufacturer protocol was then followed for isolating the RNA; briefly, 1-bromo-3-chloropropane was added to the Trizol/cell mixture to enable phase separation. The RNA from the aqueous phase was then precipitated with isopropanol, washed with 70% ethanol, dried, and resuspended in nuclease-free water. The RNA concentration was measured at 260 nm wavelength on a Nanodrop spectrophotometer (Nanodrop, Wilmington, DE). Any contaminating genomic DNA was removed and the RNA was transcribed to cDNA using the QuantiTect Reverse Transcription Kit as per the manufacturer’s instructions.

qRT-PCR was performed to determine the expression levels of chordin, noggin, Runx2, and alkaline phosphatase. The housekeeping gene glyceraldehydes 3-phosphate dehydrogenase (GAPDH) was used as an internal control. PCR was performed using SYBR green master mix. The primer sequences are listed below in Table 7.2.
amplification was performed using an ABI 7700 system (Applied Biosystems) as follows: 50°C for 2 min, 95°C for 10 min, then 40 cycles of 95°C for 15 sec, 60°C for 1 min, and 95°C for 15 sec. A denaturation curve was run consisting of 60°C for 20 sec followed by a slow ramp to 95°C for 15 sec.

<table>
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<th>Gene Name</th>
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<td>GAPDH reverse</td>
<td>GGCTGCGCCATTGCCCTCAA</td>
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<td>Runx2 forward</td>
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<td>CAGCGACACGACACTGCCCCTGG</td>
</tr>
<tr>
<td>Noggin reverse</td>
<td>AGCGCTGGTGCAGGTGGC</td>
</tr>
<tr>
<td>Chordin forward</td>
<td>GCCAGCTCAGCCTGCTAGGG</td>
</tr>
<tr>
<td>Chordin reverse</td>
<td>CAGTCGCGCTGATCCCTCCGC</td>
</tr>
</tbody>
</table>

Table 7.2 Primers used for qRT-PCR.

Calcium Assay using Alizarin Red-S

At the end of each experiment, cells were rinsed with PBS and then fixed in ice cold 70% ethanol for 1 hr. They were then rinsed with deionized water (diH₂O) and stained for 10 min with 40 mM Alizarin Red-S (AR-S), pH 4.2, at room temperature with gentle rocking. The cells were then rinsed two times with diH₂O, followed by a 15 min rinse in PBS. The stained cultures were then photographed. Next, they were destained in
10% w/v cetylpyridinium chloride (CPC) in 10 mM sodium phosphate, pH 7.0, for 15 min at room temperature. The absorbance at 562 nm of these AR-S extracts was determined using a plate reader.}

Oil Red O Staining

Oil Red O solution was prepared by dissolving 2.1 g Oil Red O in 600 ml absolute isopropanol without stirring overnight at room temperature, filtering through 0.22 µm filter, then adding 450 ml diH2O. The solution was left at 4°C overnight, then filtered twice more the following day. To stain for adipogenesis, cultures were fixed for 1 hr in 10% formaldehyde in PBS, washed with diH2O, then stained for 2 hr in the Oil Red O solution. They were then rinsed with PBS three times. Photographs of the stained cells were taken, and the amount of Oil Red O staining was then quantified by extraction in isopropanol and measurement of the absorbance at 510 nm using a plate reader.

7.3 Results

GFP Silencing Experiments

We utilized a reporter gene stably transfected in HEK293 cells, a rapidly proliferating cell line. The reporter gene is destabilized GFP (deGFP) which has a half-life of about 2 hours, compared to enhanced GFP (eGFP) which has a half-life of about 24 hours. As many proteins in the dynamic in vivo environment do not exhibit long half lives, the choice of deGFP as a “proof of principle” target gene representative of
potential knockdown when silencing a gene for a therapeutic application. When the siRNA was applied exogenously in tissue culture media to these stably transfected HEK293 cells in monolayer at three different concentrations of siRNA, deGFP expression is significantly decreased at 24 and 48 hours post-transfection (Figure 7.2). After 48 hours, the expression of deGFP has been silenced almost entirely at higher concentrations of siRNA. This shows the powerful ability of siRNA to inhibit protein expression, and verifies the use of deGFP as a reporter gene to assay knockdown in this timeframe with this cell line.

The duration of silencing in these cells was next examined. When treated with the highest concentration of siRNA (1 µM), the GFP expression was still silenced at 3 days, but by 6 days had started to increase and by 10 days post-transfection was at the same GFP expression level as the control (untreated) cells (Figure 7.3a). If a population of HEK293 cells was treated once with 1 µM siRNA, again it can be seen that the expression has started to return 6 days post-transfection (Figure 7.3b). If, however, the cells are supplied with siRNA both on day 0 and day 3, the expression of GFP is still silenced 6 days post-transfection (Figure 7.3b), indicating that repeated exposure of the cells to the siRNA is necessary to maintain the gene silencing effect.

The siRNA was uniformly distributed and encapsulated into three biopolymer hydrogel systems for sustained release: calcium-crosslinked alginate, photocrosslinked alginate, and collagen. While keeping the total amount of siRNA incorporated into each hydrogel constant, release from all three systems was quantified over the course of two weeks. Each material exhibited a different release profile (Figure 7.4a,b). siRNA was released in a sustained manner for approximately one week from the alginates and for
approximately two weeks from the collagen. The photocrosslinked alginate released the highest total amount of siRNA, followed by the calcium-crosslinked alginate, then the collagen. Alginate is a negatively charged polysaccharide, so the possibility of retaining more siRNA (also negatively charged) within the hydrogel and delaying its release by the addition of positively charged polymers was examined. Indeed, the addition of either chitosan or polyethylenimine (PEI) to the calcium-crosslinked alginate hydrogels delayed the release of the siRNA into the surrounding media (Figure 7.4a) likely due to electrostatic interactions between the nucleotides and positively charged polymers.

The released siRNA was tested for bioactivity to ensure its incorporation and subsequent release from these hydrogels at 37°C, and its prior exposure to UV in the photocrosslinked hydrogels, did not affect its ability to silence protein expression. HEK293 cells cultured in monolayer were exposed to siRNA released from hydrogels in transwell membranes over them. The GFP expression of these cells was measured after 3 and 6 days of culturing in the presence of calcium-crosslinked alginate, photocrosslinked alginate, and collagen hydrogels containing siRNA. All three materials delivered siRNA sufficient to silence GFP expression to less than 20% of the no treatment control samples (Figure 7.4c). Further, the effect was more pronounced at day 6 compared to day 3. To ensure bioactivity of the siRNA, cells were cultured in serum-free media for 3 days followed by culture in 1% serum for the next 3 days, and by day 6 the cells started to show some effects of serum deprivation. Notably, the no treatment controls have a large standard deviation in their level of GFP expression, as some cells seemed to be shutting down production of the GFP in the absence of serum, as has been previously documented. However, even when normalized to the no treatment controls, the gene
knockdown is significant in the treated samples, indicating that the serum deprivation has a minimal effect on GFP knockdown compared to the delivered siRNA. The GFP silencing of these cells due to the RNA interference is clearly seen in a histogram from representative samples measured at day 3 using flow cytometry (Figure 7.4d).

The ability of cells incorporated within the hydrogels to uptake siRNA incorporated within the same hydrogels was then examined. Two materials with very different siRNA release profiles were used: photocrosslinked alginate and collagen. The HEK293 cells were incorporated into the hydrogels, and three conditions were examined: (1) cells in the hydrogels without siRNA; (2) cells in siRNA-containing hydrogels; and (3) cells in hydrogels without siRNA incorporated, but that were exposed to an equal amount of siRNA supplied exogenously in the surrounding media for the first 24 hours only. The cells incorporated into the hydrogels without siRNA exhibited strong GFP expression after both 3 and 6 days in culture (Figure 7.5).

In contrast, cells that were co-incorporated with siRNA in the hydrogels showed significant knockdown at day 3. By day 6, the GFP expression had increased in the cell population within the photocrosslinked alginate. However, the cells in the collagen continued to display substantial GFP knockdown at day 6 compared to both the control samples and the samples supplied with exogenous siRNA. This correlates well with the release profiles of siRNA from these hydrogels, as the collagen exhibited more sustained release compared to the photocrosslinked alginate.

It is important to consider the effect of exogenously supplied siRNA at the same total amount as incorporated into the hydrogels, but only for the first 24 hours. This
would mimic the effect of a bolus delivery of siRNA to a site, where the entire amount of siRNA is injected at a single timepoint. The cells encapsulated within the photocrosslinked alginate were unaffected by the exogenous supplementation of siRNA, as the GFP expression appeared similar to the controls. In contrast, the cells within collagen did exhibit GFP silencing at day 3, although not to as great of an extent as those exposed to the hydrogel-incorporated siRNA. However, the silencing from exogenous supplementation did not last until day 6, where the GFP expression returned to levels as strong as the no treatment control cells. This demonstrates the importance of sustained delivery of siRNA as opposed to a single dosage at one timepoint.

**hMSC siRNA Experiments**

The osteogenic differentiation of hMSCs when exposed to various siRNA molecules was examined. Based on the work by Zhao and Ding, three target genes were selected to be silenced by complementary siRNAs as they showed significant promise for increasing the osteogenic potential of hMSCs: GNAS, adenosine kinase (ADK), and a purinergic receptor, P2RY11. The alkaline phosphatase (ALP) activity of these cells was used as a measure of the extent of osteogenic differentiation 6 and 12 days post-transfection. The osteogenic-control cells (labeled Dex) and all of the siRNA-treated cells which were cultured in the same osteogenic media exhibit increased ALP activity from day 6 to day 12 (Figure 7.6). However, there was no statistically significant difference between the siRNA-treated samples and those grown in the osteogenic media
without siRNA, indicating that the siRNA had little effect on the ALP activity of these cells.

As these three genes did not seem to have a significant effect in this experiment, the silencing of BMP antagonists was next examined, as this seemed a very promising route to increase the osteogenic potential of hMSCs. First, siRNA against noggin was used to treat the hMSCs that were grown in four types of media: control (DMEM + 10% FBS), Dex (control medium with the addition of ascorbic acid, β-glycerophosphate, and dexamethasone), BMP-2 (control medium with the addition of ascorbic acid, β-glycerophosphate, and BMP-2), or BMP-2 + Dex (control medium with the addition of ascorbic acid, β-glycerophosphate, BMP-2, and dexamethasone). Again, the alkaline phosphatase activity was used as a measure of osteogenic differentiation, and was examined at 1, 2, and 3 weeks post-transfection. Interestingly, the ALP activity was extremely high at 3 weeks in the BMP-2 + Dex groups, both with and without siRNA (Figure 7.7a). When examining the data only from weeks 1 and 2, it is apparent that the ALP activity did increase from week 1 to week 2 in all conditions except the cells grown in the control media, and that the BMP-2 + Dex groups showed substantially increased ALP activity at week 2 compared to any of the other groups (Figure 7.7b). Regardless, there was no significant difference at any of the time points in any of the samples grown in the same type of media whether they were treated with the siRNA against noggin or not. Thus, the single treatment of si-noggin did not have a substantial effect on the ALP activity of these cells. As another marker of osteogenic differentiation, the calcium deposition of these cells was also examined. Again it was apparent that culturing the cells in osteogenic media increased their osteogenic differentiation as demonstrated by
the increased calcium deposition of these cultures compared to the control ones (Figure 7.8). However, the si-noggin had no significant effect on the amount of calcium that these cells deposited in the matrix around them.

Interestingly, it was observed that the cells exposed to BMP-2 seemed to be undergoing adipogenesis to some extent in addition to their osteogenic differentiation, as they appeared to contain lipid vacuoles when examined using light microscopy. To quantify the extent of adipogenesis and to determine if the si-noggin was having an impact on this process, hMSCs were again grown using the same conditions, with the addition of cultures grown in standard adipogenic medium with or without the si-noggin. Three weeks post-transfection the cultures were stained with Oil Red O, which was then extracted and measured to quantitatively determine the extent of adipogenesis of the cultures. The cells grown in adipogenic media with or without siRNA and with or without BMP-2 showed increased Oil Red O staining, as expected (Figure 7.9). Additionally, the Dex + BMP-2 groups showed higher degrees of adipocyte staining than the cultures treated with either Dex or BMP-2. Again, however, it was found that treatment with the si-noggin did not impact the extent of adipogenesis of these cells in any of the media conditions examined.

Overall, the treatment of hMSCs with a single dose of siRNA does not appear to impact their osteogenic differentiation as was expected. Thus, hMSCs were next treated with siRNA throughout the course of the experiments to ensure that the gene remained silenced during the entire 2 or 3 week culture periods. Furthermore, in addition to examining si-noggin, siRNA against chordin was also examined to determine if one of these BMP antagonists had a stronger differentiation effect than the other. Quantitative
real-time PCR was used to measure the expression of ALP and Runx2, a key transcription factor for osteogenic differentiation. The cells treated with BMP-2 with or without siRNA exhibited higher levels of ALP expression than those treated with Dex or the control media (Figure 7.10a). Overall the si-noggin and si-chordin do not have a significant effect on the ALP expression except in the case of cells grown in the BMP-2 + Dex media that were treated with either si-noggin or both si-noggin and si-chordin. In this case, the siRNA treated cells exhibit significantly decreased ALP expression. When examining the Runx2 expression, it is apparent that the hMSCs grown in the media containing Dex and/or BMP-2 exhibit increased Runx2 expression, indicating increased osteogenic differentiation compared to the control cells (Figure 7.10b). However, again it is found that the siRNA has little effect on this expression, with the exception of the si-noggin in the BMP-2 media which shows significantly decreased Runx2 expression compared to the BMP-2 cells not treated with siRNA.

The calcium deposition of these cells was again examined. Similar to the ALP expression, it was observed that only the si-noggin and si-noggin + si-chordin delivered to the cells cultured in BMP-2 + Dex had any significant effect on the calcium deposition, and the siRNA treated cells were found to deposit less calcium than the non-treated cells (Figure 7.11).

To ensure that the siRNA was indeed silencing the expression of the targeted genes, qRT-PCR was used to measure the level of expression of noggin and chordin. In all types of media, the si-chordin does indeed knockdown the expression of chordin in these cells (Figure 7.12a). However, the si-noggin only significantly decreased the noggin expression of the cells grown in the BMP-2 + Dex media, although a trend of
decreased noggin expression in the Dex or BMP-2 media conditions is observed (Figure 7.12b). Using these qRT-PCR results to compare the relative expression levels of chordin and noggin in these cells, it was also found that the cells express significantly higher levels of noggin than chordin (data not shown).

Finally, as there is substantial evidence from the literature that mouse osteoblastic cells can be influenced by the expression or silencing of BMP antagonists, especially noggin, we examined the effect of si-noggin on MC3T3 pre-osteoblastic mouse cells. These cells are able to undergo osteogenic differentiation when cultured in their standard medium, α-MEM, as it contains enough ascorbic acid to signal their differentiation. This base “control” medium was compared to that supplemented with Dex and also with Dex + BMP-2. As an initial trial, for all three media types the cells were treated with siRNA at the beginning of the experiment and then not again for the duration, as several published articles using murine cells treated with siRNA only once. The alkaline phosphatase activity was used as a measure of osteogenic differentiation at 2 and 3 weeks post-transfection. The dexamethasone also did not have a significant effect on the ALP activity, but the BMP-2 + Dex was found to increase the ALP activity substantially (Figure 7.13). Again, in all conditions the siRNA did not have a significant effect on the osteogenic activity of these cells.

7.4 Discussion

To date, the development of delivery vehicles for siRNA for therapeutic or tissue regeneration purposes has been focused primarily on nanoparticles which can either be
injected into the site of interest or delivered systemically. Certainly there is promise in the use of these nanoparticles carriers for siRNA applications, but unfortunately one major disadvantage of these systems is that they can be easily dispersed away from the site of interest, or in the case of systemic delivery very few particles may actually make it to the site. This work sought to overcome these drawbacks with the development of macroscopic biopolymer hydrogels that could be injected into the site of interest and which would allow siRNA to be delivered to the surrounding cells based on diffusion and degradation release mechanisms. Three biopolymers were examined for this application: calcium crosslinked alginate, photocrosslinked alginate, and collagen.

All three systems are crosslinked to form hydrogels by different mechanisms. Alginate is a polysaccharide composed of $\alpha$-L-guluronic acid and $\beta$-D-mannuronic acid units; the guluronic acid residues can be crosslinked with divalent cations such as calcium. This method for crosslinking is gentle for cells, and is reversible in the presence of calcium chelators. However, it can be difficult to modulate the mechanical properties, swelling, and degradation of these hydrogels. To enhance control over the aforementioned properties, our group recently engineered a photocrosslinkable alginate system that exhibits more controlled degradation by hydrolysis of ester linkages. The photocrosslinking occurs following brief exposure to UV light in the presence of a photoinitiator, which has been shown to have minimal effect on the viability of cells. The third natural biopolymer examined was a commercially available acid-solubilized collagen, which is a liquid at 4°C and forms a hydrogel at 37°C after adjustment to neutral pH. Collagen is a native extracellular matrix molecule to which cells can
naturally adhere, and the gentle gelling process allows cells and bioactive factors to be easily incorporated.

One strong advantage to the use of hydrogels as biopolymer scaffolds is that they are injectable, so delivery to the site of interest is minimally invasive. Additionally, due to their hydrophilic nature and high gas permeability, which permits easy transport of nutrients and oxygen and removal of waste products, it is possible to incorporate cells within the scaffold to provide an additional population of transplanted cells to increase the therapeutic effect, especially for applications in regenerative medicine. While delivery of genetic material to transplanted cells for upregulation of specific genes has been shown to enhance the quality and rate of tissue formation in tissue engineering strategies, downregulation of gene expression at the post-transcriptional level via siRNA delivery may also be a powerful approach to regulate the function of both mature cells and stem cells and ultimately enhance the formation of new tissue.

We have demonstrated the ability to deliver siRNA in a sustained manner from several biodegradable hydrogel systems to both incorporated and surrounding cells. The choice of biopolymer hydrogel strongly influences the delivery rate of the siRNA. The mechanism of siRNA release over the time period examined is likely a combination of diffusion through the biopolymer pores as well as biopolymer degradation. The siRNA is released from both types of alginate to a greater degree than from the collagen. This is likely due to electrostatic affinity of the siRNA to the collagen, whereas both the siRNA and the alginate are negatively charged and would not have any such interaction. In all cases, the released siRNA remains highly bioactive and cells exposed to these gels exhibit significant gene silencing even after 6 days of release. All cell populations
exposed to siRNA released from any of the hydrogels exhibit decreased fluorescence as compared to the cells not treated with siRNA. This demonstrates that the released siRNA retains its bioactivity, and that the effect is sustained, as it is still strong even at 6 days after starting the release. Moreover, the siRNA incorporated into these hydrogels is also available for uptake by cells that are co-encapsulated in the hydrogels. Although all three systems were able to silence gene expression from released siRNA at least 6 days after fabrication, the cells incorporated into the photocrosslinked alginate showed GFP knockdown only at day 3, in contrast to the collagen hydrogels which still exhibited silencing at day 6. This indicates that the appropriate hydrogel system can be selected based on the desired effect to transplanted cells and surrounding host cells. Since the siRNA diffuses out from the photocrosslinked alginate hydrogels more rapidly, it is not available later for the encapsulated cells. However, this material would offer a much more potent effect to the surrounding host cells, as a higher fraction of incorporated siRNA was ultimately released as compared to the collagen. In contrast, the collagen hydrogel would allow for a more sustained effect on both surrounding host cells and transplanted cells, although a lower fraction of incorporated siRNA would be released to the surrounding host cells in the short-term. The specific application for which RNA interference will be used should be examined to determine the most beneficial delivery regimen.

As the focus of this work is the promotion of bone regeneration, the use of RNAi to influence the differentiation of mesenchymal stem cells down the osteogenic lineage was examined. Human mesenchymal stem cells were used as they are a likely cell source to be used clinically. Initially, the silencing of three genes (GNAS, ADK, and P2RY11)
that have previously been suggested to increase the osteogenic potential of hMSCs was examined. None of these were found to have a significant effect on alkaline phosphatase activity in cells grown in osteogenic media. Thus, these results did not replicate those previously reported,\textsuperscript{16} and the inhibition of other molecules that could affect osteogenesis was examined. As the BMPs are widely researched for bone regeneration, and as the presence of BMP antagonists that can impede this process is also widely reported upon, the effect of silencing noggin or chordin was examined in hMSCs. Surprisingly, the silencing of either molecule had very little effect on the osteogenic differentiation of hMSCs grown in any type of osteogenic media. In some cases, the osteogenic response of these cells was even decreased when treated with the siRNA. There are several possibilities for these findings. First, donor-to-donor variation in human MSCs may be an issue, where some donors are naturally more osteogenic or more responsive to certain molecules than others. So perhaps if enough donors were examined in all of these conditions, it would be possible to find a trend of which would be responsive to this treatment compared to those that would not. However, given that several donors were examined throughout these various studies, this explanation seems less likely. Also, even in the case that only a select few donors were able to be influenced by this treatment, the treatment would not be widely useful for clinical practice.

Another possibility for these findings is that human MSCs may not be as responsive to BMPs as MSCs from other species are. Many of the studies examining noggin are conducted with cells from mice or rats.\textsuperscript{29,31,32,44} Perhaps human MSCs are less responsive to noggin than these rodent cells are, or perhaps they are expressing other molecules that influence their differentiation aside from noggin. Also, there are relatively
few studies examining chordin; one is in chick chondrocytes\textsuperscript{45} and another is with human MSCs.\textsuperscript{33} Although the one study was using hMSCs, the results were not replicated here. As only one donor was tested with si-chordin thus far, additional experiments will be conducted to examine the silencing of chordin in hMSCs from other donors. It may also be the case that hMSCs really are not as responsive to the silencing of chordin.

Finally, another potential explanation for all of these findings is that the siRNA actually was not substantially inhibiting the targeted gene expression, or that a low-level expression still present despite the knockdown may be sufficient for determining cell fate. Using qRT-PCR, the expression of chordin was demonstrated to be silenced in hMSCs, but the expression of noggin less so. Additionally, the expression of noggin exceeded that of chordin for hMSCs grown under any of the different media conditions. Thus, it is possible that the expression of noggin in these hMSCs is so high that the amount of siRNA supplied is not sufficient to silence the expression significantly. Alternatively, it may be important to use different si-noggin sequences which may prove to be more effective. Furthermore, this finding raises the question of whether silencing the expression of a single BMP antagonist would ever be sufficient to influence the osteogenic differentiation of hMSCs, given that they are expressing more than one BMP antagonist at any given time. Perhaps the treatment of hMSCs with siRNA molecules targeted against multiple BMP antagonists would produce a stronger result. This will continue to be explored in future studies.
7.5 Conclusions

This new delivery method for siRNA offers the ability to achieve sustained release locally at the site of interest. The hydrogels are injectable and therefore can be administered in a minimally invasive manner. Furthermore, a population of cells can be incorporated within these hydrogels, allowing dual use for siRNA delivery and cell transplantation. This represents an exciting new opportunity for further development in RNAi therapeutics.

The use of RNAi to influence osteogenic differentiation of hMSCs is challenging and still requires much research to identify molecules that should be targeted. Further, the culture conditions of the hMSCs have been shown to influence their expression level of osteogenic markers, and therefore the culture conditions will also play an important role in the effects of RNAi. The complexities of stem cell growth and differentiation require further examination in order to effectively use RNAi therapeutics for regenerative medicine someday.

Future studies will include examining the effects of silencing BMP antagonists in several different donors to determine the variation that may exist from donor to donor. Additionally, these results should be compared to experiments conducted using rodent cells, as there may be a large difference in response between species, which would be important to elucidate. It is also going to be imperative to determine whether these cells experience any off-target effects from the siRNA. This can be examined by several methods such as comparing the silencing effect using siRNA sequences that are complementary to different portions of the target gene, comparing siRNA knockdown to inhibition of protein activity when an antibody against that protein is supplied, or even
using gene arrays to see the various genes that may be affected within the treated cell population.
Figure 7.1 Schematic of hydrogel formation for delivery of siRNA and subsequent inhibition of gene expression in incorporated and neighboring cells. Biomaterial solutions of alginate, photo alginate, or collagen are mixed with siRNA and GFP-positive cells, and hydrogels are then formed by ionic crosslinking, photocrosslinking, or thermogelling, respectively. The siRNA diffuses through the hydrogel to affect incorporated cells, and it also released from the hydrogel to locally affect surrounding cells that are part of the host tissue.
Figure 7.2 HEK293 cells treated with siRNA. (a,b) Fluorescent photomicrographs 48 hours post-treatment. (a) No siRNA. (b) 1 mM siRNA. Scale bars represent 100 µm. (c) Quantification by flow cytometry analysis of GFP expression at 24 and 48 hours post-transfection with three concentrations of siRNA, normalized to no treatment control group.
Figure 7.3 Quantification by flow cytometry analysis of GFP expression in HEK293 cells treated with 1 μM siRNA normalized to no treatment control group. (a) Return of GFP expression over the course of 10 days in siRNA treated cells. (b) GFP expression 6 days post-transfection when cells were treated with no siRNA, siRNA on day 0 only, or siRNA on both days 0 and days 3, indicating the ability to maintain reduced protein expression by repeated supplementation of siRNA.
Figure 7.4 Release and bioactivity of siRNA from hydrogels. (a,b) Cumulative release of siRNA from hydrogels. (a) Calcium-crosslinked alginate with or without the addition of polycationic chitosan or PEI to modulate release. (b) Photocrosslinked alginate and collagen. (c,d) Bioactivity of released siRNA. (c) Percentage of GFP-positive cells after 3 and 6 days, normalized to no treatment controls. (d) Flow cytometry histograms of samples demonstrate GFP silencing at day 3.
Figure 7.5 Confocal fluorescent micrographs of cells cultured in three-dimensional hydrogels. Cells were exposed to no siRNA, siRNA only in the hydrogels, or siRNA present in the media for only the first 24 hours of the experiment. The hydrogels examined were photocrosslinked alginate and collagen, at days 3 and 6. Substantial GFP knockdown is seen at day 3 in both hydrogels with siRNA incorporated. The cells in collagen with incorporated siRNA still exhibit decreased expression of GFP at day 6. Scale bars represent 200 µm.
Figure 7.6 Alkaline phosphatase activity of hMSCs 6 and 12 days post-transfection. Control cells were grown in media without osteogenic supplements while all other groups were grown in osteogenic media containing dexamethasone, β-glycerophosphate, and ascorbic acid. Dex group indicates hMSCs grown in osteogenic media without siRNA. Remaining three groups were grown in osteogenic media after being supplied once with siRNA against GNAS, ADK, or P2RY11.
Figure 7.7 Alkaline phosphatase activity of hMSCs treated with noggin siRNA (a) 1, 2, and 3 weeks post-transfection and (b) 1 and 2 weeks post-transfection. Control cells were grown in media without osteogenic supplements. All remaining groups were grown in media containing ascorbic acid and β-glycerophosphate. Dex groups indicate hMSCs also grown in the presence of dexamethasone, whereas BMP-2 groups contained BMP-2.
Figure 7.8 Alizarin Red S staining for calcium 3 weeks post-transfection with noggin siRNA. Control hMSCs were grown in media without osteogenic supplements. All remaining groups were grown in media containing ascorbic acid and β-glycerophosphate. Dex groups indicate hMSCs also grown in the presence of dexamethasone, whereas BMP-2 groups contained BMP-2.
**Figure 7.9** Oil Red O staining for adipogenesis 3 weeks post-transfection with noggin siRNA. Control hMSCs grown in DMEM + 10% FBS, adipo cells grown in adipogenic media, and all remaining groups grown in control media containing ascorbic acid and β-glycerophosphate and either dexamethasone or BMP-2 or both. Dex groups indicate hMSCs also grown in the presence of dexamethasone, BMP-2 groups grown in the presence of BMP-2.
Figure 7.10 (a) Alkaline phosphatase and (b) Runx2 expression as measured by qRT-PCR 12 days post-transfection with chordin or noggin siRNA. Control hMSCs were grown in media without osteogenic supplements. All remaining groups were grown in media containing ascorbic acid and β-glycerophosphate. Dex groups indicate hMSCs also grown in the presence of dexamethasone, whereas BMP-2 groups contained BMP-2.
Figure 7.11 Alizarin Red S staining for calcium 3 weeks post-transfection with noggin and chordin siRNA. Control hMSCs were grown in media without osteogenic supplements. All remaining groups were grown in media containing ascorbic acid and β-glycerophosphate. Dex groups indicate hMSCs also grown in the presence of dexamethasone, whereas BMP-2 groups contained BMP-2.
Figure 7.12 (a) Chordin and (b) noggin expression of hMSCs 12 days post-transfection with the respective siRNA as measured by qRT-PCR. hMSCs were grown in DMEM + 10% FBS with ascorbic acid and β-glycerophosphate. Dex groups indicate hMSCs also grown in the presence of dexamethasone, whereas BMP-2 groups contained BMP-2.
Figure 7.13 Alkaline phosphatase activity of MC3T3s treated with noggin siRNA 2 and 3 weeks post-transfection. Control cells were grown in α-MEM (which contains ascorbic acid) + 10% FBS. All remaining groups were grown in same media with β-glycerophosphate and dexamethasone added. BMP-2 groups contained 100 ng/ml BMP-2.
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7.7 References


Chapter 8. Conclusions and Future Directions

8.1 Primary Conclusions and Implications for Regenerative Medicine and Gene Delivery

There is great therapeutic potential in the use of localized gene delivery. DNA can be delivered to cells to upregulate their expression of growth factors or other bioactive factors that contribute to tissue regeneration. Alternatively, siRNA can be delivered in order to downregulate the expression of proteins which hinder a regenerative process. DNA or siRNA incorporated into biomaterial matrices can be implanted or injected into the site of interest to allow for sustained release of the genetic material to the surrounding cells over time. The work presented in this thesis aimed to improve upon the delivery vehicles that have been researched to date, and to provide new options for gene delivery. Overall, these studies demonstrated that it is possible to deliver bioactive DNA or siRNA from a variety of biopolymer scaffolds. Moreover, the ability to tailor the release was demonstrated whether the scaffold was made from the hydrophobic biomaterial PLGA or an alginate or collagen hydrogel. However, in each case a different method for tailoring the release had to be used. This demonstrates the importance of rational biomaterial design for gene delivery.

PLGA is a widely used biomaterial that is already FDA-approved for some applications, and so its use as a scaffold for gene delivery seems promising. However, the incorporation of DNA into PLGA matrices often results in a rapid burst release with little additional release. Here, the release from two types of PLGA matrices, gas-foamed and injectable scaffolds, was tailored to provide more prolonged delivery of the DNA.
By incorporating a polysaccharide component, alginate, into the gas-foamed PLGA scaffolds, PEI-DNA nanoparticles could be released for over one month. Also, the incorporation of alginate decreased the burst release of naked plasmid DNA from these scaffolds. The inclusion of a hydrogel component into a solid hydrophobic scaffold is a novel methodology to alter the release properties of genetic material from these scaffolds.

These solid PLGA scaffolds hold much promise in tissue regeneration, but the development of injectable biomaterial systems is also of great interest as they can be placed into the defect in a minimally invasive manner, thereby reducing the surgical interventions required. Thus the development of an injectable, porous PLGA scaffold represents an exciting advancement. This had not been accomplished to date, as the PLGA is often dissolved in highly toxic solvents due to its hydrophobic nature. However, by using a solvent that has been FDA-approved, PLGA can be safely injected. The incorporation of porosity into the scaffold design is extremely important for tissue engineering applications as it allows cells to migrate into the biomaterial, proliferate, and secrete extracellular matrix to begin regeneration of the tissue. Thus, salts or sucrose and a small amount of water were mixed in with the PLGA solution prior to injection, which allowed for the formation of porous PLGA matrices both in vitro upon injection into an aqueous solution and in vivo. Furthermore, these injectable matrices were examined for their ability to deliver DNA over time. The incorporation of naked DNA into these scaffolds resulted in a burst release of the DNA, with complete release within about one week. Thus, this injectable PLGA system was further engineered by incorporating DNA into PLGA microparticles to allow for sustained delivery for at least two months. The released DNA remained bioactive and able to transfect cells.
These developments to the PLGA scaffolds (pre-fabricated and injectable) represent vast improvements to both systems from which the DNA typically diffuses rapidly, within days. By using rational biomaterial design, these systems were engineered to provide prolonged release of genetic material. These represent significant advances to the field of gene delivery in offering options that will allow DNA to be delivered locally to cells for more than one month.

Hydrogels can also be used for gene delivery. These hydrophilic polymers can be mixed with DNA or siRNA and then crosslinked to create a scaffold from which the genetic material may diffuse out over time. Furthermore, due to their hydrophilicity cells can be easily incorporated into these materials to provide a transplanted cell population.

The work presented in this thesis focused on the use of natural hydrogel materials, especially alginate. Plasmid DNA incorporated into calcium crosslinked alginate hydrogels does not diffuse from the matrices to a very great extent, likely due to diffusional limitations based on the nanoporous structure of the matrix, and due to the slow degradation of these scaffolds observed in vitro in divalent cation-containing media. However, by incorporating a cell population into the hydrogels, transfection of these cells is possible as they are able to access the DNA that surrounds them. Furthermore, the degradation rate of alginate can be tailored by altering its molecular weight or incorporating degradable linkages; thus, different release profiles could be obtained in an in vivo environment through further tailoring of the material. Importantly, heterotopic bone formation was observed in vivo when preosteoblast cells were co-incorporated with DNA encoding for BMP-2 into alginate. This system clearly presents great promise for gene delivery to promote osteogenesis, and demonstrates that the delivery of DNA to
upregulate gene expression can be an effective tool for use in tissue regeneration strategies.

An equally powerful method to influence cellular behavior may be the delivery of siRNA to downregulate the expression of proteins that hinder the regenerative process. However, the delivery of siRNA to a specific tissue location is extremely challenging. Just like any RNA molecule, siRNA is prone to degradation by ribonucleases that are ubiquitous in the surrounding environment. Furthermore, siRNA is often sensitive to degradation by heat over time, so maintaining its bioactivity for long periods of time in the body can be challenging. Hydrogels are promising biomaterial systems for the local delivery of siRNA, as they typically do not require harsh conditions for formation, which reduces the likelihood for the siRNA to be degraded during scaffold fabrication. Also, the pores of hydrogels are often smaller than many pre-formed scaffolds, and a small pore size can be beneficial for retaining small molecules such as siRNA for longer periods of time. Furthermore, they are injectable – offering a minimally invasive delivery method to a particular location, and cells can be incorporated for approaches using cell transplantation. The ability to deliver siRNA from three different natural biopolymer hydrogels for at least 1-2 weeks was demonstrated. All of the materials released the siRNA at different rates and to different extents. Furthermore, by fabricating a composite hydrogel composed of negatively charged alginate and a polycation (chitosan or PEI), the total amount of siRNA retained within the hydrogel was increased. The released siRNA from all hydrogels was bioactive and capable of sustained gene silencing.
As the primary application of this thesis work was for bone regeneration, the ability to increase the osteogenic potential of mesenchymal stem cells through treatment with siRNA was next examined. The hypothesis was that knockdown of a protein that normally inhibited the osteogenic differentiation of these cells would lead to increased osteogenesis. Human MSCs were used in these experiments as these cells may be likely to be used in the clinical setting. Although many different experiments were conducted to look at the effect of silencing five different genes, in none of these trials was the silencing found to influence the osteogenic differentiation. This highlights the complexity of cell fate decisions, and clearly more work is necessary to elucidate molecular targets that may influence these decisions. Furthermore, there are likely to be more than one signal, and so the silencing of a single gene may not be enough to fully influence the differentiation of a population of stem cells. Both RNAi and stem cell research are hot areas of research right now, and although the combination of the two is very exciting, much work remains in terms of elucidating the molecular pathways that lead cells down a particular path, and the influence that siRNA may have on these complex pathways. There are still major challenges with RNAi research, including off-target effects and incomplete silencing of gene expression (although it is possible to achieve good gene knockdown, it is probably not possible to achieve total gene knockout using siRNA). However, despite these challenges there is great potential for the use of RNAi in bone regeneration, and these avenues will continue to be explored.
8.2 Future Directions

Gene delivery provides many challenges, as not only does the DNA need to be supplied to the appropriate cells \textit{in vivo}, but the cells need to be able to uptake the DNA and express it before there can be any therapeutic effect. The local delivery of DNA or siRNA can be accomplished from biomaterial scaffolds. However, it is important to ensure that the efficiency of uptake is high enough that a large population of the surrounding cells are transfected. To this end, increasing the transfection efficiency of all of the systems described herein will be important. This will likely need to be done by further engineering the DNA nanoparticles such that they are stable, promote cell uptake, allow the DNA to gain nuclear access for expression, and do not cause cytotoxicity. Many groups are engineering more efficient DNA-nanoparticles, for example by incorporating cell penetrating peptides or targeting moieties, and it would be interesting to use some of these in the biomaterial scaffold systems described in this thesis for future projects.

Another major challenge that remains is the ability to finely control the release of genetic material from the scaffolds. Although the ability to tailor the release profiles was certainly demonstrated in this work, many of the profiles exhibit larger amounts of release in the first few days, followed by much less release over time. This type of delivery profile may be sufficient for therapeutic effect, but it is also possible that it will be necessary to have more linear release such that a similar amount is steadily released each day to the surrounding cells. Further modification of the systems demonstrated herein will be necessary to achieve more linear release profiles over time. There are several possible avenues to explore, including possibly tethering the DNA or siRNA...
molecules to the biomaterial with degradable linkages; as these links are broken the nucleotides would be released into the surrounding environment. Another possibility for altering the release profiles could be the use of affinity interactions to tailor the delivery of the nucleotides. If the DNA or siRNA were covalently modified with a functional group that had an affinity for either the scaffold material itself or a different functional group that could be covalently bound to the biomaterial, then the release would be driven not only by diffusion and degradation but also by the affinity interactions of these modified nucleotides with the biomaterial system. Also, another option could be to incorporate the DNA or siRNA into polymeric micro- or nano-spheres which would then be incorporated into the scaffold. This would likely slow the release of the genetic material and further protect it from environmental factors which could degrade it.

Additionally, this work has highlighted the complexity of stem cell differentiation and the various molecular pathways that are involved therein. The variation in response to growth factors and other inductive signals among cells from different species is important to highlight and deserves significant attention in the field. If stem cells are the desired cell type to use in the ultimate clinical application, much more needs to be learned about the signaling pathways that influence their cell fate decisions. Similarly, these studies also should be conducted using fully differentiated cells to determine if these may ultimately have more promising clinical utility based on their response to the delivered DNA or siRNA. It would also be interesting to look at how co-cultures of stem cells and fully differentiated cells respond to the gene delivery treatments. It is likely that the fully differentiated cells naturally secrete bioactive factors that would influence the differentiation of stem cells. Perhaps the presence of a smaller number of fully
differentiated cells in combination with the inductive signals provided by the delivered nucleotides would have a greater effect on guiding the differentiation of the stem cells and ultimately serve to provide increased tissue growth.
Appendix: Select Detailed Protocols

RNA Isolation Using Tri-Reagent (Invitrogen)

(This protocol assumes cells grown in 24-well plates. Scale up or down accordingly as needed.)

Sample Preparation

1. Lyse cells on culture dish by adding 1 ml Tri Reagent per cm² plate surface area (for 24-well dish, 1.9 cm², add 200 ul). Pipette up and down to efficiently lyse all cells. Transfer to RNase-free microcentrifuge tube. At this point, sample can be stored at -80 °C for up to 1 month.
2. Allow samples to stand at room temperature 5 minutes.
3. Add 20 ul 1-bromo-3-chloropropane or 40 ul chloroform.
4. Shake vigorously for 15 seconds, then allow to stand at room temperature for 2-15 minutes.
5. Centrifuge 12,000xg, 15 min, 4 °C.

RNA Isolation

Note: be sure to use all RNase-free pipet tips, tubes, water, etc. and work in RNase-free area.

1. Transfer colorless upper aqueous phase to fresh RNase-free microcentrifuge tube, being very careful to not get any of the organic or interphases. (Store the organic / inter phases at 4 °C until used for DNA and protein isolation.)
2. Add 100 ul isopropanol (2-propanol) and mix. Let stand 5-10 minutes room temperature.
3. Centrifuge 12,000xg, 10 min, 4 °C to pellet the RNA.
4. Remove the supernatant and wash the RNA pellet by adding at least 200 ul of 75% ethanol. Vortex, then centrifuge 7500xg (or 12,000xg for more stable but possibly more difficult to resuspend pellet), 5 min, 4 °C.
5. Remove the supernatant and briefly dry the RNA pellet for 5-10 minutes by air-drying or under a vacuum. Do not let pellet dry completely.
6. Add ~10-20 ul nuclease-free water, pipet up and down to dissolve pellet.
7. Measure the ng/ul concentration and 260/280 ratio on the Nanodrop.
8. Run the isolated RNA on a Bioanalyzer chip to examine.
**qRT-PCR Protocol using SYBR Green**

1. Plan plate layout.
2. Calculate amounts of everything needed. Make 10% more than you need.
   a. Each well will contain:
      - 25 ul SYBR Green PCR Master Mix
      - 5 ul of 5 uM primer F
      - 5 ul of 5 uM primer R
      - 10 ng of cDNA
      - (15 ul – volume cDNA) RNase-free water
   b. Account for 3 replicate wells for each gene / each condition.
3. Fill ice bucket. Wipe down RNA / PCR area with 70% ethanol and then DNA Away. Also wipe centrifuge tube racks and pipets with DNA Away.
4. Make master mixes for each gene to be assayed. These will contain the SYBR Green PCR Master Mix, primer F, primer R, and water (assuming same volume of cDNA in each well). Place on ice.
5. Place PCR plate on ice.
6. Pipet Master Mix into appropriate wells as per plate layout. Volume will be (50 ul – volume of cDNA).
7. Pipet cDNA into appropriate wells, changing pipet tip between every single well. Pipet up and down once or twice in the master mix to ensure mixing.
8. Cover plate with clear plastic cover.
9. Weigh plate with cover on the larger scale, remove. Take empty PCR plate that is labeled BALANCE and place it on scale. Fill wells with water until total weight is equal to your PCR plate.
10. Centrifuge the plates briefly to collect all liquid to bottom of wells. Usually can just hold the ►► button until reach ~300xg, then release to stop the centrifuge.
11. Place PCR plate into the ABI 7700 real time PCR machine, with well A1 in the top left corner. Cover plate with the 96-well foam pad, gold side up. Pull heated lid closed and screw shut.
12. Set up software, save your plate layout, then Run.
Preparation of Cells for Flow Cytometry

(This protocol assumes cells are confluent in 24-well plate)

1. Remove media from well, add 0.25 ml of PBS to rinse. Repeat for all wells in 1 plate.
2. Remove PBS from well, add 0.25 ml trypsin. Repeat for all wells in 1 plate.
3. Incubate plate at 37°C for 5-10 minutes, until cells have detached from wells and are floating. Tap plate lightly if needed.
4. Add 0.25 ml of serum-containing media to neutralize the trypsin.
5. Transfer the 0.5 ml cell suspension to a 1.5 ml microcentrifuge tube. Spin tubes at 200xg for 5 minutes in microcentrifuge.
6. Carefully aspirate off media from each tube, leaving the cell pellet. It is fine to leave a little media to be sure the cell pellet is not aspirated.
7. Resuspend the cell pellet in 0.5 ml PBS, pipetting up and down to resuspend.
8. Transfer to a 12x75 mm polystyrene round-bottom culture tube for flow analysis. Put tubes on ice until analysis.
Preparation of PEI-DNA-alginate PLGA Gas-Foamed Scaffolds

1. 30 minutes before starting, set PLGA at room temperature. Keep PEI-DNA-alginate mixtures at -20°C until immediately before use.
2. Weigh out 80 mg PLGA.
3. Weigh PEI-DNA-alginate mixtures; record weight.
4. Weigh out sucrose at (720 mg minus the weight of the PEI-DNA-alginate).
5. Mix all three components thoroughly with clean spatula for 1 minute, transfer into 13 mm die.
6. Press 3.5 tons for 1 minute.
7. Store scaffolds at -20°C as you go.
   *Make sure to clean the die thoroughly between each scaffold with acetone and diH2O.
8. Gas foam pressed scaffolds in pressure vessel at 800 psi for 20 hours.
9. Release CO2 over the course of 30 seconds – 1 minute.
10. Place scaffolds into 5 ml 0.1 M CaCl2 for 1 minute (do this for only one or two scaffolds at a time).
11. Transfer to tube with 10 ml DMEM-HG.
12. After 4 hours, remove this media and replace with 5 ml fresh DMEM-HG. (Save the spent media if you will be measuring release of the DNA.)
13. Leave overnight at room temp (RT) to finish leaching the porogen.
14. Remove DMEM, replace with 10 ml 70% ethanol to sterilize. Leave in ethanol for 30 min.
15. Transfer scaffolds to fresh tubes containing 10 ml PBS. Leave at RT 30 min.
16. Remove PBS, replace with 10 ml DMEM-HG. Leave at RT 30 min.
17. Transfer scaffolds to 24-well plates (1 plate per timepoint), seed cells at desired concentration and incubate for 4 hours. Add media to 1 ml total volume.
18. Replace media every other day.
**X-Gal Staining Protocol**

**X-Gal staining solution in PBS (phosphate buffered saline)**
5 mM K$_3$Fe(CN)$_6$
5 mM K$_4$Fe(CN)$_6$
2 mM MgCl$_2$

**X-Gal stock solution:**
50 mg/ml X-Gal in dimethyl formamide (DMF)

Just before staining, aliquot enough staining solution for all samples into a separate tube (use same volume as is typically used for cell culture media in that dish). Add X-Gal stock solution so it has a final concentration of 1 mg/ml (50x dilution).

1. 48 hours post-transfection, remove cells from incubator. Work in chemical hood.
2. Remove media, and rinse cells once with cold PBS.
3. Fix cells with 0.2% glutaraldehyde (in PBS) at 4°C for at least 10 minutes.
4. Remove fixative and rinse cells 1-2 times gently with cold PBS.
5. Incubate cells in staining solution for up to 24 hours at 4°C.
6. Remove staining solution and add PBS.
7. Look under light microscope for dark blue cells.

**Notes:**
- X-Gal can also be dissolved in DMSO for stock solution, but this increases the chance that it will crystallize when in staining solution.
- Staining can also be done at room temperature or 37°C. However, I saw more crystal formation when it was at 37°C.
- If you do get X-Gal crystals, they can be dissolved in ethanol or in PBS (but PBS will take much longer).
- Do not stain in a CO$_2$ incubator as this can halt the staining process.
- Note that you should fix cells in the chemical safety hoods, not in the biological safety hoods.
- The fixative solution interferes with the staining solution, which is why you need to carefully wash your cells after fixation. However, you must be careful during the washings that your cells do not start to come off the dish.
- Making the staining solution in PBS rather than water is supposed to enhance the staining.
- Cells can turn blue after several hours, and usually all cells that are lacZ+ will be blue by 16-18 hours. The last few hours of incubation will only serve to increase the blue color.
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