8-2012

The Formulation of a Bone Targeted Drug Delivery System of Poly(Glycolic Acid)-Poly(Ethylene Glycol) Coated Hydroxyapatite Nanoparticles for the Delivery of Statins

Erika Jelen
Clemson University, erika.jelen@gmail.com

Follow this and additional works at: https://tigerprints.clemson.edu/all_theses
Part of the Biomedical Engineering and Bioengineering Commons

Recommended Citation
https://tigerprints.clemson.edu/all_theses/1503

This Thesis is brought to you for free and open access by the Theses at TigerPrints. It has been accepted for inclusion in All Theses by an authorized administrator of TigerPrints. For more information, please contact kokeefe@clemson.edu.
THE FORMULATION OF A BONE TARGETED DRUG DELIVERY SYSTEM OF POLY(GLYCOLIC ACID)-POLY(ETHYLENE GLYCOL) COATED HYDROXYAPATITE NANOPARTICLES FOR THE DELIVERY OF STATINS

A Thesis
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Master of Science
Bioengineering

by
Erika Kirby Jelen
August 2012

Accepted by:
Dr. Frank Alexis, Committee Chair
Dr. Martine LaBerge
Dr. Yanzhang Wei
ABSTRACT

Bone is a form of mineralized connective tissue that provides strength and rigidity to the skeleton \(^1,^{10}\). The two primary components within bone tissue are an organic extracellular matrix, containing type I collagen, and an inorganic mineral component composed mainly of calcium phosphate hydroxyapatite crystals \(^1,^{22,32}\). Over time the microarchitecture of bone can break down due to a variety of different factors, mainly the onset of osteoporosis in post-menopausal women, Paget’s disease, and the experience of a loss of gravity during space flight. Currently there are about ten million people in the United States alone suffering from osteoporosis \(^10\).

The prevention of further damage as well as the replacement of lost bone tissue is the focus of many therapeutic approaches. A large number of available treatments rely on the concept of blocking further bone loss by inhibiting the natural resorption process. Bisphosphonates are the most popular drug therapy in this category \(^43\). But there are many other treatments that strive to prevent further bone resorption such as hormone therapy, estrogen agonist / antagonists, calcitonin, and denosumab. Other therapies approach the problem of bone loss by inducing the formation of new bone tissue to replace that lost to these pathologies. These include teriparatide, strontium ranelate, and statins, which are the focus of this research.

The goal of this research was to formulate a targeted nanoparticle drug delivery system for the treatment of bone diseases. A hydroxyapatite nanoparticle conjugated with poly(glycolic acid)-poly(ethylene glycol) diblock copolymer was created to deliver statin drugs. These drugs act as competitive inhibitors of 3-hydroxy-3-methylglutaryl coenzyme
A reductase, for the lowering of serum cholesterol $^{12,49}$. Recently, statins have been investigated for their ability to induce bone formation by enhancing expression of bone morphogenic protein-2 $^{12,49}$.

In addition to formulation, studies were completed to prove the particle’s low toxicity, loading abilities, and release pharmacokinetics. In order to enhance the specificity with which these particles are delivered to bone, targeting peptides were tested for \textit{in vitro} and \textit{in vivo} targeting efficiency and exclusivity.
DEDICATION

I would like to dedicate this work to my parents, Robert and Susan Jelen, and my brother, Michael Jelen, for their support throughout my academic career.
ACKNOWLEDGMENTS

I would like to thank my advisor, Dr. Frank Alexis, for his mentoring and support with my research and the completion of this thesis. I would also like to thank all the members of the Nanomedicine Lab for their help with my research. I would especially like to thank Thomas Moore for his mentoring and help.

I am also grateful to the members of my committee, Dr. Martine LaBerge and Dr. Yanzhang Wei. I would also like to thank Kim Ivey for her help with the TGA.

I would also like to acknowledge the Clemson University Department of Bioengineering and our funding source SC EPSCOR – NASA 2098490.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>TITLE PAGE</td>
<td>i</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>ii</td>
</tr>
<tr>
<td>DEDICATION</td>
<td>iv</td>
</tr>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>v</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>viii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>ix</td>
</tr>
<tr>
<td>CHAPTER</td>
<td></td>
</tr>
<tr>
<td>I. GENERAL INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>II. BONE ANATOMY AND PATHOLOGY OVERVIEW</td>
<td>3</td>
</tr>
<tr>
<td>2.1. Anatomy</td>
<td>3</td>
</tr>
<tr>
<td>2.1.1. Bone Microstructure</td>
<td>3</td>
</tr>
<tr>
<td>2.1.2. Bone Macrostructure</td>
<td>4</td>
</tr>
<tr>
<td>2.1.3. Cellular Components</td>
<td>6</td>
</tr>
<tr>
<td>2.1.4. Remodeling Process</td>
<td>8</td>
</tr>
<tr>
<td>2.2. Pathology</td>
<td>10</td>
</tr>
<tr>
<td>2.2.1. Osteoporosis</td>
<td>10</td>
</tr>
<tr>
<td>2.2.2. Paget’s Disease</td>
<td>14</td>
</tr>
<tr>
<td>2.2.3. Microgravity Bone Loss</td>
<td>16</td>
</tr>
<tr>
<td>III. AVAILABLE BONE THERAPEUTICS AND CURRENT TREATMENT AND DELIVERY RESEARCH</td>
<td>18</td>
</tr>
<tr>
<td>3.1. Anti-Catabolic Therapeutic Approaches: An</td>
<td>18</td>
</tr>
<tr>
<td>In Vitro / In Vivo and Clinical Overview</td>
<td></td>
</tr>
<tr>
<td>3.1.1. Bisphosphonates</td>
<td>19</td>
</tr>
<tr>
<td>3.1.2. Hormone Therapy</td>
<td>25</td>
</tr>
<tr>
<td>3.1.3. Estrogen Agonist / Antagonists</td>
<td>27</td>
</tr>
<tr>
<td>3.1.4. Calcitonin</td>
<td>29</td>
</tr>
<tr>
<td>3.1.5. Denosumab</td>
<td>30</td>
</tr>
<tr>
<td>3.1.6. Benefits and Limitations of Anti-Catabolic Treatments</td>
<td>32</td>
</tr>
</tbody>
</table>
Table of Contents (Continued)

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.2. Anabolic Therapeutic Approaches: An In Vitro / In Vivo and Clinical Overview</td>
<td>33</td>
</tr>
<tr>
<td>3.2.1. Teriparatide</td>
<td>33</td>
</tr>
<tr>
<td>3.2.2. Statins</td>
<td>36</td>
</tr>
<tr>
<td>3.2.3. Strontium Ranelate</td>
<td>37</td>
</tr>
<tr>
<td>3.2.4. Benefits and Limitations of Anabolic Treatments</td>
<td>39</td>
</tr>
<tr>
<td>3.3. Controlled Delivery</td>
<td>39</td>
</tr>
<tr>
<td>3.3.1. Nanoparticle Delivery Vehicles</td>
<td>39</td>
</tr>
<tr>
<td>3.3.2. Targeting Approaches</td>
<td>42</td>
</tr>
<tr>
<td>IV. FORMULATION OF TARGETED HYDROXYAPATITE NANO PARTICLES FOR THE DELIVERY OF STATIN DRUGS</td>
<td>45</td>
</tr>
<tr>
<td>4.1. Introduction</td>
<td>45</td>
</tr>
<tr>
<td>4.2. Materials</td>
<td>46</td>
</tr>
<tr>
<td>4.3. Experimental Methods</td>
<td>47</td>
</tr>
<tr>
<td>4.3.1. Synthesis of nHA-PGA-PEG</td>
<td>47</td>
</tr>
<tr>
<td>4.3.2. Toxicity</td>
<td>48</td>
</tr>
<tr>
<td>4.3.3. Dye Loading</td>
<td>49</td>
</tr>
<tr>
<td>4.3.4. Lovastatin Release</td>
<td>49</td>
</tr>
<tr>
<td>4.3.5. Peptide Targeting</td>
<td>51</td>
</tr>
<tr>
<td>4.4. Experimental Results and Discussion</td>
<td>52</td>
</tr>
<tr>
<td>4.4.1. Synthesis of nHA-PGA-PEG</td>
<td>52</td>
</tr>
<tr>
<td>4.4.2. Toxicity</td>
<td>54</td>
</tr>
<tr>
<td>4.4.3. Dye Loading</td>
<td>55</td>
</tr>
<tr>
<td>4.4.4. Lovastatin Release</td>
<td>56</td>
</tr>
<tr>
<td>4.4.5. Peptide Targeting</td>
<td>57</td>
</tr>
<tr>
<td>4.5. Conclusion of Results</td>
<td>60</td>
</tr>
<tr>
<td>V. CONCLUSIONS</td>
<td>62</td>
</tr>
<tr>
<td>VI. RECOMMENDATIONS FOR FUTURE RESEARCH</td>
<td>63</td>
</tr>
<tr>
<td>VII. REFERENCES</td>
<td>64</td>
</tr>
</tbody>
</table>
LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. World Health Organization Osteoporosis Scoring</td>
<td>11</td>
</tr>
<tr>
<td>2. Bisphosphonate Overview</td>
<td>21</td>
</tr>
<tr>
<td>3. Available Anti-Catabolic Treatments</td>
<td>32</td>
</tr>
</tbody>
</table>
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Anatomy of Native Bone Tissue</td>
</tr>
<tr>
<td>2.</td>
<td>Diagram of Active Phase of Bone</td>
</tr>
<tr>
<td>3.</td>
<td>Prevalence of Osteoporosis and/or Low Bone Mass</td>
</tr>
<tr>
<td>4.</td>
<td>Successful Variation of PGA Coating Thickness</td>
</tr>
<tr>
<td>5.</td>
<td>Conjugation of PEG to nHA-PGA</td>
</tr>
<tr>
<td>6.</td>
<td>TEM Images of nHA and nHA-PGA-PEG</td>
</tr>
<tr>
<td>7.</td>
<td>Toxicity of nHA and nHA-PGA-PEG in HUVEC Cells</td>
</tr>
<tr>
<td>8.</td>
<td>Dye-Loading as a Proof of Concept of Nanoparticle Loading Abilities</td>
</tr>
<tr>
<td>9.</td>
<td>Lovastatin Release from nHA-PGA-PEG Nanoparticles</td>
</tr>
<tr>
<td>10.</td>
<td>Targeting Peptide Binding to HA Discs and Chicken Bone</td>
</tr>
<tr>
<td>11.</td>
<td><em>In Vivo</em> Testing of Bone-Targeting Peptide Binding</td>
</tr>
<tr>
<td>12.</td>
<td>Preferential Binding of HA-1 Peptide to Bone Tissue</td>
</tr>
</tbody>
</table>
CHAPTER ONE
GENERAL INTRODUCTION

The research presented in this thesis focuses on the treatment of bone diseases such as osteoporosis, Paget’s disease, and microgravity bone loss. All of these pathologies are similar in that they result from a dysfunction or uncoupling of bone’s natural resorption and formation processes. These diseases of bone can result in traumatic fracture, deformity, and pain.10, 23.

An overview is provided outlining the normal anatomy of bone tissue, including the microstructure, macrostructure, cellular components, and the remodeling process. Following the anatomical overview each disease, osteoporosis, Paget’s, and microgravity bone loss, is detailed. Though each disease has different causes and physiological changes, they all results in a decreased bone density as well as an increased risk of fracture.

A literature review was performed to provide information on the all the currently marketed and researched treatment options. These treatments fall into one of two categories. Anti-catabolic treatments focus on the cessation of the resorption process and the retention of current bone mass and structure. While anabolic therapies act to induce bone formation to replace lost bone mass. Many different treatment options are detailed with in vitro, in vivo, and clinical findings, as well as the currently researched delivery options.

The focus of this research was an anabolic treatment option of using statins, a commonly prescribed cholesterol-lowering drug, for the induction of bone formation. The
goal was to deliver these drugs with a biocompatible, targeted nanoparticle delivery
system. Hydroxyapatite nanoparticles were made with a polymeric coating for increased
compatibility and circulation time. These particles were shown to encapsulate and release
drug with a linear release profile. Targeting options were also investigated to ensure these
particles were delivered exclusively to the bone.

This paper provides both an overview of current research as well as a detailed
procedural overview of the formulation and testing of these hydroxyapatite nanoparticles.
CHAPTER TWO

BONE ANATOMY AND PATHOLOGY OVERVIEW

2.1 Anatomy

Bone is a complex living form of specialized connective tissue that serves many vital functions in the body. The mineralized extracellular matrix, ECM, gives bone its rigidity such that it can provide mechanical support, protection of vital organs, mobility, as well as act as a reservoir for vital minerals $^1,^{10},^{27},^{30}$. Due to bone’s multi-functionality, healthy bone tissue is critically important to overall health and quality of life $^{10}$.

2.1.1. Bone Microstructure

Bone tissue consists primarily of two different components, an organic portion, mainly cells and the ECM. Also an inorganic, or mineral, component comprised of calcium phosphate in the form of hydroxyapatite rod shaped nano-crystals twenty-five to fifty nanometers in length $^1,^{22},^{32}$. Collagen is the main protein providing structural integrity to the ECM; this network of triple helical collagen molecules encompasses ninety percent of the total bone mass $^1,^{24},^{30},^{32}$. The ECM is impregnated with the mineral component, thus providing rigidity to the tissue $^1,^{32}$. These hydroxyapatite crystals, $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$, not only give bone its hardness, but it also allows for the tissue to be used as a storehouse for both calcium and phosphate. In order to maintain homeostatic blood calcium and phosphate levels, the bone can be broken down such that these minerals can be utilized elsewhere in the body $^1,^{10},^{30}$. In addition to collagen and hydroxyapatite, the bone matrix contains proteoglycan molecules. These molecules
consist of a core protein with covalently bound glycosaminoglycan (GAG) side chains. These proteoglycans allow for the binding of growth factors as well as lend to the tissue’s compressive strength \(^1,^{30}\). Other common molecules found in the bone ECM are glycoproteins that mediate attachment between various components of the matrix \(^1,^{30}\).

2.1.2. Bone Macrostructure

Bone tissue, both microscopically and macroscopically, is structured such that it provides maximum strength in order to prevent fracture. In bulk, bone contains two distinct layers. The outer layer, made of dense highly organized structures, is known as compact, or cortical bone. Cortical bone has cylindrical structures known as osteons containing concentric rings of bone tissue, each layer have a different collagen fiber orientation \(^1,^{32}\). Osteocytes reside in small spaces within the concentric rings of bone matrix called lacunae. Extending out from these voids are small tunnels known as canaliculi, these tunnels act as pathways through which extensions of osteocytes can travel and connect to neighboring cells \(^1,^{32}\). The second, inner layer bone tissue is trabecular or cancellous bone. This type of tissue is much less dense and forms a network of small struts, called trabeculae, of bone tissue extending in various directions with a continuous interstitial space. Between the concentric arrangement within the cortical bone and the random arrangement of struts allowing for even stress distribution, bone tissue is specially formed to provide extensive structure and strength to the body \(^23\). A layer of dense connective tissue surrounds the bone, both superficially and deep, to allow for cellular attachment during remodeling and enhanced vascularization. The superficial
layer is known as the periosteum, this layer of dense connective tissue resides on the outer surface of the cortical bone. The internal surface of the bone is surrounded by a single cell layer of osteoprogenitor cells, which can become active in times of remodeling. This endosteum internally surrounds the layer of cortical bone as well as wraps around each trabeculae. This tissue layer remains stagnant until times of remodeling when it thickens, allowing a layer of cells to reside and attach to the bone surface.

Bone is a highly vascularized tissue containing an interconnected network of vessels. This vascular network consists of many different vessels and structures including the medullary cavity, nutrient foramina, Haversian canals and Volkmann’s canals. Within the center of long bones is a cavity known as the medullary cavity, in which bone marrow, consisting of developing blood cells and reticular fiber, resides. The main route by which blood enters the bone tissue is by multiple nutrient foramina, small openings in the bone through which blood vessels pass. These incoming vessels supply blood to the medullary cavity, where the network of vessels within the tissue originates. From the medullary cavity, Volkmann’s canals provide a route of entry into the compact bone. These canals run perpendicular to the axial length of the bone. Originating from these Volkmann’s canals are Haversian canals that run through the center of each individual osteon. Within these Volkmann’s and Haversian canals are capillaries that allow for the diffusion of nutrients to bone cells due to the ease of movement across the single cellular endothelial layer of the capillaries. Haversian canals are typically 200-300 nanometers apart; this distance is such that each bone cell is close enough to
receive nutrients by diffusion. Trabecular bone is also vascularized by a similar network of capillaries that runs between the trabecular struts within the bone marrow. The cells within cancellous bone are provided nutrients from these vessels running through the interstices of the tissue. The fenestrations, or pores, within bone vasculature, facilitate this diffusion of nutrients; these openings can be up to eighty nanometers, large enough for delivery of nutrients and therapeutic treatments.

**Compact Bone & Spongy (Cancellous Bone)**

![Bone anatomy diagram](image)

**Figure 1 | Anatomy of Native Bone Tissue.** Bone contains two concentric layers of mineralized tissue. Compact bone, with cylindrical osteons and Haversian canals, reside on the periphery of the bone. Trabecular bone remains inferior with blood vessels and marrow vessels running within the trabecular struts.

### 2.1.3. Cellular Components

The cellular component of bone tissue is comprised of five different cell types. Osteoblasts are anabolic bone cells, playing a role in new bone formation. They are mononuclear cells that function to synthesize and secrete collagen, GAGs, and other bone
matrix proteins. This newly synthesized matrix, often called osteoid, will eventually be mineralized and thus new bone tissue is formed\textsuperscript{1,27,29}. These cells work in a coordinated manner to secrete sequential sheets of new osteoid\textsuperscript{29}. Once bone matrix secretion is complete, osteoblasts follow one of three paths. Some osteoblasts become flattened and remain on the periphery of the bone within the connective tissue periosteum and become bone-lining cells. Approximately ten percent of osteoblasts become embedded in the newly synthesized bone matrix and become osteocytes, and the remaining cells die by apoptosis\textsuperscript{27}. The bone-lining cells remain quiescent during times of no growth, and act to mediate biological signals and regulate bone resorption\textsuperscript{1,29}. Osteocytes remain buried in small voids, known as lacunae, within the concentric layers of bone tissue that form osteons and function in osteocytic osteolysis, breaking down mineralized bone tissue, bone formation, and the transmission and response to mechanical stimuli\textsuperscript{1,29}. Bone tissue also contains catabolic cells, known as osteoclasts, which act to break down mineralized bone. Osteoclasts attach to areas of bone, mainly the surface of the cortical bone or trabecular struts, at which point the cells dissolve the mineral components and hydrolyze the organic matrix\textsuperscript{1,27,31}. Osteoclasts are often an area of research in treating osteoporosis. The main two osteoclastic molecules researched are a ligand known as RANKL and macrophage colony stimulating factor (M-CSF)\textsuperscript{78,79}. These are membrane bound molecules on the surface of osteoclast precursors\textsuperscript{78,79}. The last type of bone cell is an osteoprogenitor cell; a derivative of a mesenchymal stem cell that eventually gives rise to an osteoblast\textsuperscript{1}. 
2.1.4. Remodeling Process

Bone is a living tissue that is constantly changing and remodeling in order to maintain mechanical competence and support body mass\textsuperscript{27}. The skeleton is constantly adapting to the external loading and unloading seen throughout life. This adaptation manifests in changes to the bone architecture and mass in response to exercise, immobilization, or weightlessness\textsuperscript{27, 31}. These changes in the bone are mainly governed by a group of biological molecules, mainly, systemic hormones such as calcitonin, parathyroid hormone (PTH), and glucocorticoids as well as local factors like cytokines and growth factors\textsuperscript{27}. An increase in stress on the bone increases the rate of bone formation while reducing that of bone resorption, resulting in an increase in bone mass\textsuperscript{27}. The body creates the physiological response to increase bone mass in order to support the increased load as well as prevent fracture\textsuperscript{27}. This process also occurs in reverse, with a decrease in activity bone formation will slow or cease and the bone will begin to resorb since the high bone mass is no longer needed. When bone loss occurs due to a lack of mechanical stimulation it leads to a reduction in density, changes in spatial orientation as well as connectivity of trabecular struts. All of these changes decrease bone strength and increase the risk of fracture\textsuperscript{27}. 

Even when bone does not experience changing load levels, it is still constantly being remodeled without any change in mass or density. In fact, in a mature adult approximately twenty-five percent of trabecular and three percent of cortical bone is renewed annually\textsuperscript{31}. There are five main phases of bone remodeling: activation, resorption, reversal, formation, and quiescence. Activation is the initial event in which
the bone surface is converted from a quiescent state to one in which mononuclear cells are circulating to eventually fuse and form osteoclasts. The specific events that initiate this phase of bone remodeling are unknown. The resorption phase begins and osteoclasts dissolve the mineral bone components and hydrolyze the organic matrix. This portion of the bone resorption process lasts forty-two days in cancellous bone and twenty-seven days in cortical bone. After osteoclasts have broken down the bone tissue, the reversal phase begins and allows transition time between resorption and formation. This stage lasts nine days in cancellous bone and four days in cortical bone, during this time preosteoblasts develop a presence in the resorption cavities. Once the

Figure 2 | Diagram of Active Phase of Bone Remodeling. Displays the progression of bone remodeling from the activation of osteoclasts and the removal of bone tissue to the recruitment of osteoblasts to the previously resorbed site to lay down new bone tissue.
transition is complete, the formation period begins. Osteoblasts differentiate and lay down new unmineralized osteoid. After twenty-five or thirty-five days in trabecular and cortical bone respectively, the osteoid begins to mineralize. Once the remodeling is complete, bone returns to a stable quiescent state characterized by only a thin layer of lining cells surrounding the bone. Overall, the control and regulation of remodeling is done by the balance and coupling of osteoblast and osteoclast cells. It is this balance that can cause pathological changes in the bone.

2.2 Pathology

Most bone diseases are characterized by bone loss due to an imbalance between the bone resorption and formation processes. The three main causes of decreased bone mass are osteoporosis, Paget’s disease, and microgravity bone loss. All three have different causes but a similar outcome, a decreased bone mineral density and increased risk of bone fracture.

2.2.1. Osteoporosis

Osteoporosis is a disease characterized by low bone mass and deterioration of bone structure that causes bone fragility and leads to an increased risk of fracture. The World Health Organization has defined osteoporosis as a bone mineral density (BMD) value more than 2.5 standard deviations below the mean for a normal young Caucasian woman. The severity of the disease is determined by a rating system developed by the
World Health Organization, called a T score. Below is a table defining the scoring system

<table>
<thead>
<tr>
<th>T Score</th>
<th>Diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 to -0.99</td>
<td>Normal</td>
</tr>
<tr>
<td>-1 to -2.499</td>
<td>Osteopenia</td>
</tr>
<tr>
<td>≤ -2.5</td>
<td>Osteoporosis</td>
</tr>
<tr>
<td>≤ -2.5 accompanied by fracture or history of fracture</td>
<td>Severe osteoporosis</td>
</tr>
</tbody>
</table>

Currently, there are roughly ten million Americans over the age of fifty with osteoporosis, and another thirty-four million at risk of developing the disease.  

Osteoporosis is the most common bone disease, and is the most important underlying cause of fractures in the elderly. Though very few people die from this disease, an estimated 1.5 million individuals suffer from an osteoporotic fracture each year. In fact, forty percent of all white women, and thirteen percent of white men, over fifty years of age will experience a fracture of the hip, spine, or wrist within the remainder of their lifetime. This disease can affect anyone, but it is two to three times more likely to develop in women, partially due to the increased rate of bone loss at the onset of menopause. Women accounted for over seventy-five percent of all cases of osteoporosis of the hip in 2002. Aside from injuries and pain, osteoporosis is also a drain on the healthcare system. It has been estimated that the direct healthcare expenditures for osteoporotic fractures range from twelve to eighteen billion dollars per

---

Table 1 | World Health Organization Osteoporosis Scoring. T scores are used by physicians to determine the severity of the osteoporosis was well as the risk of future fracture.
year. The addition of indirect costs, such as caregivers and lost productivity, is thought to increase the figure by billions\textsuperscript{10}.

Primary osteoporosis is a result of the cumulative bone loss and deterioration that occurs throughout life\textsuperscript{10}. This steady bone loss leads to compromised bone strength, predisposing the bone to an increased risk of fracture\textsuperscript{10}. During childhood bone mass increases linearly, reaching a peak in both males and females around twenty or thirty years of age. At this time the trabecular layer within the bone begins to slowly thin, resulting in a slow, linear loss of cancellous bone mass with age\textsuperscript{1, 10, 27, 31}. It is generally thought that this thinning of the trabecular layer is associated with an age-related decline in the amount of matrix synthesized by local osteoblasts\textsuperscript{31}. Cortical bone is also slowly

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{prevalence_of_osteoporosis.png}
\caption{Prevalence of Osteoporosis and/or Low Bone Mass. Data was collected by The National Center for Health Statistics, part of the Center for Disease Control, displaying the number of men and women over the age of fifty with osteoporosis\textsuperscript{10}.}
\end{figure}
lost over this time. The change in cortical bone mass is more related to an increase in porosity rather than thinning. With age the porosity of cortical bone increases due to a decrease in radial closure of osteons, a reduction in osteon wall thickness, an increase in Haversian canal diameter, and the number of remodeling units aborted at the reversal phase 31.

In men, this loss remains linear, but in females, the amount of bone loss accelerates with the onset of menopause. The exact mechanism behind bone loss in post-menopausal women is unknown but it is thought that since hormones play an important role in the regulation of bone tissue remodeling, the marked decrease of estrogen levels in women during menopause has a great effect on this, usually regulated, process 10. This enhanced rate of bone loss in post-menopausal women is associated with an increase in bone turnover, not simply an acceleration of the normal age-related bone loss. In post-menopausal women the loss of bone mass involves the complete removal of trabecular plates and a significant disruption in the trabecular lattice 31. Whole trabecular struts are resorbed, reducing the connectivity of the bone tissue and limiting the stress distribution within the tissue itself 31. The sudden and exponential decrease in estrogen levels within the body after the onset of menopause results in a fifteen percent decline in bone density 10, 33, 34. It is this increase in trabecular resorption that enhances women’s chance of developing osteoporosis and experiencing a fracture during their lifetime.

The exact pathology of osteoporosis is unknown, but there are many contributing factors causing the increased bone loss. One main factor in the reduction of bone mass is the inadequate intake and retention of calcium 33. As people age the intestinal absorption
and renal conservation of calcium is insufficient to conserve enough calcium to sustain blood levels. This retention rate is low due to the amount of calcium lost daily through shed skin, nail, hair, sweat, and urine. Because of all these factors, only four to eight percent of calcium is absorbed \(^{33}\). This inadequate calcium intake contributes to osteoporosis by resulting in the thinning of the cortical layer as well as a decrease and thinning of trabeculae \(^{33}\). In addition to changing estrogen and calcium levels, there are many age-related highly interdependent hormonal and nutritional factors that attribute to osteoporotic bone loss \(^{35}\). Lastly, though the biochemical basis behind bone loss and osteoporosis is unknown, it has been discovered that there are many other diseases attributing to the increased bone loss. Some of these pathologies are hypercortisolism, gonadal insufficiency, thyroid disease, diabetes mellitus, hyperparathyroidism, as well as some gastrointestinal disorders \(^{36}\).

2.2.2. Paget’s Disease

The second most common bone disease, Paget’s disease, is a progressive, and often crippling disorder that most often manifests in the sacrum, spin, femur, skull, sternum, or pelvis \(^{10,23}\). Paget’s disease results in an extremely disorganized bone structure that leads to an increased risk of fracture \(^{23}\).

Though Paget’s disease is the second most common bone disease, it affects far fewer people than osteoporosis. An estimated one million individuals in the United States suffer from Paget’s disease; affecting approximately 1.3 out of every one hundred people \(^{10}\). This disease is most often found within the spine, particularly the lumbosacral region,
and other bones within the axial skeleton\textsuperscript{23}. As with osteoporosis, Paget’s disease can lead to an increase in fracture risk, characteristically common in the femur and tibia\textsuperscript{23}.

The histopathology of Paget’s disease is characterized by three main physiological changes. Firstly, the bone tissue shows an abundance of osteoclasts and osteoblasts, resulting from an increase in bone turnover. The bone marrow is also invaded by a fibrous connective tissue with embedded blood vessels. Lastly, both cancellous and cortical bone display a disorganized structure, which has been termed “mosaic”\textsuperscript{23}. In early phases of the disease there is an increase in bone resorption activity. Both osteoclasts and osteocytes act to break down apatite crystals and collagen fibrils\textsuperscript{23}. This causes an increase in bone loss as well as a disruption of the bone structure within affected areas\textsuperscript{10}. In the second phase of the disease, bone formation amplifies to compensate for the induced bone resorption\textsuperscript{10,23}. Due to the speed with which this new bone is laid down, mature bone is not formed; instead woven bone is present\textsuperscript{10,23}. Woven bone is a more primitive bone tissue not normally found within the adult skeleton. This immature bone tissue has a disorganized alignment of apatite crystals and collagen fibrils\textsuperscript{10,23}. This woven bone that is laid down in response to the accelerated rate of bone resorption, causes the chaotic “mosaic” pattern within the bone structure\textsuperscript{23}. Also, within the active phase of Paget’s disease, the bone marrow is invaded by fibroblasts and mesenchymal cells acting to form a fibrous connective tissue within the bone marrow cavity\textsuperscript{23}. In the final phase of the disease, cellular activity ceases, but the affected area consists of dense woven bone tissue. This, now permanent, tissue is extremely susceptible to deformity and fracture\textsuperscript{10}. As such, common deformities seen with Paget’s
disease are the bowing of weight bearing long bones and an enlargement of the skull. Most often Paget’s disease is painless, so it often goes undiagnosed until the occurrence of a traumatic fracture or the presence of severe deformity.

As with osteoporosis, the exact cause of Paget’s disease is unknown. It is thought that Paget’s disease is caused by a combination of genetic and environmental causes, or possibly a virus. No single genetic abnormality has been discovered but between fifteen and forty percent of persons affected have a relative suffering from Paget’s disease.

2.2.3. Microgravity Bone Loss

The last pathology resulting in decreased bone mass and compromised structural integrity is caused by exposure to a weightless environment. The primary factor that causes bone remodeling is a change in mechanical stimulation, and when astronauts are exposed to the weightless environment of space, the induced bone remodeling leads to decreased bone mineral density.

Virtually every astronaut on a mission longer than thirty days has experienced bone loss in a region of the skeleton. Prolonged space flight can cause a decrease in bone mass in the vertebrae, femur, pelvis, and hip at a rate up to 0.5% of bone mass lost per month. The lack of loading on the bones can cause a significant amount of bone loss, and the ensuing recovery takes two to three times the duration of the mission.

Microgravity bone loss does not involve specific pathological changes within the body. The decrease in bone density occurs as part of a natural remodeling process,
without mechanical loading on the bone, the body resorbs the tissue due to a lack of need
2, 27. Smith et al. showed that bone resorption increased approximately fifty percent
during space flight 4. Weight-bearing bones experience the most loss due to the severe
change in the loading conditions, while nonweight-bearing bones undergo less resorption
27. A study performed in conjunction with a three-month space flight displayed an
average decrease in bone mineral density of 2.5%, 8.2%, 5.0%, and 6.2% in the tibia,
greater trochanter, femoral neck, and lumbar vertebrae respectively 3.

In addition to a lack of mechanical stimulation there are a few other conditions of
space travel that lend to a decrease in bone mass. During spaceflight there is a lack of
exposure to UV light, diminishing the reserves of vitamin D within the body 2. Also
decreased levels of calcium absorption and hormones in the blood occur in response to
bone resorption, limiting subsequent formation 2. The body loses about 250mg of calcium
per day, mainly due to increase in urinary calcium excretion 4. NASA and other
spaceflight organizations have implemented many different countermeasures to prevent
bone loss. Mainly exercise, increased calcium or phosphate intake, vitamin D
supplementation, exposure to UV light, and administration of early generation
bisphosphates, but all of these measures proved ineffective 2.
CHAPTER THREE
AVAILABLE BONE THERAPEUTICS AND CURRENT TREATMENT AND DELIVERY RESEARCH

3.1. Anti-Catabolic Therapeutic Approaches:

An In Vitro / In Vivo and Clinical Overview

Diseases of bone resulting in mineral loss and an increased risk of fracture can be detrimental to the patient as well as the health care system, due to such high costs. But there are many ways to treat these diseases including some diet and lifestyle changes. There are many nutrients that can affect bone and calcium homeostasis, such as calcium, vitamin D, phosphorous, and sodium. Adequate dietary intake of calcium is essential for sufficient bone mineral density for it slows the rate of bone resorption. Vitamin D plays a major role in preventing calcium deficiency through direct effects on the intestines, kidney, parathyroid gland, and bone. Circulation of 25-hydroxyvitamin D (25 [OH]D) indicates the status of vitamin D activity, levels of which should be between seventy-five and one hundred nanomolar per liter for optimal fracture prevention. In addition to dietary changes, non-pharmalogical lifestyle changes can aid in the prevention and treatment of such bone diseases. It has been shown that weight-bearing exercises such as aerobics can help postmenopausal women prevent at least two percent of bone mineral density loss.

Though dietary and lifestyle changes can help with treatment, many times patients need a therapeutic approach to either prevent bone loss, or help lay down new bone
tissue. There are three basic criteria used in the initiation of pharmacologic treatment of osteoporotic bone loss. [1] History of an osteoporotic vertebral or femoral fracture, [2] a T score equal to or worse than -2.5 in the lumbar spine, femoral neck, or total hip region, lastly [3] a T score from -1.0 to -2.5 with a risk of osteoporotic fracture of at least twenty percent. The National Osteoporosis Foundation recommends that a patient meeting any one of the aforementioned three criteria be considered for pharmacological treatment.

There are two main methods for the treatment of bone diseases such as osteoporosis, Paget’s disease, and microgravity bone loss; these are an anti-catabolic and an anabolic approach. Anti-catabolic bone treatments block the activity of osteoclast cells to prevent bone resorption and remodeling. This approach does not increase the amount of bone; its focus is to prevent any further pathological loss of bone structure. Anabolic methods of treatment focus on inducing osteoblasts to lay down new ECM, to be mineralized and increase the skeleton bone mineral density. The goal of these therapies is to replace any lost tissue with new mineralized bone. There are multiple anti-catabolic and anabolic therapies either in current use or being researched.

3.1.1. Bisphosphonates

The most common drug therapy available for treatment of bone loss is a class of anti-catabolic drugs called bisphosphonates, these therapies are considered as a first-line treatment. They work to inhibit bone remodeling, including both the resorption and formation processes. This results in reduced cortical porosity, improved bone mineralization, and structural maintenance of trabecular and cortical bone.
Bisphosphonates act to prevent future bone loss and retain the current bone structure. An overview of current commercially available bisphosphonates, such as alendronate, risedronate, ibandronate, and zoledronic acid, can be found in the table below. Some additional bisphosphonate formulations are clodronate, etidronate, incadronate, minodronate, olpadronate, pamidronate, and tiludronate.

Bisphosphonates have a marked affinity to bind to the surface of calcium phosphate hydroxyapatite crystals. Bisphosphonate molecules thermodynamically prefer to be bound to calcium phosphate, creating a strong binding preference. These bisphosphonates surround the bone and act directly on osteoclasts by inhibiting recruitment and adhesion to the mineral matrix, shortening the osteoclast lifespan and directly inhibiting the cellular activity. Through all these methods, bisphosphonates prevent further bone loss once engulfed by the catabolic cells during remodeling. Bisphosphonates contain a phosphate – carbon bond that is resistant to most chemical agents and is inert to enzymatic degradation. Since these molecules cannot be broken down, they accumulate in the osteoclasts and result in substantial toxicity. In vitro bisphosphonates alter the osteoclastic activity by inhibiting the initial act of bone remodeling, formation of pits in the mineralized substrate. Bisphosphonates produce the same effect in vivo, but there is a poor correlation between in vitro and in vivo bisphosphonate efficacy. In normal animal models bisphosphonates blocked the degradation of both bone and cartilage tissue by suppressing the remodeling process. This effect results in a radiologically denser club-shaped bone. Though bisphosphonates do
**Table 2 | Bisphosphonate Overview.** Currently available bisphosphonate therapies.

<table>
<thead>
<tr>
<th>Bisphosphonate</th>
<th>Brand Name / Company</th>
<th>Delivery</th>
<th>Indications</th>
<th>Bioavailability</th>
<th>Adverse Effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alendronate</td>
<td>Fosamax / Merck</td>
<td>Oral</td>
<td>Prevention</td>
<td>5mg daily or 35mg once weekly</td>
<td>Esophageal and gastric irritation, esophageal ulcerations, perforations and bleeding events. Cautious use with ASA or NSAIDs</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Prevention and treatment</td>
<td>10mg daily or 70mg once weekly</td>
<td></td>
</tr>
<tr>
<td>Alendronate +</td>
<td>Fosamax Plus D /</td>
<td>Oral</td>
<td>Treatment</td>
<td>70mg + 2800 units per week or 70mg +</td>
<td>Musculoskeletal pain</td>
</tr>
<tr>
<td>cholesterol</td>
<td>Merck</td>
<td></td>
<td></td>
<td>5600 units per week</td>
<td></td>
</tr>
<tr>
<td>Risedronate</td>
<td>Actonel / Warner</td>
<td>Oral</td>
<td>Prevention and treatment</td>
<td>5mg daily, 35mg once weekly, 75mg in</td>
<td>Esophageal and gastric irritation, esophageal ulcerations, perforations and bleeding events. Cautious use with ASA or NSAIDs</td>
</tr>
<tr>
<td></td>
<td>Chilcott</td>
<td></td>
<td></td>
<td>two consecutive days of the month, or 150mg once monthly</td>
<td></td>
</tr>
<tr>
<td>Risedronate +</td>
<td>Actonel with calcium /</td>
<td>Oral</td>
<td>Prevention and treatment</td>
<td>Weekly dosing: 35mg Actonel then 6 days 1250mg calcium</td>
<td>Muscular / joint pain, constipation, nausea</td>
</tr>
<tr>
<td>calcium carbonate</td>
<td>Warner Chilcott</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ibandronate</td>
<td>Boniva / Genentech</td>
<td>Oral</td>
<td>Prevention and Treatment</td>
<td>150mg once monthly</td>
<td>Esophageal and gastric irritation, esophageal ulcerations, perforations and bleeding events. Cautious use with ASA or NSAIDs</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Intravenous</td>
<td>Treatment</td>
<td>3mg every 3 month</td>
<td>Musculoskeletal pain, pain in extremity, diarrhea, headache</td>
</tr>
<tr>
<td>Zoledronic Acid</td>
<td>Reclast / Novartis</td>
<td>Intravenous</td>
<td>Prevention and treatment</td>
<td>Prevention: 5mg every two years Treatment: 5mg annually</td>
<td>Muscular / joint pain, pyrexia, flu-like illness, pain in extremity, nausea, vomiting, diarrhea, eye inflammation</td>
</tr>
</tbody>
</table>
not act to increase bone formation, they often result in a small increase in bone mineral density. This is caused from an uncoupling of the resorption and formation processes. In normal bone, the amount of resorption and formation are kept in check with each other. But when the resorption process is blocked, these two processes because temporarily uncoupled, allowing formation to continue for a period of time, resulting in a small increase in bone density. X-ray imaging is commonly used to determine the effectiveness of bisphosphonates by viewing the change in density and shape, known as the Schenk assay. This effect was also seen with all available bisphosphonate formation in in vivo osteoporotic mouse models. The mechanical properties of bone treated with bisphosphonates were tested in both normal and osteoporotic animal models. It was shown that, when given in appropriate doses, an improvement in bone biomechanical properties in both normal and diseased models was observed. Bisphosphonates are shown to improve torsional torque, ultimate bending strength, stiffness, maximum elastic strength and Young’s modulus of elasticity.

Bisphosphonates are the most common treatment in its drug class, coming in both oral and intravenous formulations. They are seen as an appropriate and cost-effective therapy for both the treatment and prevention of osteoporosis. Clinical studies have shown that bisphosphonates can increase vertebral bone mineral content by 5.3%, while patients receiving the placebo experienced a 2.7% loss of mineral density. These drugs also efficiently reduce the rate of fracture after only fifty weeks of treatment; fracture rates were decreased from fifty-four to six fractures per every 100 patients. Since these drugs have such a high affinity for the calcium phosphate crystals within the bone, it has
been shown that once treatment ceases, the beneficial effects can been seen for up to five additional years. This high binding affinity due to a thermodynamic driving force is enough to allow the bisphosphonates to efficiently target bone, due to the exponentially higher calcium phosphate concentrations than elsewhere in the body. Though these molecules effectively target bone tissue, oral bisphosphonates have a poor bioavailability, with less than one percent of drug reaching the bone. Intravenous bisphosphonate formulations, on the other hand, allow about half of the dose to be taken up and retained by the skeleton.

In addition to the reduction of bone resorption, bisphosphonates are also used to treat hypercalcemia, or increased blood calcium levels, in metastatic cancer patients to prevent osteoid deposit in cancerous tumors. These drugs have been shown to lower blood serum calcium levels by preventing osteoclasts from removing calcium reserves from the bone and depositing them in the blood supply. A side effect of this therapy though can be a pathological lowering of the blood calcium levels, hypocalcemia, which can cause uncontrolled muscle contractions and cardiac arrhythmias.

These drugs are approved for long-term use, but there are some adverse effects that have been seen. The only side effect common to oral bisphosphonates is the inflammation and ulceration of the upper gastrointestinal tract, which can be avoided by using intravenous formulations. The most common side effect with bisphosphonate use is acute inflammatory reactions, characterized by mainly by a low-grade fever. Recently it was reported that fifteen to thirty percent of patients receiving bisphosphonates experience a fever after an intravenous injection, with the risk varying due to patients'
underlying diseases. More serious systemic side effects associated with bisphosphonates are ocular inflammation, atrial fibrillation, idiopathic fractures of the femoral diaphysis, renal failure, and osteonecrosis of the jaw. In clinical trials these complications generally occurred in less than two percent of patients, but recently the occurrence has increased. Ocular complications such as conjunctivitis, scleritis, eyelid edema, and inflammation have an estimated incidence of 0.05%. Bisphosphonates have also been linked to increasing the chance of atrial fibrillation from a 0.5% risk to a 1.3% risk. Patients have also experienced subtrochanteric and diaphyseal fractures, both of which have an unknown cause, but a link has been made to bisphosphonate use. Though uncommon, the FDA has recommended bisphosphonate manufacturers alter the warning label on currently available medicines. Some of the more serious complications, renal failure and osteonecrosis of the jaw, are more commonly seen in patients receiving high bisphosphonate doses for the treatment of metastatic cancer. Bisphosphonates are excreted unchanged by the kidneys, processing of these molecules is shown to cause damage to the proximal tubule and cause acute tubular necrosis. With continued bisphosphonate use, renal failure can commonly occur, but with careful monitoring and dosing these chances can be lowered. In addition to renal failure, the higher doses of bisphosphonate treatments commonly given to cancer patients are known to cause osteonecrosis of the jaw. This side effect is only seen in healthy patients at a rate of one out of every 10,000 to one of every 100,000 cases. But recent statistics estimate that approximately two percent of cancer patients being treated with intravenous bisphosphonates develop osteonecrosis of the jaw. Despite the recent increase in
systemic side effects, bisphosphonates are still the most commonly prescribed treatment for bone diseases due to the effective retention of osteoporotic bone mass, slowing of bone turnover in Paget’s disease, and lowering of blood calcium levels in patients with cancer.\textsuperscript{43, 47, 65, 66}

3.1.2. Hormone Therapy

Estrogen, and the lack thereof, plays a large role in the onset of post-menopausal osteoporosis, and can also be used as a treatment option to prevent further development of the disease.\textsuperscript{43} Estrogen hormone therapy is an anti-catabolic treatment shown to not only preserve current bone mass, but the first one to two years of treatment often result in an increase in measured bone density.\textsuperscript{53} Currently, estrogen treatment therapies come in various formulations and methods of delivery.\textsuperscript{43, 53}

On the cellular level, estrogen binds to osteoclast receptors and stimulates the release of mediators that block further osteoclast activity.\textsuperscript{43} In the initial weeks of therapy, biochemical markers of bone resorption slowly decline, followed by a slow and delayed decline in bone formation markers.\textsuperscript{53} In addition to a decreased resorption, estrogen also acts to rapidly reduce the activation of new remodeling sites within the bone.\textsuperscript{53}

Estrogen hormone therapy can be used for both the prevention and treatment of osteoporosis in post-menopausal women. It is shown to reduce the occurrence of both vertebral and non-vertebral fractures by thirty percent. Since estrogen is a naturally occurring molecule, it is distributed evenly throughout the body protecting the whole
Estrogen therapy has many proven benefits, but recently many serious side effects have been discovered. The Women’s Health Initiative performed a randomized trial to investigate potential effects of long-term estrogen therapy (HERS); their results eliminated hormone therapy as a first-line option. Hormone replacement therapy was linked to an increase in thromboembolic event and strokes, as well as an increased risk of breast cancer \(^\text{10, 44, 56, 68, 69}\). Though estrogens used for the treatment of postmenopausal osteoporosis only have the biological potency of one-fourth to one-fifth of modern contraceptives, they have similar thrombotic risks \(^68\). In a placebo-controlled study, women in the placebo group had thromboembolic events occur 2.3 times per 1000 women-years. While the group receiving hormone therapy experienced an elevated risk of 4.0 per 1000 women-years \(^68\). As for the increased risk of breast cancer development there is no direct evidence, only observational correlations. It has been proposed that hormone replacement therapy can act as a promoter of already initiated breast tumors \(^70\). This is evidenced by the 8.8% annual decline in breast cancer occurrence with a 6.8% reduction in the use of hormone replacement therapy \(^70\). One group proposed that women receiving hormone replacement therapy have a 2.5 times higher risk of developing breast cancer \(^69\), while another estimated a twenty to fifty percent higher risk \(^80\) than those who do not receive this treatment. Because of these results, The North American Menopause Society and The Endocrine Society recommend hormone replacement therapy not be used for chronic disease prevention because the risks far out weight the benefits \(^43, 56\). They also recommend that estrogen therapy only be used for women at low risk for coronary heart disease, various cancers, and stroke who
are recently menopausal, these patients have a more favorable benefit to risk ratio \(^{43,56}\).

After the HERS study the prescribing of hormone therapy has steadily decreased, and more emphasis has been put on other forms of osteoporosis treatment \(^{73}\).

### 3.1.3. Estrogen Agonist / Antagonist

Another type of hormone-related anti-catabolic therapy for the treatment of various bone diseases is an estrogen modulator, raloxifene. Raloxifene, made by Eli Lilly & Co., marketed under the name Evista, is approved as a first-line treatment of both prevention and treatment of bone loss \(^{43}\). This benzothiopene derivative reduces bone resorption by mimicking estrogen’s beneficial effects within bone, while having an anti-estrogen effect on the breast and uterus. These estrogen modulators also tend to lower serum cholesterol levels \(^{43,57}\). Raloxifene is commonly prescribed because it provides the beneficial aspects of estrogen hormone therapy without the associated serious adverse effects.

Raloxifene acts by binding to estrogen receptors in the body and inhibiting the effects of estrogen on the uterus, while inducing estrogen-like effects on bone tissue \(^{57}\). In osteoporotic mouse models, raloxifene blocked the decline of bone mineral density, when compared to non-treated controls, at concentrations as low as 0.1mg/kg \(^{57}\). The ensuing effect on the bone was indistinguishable from those in animals treated with estrogen therapy \(^{57}\). While this drug had similar effects on the bone as estrogen, the two treatments diverged because raloxifene lacked a significant effect on the uterus and breast in that they do not increase the risk of cancer in these tissues \(^{57}\). Hormone therapy is known to
increase the risk of breast cancer, endometrial, or uterine, cancer was also common before the addition of progestin to estrogen treatments 57.

Raloxifene has been shown to improve bone mineral density in the lumbar spine, lowering the risk of vertebral fractures thirty to fifty percent, but there is no evidence for the protection from non-vertebral fractures 43. In addition to raloxifene’s effect on the bone, it has also been shown to decrease the chance of cancer in the breast and uterus 43. A study on breast cancer, called the Continuing Outcomes Relevant to Evista (CORE), found a fifty-nine to sixty-six percent reduction in the risk of breast cancer after eight years of treatment due to the drug’s anti-estrogen effects 46. Though this treatment reduces cancer risk, unlike estrogen, due to raloxifene’s similarity to estrogen, it has some of the same associated risks such as an increase in thromboembolic events 43, 56. For this reason The North American Menopause Society recommends the use of raloxifene for younger women who are more at risk of vertebral, rather than hip fractures. Also younger women are much less likely to have comorbid illnesses that can contribute to the risk of a thrombus formation 43, 56.

Currently, Pfizer, in collaboration with Wyeth and Ligand Pharmaceuticals, is performing clinical trial to get approval by the Federal Drug Administration for two new estrogen agonist / antagonist. Pfizer and Ligand Pharmaceuticals have been working to get FDA approval on a new hormone treatment called lasofoxifene 43. Under the brand name Fablyn, lasofoxifene has been sold in the European Union since its approval in March 2009 43. In clinical trials lasofoxifene was associated with a reduction in the risk of vertebral fractures, non-vertebral fractures, estrogen receptor-positive breast cancer,
major coronary heart disease events and stroke. Lasofoxifene has the potential to be a better treatment than raloxifene because it has proven beneficial effects on the non-vertebral fracture occurrence. Pfizer is also working with, their now subsidiary, Wyeth to obtain approval on another estrogen modulator bazedoxifene. Bazedoxifene has been shown to have similar results as raloxifene in reducing vertebral fractures up to forty-two percent but shows no reduction in the risk of non-vertebral fracture in average osteoporotic patients. Bazedoxifene does lower the incidence of non-vertebral fracture in patients at elevated risk, an advantage over raloxifene. Currently in Phase III clinical trials, bazedoxifene will be marketed in the United States with the brand name Vivant. Again, this therapy is already approved for sale and use in the European Union under the name Conbriza.

3.1.4. Calcitonin

Calcitonin is a polypeptide hormone used as an anti-catabolic second-line treatment for patients who do not respond or have intolerable reactions to first-line treatments. Calcitonin is used to inhibit further bone resorption normally in women who are more than five years post-menopausal. Calcitonin was also the first effective treatment for Paget’s disease; it lowers biochemical markers for bone turnover thirty to fifty percent, relieves bone pain, and also leads to healing of pagetic lesions. Calcitonin is commercially available as a nasal spray under the names Fortical and Micacalcin by Upsher-Smith Pharmaceuticals and Novartis, respectively. But these therapies are not
commonly used because though they are one of the safest therapies, they are also far less effective and have a much shorter duration than other treatments 54-56.

Calcitonin treatment acts directly on osteoclasts to inhibit both basal and stimulated resorption by causing a rapid loss of the cell’s ruffled border. This peptide induces an acute cessation of osteoclast cytoplasmic motility by gradual pseudopodial retraction, leading to an inability to transform the plasma membrane to an active conformation 54. Calcitonin also inhibits osteoclastic acidic secretion of tartrate-resistant acid phosphatase (TRAP) and Na⁺-K⁻-ATPase necessary for mineral and collagen breakdown 54. Studies have shown that with an extended treatment term, the number of osteoclasts eventually decreases 54. This defined cascade of biological responses to calcitonin results in a reproducible repression of bone resorption 54.

Calcitonin produces effective cessation of bone resorption, but this effect generally does not last more than 24 hours, of which the direct affect on osteoclasts’ function membrane and secretions only lasts for several hours 54. Osteoclasts resume normal function in between calcitonin uses, causing this reproducible biochemical process to have variable results 54, 55. When prescribed and used correctly, calcitonin is able to prevent bone loss and lower the incidence of vertebral fracture, but it is seen as clinically impractical due to a need for strict patient compliance 54. It is generally only used for women at least five years post-menopausal who either cannot or choose not to use more potent therapies 43.

3.1.5. Denosumab
The last anti-catabolic therapy for the treatment of bone loss is denosumab, an antibody that interacts with biological proteins in order to prevent bone resorption\textsuperscript{43}. This treatment is made by AMGEN and marketed under the brand names Prolia and Xgeva. Though this isn’t a common treatment for osteoporosis, it has been shown to be very effective at blocking bone loss.

Denosumab is a human monoclonal antibody that binds to the receptor activator of nuclear factor-kappa $\beta$ ligand (RANKL). The protein RANKL is essential to the formation and function of osteoclasts, with the binding of denosumab this protein is deactivated\textsuperscript{43}. The inhibition of this protein leads to a blockage of bone resorption\textsuperscript{43}. Denosumab works in a similar way as bisphosphonates, this antibody targets a different step in the bone remodeling process, but had produced very similar results in animal trials\textsuperscript{43}.

A clinical trial was performed called The Fracture Reduction Evaluation of Denosumab in Osteoporosis Every Six Months (FREEDOM) evaluating the efficacy of the therapy. A three-year randomized double-blind study with 7868 participants reported significant success. Denosumab reduced the risk of vertebral fracture by sixty-eight percent, hip fracture by forty percent, and resulted in a twenty percent reduction in all other non-vertebral fractures\textsuperscript{45}. This anti-catabolic treatment only has a few side effects, mainly back and muscle pain\textsuperscript{56}. A possible complication associated with denosumab, is the lack of studies on the long-term effects of disrupting the RANKL receptor sites. It is known that these ligands also play a role in the immune system\textsuperscript{43}.
### Table 3 | Available Anti-Catabolic Treatments

Currently available anti-catabolic therapies.

<table>
<thead>
<tr>
<th>Therapy</th>
<th>Brand Name / Company</th>
<th>Delivery</th>
<th>Indications</th>
<th>Bioavailability</th>
<th>Adverse Effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hormone Therapy</td>
<td>Many</td>
<td>Oral</td>
<td>Treatment</td>
<td>0.625mg daily</td>
<td>Abdominal pain, headache, heart attach, stroke, pulmonary emboli, breast cancer</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Transdermal</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Estrogen Agonist / Antagonist</td>
<td>Evista / Eli Lilly &amp; Co.</td>
<td>Oral</td>
<td>Prevention and treatment</td>
<td>60mg daily (up to 40 months)</td>
<td>Leg, cramps, hot flashes, thromboembolic events</td>
</tr>
<tr>
<td>Calcitonin</td>
<td>Miacalcin / Novartis Pharmaceuticals</td>
<td>Subcutaneous/intramuscular</td>
<td>Treatment</td>
<td>100IU daily</td>
<td>Nausea, local inflammatory reaction, vascular flushing</td>
</tr>
<tr>
<td></td>
<td>Fortical / Upsher-Smith Laboratories</td>
<td>Nasal</td>
<td></td>
<td>200IU daily</td>
<td>Nasal discomfort</td>
</tr>
<tr>
<td></td>
<td>Miacalcin / Novartis Pharmaceuticals</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Denosumab</td>
<td>Prolia / AMGEN</td>
<td>Subcutaneous</td>
<td>Treatment</td>
<td>60mg every 6 months</td>
<td>Muscular pain</td>
</tr>
<tr>
<td>Teriparatide</td>
<td>Forteo / Eli Lilly &amp; Co.</td>
<td>Subcutaneous</td>
<td>Treatment</td>
<td>20µg daily (up to 2 years)</td>
<td>Nausea, hypotension, hypercalcemia</td>
</tr>
</tbody>
</table>

### 3.1.6. Benefits and Limitations of Anti-Catabolic Treatments

Each year 1.5 million people in the United States suffer from an osteoporotic-related fracture, making it essential to preserve bone mass and structural integrity while reducing the risk of a traumatic fracture. Anti-catabolic therapies are the most widely prescribed treatments in the United States for the treatment and prevention of...
osteoporosis, mainly bisphosphonates. These anti-catabolic treatments are extremely effective in lowering the risk of both vertebral and non-vertebral fractures and ceasing the bone resorption process. Risk of fracture is lowered up to sixty-eight percent and in some cases the bone mineral density can even increase around five percent. In some treatment forms, such as estrogen agonist / antagonists, these effects can be seen with very low dosages and after less than one year of treatment. Though these treatments are very effective and commonly used, they focus on retaining the density and structure the bone has at the initiation of treatment. If therapy is started at a more progressed stage of the disease, anti-catabolic treatments may not be as effective. Since these drugs act to prevent further damage they are very effective when administered early in the disease progression, but for patients with severe bone loss, an anabolic therapy may be more desirable.

3.2. Anabolic Therapeutic Approaches:

An In Vitro / In Vivo and Clinical Overview

3.2.1. Teriparatide

Parathyroid hormone naturally plays a role in the body by maintaining calcium levels and stimulating both bone formation and resorption. As a therapeutic treatment, recombinant forms of parathyroid hormone have been shown to have anabolic properties and act to rebuild bone tissue. Currently, the available parathyroid hormone therapy is a recombinant intravenous injectable drug teriparatide, made by Eli Lilly &
Co., with the brand name of Forteo. There are also competitor forms of recombinant parathyroid hormone in clinical trials 43, 58.

Teriparatide and recombinant forms of parathyroid hormone act in a similar manner as the natural molecule, they act to activate bone turnover throughout the body 43, 56, 58, 59. Studies have shown that biochemical markers for bone turnover are elevated throughout the blood indicating a global response to the treatment. Also the increase in bone mineral density seen as a result of the therapy proves a continuous effect on the coupling of formation and resorption, causing a balance in the favor of formation 59. The early increase in bone mineral density suggests a rapid stimulation of osteoblastic activity through the activation of existing osteoblasts and the induction of differentiation of bone-lining cells 59. Another advantageous effect seen from the use of teriparatide is the lack of influence of other hormones. Fluctuating levels of estrogen and testosterone constantly influence natural parathyroid hormone, while the responsiveness of osteoblasts was unchanged with teriparatide 59.

In application, teriparatide has shown strong clinical success in the induction of bone growth and remodeling. It has been shown that the bone mineral density of the spine is increased by 8.6% and that of the femoral neck increased by 3.5% 43. Also, more significantly, a sixty-five percent reduction in vertebral fracture and a fifty-three percent decrease in non-vertebral fracture risk were seen with the use of teriparatide treatment 43, 76. In a study comparing teriparatide and bisphosphonate, the bone mineral density measurements and biochemical markers for bone turnover were higher with teriparatide treatment 86. It was thought that the anti-resorptive action of bisphosphonates could be
combined with the anabolic effects of teriparatide. But this same study demonstrated that when used together, bisphosphonates hinder teriparatide’s ability to increase bone formation, indicating that resorption plays a large role in teriparatide’s ability to enhance bone formation. A possible issue with this form of treatment may arise from future studies. There are currently no studies proving the long-term efficacy and safety of teriparatide. Because of this lack of data, teriparatide is generally only prescribed to patients with extremely low bone mineral density and for only a maximum of two years. Upon the cessation of this hormone treatment, without further treatment many patients experience substantial bone loss. Commonly, bisphosphonates are required to prevent loss of the newly formed bone tissue. In a study of patients undergoing one year of treatment with teriparatide then one year of alendronate therapy showed increased in bone mineral density in both cortical and trabecular bone. Cancellous bone experienced a thirty-one percent increase in volumetric mineral density, all of which was retained due to the anti-resorptive bisphosphonate administration.

Another form of recombinant parathyroid hormone is under investigation for possible approval by the FDA for use in the United States. NPS Pharmaceuticals formulated a fully human recombinant hormone, PTH (1-84). This new drug is shown to reduce the risk of new or worsened vertebral fractures. In a study of 2532 postmenopausal women, PTH (1-84) increased the bone mineral density of the spine and hip 6.9% and 2.1%, respectively. These increases in density were accompanied by a concurrent loss in bone mineral density of the forearm as well as an increase in
hypercalciuria, hypercalcemia, and nausea by 24%, 23%, and 14% respectively\textsuperscript{58}. This treatment is currently approved for use in the European Union\textsuperscript{43}.

3.2.2. Statins

Statins are a class of commonly prescribed drugs that act as competitive inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase in order to decrease hepatic cholesterol biosynthesis. These molecules acts as a rate limiting step in cholesterol synthesis by blocking conversion of HMG-CoA to mevalonate, resulting in lowered serum cholesterol levels for the treatment of high cholesterol and coronary heart disease\textsuperscript{12,49}. Recently, studies have shown that statins also increase the expression of bone morphogenic protein 2 (BMP-2) in osteoblasts, inducing osteoblast differentiation, and subsequently stimulating bone formation\textsuperscript{8,25,49}. These treatments are currently being investigated and researched by many different groups for possible use as a therapy for bone loss, reversing the acquired skeletal fragility.

Statins induce bone formation by enhancing expression of various biological proteins associated with bone cells. In culture of both murine (2T3) and human (MG-63) bone cells, statins enhanced the expression of the BMP-2 mRNA, which has been shown to be the most potent inducer of osteoblast differentiation\textsuperscript{12,25}. These cholesterol-lowering drugs also increased the expression of type 1 collagen mRNA while concomitantly lowering the expression of collagenase in MC3T3-E1 mouse osteoblast cells\textsuperscript{18}. Increased collagen availability further enhances the ability for newly differentiated osteoblasts to lay down osteoid. Alkaline phosphatase, necessary for bone
mineralization, concentration was also elevated in cells treated with statin drugs\textsuperscript{18, 20}. Osteoblastic differentiation, availability of collagen, and presence of alkaline phosphatase all act to increase the number of bone forming regions to allow an increase trabecular bone volume between thirty-nine and ninty-four percent\textsuperscript{12}. When tested in animal models, lovastatin, simvastatin, fluvastatin, and mevastatin all increased new bone formation two to three fold. This increase is comparable to treatment with BMP-2, the main component inducing osteoblast activity\textsuperscript{12}. While BMP-2 is highly effective in inducing bone formation, it is not an ideal treatment because it is very expensive to manufacture\textsuperscript{84}.

One of the few clinical studies performed with statins consisted of 1003 postmenopausal women in the United Kingdom. The results showed that the bone mineral density seen in both the hip and the spine remained significantly higher in the experiment group as compared to the control\textsuperscript{42}. Though much of the clinical information on statins comes from observational studies, it is enough to provide a solid correlation between the use of statins and the reduced risk of fracture and increased bone mineral density.

3.2.3. Stronium Ranelate

The last treatment option widely researched is strontium ranelate, a conjugate of strontium and ranelic acid\textsuperscript{43, 60-63}. The mechanism of action behind strontium ranelate is not well known. \textit{In vitro} and \textit{in vivo}, it has shown both anabolic and anti-catabolic properties, it is proven to both increase bone formation and decrease resorption\textsuperscript{43, 60-63}. 
While not approved for sale in the United States, an oral form of strontium ranelate is marketed for use in prevention of vertebral and hip fractures abroad by Servier under the name Protelos 43.

Research into the direct mechanism of strontium ranelate has shown that treatment of mouse calvaria bone cells for twenty-two days resulted in increased expression of multiple osteoblast markers, as well as an increase in number of bone nodules 60. Strontium ranelate has also been associated with a decrease in the numbers of mature osteoclasts and bone resorption 60. It is thought that this decrease in bone resorption is achieved by strontium ranelate’s total disruption of the actin cytoskeleton of osteoclasts. The disruption of this protein structure prevents the cells from attaching to the apatite minerals, a necessary step in the mineral breakdown 60. Animal studies have also proven the efficacy of strontium ranelate as an anabolic bone agent. Intact rats given a dose of ninety milligrams per kilogram per day showed an increase in cortical and trabecular bone volume, microarchitecture, and total alkaline phosphatase activity, an indication of bone-forming activity 63. Strength of both cortical and trabecular bone increased, this was associated with improvements in the micro-architecture in both tissues 60.

In clinical trials, strontium ranelate decreased the risk of fracture as well as increased patients’ bone mineral density 61, 62. It was shown that a dose of two grams per day decreased the relative risk of non-vertebral fractures by sixteen percent 62. Among women with a high risk of fracture, the prevalence was reduced thirty-six percent in the hip and thirty-nine percent in the vertebrae 62. Strontium ranelate, at the same dosage,
increased the bone mineral density in the lumbar spine by 14.4% and in the femoral neck by 8.3% after thirty-six months of treatment 61.

3.2.4. Benefits and Limitations of Anabolic Treatments

The ability to enhance bone mineral density and repair pathological damage is an optimum treatment path for bone disease therapies. The only anabolic treatment approved for use in the United States is a form of recombinant parathyroid hormone, teriparatide 10, 43, 58. Teriparatide is approved for up to two years of use, resulting in increased bone mineral density up to 8.6% 43. Though teriparatide is very effective at increasing bone density, upon cessation of treatment bisphosphonates are needed to prevent continued breakdown of the tissue 74. Statins have shown remarkable success in the induction of bone formation through the increased expression of biological markers, but much more research must be completed before this can be considered a potential treatment for bone pathologies 12, 18, 25. Lastly, strontium ranelate acts in two ways to increase formation and decrease resorption and has shown increases in bone density 61. The mechanism behind this therapy is not well known or understood, but with further research strontium ranelate may be approved as an effective therapy by the FDA 60.

3.3. Controlled Delivery

3.3.1. Nanoparticle Delivery Vehicles
Nanoparticles are commonly investigated for their ability to transport drug therapies and release the treatments directly into the cell. These nanoparticles are so effective because they are much smaller than a cell; these particles are taken up by the cell, transported across the cell membrane, and released into the cytosol for delivery to cell organelles. Many materials are being researched for the fabrication and loading of nanoparticles. A large focus has been placed on polymers because of the lack of inflammatory or immune response, and the proven biodegradability of some bioinert polymers. For therapeutic delivery to bone tissue, an emphasis has been placed mainly on three different polymers, N-(2-hydroxypropyl)methacrylamide copolymer (HPMA), polyurethane (PU), and poly(lactide-co-glycolide)-poly(ethylene glycol) copolymer (PLGA-PEG).

HPMA copolymer nanoparticles were successfully formulated with a proper molecular size such that they were efficiently cleared from the body by the liver, with minimal accumulation in the heart and lungs. It has been shown that a larger molecular weight increased the particle accumulation in bone, but decreased the specificity of the targeted delivery. These HPMA particles were also used to deliver prostaglandin, known to have anabolic effects on bone, to the tissue with a large portion of the drug being release in the cellular osteoclast environment. Biodegradable PU scaffolds have also been a topic of research for the delivery of statins to bone tissue. The use of a scaffold prevents a large amount of the drug from being lost due to first-pass metabolism. It was shown that two hundred micrograms of lovastatin was a sufficient dose to enhance new bone formation without any adverse inflammatory reactions. Groups are
also looking into using known biocompatible polymers for bone drug delivery such as PLGA-PEG. PEG is commonly used in nanoparticle formulations to provide additional biocompatibility and increased blood circulation time compared to non-PEGylated particles. Avgoustakis et al. showed that PLGA-PEG particles remained in systemic circulation for hours, while particles without PEG conjugation were removed from circulation within minutes. It was also showed that altering the proportions of PLGA to PEG within the copolymer could change the biodistribution of the particles.

Rather than using inert polymers, an emphasis has also been placed on the use of physiological materials such as hydroxyapatite and other calcium orthophosphates for the delivery of drug therapies, especially to bone tissues. Hydroxyapatite and other calcium phosphate molecules are chemically similar to the inorganic component of bone, eliciting no immune response and allowing them to chemically bond to the bone tissue. Because of the chemical likeness to native bone tissue, the use of hydroxyapatite as a particle allows for targeted delivery for, these particles preferentially deposit in bone tissue. Once these particles bind to the bone they are actively transported into bone cells where the loaded drug can be released for treatment of various conditions. It has been shown that a portion of the particles escapes the phagocytic pathway and directly enters the cytoplasm for efficient quick delivery. In addition to allowing for targeted delivery of drug, the nano-crystalline structure of hydroxyapatite particles has been shown to stimulate bone proliferation and growth. For all these reasons hydroxyapatite and other calcium phosphate materials are seen as potential candidates for slow release vectors, while being investigated for drug delivery and bone stimulation.
3.3.2. Targeting Approaches

In order to enhance specificity of polymer and hydroxyapatite delivery to bone, many different targeting techniques have been investigated. Delivery specificity is extremely important because drug absorption in unwanted tissues can cause serious adverse effects, as was the problem with estrogen therapy \(^98\). There are two main approaches to locally targeted drug delivery with nanoparticles, passive and active targeting \(^11\). Passive targeting is often used for delivery to tumors because it utilizes characteristic physiological tumor biology. Tumors experience an increase in vascular fenestrations due to the enhanced permeability and retention effect, allowing for the increased delivery of nutrients to the tumor due to the accelerated growth rate \(^11\). Passive targeting relies on this increased permeability to allow for more nanoparticles to be delivered to the tumor site \(^11\). Though effective, passive targeting is difficult to control because of the randomness of the approach. For delivery to non-tumorous tissues active targeting must be utilized. Active targeting approaches use the conjugation of small molecules to the surface of the nanoparticles. These molecules are specially chosen to bind to overexpressed antigens or receptors at the site of targeted delivery \(^11\). Targeting agents generally fall within one of the following categories: proteins, including antibodies, nucleic acids, peptides, vitamins, or carbohydrates \(^11\).

Targeting of bone tissue would allow for particles to accumulate in the skeleton, improving the pharmacokinetic profile and therapeutic effects of the drug \(^98\). Bone is seen as a difficult target due to the low blood flow rate, only 0.05-0.2ml/min/g, and the tissue
Bone contains a large portion of type I collagen, but this would not be a suitable molecular target because it is ubiquitous throughout the body, so the mineral component, hydroxyapatite, remains the most suitable target. There are many molecules used to provide bone targeting specificity, mainly tetracycline, bisphosphonates, peptide sequences, and various bone proteins. Tetracycline is a crystalline amphoteric substance that has the ability to bind to the surface of bone apatite crystals by chelation with surface calcium ions. These molecules bind strongly to bone mineral with minimal dissociation, but tetracycline has a poor chemical stability during modification. This instability eliminates the ability to conjugate drugs to the targeting molecule, making tetracycline an unusable targeting agent. In addition to their therapeutic effects, bisphosphonates have such a high affinity for bone they are also used as a targeting molecule to deliver other drugs. Bisphosphonates have been successfully conjugated to free drug, HPMA, and PEG for controlled drug delivery. Bisphosphonates have also been altered to form a liposome to control the release of therapeutic agents from a scaffold material. The use of bisphosphonates allows for a cost effective and efficient method to target bone tissue, but these delivery vehicles must be specially designed to block the anti-resorptive effects of the bisphosphonate itself. Another common bone targeting agent is a D-aspartic acid octapeptide (Asp₈). This peptide sequence can distinguish between functional domains of the skeleton and preferentially bind to resorptive sites. In recent studies targeting with Asp₈ resulted in longer circulation half-lives, preferential binding to eroded surfaces, and targeted biodistribution with over sixty percent of particles binding to the skeleton.
Polymalonic acid has also been under investigation for use in a targeted drug delivery system because it has a strong affinity to bind to hydroxyapatite. Though it has been successfully modified on the surface of drugs, no therapeutic effects have been reported. Lastly, a few native bone proteins have been used as targeting agents for their ability to bind to available calcium ions. Osteocalcin and osteopontin are both negatively charged proteins that can bind to the positively charged calcium ions on the bone surface, though effective, these are rarely used.
CHAPTER FOUR

FORMULATION OF TARGETED HYDROXYAPATITE NANOPARTICLES FOR THE DELIVERY OF STATIN DRUGS

4.1. Introduction

The goal of this research was to develop a hydroxyapatite nanoparticle targeted drug delivery system for the treatment of pathological loss of bone mineral density with statin therapies. In order to enhance biocompatibility and prolong systemic circulation time the hydroxyapatite particles were functionalization with poly(ethylene glycol) (PEG) with a poly(glycolic acid) (PGA) linker. The final formulation of the nanoparticles consists of a hydroxyapatite nanoparticle functionalized with a diblock copolymer of PGA-PEG, resulting in nHA-PGA-PEG. It has been proven that alterations in the ratio between components of polymeric diblock copolymers can alter the accumulation, biodistribution, and circulation time in vivo. For this reason, alterations were made in the formulation of these coated nanoparticles as a proof of concept that the polymer ratios can be changed such that an optimum ratio can be determined. After synthesis toxicity studies were completed on control, non-cancerous human umbilical vascular endothelial cells (HUVEC) to prove the particles non-toxic on their own.

In order to develop a drug delivery system, the nanoparticle must be able to be loaded with the particular drug. The nHA-PGA-PEG particles were first loaded with a fluorescent dye and imaged to prove the ability to load the nanoparticles. Lovastatin, a common anabolic statin drug, was loaded into the nHA-PGA-PEG particles. A release
study was completed to determine the pharmacokinetics of the drug release from the newly formulated particles.

Lastly, another goal was to use a targeting molecule on the surface of the nanoparticles to enhance delivery to bone tissue. Experiments were performed on various bone tissue samples followed by an in vitro study proving that the particular targeting peptide chosen in fact preferentially binds to calcium phosphate minerals.

4.2. Materials

For the synthesis of nHA-PGA-PEG, nano-hydroxyapatite was purchased from SkySpring Nanomaterials. Also glycolide was bought from Sigma-Aldrich and methoxy-poly(ethylene glycol)-isocyanate was from Nanocs, Inc. The anhydrous dimethylformamide and phosphazene base P₂-t-Bu were also purchased from Sigma-Aldrich. Lastly, molecular weight cutoff centrifugal filter units were purchased from Millipore.

Toxicity studies were completed using well plates from Corning, endothelial growth media purchased from Lonza, and Presto Blue Cell Viability Reagent from Life Technologies.

In addition to the materials mentioned above, dye loading was completed with an Alexa Fluor 647 cadaverine from Life Technologies and acetonitrile purchased from Sigma-Aldrich.

Additional materials utilized for the drug release studies include lovastatin purchased from TCI America. HyPure water was purchased from Fisher Scientific, 3.5
kc MINI Dialysis Units from Thermo Scientific, and methanol was from Sigma-Aldrich. The HPLC Alltima C18 column was purchased from Grace Davison.


4.3. Experiment Methods

4.3.1. Synthesis of nHA-PGA-PEG

In order to synthesize the nHA-PGA-PEG particles 40 milligrams of nano-hydroxyapatite (nHA) and 464 milligrams of glycolide were weighed out and dried overnight under a 32 inches of mercury vacuum, in the same vial. Concurrently, 100 milligrams of methoxy-poly(ethylene glycol)-isocyanate (mPEG-isc) was dried in a separate container, also overnight. The following day, the dried nHA and glycolide were dissolved in 3 milliliters of anhydrous dimethylformamide (DMF), and stirred for 30 minutes at room temperature. Following stirring, 25 microliters of phosphazene base P$_2$-t-Bu solution was added. Both the reaction vessel and the dried mPEG-isc were purged with N$_2$, and the reaction was stirred overnight. The next day, the dried mPEG-isc was dissolved in 1 milliliter of anhydrous DMF and added to the reaction vessel. The vial
containing the reactive components was purged under N$_2$ and the reaction was stirred for an addition 24 hours. After the final stirring period, the contents of the reaction were washed twice in DMF using molecular weight cutoff (MWCO) centrifugal filter units at a speed of 5000 rpm for 5 minutes. After centrifuging, the supernatant was removed and the remaining pellet was re-dispersed before centrifuging again. Following both wash cycles, the samples were lyophilized for further analysis by thermogravimetric analysis (TGA) and Fourier transform infrared spectroscopy (FTIR).

4.3.2. Toxicity

HUVEC cells were seeded in 25 wells of a black, clear bottom, cell bind 96-well plate at a density of 10,000 cells/well in 200 microliters of endothelial growth cell media. Plates were then incubated overnight at 37°C and 5% CO$_2$ to allow the cells to adhere. The next day, 1 milligram of lyophilized nHA-PGA-PEG was weighed out. Solutions were made at concentrations of 0, 50, 100, 250, and 500 microgram nHA-PGA-PEG/milliliter cell media in a sterile hood. Then, 200 microliters of each nanoparticle concentration was added to each of 5 wells with HUVEC cells. The well plate was then incubated for 24 hours at 37°C and 5% CO$_2$. After 24 hours, the cell media containing nanoparticles was removed and 200 microliters of fresh media was added to each well. The plates were then incubated at 37°C and 5% CO$_2$ for another 48 hours. After the final incubation, the cell media is removed and 100 microliters of a 10:1 solution of cell media to Presto Blue Cell Viability Reagent was added to each well. The well plate was then incubated at 37°C and 5% CO$_2$ for 40 minutes covered from light. The fluorescence signal is then read using a
Biotek Synergy 4 fluorescent plate reader with Gen5 1.11 software with an excitation wavelength of 560 nanometers and an emission wavelength of 590 nanometers. The data is then analyzed by comparing the average fluorescent intensity of the samples with 0 microgram/milliliter nanoparticle concentration as 100% viability to the average intensity of samples incubated with nanoparticles.

4.3.3. Dye Loading

In order to load a fluorescent dye, Alexa Fluor 647 (AF 647) cadaverine was dissolved at 1 milligram/milliliter in an 80:20 solution of DMF to acetonitrile (ACN). nHA-PGA-PEG particles were the dissolved at 1 milligram/milliliter in the AF 647-DMF/ACN solution and stirred for 1 hour. The reaction solution was then dropped into water at a 1:2 nHA-PGA-PEG to water ratio and stirred for an additional 2 hours to evaporate the organic solvent. The nanoparticle solution was then washed twice in water with MWCO centrifugal filter units at 5000 rpm for 5 minutes. Again, after centrifugation the supernatant was removed and the remaining particles were resuspended in water before centrifuging again. Once washed, the nHA-PGA-PEG particles were re-suspended in phosphate buffered saline (PBS) and imaged using the IVIS small animal imaging system located in the Godley-Snell Research Center. An excitation wavelength of 640 nanometers was used with a Cy.5.5 emission filter. Fluorescent imaging was used to detect the presence of AF 647 dye encapsulated within the nHA-PGA-PEG particle.

4.3.4. Lovastatin Release
For the completion of the drug release study, 5 milligrams of lovastatin was dissolved in 1 milliliter of ACN. Then 1 milligram of nHA-PGA-PEG was weighed out and distributed evenly into five 1.5 milliliter microcentrifuge tubes. Then 200 microliters of lovastatin/ACN was added to each microcentrifuge tube, they were then wrapped in aluminum foil to protect them from light and rotated on a rotisserie for 1 hour. Next, 200 microliters of the nHA-PGA-PEG-lovastatin/ACN solution was dropped into each of five vials containing 400 microliters of HyPure water. These solutions were then stirred for 2 hours protected from light. The samples were then washed twice in water using MWCO centrifugal filter units as described previously. After washing, each sample was re-dispersed in 200 microliters of water.

The lovastatin-loaded nHA-PGA-PEG was added to the top of a 3.5 kD MINI Dialysis unit. The dialysis unit was then loaded into the top of a vial containing 7 milliliters of HyPure water. Samples were placed in an incubator at 37°C and 5% CO₂. At each designated time point aliquots were taking from the bottom of the vial, and the sample was then lyophilized.

High performance liquid chromatography (HPLC) was used to determine the amount of released lovastatin in each aliquot. The lyophilized samples were dissolved in 1 milliliter of methanol, then filtered with a 0.2 micrometer nylon syringe filter and extruded into amber 2 milliliter HPLC vials. HPLC was then performed with a Waters 1525 Binary HPLC pump with a 2998 photodiode array detector. An Alltima C18 column with 5 micrometer pores and dimensions of 250 x 4.6 millimeters was used. The mobile phase used was a 71% ACN, 29% 0.05 molar ammonium acetate at a pH of 4.0, titrated
with acetic acid. The flow rate of the HPLC system was set to 1 milliliter/minute and lovastatin was detected at a wavelength of 238 nanometers. Data is graphed at a cumulative release of lovastatin in micrograms over time as well as a percent cumulative release over time.

4.3.5. Peptide Targeting

A stock peptide-fluorescent molecule solution of 1 milligram/milliliter was diluted down to 0.2 milligram/milliliter in water. The peptide solution was then added to the deparaffinized tissue array and/or the hydroxyapatite and chicken bone sample. 5.5 microliters was added to the each sample on the tissue array and 50 microliters was added to the bone samples held within a clear non-sterile 96-well plate. The samples were then covered from light and incubated at room temperature for 2 hours to allow for peptide binding. After the incubation, the peptide solution was pipetted off the array sample and/or out of the well plate. The samples were then washed 3 times by pipetting water on and off the samples. The samples were then covered from light until imaging was done. Imaging was completed with the IVIS small animal imaging system at the Godley-Snell Research Center with an excitation wavelength of 535 nanometers and an emission filter of DSRed with a 1 second exposure. Data was analyzed by subtracting the fluorescent signal from blank samples, without peptide addition. In vivo studies were performed by injecting 1 milliliter of 0.2 milligram/milliliter fluorescent peptide solution intravenously according to approved animal protocols. Imaging was done 3 days after injection using the IVIS small animal imaging system with the same settings as mentioned above.
4.4. Experiment Results and Discussion

4.4.1. Synthesis of nHA-PGA-PEG

Synthesis of hydroxyapatite nanoparticles with a copolymeric coating of PGA-PEG was successful, resulting in biocompatible particles for the use in drug delivery applications. Figure 4 shows the results of a TGA of the nHA nanoparticles with a PGA coating. Various monomer-to-catalyst ratios were used in order to alter the thickness of the PGA coating on the nHA particles. The various decaying profiles display that each ratio resulted in a different thickness coating. This experiment was used as a proof of concept that the thickness of the PGA coating can be controlled during synthesis. This control is important because the size and molecular weight of particles can greatly affect the circulation time, biodistribution, and accumulation of particles\textsuperscript{98}. With the ability to control the coating thickness, an optimum thickness, and therefore molecular weight, of the nanoparticles can be determined. After successful functionalization of PGA on the nanoparticle surface, PEGylation was done in order to increase biocompatibility and circulation time. Figure 5 shows a TGA of just nHA, nHA-PGA, and nHA-PGA-PEGmal. The different decomposition temperatures of each sample prove that PEG was conjugated to the PGA. The PEG used in this formulation had a maleimide functional group; this end group was needed such that a targeting molecule can be further added to the nanoparticle coating.
**Figure 4 | Successful Variation of PGA Coating Thickness.** Various monomer to catalyst ratios were used to prove polymeric coating thickness can be controlled.

**Figure 5 | Conjugation of PEG to nHA-PGA.** PEGylation of nHA-PGA particles is successful, evident by the difference in decomposition temperature.
After successful synthesis of nHA particles with a PGA-PEG coating, TEM images were taken in order to view the size change due to the polymer coating. The image of the left in Figure 6 is a TEM of just nHA, as shown these structures have a much smaller diameter. The image on the right is of nHA-PGA-PEG particles. The thickness change is evident indicated a successful conjugation of PGA-PEG on the surface of the nHA particles.

4.4.2. Toxicity

Toxicity studies were performed to determine the interaction these nHA-PGA-PEG particles would have with a normal cell. HUVEC cells were used as a control for a noncancerous cell. These studies showed that these particles showed a slight, but not
detrimental toxicity in cells. The viability remained around 80% even at higher nanoparticle concentrations such as 500 micrograms/milliliter. For the purposes of this study, 80% viability was viewed as an acceptable toxicity level.

![Graph showing toxicity of nHA and nHA-PGA-PEG in HUVEC Cells](image)

**Figure 7 | Toxicity of nHA and nHA-PGA-PEG in HUVEC Cells.** Cell viability around 80% for elevated nanoparticle concentration indicates an acceptable toxicity.

4.4.3. Dye Loading

Upon successful formulation of nHA-PGA-PEG nanoparticles, it must be shown that these particles can be loaded with substance for eventual release. A fluorescent dye was used as a proof of concept that these particles can be loaded. The image below shows a solution of dispersed nHA-PGA-PEG particles loaded with fluorescent dye. As compared to the solution of non-loaded nHA-PGA-PEG, the fluorescent signal is much higher indicating that the dye was successfully encapsulated within the nanoparticles. On
the right is a chart comparing the actual fluorescent signal intensities, as shown the 
increase in signal is a direct correlation to the fluorescent dye loaded within the particles.

![Image of fluorescent dye comparison]

**Figure 8 | Dye Loading Used as Proof of Concept for Nanoparticle Loading Abilities.** Successful loading of a fluorescent dye proves the ability of the formulated nHA-PGA-PEG nanoparticles to be loaded with drug.

4.4.4. Lovastatin Release

The next step in the formulation of nHA-PGA-PEG nanoparticles for drug delivery was to load these particles with a desired therapy and monitor the pharmacokinetics of the release. An HPLC release study was conducted to determine the release profile of the newly formulated nHA-PGA-PEG nanoparticles. Figure 9 shows the cumulative release of lovastatin over a two-week time period. It was shown that the lovastatin was released linearly over time, with the entire drug reservoir being released within two weeks. This experiment was also done with three different PGA thicknesses.
The varying thickness resulted in different release profiles, proving that the release of lovastatin from these particles can be controlled by the thickness of the polymer coating.

**Figure 9 | Lovastatin Release from nHA-PGA-PEG Nanoparticles.** HPLC release study showed that nHA-PGA-PEG release kinetics can be controlled with variations in PGA coating. G200-PEG is 20 wt% PGA, G100-PEG is 40 wt% PGA, and G50-PEG is 60 wt% PGA.

4.4.5. Peptide Targeting

The final goal of this research was the use of a targeting molecule on the surface of the particles to enhance the specificity of the delivery to bone tissue. A peptide solution was used as a proof of concept that these peptides can bind to bone and will do
so preferentially. Experiments were first conducted using hydroxyapatite disks and samples of decellularized chicken bone to prove the ability of the peptide to bind to the bone surface. The binding abilities were then tested *in vivo* with a mouse model, the images on the right of Figure 11 show that even within the physiological environment these two peptides were able to bind to the bone surface. Though both peptides have binding abilities the HA-1 peptide had a stronger fluorescent signal. Moving forward the HA-1 peptide became the focus as a targeting molecule.

![Fluorescence graph](image)

**Figure 10 | Targeting Peptide Binding to HA Discs and Chicken Bone.** Binding abilities of bone-targeting peptides were tested using hydroxyapatite discs and decellularized chicken bone samples.
It is necessary for a targeting molecule to not only bind to the preferred tissue, but it must do so preferentially. An array of human tissue samples was used in order to determine the specificity of the HA-1 peptide. Figure 12 shows that the peptide binding to bone tissue was much higher than that of any other tissue. Proving the specificity of the HA-1 peptide was sufficient to provide a targeted delivery. This experiment also proved that this peptide does not have a tendency to accumulate in tissues such as the heart, lungs, or brain where the accumulation of unwanted particles could result in serious side effects.

**Figure 11 | In Vivo Testing of Bone-Targeting Peptide Binding.** *In vivo* experiments were performed to determine the binding abilities of targeting peptides in the physiological environment.
4.5. Conclusion of Results

The synthesis of nHA-PGA-PEG nanoparticles able to encapsulate and deliver drugs with controlled pharmacokinetics was achieved. Not only was a polymeric surface coating conjugation demonstrated, but also the thickness of the PGA can be controlled. This controllable thickness directly correlates to an ability to control the release profile of the nanoparticles, as shown with the variation in release profiles determined by an HPLC release study of lovastatin. The next step in the fabrication of these nanoparticles is the development of a targeting mechanism to prevent the delivery of statin drugs to other tissues in the body. Two peptides, made by members of the nanomedicine lab, were

Figure 12 | Preferential Binding of HA-1 Peptide to Bone Tissue. A human tissue array was used to determine the specificity of HA-1 binding to bone tissue.
tested for their efficacy and specificity of bone targeting. The HA-1 peptide demonstrated both *in vitro* and *in vivo* targeting abilities for the calcium phosphate mineral component of bone tissue. These were assessed using hydroxyapatite discs and decellularized samples of chicken bone as well as an *in vivo* study with the peptide solution injected intravenously. The *in vivo* images not only show that these peptides can bind in the physiological environment, but because these images were taken three days after injection, they also prove the peptides remain bound to the surface. These particles show promise for a potential therapeutic tool for the targeted delivery of drugs to the skeleton.
CHAPTER FIVE

CONCLUSIONS

The high prevalence of osteoporosis, as well as the deformity and pain of Paget’s and microgravity bone loss, is evidence that effective treatments are of a highest priority. Presently, there are many treatment options that can prevent further bone loss and even increase the bone mineral density slightly. More focus must be placed on anabolic treatments to allow the recovery and rehabilitation of lost tissue. Statins have promising results for the induction of bone formation by the increase in BMP-2 mRNA. With these nHA-PGA-PEG nanoparticles, these statin molecules could be delivered directly to the skeleton to allow for a quick and efficient increase in the natural formation process. These particles have proven abilities to be loaded with drug and release that loaded drug with a controllable profile. Hydroxyapatite particles are known to target bone due to the chemical likeness of the molecules, but to enhance the targeting, peptide sequences have been tested for binding efficiency and specificity. It is shown that both peptides tested can exclusively target bone and remain bound to the surface.

This research has many implications for potential drug delivery applications. The use of a hydroxyapatite core provides a particle with minimal immune response; then the addition of a polymeric coating enhances the biocompatibility and circulation time within the body. These developed nanoparticles provide an ability to controllably deliver lovastatin to increase bone formation with a targeting mechanism that allows these anabolic bone agents to be delivered to bone with both efficiency and specificity.
In all, these particles show promise for future development as a potential targeted drug delivery vehicle for the delivery of statins to the skeleton.
CHAPTER SIX
RECOMMENDATIONS FOR FUTURE RESEARCH

1. Conjugation of HA-1 peptide to the maleimide group at the end of the PEG segment of the polymeric PGA-PEG surface coating.
   a. Conjugation has been successful but the process must be optimized to provide both consistent and reproducible peptide conjugation.

2. Targeting and non-targeting uptake into osteoblast and control, HUVEC, cells.
   a. The goal of this experiment is to prove the efficacy of the targeting approach. The uptake of targeted particles into cells should be higher than that of untargeted particles.

3. In vivo animal studies of HA-1 targeted nHA-PGA-PEG particles.
   a. These animal studies will be used to prove that the targeted nanoparticles predominantly accumulate in the bone, while also having minimal accumulation in the other tissues such as the heart, brain, and lungs where calcification can be dangerous.
CHAPTER SEVEN

REFERENCES


