Electrospun Chitosan Membranes Loaded with Raspberry Ketone to Induce Differentiation in Preosteoblasts

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ELECTROSPUN CHITOSAN MEMBRANES LOADED WITH RASPBERRY KETONE TO INDUCE DIFFERENTIATION IN PREOSTEOBLASTS

by

Paul K. Cameron

A Thesis

Submitted in Partial Fulfillment of the Requirements for the Degree of

Master of Science

Major: Biomedical Engineering

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DEDICATION

This thesis is dedicated in loving memory to my grandmother, Elizabeth Cameron, and also to my parents who have supported me throughout my educational career.
ACKNOWLEDGEMENTS

Firstly, I would like to thank my advisor, Dr. Joel D. Bumgardner. Without his guidance and patience, this thesis would not have been possible. I would also like to thank the other members of my thesis committee, Dr. J. Amber Jennings and Dr. Judith Cole, for their invaluable knowledge and support. I also thank the entire Biomedical Engineering faculty at the University of Memphis for building a fantastic graduate program. My labmates, Dr. Fernanda Delbuque Guerra, Priya Murali, Hengjie Su, Blass Watson, and Kevin Patel, taught me how to use lab software, culture cells, and run assays. To them I extend my sincere gratitude. Lastly, I would like to thank my close friends and family, who were always there to remind me what I was capable of.
PREFACE

The main body of this thesis is a journal article entitled “Electrospun Chitosan Membranes Loaded with Raspberry Ketone to Induce Differentiation in Pre-Osteoblasts.” This manuscript will be submitted to the Journal of Tissue Engineering and Regenerative Medicine.
ABSTRACT

This study evaluated the potential of adding raspberry ketone (RK) to electrospun chitosan membranes (ESCMs) to create a bioactive guided bone regeneration (GBR) membrane capable of stimulating bone cell differentiation. In this study, W-20-17 cells exposed to 50-200 μg/ml RK showed an increased expression of alkaline phosphatase (ALP). RK was loaded onto ESCM discs modified by one of three different fatty acid anhydrides and release profiles were examined. It was found that the RK release profile was dependent on the type of fatty acid treatment used. RK loaded membranes were then evaluated for cytocompatibility and osteodifferentiation potential. While the RK released from the membranes had no cytotoxic effect on the cells, it did not induce osteodifferentiation. It is speculated that RK levels were not sustained at high enough concentrations to affect the cells. ESCMs, with a more sustained RK release profile, may have potential to be used in GBR applications.
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CHAPTER 1: INTRODUCTION

Problem Statement

Periodontal disease or trauma can lead to significant craniofacial bone loss. In most cases, to regenerate lost craniofacial bone, a bone grafting procedure is required. However, soft tissue grows faster than bone and tends to migrate into the graft site, leading to incomplete regeneration. To prevent this, a procedure known as guided bone regeneration (GBR) was developed in which a barrier membrane is placed over the graft site to prevent ingrowth of the faster-growing soft tissue. There are around 5,000,000 dental implants placed each year in the U.S. and it is estimated that 40% of these require GBR procedures\textsuperscript{1,2}. Current membranes made out of expanded polytetrafluoroethylene (ePTFE), collagen or aliphatic polyesters are not ideal, either being non-degradable, or having unpredictable degradation rates. Aliphatic polyester membranes such as poly(lactic acid) produce acidic degradation products, causing inflammation. Currently, GBR membranes have a relatively high (23-50%) complication rate\textsuperscript{3}. Complications include membrane exposure and infection which can lead to inadequate bone regeneration and longer treatment times\textsuperscript{3}.

Attempts to enhance bioactive properties of the membranes to augment bone regeneration, particularly with collagen membranes have been through addition of bone morphogenetic protein-2 (BMP-2). However, while BMP-2 is effective in accelerating bone growth, it is extremely expensive, shows poor release patterns, and has been associated with side effects such as ectopic bone formation, ankylosis, and bone resorption\textsuperscript{4,5}. Thus there is a clinical need to improve GBR membranes to provide effective barrier function while helping stimulate bone regeneration at the graft site.
Electrospun chitosan membranes have emerged as a promising alternative to current GBR membrane materials. Chitosan is biocompatible, biodegradable and has non-acidic degradation products. Electrospun chitosan membranes have been shown in *in vivo* rodent models to be biocompatible, provide effective barrier function, and have appropriate degradation rates. Furthermore, the nanofiber structure of the electrospun membranes provides high surface area that is potentially advantageous for drug loading and delivery. Recently, the natural compound raspberry ketone (RK), a hydroxy-phenolic compound, has shown potential to stimulate osteoblast differentiation *in vitro*. RK obtained from raspberries, is inexpensive and readily available as a nutraceutical. However, the local delivery of raspberry ketone as an alternative to BMP-2 for stimulating and enhancing bone healing and regeneration has not been explored.

The long range goal of this research will be to examine the potential of RK-loaded electrospun chitosan membranes to be used for guided bone regeneration applications. Improved bone regeneration in traumatic injuries or implant sites will lead to improved speech, masticatory function, aesthetics, and overall quality of life for patients. In some regions of the world, mammalian-derived GBR materials such as collagen are sometimes met with resistance due to religious or lifestyle beliefs as well as the fear of disease transmission. Since chitosan is derived from invertebrates, it can be provided as an alternative natural GBR material to mammalian-derived materials. Using RK as an alternative to BMP-2 could dramatically reduce the costs of bone regeneration and craniofacial/dental implant procedures. If found to be effective in GBR applications, RK could also be used in other orthopedic applications such as large segmental bone defects where osteogenesis is important.
Hypothesis & Rationale

This work aims to take advantage of the biocompatible, biodegradable, osteoconductive properties and nanofiber structure of electrospun chitosan membranes and the potential osteogenic properties of RK to create a bioactive guided bone regeneration membrane that not only provides effective barrier function, but also stimulates bone growth and differentiation. Therefore the hypothesis of this work is that RK released from chitosan membranes is effective in stimulating osteo-differentiation and mineralization in pre-osteoblast cells. The goals are to determine the effects of chitosan membrane modifications on RK release profiles, the cytocompatibility of RK loaded membranes, and whether RK released from the membranes has bioactive properties that induce differentiation of and mineralization by osteoblasts.

The rationale for using electrospun chitosan membranes is that they have been shown in *in vivo* rodent models to be biocompatible, provide effective barrier function, and biodegrade over 3-4 months into non-acidic degradation products. When chitosan is electrospun, a non-woven nanofibrous structure is created. This structure has interconnected pores small enough to prevent cell migration through the material, but large enough to allow for cell-to-cell communication between compartments. The high surface area of the nanofibers also provides advantages for drug loading and release. Since RK has shown potential to differentiate stem cells into osteoblasts, the addition of RK to the electrospun chitosan membranes may result in a bioactive membrane capable of accelerating the differentiation of a pre-osteoblast cell line into mature osteoblasts.
Clinical Background

Craniofacial Bone Loss

Periodontal disease is an infection of the tissue surrounding the teeth, particularly the gums and jaw bone. There are many causes of periodontal disease, but the most common is poor oral hygiene, which leads to failure to remove plaque. The buildup of plaque eventually leads to infection. Symptoms of periodontal disease include red, swollen, painful gums, loose teeth, and painful chewing. The infection can also result in tooth and/or bone loss in the jaw, affecting mastication, speech, and appearance of patients. Periodontal disease is a large problem, especially in older populations, affecting 8.5% of adults ages 20-64 and 17.2% of adults over the age of 65\textsuperscript{13}. These numbers are expected to increase with our country’s aging population. There are also factors that can put individuals at a higher risk of periodontal disease. These include smoking, diabetes, some medications, and some illnesses such as AIDS\textsuperscript{13}. Tooth and bone loss may also occur due to injuries such as assaults, traumatic accidents, or military conflicts\textsuperscript{14}. Assaults are the most common cause of traumatic facial injury, making up about 70\% of cases\textsuperscript{14}. After an injury, patients can have problems with mastication, speech, and appearance, similar to periodontal disease. Craniomaxillofacial injuries and periodontal disease often lead to the use of dental implants in patients. It is estimated that about 5,000,000 dental implants are placed per year and the US and European dental implant market will reach $4.2 billion by 2022\textsuperscript{1,15}. 
Bone Grafting

Implants involving periodontal disease or craniomaxillofacial injuries often require bone graft procedures to regenerate the maxillary or mandibular bones in order to sufficiently hold the implants in place. There are four major types of materials used for periodontal bone graft procedures: autografts, allografts, xenografts, and alloplasts\textsuperscript{16}. There are relative advantages and disadvantages of these grafts, though all are used to treat craniomaxillofacial defects. Autografts are taken from the same patient, often from the mandible and sometimes the iliac crest. These grafts are the most successful osteogenic material of all the grafts, but are criticized for causing donor site morbidity and lack of graft volume\textsuperscript{16}. Allografts are taken from a different patient and are used either freshly frozen or freeze-dried. Lack of graft volume is not an issue with these grafts but they do pose a minimal risk of disease transmission. Xenografts are collected from a different species. Common xenograft species in periodontal regeneration include pigs and cows. These grafts have concerns with resorption, disease transmission, host rejection, and in some cases cultural and religious beliefs. Finally, alloplasts are synthetic biomaterials that are developed to induce bone healing. The most common alloplasts used are hydroxyapatite, tricalcium phosphate, and bioactive glasses. These materials do not have the same problems as the organic materials such as limited supply and disease transmission. However, they do not exhibit any osteogenic properties on their own\textsuperscript{16}.

The dental implant is often placed a few months subsequent to the grafting procedure, allowing the bone to partially regenerate before the implant is placed\textsuperscript{17}. However, implant failure rates of up to 10\% are still recorded\textsuperscript{17}. A major problem with this procedure is that the soft tissue surrounding the bone graft site grows faster than
bone, often leading to migration of soft tissue into the graft site\textsuperscript{12}. This prevents the bone from fully regenerating. Without sufficient bone regeneration, the implant may not be adequately held in place and could have inadequate stability\textsuperscript{17}. Loose implants often lead to additional procedures, including additional bone grafting, to repair the implant site and create a more sufficient site. These additional procedures lead to increased stress and pain for the patient as well as more expenses for the patient, doctor and staff, and insurers.

\textit{Osteoblast Differentiation}

The cell type primarily responsible for regeneration of bone is the osteoblast. Bone regeneration requires mesenchymal stromal cells (MSCs) to differentiate into osteoblasts and for these cells to actively form new bone. MSCs first migrate via blood vessels to the injury site, where they begin to differentiate\textsuperscript{18}. During differentiation, preosteoblasts first proliferate and express osteopontin and type-1 collagen, creating a matrix around the cells. This matrix is mineralized with calcium phosphate crystals in the presence of alkaline phosphatase (ALP) and other proteins such as bone sialoprotein. Osteocalcin and osteopontin are expressed following the initial mineralization\textsuperscript{19}. This process is referred to as osteogenesis. Osteoblasts are relatively slow responders, and typically do not differentiate rapidly unless signaled by either a biochemical or mechanical signal\textsuperscript{19}. Therefore, the use of synthetic bone grafting materials alone has historically shown limited success\textsuperscript{18}. Biological mediators such as BMP-2 and tissue engineering techniques including guided bone regeneration have recently been used in an attempt to accelerate the differentiation and osteogenic processes. Faster differentiation
of osteoblasts leads to faster regeneration of bone and less time between the bone grafting procedure and the implant placement.

Guided Bone Regeneration

Barrier membranes can be used to separate the soft tissue from the graft site. These membranes are referred to as guided bone regeneration (GBR) membranes\textsuperscript{12}. The membranes are placed on top of the grafting material during the grafting procedure and the soft tissue is then pulled back over the site and closed (Figure 1). The membrane acts as a mechanical barrier between the two tissue compartments, allowing slower-growing bone to fully regenerate. The concept of GBR has been around since the 1950s, when it was explored for both spinal fusion and maxillofacial regeneration applications\textsuperscript{12}. Since then the field has grown and several different materials have been used.

Figure 1. Bone graft site being covered by a guided bone regeneration barrier membrane\textsuperscript{20}.
Current Solutions

Current GBR membrane materials consist of expanded polytetrafluoroethylene (ePTFE), collagen, or aliphatic polyesters including poly(lactic acid) (PLLA) and poly(glycolic acid) (PLGA)\textsuperscript{18,21}. ePTFE (Gore-Tex\textsuperscript{®}) membranes are highly mechanically stable and extremely hydrophobic and therefore act as an effective barrier to soft tissue, showing significant periodontal regeneration in several clinical trials\textsuperscript{18}. The membranes are biocompatible and elicit minimal foreign body reaction, but they are not biodegradable. Since they are not resorbed into the surrounding tissue, ePTFE membranes require a second procedure to remove the membrane from the graft site. This second procedure increases costs as well as risks of infection or damage to the newly regenerated bone, further hindering the implant process\textsuperscript{18}. Collagen membranes, although less mechanically stable than ePTFE, are highly biocompatible and show some degree of bioactivity through the activation of surrounding fibroblast cells. Collagen is also biodegradable and therefore does not require a second procedure for removal. However, these membranes’ degradation times can vary anywhere between 1-6 months depending on the degree of crosslinking\textsuperscript{12}. Even membranes that undergo the same cross-linking methods can have degradation times that vary from 2-8 weeks\textsuperscript{16}. Due to this variability in degradation times, there have been cases where collagen membranes have been shown to degrade too quickly to provide an adequate barrier for the regenerating bone, especially in large bone graft sites consistent with traumatic craniofacial injuries\textsuperscript{18,21}. Aliphatic thermoplastics, such as PLLA and PLGA are commonly used as synthetic resorbable GBR membranes. They are popular because some properties, such as degradation time and mechanical properties, can be customized by changing the composition and chain
length of the polymers. PLLA and PLGA, like collagen, are also biodegradable, however they can cause inflammatory, foreign body responses because of their acidic degradation products. This creates a cytotoxic environment that inhibits new bone formation in the graft site\textsuperscript{21,22}. Currently, GBR membranes have a relatively high (23-50\%) complication rate. Complications include membrane exposure and infection which can lead to inadequate bone regeneration for implant placement\textsuperscript{3}.

GBR membranes are often supplemented with bioactive molecules to augment healing of bone grafting sites. The most commonly used drug for this purpose is bone morphogenetic protein-2 (BMP-2). BMP-2 is a protein found in large amounts in bone tissue and is produced by mature osteoblasts. It induces differentiation of mesenchymal stem cells (MSCs) and pre-osteoblasts through activation of Chfα1/Osf2\textsuperscript{23}. Once activated, this “master gene” causes MSCs to express an early marker for osteo-differentiation, osteopontin. A collagenous matrix is then formed around the cells. This matrix can be mineralized in the presence of cell-secreted alkaline phosphatase (ALP) and bone sialoprotein\textsuperscript{23}. Studies have shown BMP-2 can cause an increase in the rate of bone regeneration\textsuperscript{4,5}. These studies have led to the development of a commercially available BMP-2 collagen sponge system, Infuse\textsuperscript{®}, from Medtronic for dental applications\textsuperscript{24}. However, a consistent delivery system for the drug has yet to be established. BMP-2’s dosing and release patterns from collagen have been found to be unpredictable\textsuperscript{4}. The release rate is affected by degree of porosity and interconnectivity of pores, as well as the degradation mechanics of the material onto which it is loaded. Typically, supraphysiologic concentrations are used to ensure that BMP-2 is retained at the treatment site long enough for the bone-forming cells to migrate to the area\textsuperscript{5}. This
technique can cause a build-up of BMP-2 at the graft site, leading to adverse reactions such as ectopic bone formation, bone/tooth root resorption, and ankyloses\textsuperscript{4,5}. The cost of manufacturing synthetic BMPs is also extremely high, adding to the already high costs of therapies and research. Thermo-Fisher Scientific lists just 100 µg of BMP-2 Recombinant Human Protein for over $1,200. The cost limits clinical use of the protein, and makes research and development of a reliable and sufficient delivery system extremely expensive.

\textit{Chitosan}

Chitosan is a biopolymer derived from chitin, a naturally occurring polysaccharide found in crustacean shells and insect exoskeletons. Chitosan degrades into short chain oligosaccharides and simple sugars, which are non-acidic and exhibit no cytotoxicity. It is widely used as a biomaterial because it is non-toxic, osteoconductive, biodegradable, and biocompatible. In addition, it has been reported to have anti-tumor, antimicrobial, and antioxidant properties\textsuperscript{25}. Chitosan’s mechanical, degradation, and bioactive properties can be modified by changing the degree of deacetylation (DDA), or the number of acetyl groups present. Naturally occurring chitin has a DDA of 0%. The polymer is considered chitosan at a DDA of 50% or above. Increasing the DDA makes the structure more crystalline, enhancing the mechanical properties and lengthening the degradation time\textsuperscript{26}. The molecular weight of chitosan plays a large role in the viscosity and solubility of the polymer\textsuperscript{27}. Chitosan can be solubilized at relatively low concentrations in acidic solutions and formed into films, microbeads, sponges, paste,
scaffolds, or gels. This versatility is another reason it is widely used for biomedical applications.

![Image of chitin and chitosan molecules]

Figure 2. Illustration of the removal of acetyl groups from chitin to produce chitosan$^{27}$.

Electrospinning

Electrospinning is an additive manufacturing technique in which polymers can be spun into nanofibers using an electrical charge$^{29}$. An electrospinning setup typically consists of three components: a voltage source, a conductive spinneret, and a collector (Figure 3). The voltage source supplies a charge to the spinneret, which creates an electric field in which the electrostatic forces draw the dissolved polymer out of the spinneret and onto the collector in the form of nanofibers$^{29}$. The thickness and structure of the nanofibers vary depending on the set up of the spinneret and the collector as well as the charge applied, the distance of the collector, and various atmospheric conditions such as temperature and humidity.
Chitosan can be electrospun into a randomly distributed nanofibrous structure when dissolved in a dilute acid. Solvents that have been used to electrospin chitosan blends include hexa-fluoro isopropanol (HFIP), trifluoroacetic acid (TFA), and diluted acetic acid solutions. However, when spinning pure chitosan, these solvents produce fibers with non-uniform diameters and small beads were deposited on the collector. However, it has been shown that by diluting TFA with dichloromethane (DCM) 70:30, pure chitosan fibers with uniform diameter and distribution can be produced. This structure provides an extremely high surface area that is ideal for drug delivery purposes.

The structure of electrospun chitosan membranes also has a degree of porosity, allowing cell communication through the membrane while not allowing cells through. There have been multiple studies that reported new bone formation in critical-sized defects using electrospun chitosan membranes as a GBR material.

Figure 3. An illustration of a basic electrospinning setup. Not drawn to scale.
**Post-spinning Treatments**

The main drawback with electrospun chitosan membranes in GBR applications has been a high degree of swelling in the fibers, leading to a loss of nanofiber structure or morphology and porosity\(^{32-35}\). This loss of structure is due to the formation of acidic salts on the chitosan fibers during the spinning process. In an aqueous environment, these salts dissolve, lowering the pH in the surrounding area and partially dissolving the chitosan fibers\(^{32}\). To prevent swelling in the membranes, post-spinning treatments have been used to stabilize the fibrous structure of the membranes. In an attempt to combat this loss of structure, crosslinking using glutaraldehyde or genipin has been attempted\(^{10,35}\). However, these efforts were in an attempt to crosslink the amino groups of chitosan, which are the site of the acidic salts after spinning. Therefore, there are often not enough free amino groups for sufficient crosslinking\(^{32}\). Alkaline solutions have also been used in an attempt to neutralize the acidic salts. However, these solutions are aqueous and still cause swelling and loss of fibrous structure\(^{32}\). Wu et al. have shown that acylation of the membranes followed by hydrolysis can prevent deterioration of the nanofibers in electrospun chitosan while removing any harmful acidic salts introduced during the electrospinning process. The acylation process adds an acyl group to the chitosan chain, creating a hydrophobic layer around the fibers\(^{32}\). This prevents the fibers from swelling in an aqueous environment. Acidic salts can then simply be dissolved out of the fibers in water while the fibers retain their structure. These treated membranes have shown compatibility with Saos-2 bone cells in culture and in an *in vivo* rat calvarial model were well tolerated, remained cell occlusive, and supported bone healing/regeneration similar to that of commercial collagen membranes\(^{32}\).
Raspberry Ketone

RK is a natural phenolic compound found in raspberries. The molecule is used in flavored candies, soaps, and candles because of its sweet aroma. It is also sold as a dietary supplement due to manufacturer claims that it has fat-burning properties. Recently, RK has been shown to promote the differentiation of stem cells into osteoblasts in vitro. When C3H10T1/2 cells were exposed to RK, levels of osteogenic markers TGF-β, ALP, osteocalcin, and collagen type 1 were increased. This means that RK may promote new bone growth in newly differentiated cells. RK is readily soluble in ethanol and can be loaded onto the membranes in an ethanol solution. Once the ethanol evaporates, the RK is adsorbed to the surface of the fibers of the membrane. This makes RK very easy to load onto the chitosan material. The affordability of RK would make it much more accessible than BMP-2 if it can promote differentiation and mineralization at similar levels.
Research Goal & Hypothesis

The goal of this work is to evaluate whether electrospun chitosan membranes loaded with raspberry ketone (RK) have potential to induce osteo-differentiation in pre-osteoblasts. It is hypothesized that electrospun chitosan membranes loaded with raspberry ketone are effective in inducing osteo-differentiation and mineralization in pre-osteoblasts in vitro. The specific objectives are to determine:

- RK release kinetics from electrospun chitosan of different post-spinning treatments
- At what RK concentration membranes are toxic to cells
- Whether RK delivery has bioactive properties that induce differentiation of and mineralization by osteoblasts
CHAPTER 2: JOURNAL SUBMISSION

Electrospun Chitosan Membranes Loaded with Raspberry Ketone to Induce Differentiation in Pre-osteoblasts

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Abstract

Electrospun chitosan membranes (ESCM) modified with short chain fatty acids have shown promise as biocompatible and biodegradable guided bone regeneration (GBR) membranes for preventing soft tissue migration into craniofacial bone graft sites. Raspberry ketone (4-(4-hydroxyphenyl)butan-2-one; RK), a naturally occurring phenolic compound, has shown potential to stimulate the differentiation of osteoblastic precursors, thereby accelerating the bone-healing process. The aim of this study was to evaluate the potential of adding RK to ESCMs to create a bioactive membrane capable of stimulating bone cell differentiation. This study showed that W-20-17 murine mesenchymal cells exposed to 50-200 µg/ml RK over 7 days expressed higher levels of alkaline phosphatase (ALP) than cells exposed to 50-200 ng/ml bone morphogenetic protein-2 (BMP-2), suggesting that RK has potential as an osteogenic agent. RK was loaded at 100, 250 or 500 µg/1 cm diameter ESCM discs modified by one of three different fatty acid anhydrides (acetic anhydride, butyric anhydride, or hexanoic anhydride) and the release patterns were examined. Fourier transformed infrared (FTIR) spectroscopy and water contact angle measurements showed that the fatty acid anhydrides reacted with the chitosan and increased the membranes’ hydrophobicity. It was found that the RK release from the fatty acid-treated membranes followed a burst release pattern with the release profile dependent on type of fatty acid treatment. RK peak release decreased and duration of release increased with increasing length of fatty acid chain used. RK loaded membranes were then evaluated for cytocompatibility and ability to stimulate the differentiation and mineralization of the W-20-17 cells as compared to BMP-2. RK released from the membranes had no cytotoxic effect on the cells. However, RK-loaded
membranes did not induce differentiation of the pre-osteoblasts. It is speculated that the decreasing rate of release resulted in RK levels not being sustained at high enough concentrations to affect the cells. Electrospun chitosan membranes, with a more sustained RK release profile, may have potential to be used in guided bone regeneration applications.

Keywords: Guided bone regeneration, chitosan, raspberry ketone
Introduction

Guided bone regeneration (GBR) membranes are used to cover a dental or maxillofacial bone graft site to prevent ingrowth of surrounding soft tissue, and to maximize the amount of healing and regenerated bone\(^1\). The membrane acts as a mechanical barrier between the two tissue compartments, allowing slower-growing bone to fully regenerate. The concept of GBR has been around since the 1950s, when it was explored for both spinal fusion and maxillofacial regeneration applications\(^1\).

Current GBR membrane materials include expanded polytetrafluoroethylene (ePTFE), collagen, and aliphatic polyesters including poly(lactic-acid) (PLLA) and poly(glycolic-acid) (PLGA)\(^2\)-\(^3\). ePTFE (Gore-Tex\(^\text{®}\)) membranes are highly mechanically stable and hydrophobic and therefore act as a great barrier to soft tissue, showing significant periodontal regeneration in several clinical trials\(^2\)-\(^4\). However, the membranes are not biodegradable and require a second procedure to remove the membrane from the graft site, increasing costs and risk of infection\(^2\). Collagen membranes, although less mechanically stable than ePTFE, are highly biocompatible and show low immunogenicity\(^5\). Collagen membranes are biodegradable and therefore do not require a second procedure for removal but have been cited as having unpredictable, too rapid degradation, resulting in inadequate barrier function for regenerating bone\(^2\),\(^5\). Aliphatic thermoplastics, such as PLLA and PLGA, are also used as resorbable GBR membranes\(^3\). These synthetic membranes are biodegradable, but they can cause inflammatory, foreign body responses because of their acidic degradation products, inhibiting new bone formation at the graft site\(^5\),\(^6\). Currently, GBR membranes have a relatively high (23-50\%) complication rate. Complications include membrane exposure caused by thin gingival
tissues in tension or lack of blood supply and infection, which can lead to inadequate bone regeneration\textsuperscript{6}.

To help enhance bone regeneration, GBR membranes are often supplemented with bone morphogenetic protein-2 (BMP-2) to make them more bioactive. Studies have shown BMP-2 can increase rates of bone regeneration\textsuperscript{7,8}. However, BMP-2’s dosing and release patterns, particularly from collagen, have been found to be unpredictable and adverse reactions such as ectopic bone formation, bone/tooth root resorption, and ankyloses as well as high costs limit its use\textsuperscript{7,8}.

Chitosan is widely used as a biomaterial because it is non-toxic, osteoconductive, biodegradable, and biocompatible\textsuperscript{9,10}. Nanofibrous membranes can be electrospun from chitosan dissolved in acidic solutions. There have been multiple studies that reported new bone formation in critical-sized defects using these electrospun chitosan membranes as a GBR material\textsuperscript{11-15}. The main drawback with these membranes has been a high degree of swelling in the fibers\textsuperscript{16-19}. Post-spinning treatments can be used to prevent membrane swelling. Wu et al. have shown that acylation of the membranes followed by hydrolysis can prevent deterioration of the nanofibers while removing harmful acidic salts introduced during the electrospinning process\textsuperscript{16}. In an \textit{in vivo} rat calvarial model, the treated membranes were well tolerated, remained cell occlusive, and supported bone healing/regeneration similar to that of commercial collagen membranes\textsuperscript{16}.

Raspberry Ketone (RK) is a natural phenolic compound found in raspberries and is commercially available as a nutraceutical because of its reported antioxidative properties\textsuperscript{20}. RK has been shown to promote the differentiation of C3H10T1/2 murine stem cells into osteoblasts\textsuperscript{21}. RK has also been shown to increase levels of TGF-β, ALP,
osteocalcin, and collagen expressed by these cells \textit{in vitro}^{21}. This suggests that RK could promote new bone growth in newly differentiated cells. The affordability of RK would make it much more economical than BMP-2 if it can promote differentiation and mineralization at similar levels.

The goal of this work is to determine whether or not electrospun chitosan membranes loaded with raspberry ketone (RK) induce osteo-differentiation. It is hypothesized that electrospun chitosan membranes can be loaded with raspberry ketone and release it over time to promote osteo-differentiation and mineralization in the pre-osteoblasts \textit{in vitro}. Chitosan could provide an alternative natural material for GBR applications that can be made bioactive by supplementation with RK. In addition, RK could provide a much more financially accessible and safer alternative to BMP-2 as an osteogenic agent.

\textbf{Materials and Methods}

\textit{Fabrication of Electrospun Chitosan Membranes}

The electrospinning procedure used was previously reported by Wu et al.\textsuperscript{16} To summarize, chitosan (71\% DDA, 311.5 kDa, Primex) at 5.5 (w/v)\% was dissolved in a solution containing 70\% (v/v) trifluoroacetic acid (TFA) - 30\% (v/v) dichloromethane. The solution was loaded into a 10 ml syringe with a 20 gauge needle and electrospun at a voltage of 26 kV. The chitosan fibers were collected on non-stick aluminum foil (Reynolds Wrap VR) wrapped around a rotating collecting plate (38.1 cm diameter disc).
The collector was positioned approximately 15 cm from the needle tip and rotated at 8.4 RPM to encourage even and random distribution of fibers.

*Post-Electrospinning Treatments*

To remove the TFA salts that are spun into the membrane and to stabilize the fibrous structure, the membranes were treated post-spinning by acylation as described by Wu et al. One of three fatty acids were used: acetic anhydride (AA), butyric anhydride (BA), or hexanoic anhydride (HA). These fatty acids have a carbon chain length of one, four, and six carbons, respectively. The electrospun chitosan was cut into 1 cm diameter discs and placed in a solution of 1 ml pyridine - fatty acid anhydride (1:1) for every 5 mg of membrane material, adding an acyl group onto the chitosan chain and creating a hydrophobic layer around the fibers. The membranes were then placed in a 1 L distilled water bath for 72 hours, with water being refreshed every 24 hours. The water dissolved the trifluoroacetic acid (TFA) salt out of the membrane that was left behind from the chitosan being solubilized in TFA pre-spinning.

*Membrane Characterization*

After post-spinning treatments, the water contact angles were found using the VCA OptimaXE system to determine hydrophobicity (n=3 per group). Membrane composition was confirmed using Fourier transform infrared spectroscopy (FTIR, PerkinElmer Frontier). The nanofiber structure was confirmed using a scanning electron microscope (SEM, Nova NanoSEM).
**RK Loading and Elution**

An elution study was performed to determine the release profile of RK passively loaded onto electrospun chitosan membranes. Treated membrane discs (1 cm diameter) were made aseptic using a combination of 70% ethanol and UV light. They were then loaded at concentrations of either 100, 250, or 500 μg of RK per membrane. These concentrations were based on the work of Takata et al. using C3H10T1/2 murine stem cells. For loading, RK was dissolved in 100% ethanol. The amount of ethanol the membranes absorb was determined by finding the percent swelling of the membranes by weighing them before and after soaking in 100% ethanol. The percent swelling determined how much of the RK-in-ethanol stock should be added to reach the desired amount of RK per membrane. The RK-ethanol stock was added to the membranes in a Class II Biological Safety Cabinet and allowed to dry for 30 minutes. The loaded membranes (n=4 per treatment per loading) were submerged in 1 ml PBS in a 24-well tissue culture plate and placed in a 37°C incubator. The PBS was collected, frozen at -80°C, and completely refreshed at time intervals of 1, 3, 5, 7, 9, 11, 14, 17 and 21 days. At the end of the study, the PBS was analyzed for amount of RK using high performance liquid chromatography (HPLC, Thermo Scientific UltiMate 3000). A mobile phase consisting of 70% [0.124 M monopotassium phosphate (KH2PO4), 0.08 M dipotassium phosphate (K2HPO4)] and 30% Acetonitrile (CH3CN) was created for HPLC analysis. The solution was pumped through a Hypersil GOLD C8 column at a rate of 1 ml/min for 10 minutes. The solution was analyzed using UV-Vis spectroscopy at a wavelength of 209 nm.
**RK Cell Viability Study**

Cell viability and growth tests were performed over 12 days with membranes loaded with 0, 100, 250, or 500 μg of RK per disc using a trans-well system. Membranes were gas-sterilized using ethylene oxide prior to loading with RK at 100, 250, or 500 μg/membrane as previously outlined. The membranes were then placed in the bottom of 24-well plates and W-20-17 cells, osteoblast precursors, were seeded into trans-well inserts (0.4 μm pore size, Falcon) at 3200 cells/cm². The cells were not seeded directly onto the membranes because of the hydrophobic nature of membranes which could confound the response of the cells to released RK. A control group containing membranes without RK and a group with no membranes were also used. The growth medium used was Dulbecco’s Modification of Eagle’s Medium (DMEM) with 4.5 g/L glucose, L-glutamine and sodium pyruvate (Corning Inc., Corning, NY, USA) supplemented with 10% fetal bovine serum (Fisher Scientific), 500 IU/ml penicillin and 500 mg/ml streptomycin. The wells were filled with 1 ml of medium and the plates were placed in a 37°C incubator. On days 1, 3, 7, and 12 a CellTiter-Glo assay (Promega, Madison, WI, USA) was performed to estimate cell viability (n=3 per group).

**Differentiation of Pre-Osteoblasts**

The potential of RK to stimulate osteo-differentiation was examined using the W-20-17 mouse stromal cell line (ATCC CRL-2623). These cells were used since they show a dose-dependent increase in the expression of alkaline phosphatase (ALP) in response to BMP-2. The cells were seeded at 3200 cells per well in 96 well plates in complete
DMEM medium and allowed to attached overnight. The medium was removed and replaced with DMEM medium containing RK at concentrations of 0, 6.25, 12.5, 25, 50, 100, 200, and 400 μg/ml. Media were also prepared with the RK concentrations spiked with 25 ng/ml BMP-2 (GoldBio, St. Louis, MO, USA) in order to investigate the possibility of synergistic or additive effects between RK and BMP-2 on ALP production. Medium containing BMP-2 at 0, 6.25, 12.5, 25, 50, 100, 200, and 400 ng/ml media were used as control. The cells were kept in a 37°C 5% CO₂ water-jacketed incubator for 7 days with media being replaced at 3 and 5 days with the same formulations. Test plates were removed after 1, 3, and 7 days (n=4 per group per time point). At each time point, the medium was removed and replaced with fresh distilled/deionized water and then the plates were subjected to 3 freeze-thaw cycles to lyse the cells for measurement of ALP activity using QuantiChrom™ Alkaline Phosphatase Assay (BioAssay Systems, Hayward, CA, USA) and DNA using the Quant-IT PicoGreen DNA assay (Invitrogen).

Mineralization Study

Treated membranes were gas-sterilized and loaded with RK concentrations of 100, 250, or 500 μg/membrane as described. The membranes were placed in trans-well inserts and W-20-17 cells were seeded in bottom of 24-well plates. Control groups containing trans-wells with membranes without RK and wells with no membranes. Cells were cultured in 1 ml of mineralizing medium (DMEM complete growth medium supplemented with 5mM beta-glycerol phosphate and 50μg/ml ascorbic acid). Cells with no membranes grown in mineralizing media supplemented with 25ng/ml BMP-2 were also used as controls. The plates were then placed in a 37°C incubator. ALP and
PicoGreen assays were performed on Days 1, 7, 14, and 21 (n=4 per group per time point). On days 14 and 21, the amount of calcium phosphate deposition as an indicator of mineralization for each group was analyzed using a quantitative Calcium Assay (Pointe Scientific, Inc., Canton, MI). On Day 21, cells were fixed using a 3.7% formalin solution and stained with Alizarin Red S (2%, MP Biomedical) for qualitative analysis (n=1 per group).

Statistical Analysis

A mixed model linear regression was performed on the release data to detect interactions based on factors of time, treatment, and dosage. ANOVA was used to determine where differences existed between groups. Statistical significance was declared when $p < 0.05$.

Results

Water Contact Angle

The contact angles appeared to increase with increasing fatty acid chain length, although no statistical difference was found (Table I).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Contact angle (degrees)</th>
</tr>
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<tbody>
<tr>
<td>Acetic Anhydride</td>
<td>57.1±13.3</td>
</tr>
<tr>
<td>Butyric Anhydride</td>
<td>66.0±39.6</td>
</tr>
<tr>
<td>Hexanoic Anhydride</td>
<td>100.1±16.3</td>
</tr>
</tbody>
</table>
SEM images confirmed that the nanofiber structure of the membranes was maintained after post-spinning treatments (Figure 1). The AA, BA, and HA-treated membranes appeared to show minimal swelling of the fibers due to the aqueous solutions used in the treatments.

Figure 1. SEM images of an untreated membrane (A), AA-treated membrane (B), BA-treated membrane (C), and HA-treated membrane (D) (5,000x mag).

FTIR data confirmed that the post-spinning treatments removed the TFA salts from the membranes. The three small peaks at 722, 802 and 841 cm$^{-1}$ are associated with
the presence of trifluoroacetic salts in the membrane\textsuperscript{16}. The peaks are present and well-defined in the as spun membrane (Figure 2). However, for all three of the pyridine-fatty acid treatments, the peaks are not present. The peak starting around 1742 cm\textsuperscript{-1} corresponds to the acyl (C=O) groups which support the reaction of the fatty acids to the hydroxyl (OH) groups of chitosan. The peak observed between 2900 and 3000 cm\textsuperscript{-1} correspond to the methyl groups present.

**Figure 2.** FTIR data for the as spun and treated electrospun chitosan membranes. The peaks associated with methyl groups, acyl groups, and TFA salts have been highlighted. (n=1 per group).
**RK Loading and Elution**

All membranes showed a burst release profile with the largest amount of RK being released within the first day (Figure 3). However, the amount of RK loaded and the type of membrane treatment both had a statistically significant effect on amounts and duration of release (p-value < 0.0001). Membranes treated with acetic anhydride released RK the most quickly, no matter the loading dosage (Figure 3A). The AA-treated membranes loaded with 500 μg RK showed trace amounts released on Day 9, but the other dosages released all RK by Day 5. The butyric anhydride-treated membranes showed a slightly more sustained release, showing release up to Day 9 (Figure 3B). Hexanoic anhydride-treated membranes showed the longest release pattern. These membranes still showed RK release up to 14 days after loading (Figure 3C). No membranes showed any significant release on Days 17 or 21.
Figure 3. RK release from electrospun chitosan membranes loaded with 500 μg (A), 250 μg (B), or 100 μg (C). Inset graphs indicate elution for Days 7-14 (n=4 per group). Bars
are means and error bars are standard deviations. *Statistically significant difference (p<0.05).

\textit{RK Cell Viability Study}

In general, there were only minor statistical differences in the growth of the cells exposed to the membranes with and without RK loading and the controls (Figure 4). The differences were detected primarily at the Day 1 time point. Other than Day 1, only two significant differences were found between test membranes and the no membrane control group: at Day 3 for the AA-treated membrane loaded with 250 μg RK and at Day 12 for the BA-treated membrane loaded with 100 μg RK. While these were statistically different the real differences were small and may not be of practical importance. Overall, the assay showed similar viability of cells in trans-wells exposed to RK loaded membranes as compared to wells with non-loaded membranes and no membranes at all.
Figure 4. Effect of electrospun chitosan membranes loaded with 500 μg RK (A), 250 μg RK (B), and 100 μg RK (C) on growth of W-20-17 cells over 12 days (n=3 per group).

*Statistically significant difference.

Pre-Osteoblast Differentiation

The results of the differentiation study examining ALP expression are shown in Figure 5. There were no statistical differences detected between cells exposed to RK, RK spiked with BMP-2, or BMP-2 at Day 1 (Figure 5A). On Days 3 and 7, statistical differences in ALP expression by the cells exposed to different medium were detected (p<0.05). ALP expressions increased with increasing amounts of BMP-2 from 50ng/ml to
400 ng/ml. ALP expression was also increased with increasing amounts of RK from 50 μg to 200 μg. There was no effect of spiking RK solutions with 25 ng BMP-2. The expression of ALP by cells exposed to 50 μg to 200 μg RK were statistically greater than cells exposed to 50 ng to 200 ng of BMP-2 on Day 7. Only the cells supplemented with 400 ng BMP-2 showed a significantly (p<0.05) higher ALP production than cells with 400 μg RK by Day 7, even when spiked with 25 ng BMP-2. This is likely due to some degree of toxicity associated with the RK at such a high dose, which was confirmed by the PicoGreen assay.

Figure 5. ALP expression of W-20-17 cells normalized by DNA on Day 1 (A), Day 3 (B), and Day 7 (C). Cells were exposed to either RK, BMP-2, or RK spiked with 25 ng BMP-2.
BMP-2 (n=4 per group). Bars are means and error bars are standard deviations.

*Statistically significant difference (p<0.05).

Mineralization Study

ALP expressions of the W-20-17 cells showed only minor statistical differences between the groups with RK-loaded membranes and the control group containing no membranes (Figure 6). There were no statistical differences between RK loading concentrations for the HA-treated membranes. The positive control group containing 25 ng/ml BMP-2 showed approximately 50x higher ALP levels at Day 14. Overall, ALP levels were the highest on Day 14, dropping lower on Day 21.
**Figure 6.** ALP expression of W-20-17 cells exposed to AA-treated membranes (A), BA-treated membranes (B), or HA-treated membranes (C) loaded with RK and the BMP-2 control (D) over 21 days (n=4 per group).

*Calcium Assay and Alizarin Red S Staining*

As with the ALP expression, there were very few differences in calcium levels detected between the test groups and the negative control (Figure 7). The largest difference occurred in the HA-treated group loaded with 250 μg RK on Day 14.
However, on Day 21 this group was not different from the negative control. The BMP-2 group showed calcium levels about 30x higher than the other groups on Day 21. The BMP-2 group also showed approximately a 100% increase in calcium levels from Day 14 to Day 21. This increase was not observed in the other groups.

**Figure 7.** Calcium concentration of media from cells exposed to membranes loaded with 500 µg RK (A), 250 µg RK (B), 100 µg RK (C), and no RK (D) and media supplemented...
with 25 ng/ml BMP-2 (E) (n=4 per group). Bars are means and error bars are standard deviations. *Statistically significant difference (p<0.05).

Cells stained with 2% Alizarin Red S on Day 21 are shown in Figure 8. The BMP-2 group showed strong staining with Alizarin Red S (Figure 8A). The no membrane group showed little to no staining. All of the test membrane groups also exhibited little to no staining, similar to the no-membrane group, and much less staining than the BMP-2 group.

Figure 8. Cells exposed to no membrane (A), an HA-treated membrane loaded with 250 μg RK (B), and 25 ng/ml BMP-2 media (C) for 21 days stained with 2% Alizarin Red S for calcium deposition (n=1 per group).
Discussion

A GBR membrane provides a barrier to soft tissue infiltration during bone grafting procedures. Acylated electrospun chitosan membranes have been explored as potential GBR membranes because they are biocompatible, biodegradable, and their nanofiber structure allows cell-to-cell communication between compartments. RK is a molecule that has been shown to have osteogenic potential\textsuperscript{21}. After loading with RK, acylated membranes released RK for up to 14 days, with tailorable release by modification of membrane acyl group. The ability to locally deliver RK from electrospun chitosan membranes may result in a bioactive GBR membrane that is able to enhance bone regeneration.

As higher water contact angles are associated with hydrophobic characteristics, the increase observed suggests that the membranes were made more hydrophobic by treating with longer fatty acid chains. The large standard deviations are likely due to the small sample size (n=3 per group). The treatments did not have any effect on the structure of the nanofibers, as shown by the SEM images. It was also verified via FTIR that the pyridine-fatty acid treatments removed the TFA salts introduced during the electrospinning process and the addition of the fatty acid chains on the chitosan molecules was successful. These results were consistent with those obtained by Wu et al\textsuperscript{16}.

The burst release of RK from the membranes was able to be manipulated by treating with increasing fatty acid chain length. AA-treated membranes showed approximately a 90\% decrease in RK released between Day 1 and Day 3. BA-treated membranes showed an 85\% decrease and HA-treated membranes showed a 70\% decrease
in the same span. With increasing length of fatty acid chains, RK also showed a more prolonged release profile from 5 to 11 days. The decrease in peak release and the longer release time periods could be attributed to the increasing hydrophobicity of the membranes due to increasing fatty acid chain length and the hydrophobic character of the aromatic ring and butanone group of the RK molecule. A hydrophobic molecule is less likely to be released from the membrane into an aqueous environment if the membrane is also hydrophobic\textsuperscript{22}. In addition, the degree of interaction of the butanone group of RK likely increases with FA chain length resulting in slower release rates, as illustrated in Figure 9. Predictable modification of the release profile of RK from electrospun chitosan membranes makes the combination a promising system for GBR drug delivery. These membrane modifications may have advantages in the loading and release of other hydrophobic drugs, such as hydrophobic antibiotics or anti-cancer drugs\textsuperscript{23}. Selection of acyl group length could be used to design rapid release, intermediate release, or extended release patterns, depending on what dosing pattern is appropriate for biological activity.

\textbf{Figure 9.} An illustration of the possible interaction between the fatty acid chains bonded to the chitosan molecule and the butanone group of RK.
The results of the W-20-17 cell viability study showed that electrospun chitosan membranes loaded with RK at concentrations of up to 500 μg/membrane had little effect on the viability or growth of W-20-17 cells. This is significant because it suggests that electrospun chitosan membranes loaded with RK would not be toxic to osteoblastic precursors up to a loading concentration of 500 μg. Takata et al. showed that C3H10T1/2 stem cells exposed to 200 μg/ml and 500 μg/ml RK for 72 hours showed a significant decrease in viability. However, even when 500 μg RK was loaded onto membranes, the RK concentration never reached a level of 500 μg/ml and maximum concentrations above 200 μg/ml occurred only for 24 hours in our elution study. By Day 3, the RK release concentrations were at non-toxic levels of 100 μg/ml or less. By controlling the loading levels, this study showed that non-toxic elution of RK could be achieved. It is crucial that RK is not toxic to osteoblastic precursors or stem cells for maximum healing and bone regeneration. Treated membranes themselves did not release any cytotoxic materials, as cells exposed to unloaded membranes showed normal growth patterns. These results were consistent with the viability study performed by Wu et al. and Norowski et al. using SAOS-2 human osteoblastic cells. This biocompatibility compares to other GBR materials such as ePTFE and collagen.

In the experiment evaluating effects of RK on induction of ALP in the W-20-17 cells as an indicator of osteogenic effects, RK solutions were tested with and without being spiked with 25 ng/ml of BMP-2. A low concentration of BMP-2 was selected that is known have a positive effect on inducing ALP expression in the cells but not so large as to overwhelm any possible effect of RK. Increasing BMP-2 concentrations exhibited increases in ALP expression, consistent with other similar studies. There was also an
increase in ALP expression with increasing RK concentration up to 400 \( \mu g/ml \). However, the high expression of ALP due to the 400 \( \mu g/ml \) RK solution may be an artifact of low cell numbers due to a decrease in cell viability at this high RK concentration. At non-toxic concentrations of RK, the amount of ALP expressed was similar to or higher than the BMP-2 group, suggesting that RK can induce differentiation of pre-osteoblasts. These results agreed with the findings of Takata et al. that 100 \( \mu g/ml \) RK approximately doubled ALP expression in C3H10T1/2 murine stem cells in the presence of 300 ng/ml BMP-2\textsuperscript{21}. Interestingly, with W-20-17 cells there did not appear to be any additive, antagonistic, or synergistic interaction between RK and BMP-2. The group of RK spiked with BMP-2 produced similar levels of ALP to the RK group, suggesting that RK is capable of inducing differentiation on its own. The exact mechanism of RK inducing differentiation is unclear, and will need to be investigated in the future.

RK-loaded membranes did not induce higher ALP expression than the negative control in the 21 day mineralization study. Nor was there enough evidence to suggest that the RK-loaded membranes resulted in increased calcium deposition by the cells. This could be due to the burst release followed by a decreasing rate of RK release from the membranes. In the initial ALP study, cells were exposed to constant concentrations of RK for up to seven days that resulted in an increasing expression of ALP as indicator of osteoblastic differentiation. Additionally, while Takata’s study found an increase in ALP in the presence of RK, it only examined the effects of RK at constant concentrations from 0-100 \( \mu g/ml \) over 6 days\textsuperscript{21}. However, elution profiles of RK from the membranes indicated that RK levels were between 50 and 200 \( \mu g/ml \) for between 1 and 3 days, depending on membrane modification. Even though the HA-treated membranes loaded
with 500 μg released RK for up to 14 days, release levels dropped below 50 μg/ml by Day 5, which may be too soon to have stimulated ALP expression and hence osteodifferentiation. These results suggest that RK-loaded chitosan membranes may have a similar problem to BMP-2 loaded GBR membranes: the drug is released too quickly to maintain an effective dosage. Takata et al. also found that RK at constant concentrations of 50-100 μg/ml over 6 days induced upregulation of collagen I and osteocalcin in the presence of the differentiation agent all-trans-retinoic acid (ATRA). These effects were not investigated in the presence of BMP-2. However, the study provides more indication that RK may induce differentiation and mineralization in W-20-17 cells with higher concentrations at later time points. Some potential solutions to this problem include increasing the loading concentration and treating the chitosan membranes with a longer fatty acid chain. Increasing the loading concentration would increase the amount of drug released at later time points, but it could lead to unknown side effects and cytotoxicity at early time points. Treating with a longer fatty acid chain would increase the hydrophobicity of the membrane and potentially result in a longer, more sustained release of RK. Additionally, in this in vitro study, the membranes were not in direct contact with the cells. In vivo the membranes would be in direct contact with bone and may have a greater effect. In any case, RK remains a potential bioactive agent for osteo-differentiation.
Conclusion

Electrospun chitosan membranes loaded with raspberry ketone have many of the properties desired for a guided bone regeneration system. The release of RK from the chitosan membranes can be predictably manipulated based on the type of fatty acid the membranes are treated with. RK did not show any cytotoxicity to W-20-17 cells up to a dosage of 500 μg/membrane. In addition, RK-spiked media appeared to induce expression of comparable levels of ALP from W-20-17 cells as BMP-2 after 7 days. However, the mineralization study showed that the RK-loaded membranes did not have a significant effect on mineralization over 21 days. It is speculated that RK was released from the membranes too quickly to induce mineralization in the cells. Future work will explore modifications to extend delivery of RK to maintain active concentrations over a timeframe relevant to osteodifferentiation, as well as expanded preclinical evaluations in bone healing models. To conclude, electrospun chitosan membranes loaded with raspberry ketone may have potential to be used in guided bone regeneration procedures.
References


CHAPTER 3: CONCLUSIONS

Electrospun chitosan membranes loaded with raspberry ketone have shown that they are a promising guided bone regeneration system. Electrospun chitosan membranes were successfully fabricated and treated using a fatty acid and pyridine solution. RK was successfully loaded onto the membranes using 200 proof ethanol. This loading technique could be used at time of care by physicians if desired. The type of fatty acid used in the post-spinning treatment was shown to manipulate the release pattern of the RK from the chitosan membrane. This suggests some level of customization is possible and the release of RK can be either shortened or prolonged. The RK-loaded membranes also showed no cytotoxicity in vitro, indicating that dosages of up to 500 μg/membrane are safe to use and are not toxic osteoblastic pre-cursors. RK also appeared to induce expression of ALP from W-20-17 cells at similar levels as BMP-2 after 7 days of culture. These results demonstrate that RK could be effective in differentiating osteoblastic precursor cells in a bone graft site. However, RK-loaded membranes did not show an ability to induce differentiation or mineralization, likely because the RK was released too quickly to have an effect. Improvements of RK release from the membranes are necessary to determine the potential of RK-loaded chitosan membranes for GBR purposes.
CHAPTER 4: FUTURE WORK

Experiments need to be performed to determine dosing of RK needed to induce osteoblast differentiation. Then studies will be needed to determine how to deliver RK to achieve the necessary concentration and time of release. This could be examined by increasing fatty acid chain length. Alternative mechanisms could be to explore ways to link RK to chitosan fibers to provide a more sustained release. Previous un-published results by our group have shown that it may be possible to link RK to chitosan via a reaction between the ketone of RK and the amine group of the chitosan. Additionally, in vivo dosing studies will be needed to determine if dosing is the same as in vitro. RK alone helps to differentiate effects but in vivo there is a complex milieu of factors that may act in combination with RK to increase osteogenic differentiation. The mechanism of action of RK will also be investigated. Studies may involve identification of a cell surface receptor that RK activates or determining whether RK penetrates the cell membrane and activates some other pathway. Although the effects of RK and BMP-2 together were briefly investigated, there will be further experiments into whether an interaction exists that could produce an additive or synergistic effect in osteoblast differentiation and maturation. In the study performed by Takata et al. the C3H10T1/2 cells only responded to RK in the presence of 300 ng/ml BMP-2, so higher concentrations of BMP-2 may need to be tested in conjunction with RK. Other biological roles of RK may also be explored in the context of bone healing, such as effects on inflammation. RK has already been shown to have antioxidant effects on cultured monocytes\(^{37}\). The impact of RK on these cells and the normal inflammatory healing response will be important to understanding the overall potential for locally delivering RK to stimulate bone healing.
REFERENCES


APPENDICES

Appendix A

Electrospinning Methods

Chitosan from Primex (71% DDA, 311.5 kDa) at 5.5 (w/v) % was dissolved in a solution containing 70% (v/v) trifluoroacetic acid (TFA) - 30% (v/v) dichloromethane (DCM). Glass serological pipettes were used because plastic could be dissolved by TFA or DCM. This solution was gently mixed overnight on a Belly Dancer shaker (Stovall Life Science, Inc.). The next day, the solution was loaded into a 10 ml syringe with a blunt 20 gauge needle. The syringe was then loaded onto a syringe pump and the pumping rate was set for 15 μl/minute. The solution was electrospun with an applied DC voltage of 26 kV (Figure 4). The chitosan fibers were collected on non-stick aluminum foil (Reynolds Wrap VR) that was wrapped around a rotating metal collecting plate (38.1-cm-diameter circular disc). The collector was positioned approximately 15 cm from the needle tip and rotated at 8.4 RPM by an AC motor to ensure even and random distribution of fibers. After the entire solution was pumped out of the syringe, the voltage source was turned off and the foil was removed from the collecting plate. The membrane could then carefully be removed from the non-stick foil.

Figure 4. A diagram of the electrospinning set up used by our lab.
Appendix B

HPLC Methods

A mobile phase consisting of 70% [0.124 M monopotassium phosphate (KH₂PO₄), 0.08 M dipotassium phosphate (K₂HPO₄)] and 30% Acetonitrile (CH₃CN) was created for HPLC analysis. A 500 µg RK per 1 mL PBS standard was used. Serial dilutions were made using this standard to create additional standards of 250, 125, 62.5, 31.25, 15.63, 7.81, 3.91, 1.95, 0.98, 0.49 and 0 µg/ml. The solution was pumped through a Hypersil GOLD C8 column at a rate of 1 ml/min for 10 minutes. RK showed a peak at approximately 3.88 minutes, as shown by Figure 10. Using the standard curve, the average amount of RK (µg/mL) in the PBS and standard deviation were found for each day and a bar graph was created for each treatment and dosage to observe the release.

![Chromatogram](attachment:chromatogram.png)

**Figure 5.** The chromatogram produced by running the 500 µg/ml RK standard through the HPLC at a rate of 1 ml/min.
Appendix C

Cumulative Release of RK

The elution data were graphed as cumulative release over time (Figure 6). These graphs were not used because some of the lines overlapped, making it difficult to distinguish between groups. However, it does illustrate how quickly the membranes released RK.
**Figure 6.** Cumulative release of RK from treated membranes over 14 days (n=4 per group).
Appendix D

Additional RK Elution Data

The following data are from a previous RK elution study performed with the help of Dr. Delbuque Guerra. These study data were not used because the time points were not consistent with media changes in cell culture. Other than the time points, the procedure was identical to the methods outlined previously in the thesis. The study was repeated using time points modeling when growth media would be changed during a culture. This gives a better estimate of the amount of RK the cells were exposed to in later experiments. The release profiles of this preliminary data, though, are consistent with the follow-up study (Figure 7).
**Figure 7.** Elution data from preliminary RK release study. Error bars are standard deviations (n=4 per group).
Appendix E

Additional ALP Results

The ALP study was attempted previously but the standard deviations were far too large to use the data. These large standard deviations were likely due to something like pipetting error. The study was repeated to attempt to decrease error. The data shown below are from the previous ALP study (Figure 8). The standard deviations of the data used in the thesis were much smaller.

Figure 8. ALP data from previous study. Data were not used because standard deviations were too large.