Developing a Drug Delivery System for Treatment of Vocal Fold Scarring

Aaron Michael Kosinski
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By  Aaron M. Kosinski

Entitled
Developing a Drug Delivery System for Treatment of Vocal Fold Scarring

For the degree of  Doctor of Philosophy

Is approved by the final examining committee:

Alyssa Panitch
	Chair
Preeti M. Sivasankar

Albena Ivanisevic

Kinam Park

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Head of the Graduate Program  Date
DEVELOPING A DRUG DELIVERY SYSTEM FOR TREATMENT OF VOCAL FOLD SCARRING

A Dissertation
Submitted to the Faculty
of
Purdue University
by
Aaron M. Kosinski

In Partial Fulfillment of the
Requirements for the Degree
of
Doctor of Philosophy

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ABSTRACT

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Vocal fold scarring is an affliction that results in the formation of a disorganized and stiff extracellular matrix (ECM) with abnormal ECM component densities & structures including a significant increase in collagen deposition. It is caused by improper healing post injury and results in profound changes in the biomechanical properties of the vocal folds impairing their ability to generate a normal mucosal wave during phonation.

Finding an effective treatment for vocal fold scarring has been elusive. Currently, treatments seek temporary solutions that correct glottal incompetence and reduce stiffness caused by the scar through the augmentation of the vocal folds using various injectable materials, such as hyaluronic acid or calcium hydroxylapatite. These solutions do not actually treat the actual scar, but only relieve its effects over the short term seeing as current injectable materials readily degrade.

To solve this problem researchers have been testing the impact various therapeutics have on helping prevent or regress vocal fold scarring. Unfortunately, realizing the full potential of such therapeutics has not been fully achieved due to many barriers. The
largest being that, even if anti-scarring agents are directly injected into a vocal fold scar, these therapeutics are readily cleared or metabolized by the vocal fold tissue over a short period of time. This necessitates the need to re-inject the therapeutic multiple times in order to maintain its efficacy. Though this is possible, issues of patient safety and tolerance quickly become significant problems one must address if one desires to pursue such a rigorous treatment method.

The overall goal of my research was to address this issue of effectively delivering therapeutics to an area of vocal fold scarring to facilitate healthy unscarred tissue regeneration. To accomplish this goal my early work focused on the development of *in vitro* unscarred and scarred culture systems in which to eventually test our newly developed delivery systems. Initial work with these systems focused on characterizing them in order to better understand the role RGD integrin binding site concentration and cell phenotype had on directing extracellular matrix gene expression in vocal fold fibroblasts. What we learned was that indeed RGD integrin binding site concentration does affect both depending on the circumstances. With changes in fibroblast phenotype from unscarred to scarred resulting in ECM gene up-regulation for all genes tested, except for HAS2 and decorin. Meanwhile, changes in RGD concentration only increased elastin and collagen type 3 alpha 1 expression as RGD concentration was increased in scarred vocal fold fibroblasts only. HAS2 was also down-regulated in scarred fibroblasts at the highest RGD concentration. However the other genes tested were unimpacted by RGD concentration changes in scarred or unscarred fibroblasts. This knowledge is critical in developing bioactive materials that, when implanted into sites of tissue damage
and scarring, direct cells to regenerate healthy tissues with normal ECM ratios and morphologies. Next, we moved on to developing our drug delivery system initially utilizing a nanoparticle based system. Our nanoparticle system was composed of a poly-lactic-co-glycolic acid (PLGA) core encapsulated within a shell of poly(N-isopropylacrylamide (pNIPAM) and had unique properties that were designed to help better facilitate more effective delivery of therapeutics including active targeting and the ability to respond to dynamic environmental stimuli. However, concerns arose that the small size of the nanoparticles would not allow for an effective amount of drug to be loaded into them and that they would more readily degrade and release loaded therapeutics due to their high surface to volume ratio. We, therefore, transitioned to using larger hydrogel templated PLGA microparticles instead. We also moved on to testing our ability to actually deliver an effective anti-fibrotic and anti-inflammatory agent to scarred vocal fold fibroblasts over the long term. To do this, the PLGA microparticles were loaded with the corticosteroid dexamethasone. Real-time PCR showed that only dexamethasone loaded microparticles proved effective at maintaining down regulation in the expression of COL3A1 and COL1A2 over the long term. While an ELISA showed that dexamethasone only decreased the deposition of the pro-inflammatory cytokine interleukin-6 over the long term, but had no impact on the other three pro-inflammatory cytokines tested.

Future work will look at trying to continue to improve the drug delivery platform for use in vocal fold scarring by combining the pNIPAM-co-acrylic acid shell, from our core + shell nanoparticle work, with the PLGA microparticle delivery system. Such a
combination would meld the advantages of the larger microparticle size and its ability to more readily deliver an effective dose of therapeutic with the many beneficial attributes, like targeting and responsiveness to environmental stimuli, of the pNIPAM-co-acrylic acid shell. Furthermore, with the addition of a shell, another level of control over therapeutic release would be available.
CHAPTER 1. INTRODUCTION

1.1 Background and Significance

It is estimated that 5-10% of the United States population suffers from some form of communication disorder resulting in estimated economic losses of $154 to $186 billion per year.\[^{10}\] These communication disorders can stem from many different sources including the impairment of the vocal folds, which are responsible for the generation of most human phonation capabilities. Due to this unique functional role, the vocal folds have evolved a compositional structure that provides them with strength,\[^{1, 2}\] which allows them to undergo significant stresses stemming from conversational phonation frequencies that range from \(\sim 100\text{-}300\text{Hz} \) at amplitudes of 1-2mm.\[^{2}\] While at the same time maintaining a high degree of flexibility that permits low effort generation of a wide range of sounds.\[^{1, 2}\]

1.2 Vocal Fold Structure

Human vocal folds are composed of many different layers.\[^{1, 2}\] The first is the epithelium layer which serves as a defensive barrier against external environmental factors like bacteria or air pollutants.\[^{1, 2}\] It is composed of stratified squamous epithelia unlike the rest of the respiratory tract, which is composed of ciliated columnar epithelia.\[^{1, 2}\] This epithelium layer is then anchored in place by the basement membrane zone (BMZ)
The BMZ is mainly composed of ECM components such as high amounts of proteoglycans like decorin, heparin sulfate, & chondroitin 4-sulfate as well as collagen type I & III and some elastin. Inferior to the BMZ is the superficial lamina propria layer. It is one of the most important layers in the vocal folds as this is where the mucosal wave is generated and allowed to then propagate through the other layers. As a result, it must be highly flexible, and is, therefore, composed of only limited amounts of mature organized elastin fiber networks & thin bundles of collagen type I & III fibers. It also contains significant amounts of the proteoglycan decorin.

Moving deeper we come to the intermediate lamina propria layer, which is composed of high levels of elastin, organized into mature organized fiber networks, as well as hyaluronan. Little collagen type I or III and proteoglycans are present in the intermediate lamina propria layer, although there is some versican. Inferior to the intermediate layer is the deep lamina propria layer, which is composed of significant amounts of collagen type I & III and elastin in the form of mature organized collagen or elastin fiber networks. Proteoglycans like versican, decorin, chondroitin 4-sulfate, and biglycan are also present in sizable amounts in the deep lamina propria layer. The final layer of the vocal folds is the vocalis muscle which, in tandem with other muscles, plays a significant role in controlling the overlying vocal fold layers in order to facilitate and control the dynamic range of sounds generated during phonation.
1.3 Vocal Fold Scarring

As the previous section elucidated, the structure of the vocal folds is highly complex due in part to the unique functional role they play in allowing the controlled generation of the wide range of sounds in human phonation. Unfortunately, this also means that any damage to the vocal folds can result in significant, if not complete, loss of a human’s ability to phonate. Such damage can come from many sources including intubation, vocal misuse or phonotrauma, gastroesophageal reflux disease, chemical irritants like smoking or air pollution, blunt/penetrating trauma, cancer, or various surgical treatments. How the vocal folds respond to these damaging events depends on the extent of the damage as well as how often the damaging event occurs. If the damaging event is mild and occurs infrequently then the normal vocal fold wound healing process will be able to regenerate the highly tuned ECM structure with no adverse complications. However, if the damaging event is significant or occurs frequently then the normal vocal fold wound healing process will be unable to regenerate the highly tuned ECM structure causing pathological disorders to manifest.

One such pathological disorder is vocal fold scarring and it is one of the most difficult to treat. Vocal fold scarring usually occurs due to external trauma, chronic vocal misuse, a major or chronic inflammation event, or iatrogenic causes. Once formed the vocal fold scar effects the patient by manifesting as vocal fatigue, increased phonation effort, hoarseness, breathing difficulties or shortness of breath usually due to excessive air loss during phonation, glottal incompetence, or if scarring is severe enough, complete loss of phonation capabilities. All of these manifestations stem from the fact that post...
injury the vocal fold wound healing process is unable to properly regenerate the normal ECM structures that make up the layers of the vocal fold.\textsuperscript{[2, 16, 17]}

Instead, what occurs is the formation of a disorganized ECM that does not exhibit the normal layered structure or ECM component distribution.\textsuperscript{[2, 16, 17]} This change in relationship between the various ECM component densities and organization results in profound changes in the biomechanical properties, like viscosity and shear strength, of the vocal folds, thereby resulting in impairment of normal mucosal wave generation.\textsuperscript{[2, 16, 17]} Since the mucosal wave is initially propagated in the superficial layer of the lamina propria, it is this layer that is most affected by changes in its ECM composition.\textsuperscript{[1, 2, 16, 17]}

Although we know that vocal fold scarring causes dysphonia (loss of normal vocal function) due to improper regeneration of the ECM, much research is still being done in order to better understand the biological processes behind vocal fold scar formation. To this end, researchers have learned from various animal models that the regeneration of the ECM post injury is a dynamic process with various ECM components being expressed at various time points during the wound healing process.\textsuperscript{[16, 17]} Overall, it has been shown in these animal models that vocal fold scar tissue is characterized by an increase in collagen that is present in thick disorganized bundles and low levels of elastin that is present in a fragmented network of disorganized fibers.\textsuperscript{[17]} High levels of fibronectin and low levels of hyaluronan are also present.\textsuperscript{[17]} Decorin and fibromodulin are also decreased in the rabbit model.\textsuperscript{[17]} These results will prove invaluable in leading researchers to a better
understanding of vocal fold scarring and potentially better treatments that prevent scar formation or are able to revert scarred tissue back to a healthy state.

1.4 Current Treatments for Vocal Fold Scarring

Once a patient has been diagnosed with a vocal fold scar, the severity of the scarring and its subsequent impact on phonation must be determined. To do this a comprehensive voice evaluation must be conducted by a speech language pathologist in conjunction with a physician specializing in otolaryngology. The evaluation includes a detailed review of the patient’s past medical history including voice use profile, instrumental voice assessment, and most importantly a laryngeal videostroboscopy. Videostroboscopy allows the pathologist and the physician to visualize the vocal folds during vibration helping them identify decreased mucosal wave or adynamic segments of the vocal folds caused by the scar.[3, 16] Depending on these findings direct laryngoscopy using high-powered microscopy may be warranted in order to fully characterize the nature and extent of the vocal fold scar.[3]

Once the severity of the vocal fold scar has been ascertained through these diagnostic procedures a treatment regimen can be developed. For example, if vocal fold scarring is not severe then voice therapy can be used to alleviate dysphonia caused by the scar. Voice therapy may involve patient education aimed at reducing or eliminating maladaptive compensatory behaviors that arise due to the scar.[3, 16] It can also be helpful in improving the efficiency of vocalization in patients with vocal fold scarring.[3]
However, if vocal fold scarring is severe or voice therapies fail then surgical treatment may be necessary.

Surgical treatments mainly revolve around vocal fold augmentation medialization.\cite{3, 16} This procedure seeks to correct glottal incompetence, that commonly occurs due to vocal fold scarring, by injecting a material into the scarred vocal folds in order to medialize them and thereby improve phonation capabilities in the patient.\cite{3, 16} The most common types of materials used in the clinic as medialization agents include calcium hydroxylapatite, human collagen, micronized dermis, hyaluronic acid, methylcellulose, or bovine collagen.\cite{18}

1.5 **Disadvantages of Current Treatments**

Though vocal fold augmentation medialization with the aforementioned materials has proven promising, this treatment for vocal fold scarring induced glottal incompetence is not the optimal form of treatment. First off, the current materials used to medialize the vocal folds are all of a temporary nature with the body reabsorbing these materials over a rather short period of time, usually within a few months to a year. This necessitates reinjection of these materials into the vocal fold in order for the patient to continue to reap the benefits from vocal fold augmentation medialization.\cite{2} Second, some of the aforementioned treatments can also cause undesired immune response reactions.\cite{18} For example, if bovine collagen is not correctly processed not all of the bovine antigens will be removed from it. As a result, when it is then injected into the human vocal folds, it will illicit an undesirable inflammatory immune response potentially causing further
scarring. Finally, and most importantly, none of these current materials actually facilitate the regeneration of the vocal folds back to their normal unscarred state nor could they be used to prevent vocal fold scar formation. As a result, they provide no means of achieving long-term repair and restoration of function of scarred vocal folds.\(^2\)

1.6 Developing Better Treatments

To overcome these disadvantages researchers have been trying to engineer better materials/treatments utilizing a variety of different methods. One such method has used complex natural decellularized ECMs as opposed to individual ECM derived components, like collagen, to facilitate the regeneration of damaged or scarred vocal folds. These decellularized matrices are usually obtained from xenogeneic or allogeneic sources. For example xenogeneic porcine liver-derived ECM has been used to facilitate the successful regeneration of injured canine vocal folds.\(^{19}\) The major advantage of these types of matrices is the fact that they inherently contain a heterogeneous mixture of biological components that are critical in facilitating normal tissue regeneration. Disadvantages include the fact that current decellularization processes can remove some of the natural decellularized ECM’s unique biological components. However, care must be taken to ensure that the decellularization process is still rigorous enough to remove potential immunogenic factors, that could induce an undesirable immune response, or viruses, that could cause disease. The most difficult disadvantage to overcome will be how best to implant these matrices without damaging the matrix itself or healthy tissue that already resides at the implant site.
A second method being explored is the engineering of multi-component synthetic hydrogels in order to form a hybrid material that contains the beneficial attributes of each component. For example, chemically modified hyaluronic acid (HA) and gelatin were combined to form a hybrid HA-gelatin hydrogel.\textsuperscript{[20]} This hybrid material has beneficial attributes of both HA and gelatin. In the case of HA, one can vary the amount of HA crosslinking to tune the biomechanical properties of the hybrid hydrogel.\textsuperscript{[20]} While gelatin’s usage can enhance the poor adherence properties of HA.\textsuperscript{[20]} The major advantage of this approach is that it allows one, in a highly controlled and reproducible manner, to mix, match, and tune mechanical and biological properties to treat vocal fold disorders like scarring. The disadvantages consist of the fact that residual unreacted components can prove toxic to cells. Of greater concern is the number of challenges, like scale and cost, that exist with engineering such highly complex multi-component hybrid hydrogel systems.

A third method under development involves combination treatments. Here researchers are creating complex systems that combine synthetic or natural materials and biologically relevant cell lines or molecules, such as growth factors, in order to address many different vocal fold disorders like scarring. A current example of a combination treatment involved combining bone marrow mesenchymal stem cells with a synthetic ECM which, when administered to damaged rat vocal folds, promoted healthier deposition of ECM versus controls.\textsuperscript{[21]} The advantage of these combination treatments is that they will better facilitate the healthy integration of the implant with the host tissue. The disadvantages include the fact that cells and some molecules can cause undesired immune responses.
Furthermore, biologically active components could have undesired off target effects outside the vocal folds.

The fourth, and final method discussed here, involves the use of nanoparticles or other nano- or micro-structures in order to facilitate the delivery and controlled release of relevant biological agents to damaged or diseased vocal folds.\textsuperscript{22, 23} For example, PLGA nanoparticles loaded with Texas Red-dextran have shown successful release of the Texas Red-dextran when injected \textit{in vivo} into murine vocal folds.\textsuperscript{22} While hepatocyte growth factor loaded PLGA nanoparticles were shown to reduce procollagen expression \textit{in vitro} by 3T3 fibroblasts signifying successful release of functional hepatocyte growth factor from the PLGA nanoparticles.\textsuperscript{22} The advantages of this method are that therapeutics administered to the vocal folds via nano- or micro-structured delivery systems will help protect them from degradation and provide a depot from which the therapeutic can be released in a controlled manner over time. Disadvantages include the fact that the use of these nano- or micro-structures as bulking agents, in order to correct the glottal incompetence that sometimes results from vocal fold scarring, is questionable as such structures are not very conducive for this use.

1.7 Specific Aims

The goal of this dissertation is to address the gap in effective clinical treatments for vocal fold scarring by developing a particle based therapeutic delivery system that will both target and deliver therapeutics that can prevent or treat vocal fold scarring. Such a system will potentially enable us to deliver multiple therapeutics in a highly controlled
manner. This is important when one wants to facilitate proper tissue regeneration. To achieve this three different specific aims were proposed and completed.

1.7.1 Aim #1
Characterize the role different concentrations of RGD, an integrin binding site, plays in directing extracellular matrix gene expression in scarred and unscarred in vitro culture systems of immortalized human vocal fold fibroblasts.

Much work has been done pertaining to the role RGD integrin binding sites play in cell growth, proliferation, and motility.[4-7] However, little work has been done to elucidate the role that RGD integrin binding sites play in directing ECM gene expression. Little is also known pertaining to how ECM expression changes when a cell’s phenotype changes from unscarred to scarred. This aim sought to address this issue by assessing how relative gene expression profiles of ECM components expressed by immortalized human vocal fold fibroblasts (I-HVFFs) change based on phenotype, unscarred versus scarred, and on the concentration of RGD on which they are grown.

1.7.2 Aim #2
Develop and characterize a nanoparticle system that resists aggregation caused by lyophilization, responds to environmental based stimuli, and can be modified with targeting ligands.
A novel poly(lactic-co-glycolic acid) (PLGA) core + poly(N-isopropylacrylamide) (pNIPAM)-co-acrylic acid (AAc) shell nanoparticle system was synthesized and characterized. A critical characteristic of this system is the ability of the pNIPAM-co-AAc shell to prevent the aggregation of the PLGA cores during lyophilization without the need of cryo-protectants. Secondly, the addition of the pNIPAM-co-AAc shell imbues the overall PLGA core + pNIPAM shell nanoparticle system with more dynamic properties including the ability to respond to environmental stimuli. Thirdly, the addition of the acrylic acid monomer to the pNIPAM shell polymer provides a functional handle used to append targeting ligands in an acrylic acid monomer concentration dependent manner. Finally, results from in vitro experiments demonstrate the biocompatibility of these PLGA core + pNIPAM-co-AAc nanoparticles with cells.

1.7.3 Aim #3

Evaluate the long-term anti-fibrotic and anti-inflammatory capabilities of the corticosteroid dexamethasone on scarred I-HVFFs when released from PLGA hydrogel templated microparticles.

Controlling the release of various therapeutics that show promise treating scarring may improve their efficacy. To shed light on this issue we loaded dexamethasone (dM), an anti-fibrotic and anti-inflammatory therapeutic, into a hydrogel templated poly-lactic-co-glycolic acid (PLGA) microparticle (mP) delivery vehicle. Release studies showed successful loading and release profiles of dM from three types of PLGA microparticles whose polymer chain terminating moiety, acid or ester, and molecular weight, low or
high, were varied. To ascertain dM’s anti-fibrotic and anti-inflammatory effectiveness when released from PLGA mPs over the long term, they were exposed to scarred human vocal fold fibroblasts in vitro. Marked down regulation in collagen in scarred fibroblasts was only seen in those treated with dM loaded mPs, indicating dM maintained its anti-fibrotic activity over the long term. While ELISAs illustrated that of the 4 pro-inflammatory cytokines tested dM’s anti-inflammatory affects over the long term were only maintained for one, IL-6, of the cytokines tested.
1.8 List of References


CHAPTER 2. CHARACTERIZING THE IMPACT RGD CONCENTRATION PLAYS IN DIRECTING ECM GENE EXPRESSION IN SCARRED AND UNSCARRED IN VITRO VOCAL FOLD FIBROBLAST CULTURE SYSTEMS

2.1 Abstract

The impact of RGD integrin binding site concentration and cell phenotype on directing extracellular matrix (ECM) gene expression in vocal fold fibroblasts is little understood. Less is known about cell response to RGD concentration on a biomaterial when fibroblasts are in a scar-like environment compared to a healthy environment. We investigated the effects of varying RGD integrin-binding peptide surface concentration (0.01mM – 1mM), on ECM gene expression of elastin, collagen type 3 alpha 1, decorin, fibronectin, hyaluronan synthase 2, and collagen type 1 alpha 2 in scarred and unscarred immortalized human vocal fold fibroblasts (I-HVFFs). Phenotype and RGD concentration affected ECM gene expression. Phenotype change from unscarred to scarred resulted in ECM gene up-regulation for all genes tested, except for decorin. Systematically altering RGD concentration affected the expression of elastin and collagen type 3 alpha 1 in a scarred phenotype but not unscarred. Specifically greater up-regulation in gene expression was observed with higher RGD concentrations. This research demonstrates that controlling RGD concentration may influence ECM gene expression levels. Such knowledge is critical in developing bioactive materials that, when
implanted into sites of tissue damage and scarring, direct cells to regenerate healthy tissues with normal ECM ratios and morphologies.

2.2 Introduction

Much work has been done on the role of RGD integrin binding sites in cell binding, growth, proliferation, and motility.\cite{1-4} As a result RGD has been incorporated into many different types of materials in order to facilitate the binding and proliferation of cells to normally non-adherent materials.\cite{5-7} For example, non-adherent hyaluronic acid has been modified with RGD to develop an adherent, bioactive material for correcting vocal fold defects.\cite{7} However, improving cell adherence does not usually result in the proper full restoration of healthy tissues, vocal folds or otherwise. That is because healthy tissues are complex heterogeneous structures that require the expression and deposition of ECM components in normal ratios and morphologies in order to maintain their proper function.

Researchers have been working to develop materials that facilitate such regeneration via many different methods. One such method is the use of RGD to modulate the expression of genes that play key roles in regeneration of the tissue being studied. For example TiO$_2$ nanotube surfaces have been modified with varying amounts of RGD.\cite{8} Rat bone marrow stromal cells when grown on these substrates showed a dramatic enhancement in the expression of osteogenic genes on nanotube surfaces modified with higher concentrations of RGD versus lower.\cite{8} This same trend was also seen in a 3D environment. Here, goat bone marrow stromal cells were grown in
poly(ethylene glycol) diacrylate hydrogels modified with varying amounts of RGD.\[^{[9]}\]

Again, as RGD concentration increased, bone-related marker expression also increased.\[^{[9]}\] Finally, researchers have also looked at how cell lines from soft tissue sources behaved when both RGD concentration and integrin type were varied.\[^{[10]}\]

They found that as the adhesiveness of the surface increased, either due to the increased RGD or the use of a more adherent integrin, a decrease in overall ECM production by the cell lines tested was observed.\[^{[10]}\]

Although RGD’s impact on gene expression has been studied in relation to bone cell differentiation and impact on overall ECM production of cells from soft-tissue, gaps still exist.\[^{[8-10]}\] For one, though we have a gross understanding, as mentioned in the third example above, on how RGD concentration impacts overall ECM deposition. A finer break-down of how RGD concentration impacts the expression of individual ECM genes would be of greater help in designing better biomaterials that facilitate the expression of individual ECM components in healthy ratios. Furthermore, little is understood with regards to how ECM component gene expression is affected by changes in a cell line’s phenotype brought on by changes in the environment. This knowledge is especially important to have so one can understand how cells growing on RGD modified materials might behave when implanted into a site of tissue damage and scarring.

Here we begin to address these issues by growing adherent immortalized human vocal fold fibroblasts (I-HVFFs) in scar-like or healthy environments on NHS-ester
polyethylene glycol (PEG) thin film coated glass substrates modified with varying concentrations of RGD integrin binding peptide. Treated I-HVFFs were then probed utilizing RT-qPCR to assess the impact of varying RGD concentration and cell phenotype on the expression of six ECM genes including elastin (ELN), decorin (DCN), fibronectin (FN), collagen type 1 alpha 2 (COL1A2), collagen type 3 alpha 1 (COL3A1), and hyaluronan synthase 2 (HAS2). These ECM genes were targeted as they are the primary constituents that dictate the biomechanical properties of vocal fold connective tissue.[11-13]

2.3 Materials and Methods

2.3.1 Peptide Synthesis

All peptides were synthesized using a 0.4mmol scale Knorr-amide resin (Synbiosci Corp.) and standard FMOC (9-fluorenylmethyloxycarbonyl) chemistry. Once synthesized peptides were capped with an acetyl group, they were then cleaved from the resin using 95% trifluoroacetic acid (Sigma-Aldrich), 2.5% water, 1.25% triisopropylsilane (Sigma-Aldrich), and 1.25% ethanedithiol (Sigma-Aldrich). Cleaved peptides were precipitated in cold ether and then recovered utilizing centrifugation. The crude peptides were then further purified using an acetonitrile gradient on an AKTA Explorer FPLC (GE Healthcare) equipped with a 22/250 C18 reversed phase column (Grace Davidson). Molecular weight and purity was confirmed by time of flight MALDI mass spectrometry using a 4800 Plus MALDI TOF/TOF Analyzer (Applied Biosystems).
2.3.2 Substrate Modification

NHS-ester PEG thin film coated glass slides, termed Nexterion H, were purchased from Schott. These substrates once cut to 10x10mm were rinsed with 50mM sodium borate buffer pH 7.5. They were then incubated, according to the literature,[14] in varying concentrations of peptide resuspended in 50mM sodium borate buffer pH 7.5 for ~21 hours with 300rpm shaking on a plate shaker at room temperature. Post incubation substrates were dried with dry nitrogen gas and used immediately or stored at -20°C.

2.3.3 Cell Culture

I-HVFFs were developed and provided to us by Dr. Susan Thibeault at the University of Wisconsin, Madison.[15] These I-HVFFs were kept in an unscarred phenotypic state by growing them in Dulbecco’s Modified Eagles Medium (Sigma-Aldrich) supplemented with 10% FBS (Sigma-Aldrich), 1% penicillin/streptomycin (Sigma-Aldrich), 1% MEM non-essential amino acids (Sigma-Aldrich), and 200ug geneticin (G418; Teknova) per mL of media. Unscarred I-HVFFs were induced into a scarred phenotypic state by utilizing induction media. This media contained all the same aforementioned media components except the 10% FBS was removed and 10ng/mL of transforming growth factor – beta 1 (TGF-β1; Biosource) was added. Successful transition of unscarred I-HVFFs to a scarred phenotype was confirmed using immunohistochemistry.
2.3.4 Peptide Functionality

NHS-ester PEG thin film substrates modified with 0.1, 0.01, or 0.001mM of RGD or RGE were incubated with 20,000 I-HVFFs/mL in normal unscarred media for 4hrs at 37°C at 5% CO₂. Post-incubation all substrates were briefly rinsed in PBS pH 7.4 to dislodge any non-adhered I-HVFFs. Ten random images of each substrate’s surface were then taken using a Nikon Eclipse TS100 optical microscope & digital camera. Finally, the number of I-HVFFs on the differently modified substrates was manually counted & then this quantitative data was analyzed using basic statistical software.

2.3.5 Immunohistochemistry

20,000 I-HVFFs/mL in normal unscarred media were incubated with NHS-ester PEG thin film substrates previously modified with 0.01 or 1mM RGD for 4hrs at 37°C & 5% CO₂. All substrates were then briefly rinsed in PBS pH 7.4 to dislodge any non-adhered I-HVFFs post incubation, and placed back in normal unscarred media until I-HVFF confluency reached ~50%. Once reached, half were rinsed and placed back in normal unscarred media while the other half were placed in the aforementioned induction media. They were then allowed to grow/induce for 5 days in their respective media at 37°C & 5% CO₂ with a media change on day 2. The cells were then fixed for 30 minutes in ~10% formaldehyde (Mallinckrodt Chemicals) and permeabilized with 0.5% Triton-X 100 (Sigma-Aldrich) for 20 minutes. Blocking occurred in 3% bovine serum albumin (BSA; SeraCare Life Sciences) for 1hr at room temperature. Next all substrates were incubated for 90 minutes at room temperature with primary 1:200 α-smooth muscle actin antibody (α-SMA; Invitrogen) in 0.1%
BSA + PBS pH 7.4. Subsequently, substrates were incubated with 5μg/mL of AlexaFluor 488 goat anti-mouse secondary antibody (Invitrogen) in 2% BSA + PBS pH 7.4 for 45 minutes at room temperature. Finally, after rinsing with 0.05% Tween 20 and then plain PBS pH 7.4 the I-HVFFs were imaged on an Olympus FV1000 confocal microscope.

2.3.6 RT-qPCR

NHS-ester PEG thin film substrates were modified in the same aforementioned fashion with 0.01, 0.1, or 1mM of RGD peptide. Following modification I-HVFFs were grown on them using techniques described earlier. Post growth, RNA was isolated from each sample utilizing the Nucleospin total RNA isolation kit (Clontech). Purified RNA was then quantified using a NanoDrop 2000 spectrophotometer (Thermo). Reverse transcription of the pure RNA was achieved using a High-Capacity cDNA reverse transcription kit (Life Technologies). Finally, real-time or quantitative polymerase chain reaction (qPCR) was conducted utilizing an Applied Biosystems 7500 real-time PCR machine. Briefly, qPCR reactions were set-up utilizing the TaqMan gene expression master mix (Life Technologies), six ECM gene-specific probes (Life Technologies) [Including human ELN, DCN, FN, COL1A2, COL3A1, and HAS2.], and template cDNA from the different samples. qPCR reaction conditions were as follows: 50°C for 2min, 95°C for 10min, and 40 cycles of 95°C for 15sec, and 60°C for 1min. Human β-actin was used as the endogenous control. The average cycle threshold (Ct) of each sample was then used for the calculation of expression levels using the relative comparative Ct method.
2.4 Results and Discussion

Peptide and NHS-ester PEG thin film functionality was confirmed. Substrates modified with 0.1mM RGD had an average of ~35 I-HVFFs per mm$^2$ adhered while 0.001mM RGD had only an average of ~18 I-HVFFs per mm$^2$ (Figure 2.1A). Furthermore, for each concentration of RGD and RGE more I-HVFFs per mm$^2$ adhered to the integrin-binding peptide, RGD, then the non-integrin binding control, RGE, peptide (Figure 2.1A). Finally, the fewest number of I-HVFFs, at an average of ~2 I-HVFFs per mm$^2$, bound to unmodified control NHS-ester PEG thin film substrates (Figure 2.1A) confirming PEG’s anti-fouling capabilities. These data overall suggest that the PEG, RGD, and RGE peptides all functioned according to the literature,$^{[14,16]}$ and that I-HVFF binding mainly occurred through the RGD integrin binding site. Immunohistochemistry confirmed that I-HVFFs grown in a healthy environment, without TGF-β1, were unscarred & did not express α-SMA (Figure 2.1B). While those growing in a scar-like environment, with TGF-β1, became myofibroblastic/scared & expressed α-SMA (Figure 2.1D and 1E)$^{[17]}$. Furthermore, increasing RGD concentration from 0.01mM (Figure 2.1D) to 1mM (Figure 2.1E) resulted in greater spreading of the scarred I-HVFFs.$^{[16]}$
Figure 2.1 Peptide and NHS-ester PEG thin film functionality confirmed. High concentrations of RGD, 0.1mM, bind more I-HVFFs than lower concentrations (A). PEG thin film has anti-fouling capabilities with unmodified control binding the lowest number of I-HVFFs per mm² (A). Immunohistochemical α-SMA staining verified unscarred I-HVFFs (B) were induced into a scarred myofibroblastic state (D & E). Furthermore, I-HVFFs spread more readily on high 1mM RGD (E) versus low 0.01mM RGD (D). B = α-SMA stained unscarred I-HVFFs on 0.01mM RGD, C = brightfield image of unscarred I-HVFFs on 0.01mM RGD, D = α-SMA stained TGF-β1 scarred I-HVFFs on 0.01mM RGD, & E = α-SMA stained TGF-β1 scarred I-HVFFs on 1mM RGD. Scale bars = 20um. Treatments in A with non-matching symbols are statistically different from each other according to a one-way ANOVA + Tukey post-hoc test; p < 0.05.
RGD concentration and fibroblast phenotype affected the expression of ECM genes. With respect to the control, no RGD unscarred I-HVFFs which was set equal to 1, changes in the I-HVFF growing environment caused a change in phenotype, from unscarred to scarred, resulting in up-regulated gene expression of all six ECM genes except DCN & HAS2 (Figure 2.2). Variations in RGD integrin concentration impacted ECM gene expression only in specific phenotypes. Unscarred I-HVFFs were unaffected by changes in RGD integrin concentration for all six ECM genes except HAS2 which saw down-regulation with addition of any RGD (Figure 2.2). Scarred I-HVFFs were affected, with ELN gene expression being up-regulated as the concentration of RGD increased (Figure 2.2). Similar results, albeit of smaller magnitude were obtained for COL3A1 gene expression as well (Figure 2.2). HAS2 expression was also impacted with a further down regulation in its expression at the highest RGD concentration in scarred I-HVFFs (Figure 2.2). DCN, FN, & COL1A2 remained unchanged in scarred I-HVFFs with respect to changes in RGD concentration (Figure 2.2).
Figure 2.2 Illustrates that varying both RGD concentration and cell phenotype, from unscar to scar, has various impacts on the expression of six ECM genes including elastin (ELN), collagen 3 alpha 1 (COL3A1), hyaluronan synthase 2 (HAS2), decorin (DCN), fibronectin (FN), collagen 1 alpha 2 (COL1A2). Of note is the major impact scarring and varying RGD has on ELN & COL3A1 expression. While the phenotype change from unscarred to scarred I-HVFFs results in up-regulation in all six ECM genes tested except for HAS2 & DCN. A = 0.01mM RGD Unscar, B = 1mM RGD Unscar, C = No RGD Scar, D = 0.01mM RGD Scar, E = 0.1mM RGD Scar, F = 1mM RGD Scar. Treatments within each ECM gene with non-matching symbols are statistically different from each other according to a one-way ANOVA + Tukey post-hoc test; p < 0.05.
In summary, our results demonstrate that RGD concentration and cell phenotype impact gene expression of the ECM genes investigated here. This suggests that controlling RGD concentration with respect to cell phenotype may improve a material’s bioactivity and thereby improve its regenerative properties when implanted in any type, vocal fold or otherwise, of damaged or scarred tissue. Future work will focus on ascertaining if these changes in ECM gene expression also result in changes in protein expression.
2.5 List of References


CHAPTER 3. DEVELOPMENT AND CHARACTERIZATION OF A NANOPARTICLE SYSTEM THAT RESISTS LYOPHILIZATION BASED AGGREGATION, RESPONDS TO ENVIRONMENTAL STIMULI, AND CAN BE MODIFIED WITH TARGETING LIGANDS

3.1 Abstract

Poly(lactic-co-glycolic acid) (PLGA) is a popular material used to synthesize nanoparticles for drug delivery. However, PLGA nanoparticles lack desirable attributes including active targeting abilities, resistance to aggregation during lyophilization, and the ability to respond to dynamic environmental stimuli. To overcome these issues, we synthesized a nanoparticle consisting of a static PLGA core encapsulated within a dynamic shell of poly(N-isopropylacrylamide). Dynamic light scattering and transmission electron microscope imaging were used to characterize the nanoparticles, while an MTT assay and ELISA suggested biocompatibility in THP1 cells. Finally, a collagen type II binding assay showed successful modification of these nanoparticles with an active targeting moiety.

3.2 Introduction

Poly(lactic-co-glycolic acid) (PLGA) is a biocompatible, biodegradable synthetic polymer that is easy to fabricate into size-specific nanoparticles and has a well-documented ability for sustained therapeutic release.[1-3] Although many investigators and commercial entities have embraced these desirable attributes to construct PLGA
nanoparticles for use in research and commercial products.\textsuperscript{[1, 3-5]} PLGA nanoparticles still have significant limitations when it comes to using them to deliver therapeutics to a specific disease site. One such limitation is that PLGA nanoparticles, when delivered intravenously, have no active targeting capabilities and are restricted to passive targeting via the enhanced permeability and retention (EPR) effect seen in cancerous and inflamed tissues.\textsuperscript{[6-8]} Tissue accumulation via the EPR effect can provide potential benefits for cancer drug delivery,\textsuperscript{[6]} but it renders drug delivery for other disease states that lack the EPR effect problematic. Other investigators have attempted to overcome this challenge by modifying PLGA nanoparticles for “active” targeting with the addition of monoclonal antibodies.\textsuperscript{[9]} Another challenge is the long-term storage of aqueous suspensions of PLGA nanoparticle systems, due to hydrolytic degradation of the polymer and subsequent release of the encapsulated therapeutic.\textsuperscript{[10, 11]} To overcome this issue, researchers have used lyophilization to prepare therapeutic-containing PLGA nanoparticles for long-term storage.\textsuperscript{[10, 11]} However, this simple solution introduces another problem: lyophilization causes the PLGA nanoparticles to aggregate into clumps that upon rehydration, readily fall out of solution.\textsuperscript{[10, 11]} Finally, PLGA nanoparticles are limited in their responses to environmental stimuli like temperature or pH. Although environmental sensitivity is not a requirement for nanoparticle systems, it does provide advantages, such as the ability to control therapeutic release from nanoparticle carriers only when they are exposed to environments inherent to particular disease states.\textsuperscript{[12]}

In an effort to overcome the limitations associated with PLGA nanoparticles, as well as increase their functionality, we developed a method to encapsulate PLGA nanoparticles
within a shell of poly(N-isopropylacrylamide) (pNIPAM). pNIPAM was chosen because it is a versatile material with some inherently unique properties. The pNIPAM shell can be synthesized as a homopolymer, or as a copolymer with incorporation of a variety of chemical moieties with defined concentrations that can then be further modified with active targeting functional groups.\textsuperscript{[13-15]} Additionally, pNIPAM can tolerate lyophilization;\textsuperscript{[16]} we hypothesized that a pNIPAM shell would protect encapsulated PLGA nanoparticles from aggregation during lyophilization. Finally, pNIPAM is a temperature responsive polymer that can undergo a phase transition at a lower critical solution temperature (LCST) around 31-32°C.\textsuperscript{[17]} At temperatures below its LCST, pNIPAM nanoparticles are swollen and hydrophilic.\textsuperscript{[13]} However, once the temperature increases above the LCST, pNIPAM nanoparticles collapse as the polymer becomes hydrophobic.\textsuperscript{[13]} Thus, by encapsulating PLGA nanoparticles in a pNIPAM shell, the nanoparticles should have the ability to respond to environmental stimuli, such as temperature. This response could then be used for loading and/or controlling the release of therapeutics from the pNIPAM layer.\textsuperscript{[18]} Here, we characterize this novel PLGA core + pNIPAM shell nanoparticle system.

3.3 Materials and Methods

3.3.1 Core + Shell Nanoparticle Synthesis

Synthesis of the core + shell nanoparticles occurred in two steps: synthesis of the PLGA cores followed by the addition of the pNIPAM shell (Figure 3.1). First, PLGA cores were synthesized using a single emulsion technique.\textsuperscript{[26]} Briefly, 200mg of poly(dl-
lactide/glycolide) 50:50 (PLGA; Polysciences Inc.) was dissolved in 5mL of
dichloromethane (DCM; Sigma-Aldrich), added to 20mL of 5% polyvinyl alcohol (PVA;
Alfa Aesar; Avg. MW = 11,000 – 31,000), and homogenized for 30s using a probe
sonicator (Branson Sonifier 450) to generate a single emulsion. The emulsion was then
added to 100mL of rapidly stirred distilled water and left overnight to allow for full
evaporation of the DCM. The PLGA nanoparticles were further purified via
centrifugation washes with distilled water. Any clumps of PLGA nanoparticles that
remained after centrifugation were disrupted using brief sonication. The PLGA cores
were then encapsulated in pNIPAM shells using aqueous free radical precipitation
polymerization under a nitrogen atmosphere. Briefly, 0.27g N-isopropylacrylamide
[2.385mmol] (Polysciences Inc.), 0.021g N-N’-methylene bisacrylamide [0.136mmol]
(Fluka), 0.012g sodium dodecyl sulfate [0.042mmol] (Sigma Aldrich), and 0.015g
ammonium persulfate [0.066mmol] (Sigma Aldrich) were dissolved in 30mL of distilled
water and purged of oxygen by nitrogen bubbling. For nanoparticle targeting, either
1.67µL [0.024mmol or 1 mol%] or 8.35 µL [0.121mmol or 5 mol%] of acrylic acid (AAc;
Alfa Aesar) were included. Meanwhile, 20mL of the PLGA nanoparticle cores were
added to a 250mL three-neck round bottom flask and equilibrated to 70°C for 20 minutes
under nitrogen with stirring. Then, 10mL of the shell solution was added to the 70°C
equilibrated PLGA nanoparticle cores and allowed to polymerize. Additional 5mL
aliquots of shell solution were added 30, 50, 70, and 90 minutes after the initial
polymerization. Polymerization continued for 6 hours after the final addition of shell
solution. Purification was achieved through dialysis of the PLGA core + pNIPAM shell
nanoparticles against distilled water for 7 days in 15,000 MWCO dialysis tubing
(Spectrum Laboratories, Inc.). Then centrifugation washes were performed in order to further isolate the core + shell nanoparticles. Any clumps of core + shell nanoparticles post centrifugation were dispersed using brief sonication. Finally, a portion of the samples were lyophilized and then rehydrated in distilled water.

3.3.2 Transmission Electron Microscopy (TEM) Characterization

Images were taken of the pre- and post- lyophilized nanoparticle samples stained with 2% uranyl acetate. The stained nanoparticles were then placed on a glow-discharged 400 mesh coated with formvar + carbon film, and then placed in a Philips CM-100 TEM where images were captured on Kodak SO-163 electron image film.

3.3.3 Nanoparticle Sizing and Zeta Potential

Measurements were taken with a Malvern Zetasizer Nano ZS90. Pre- and post-lyophilized nanoparticles were suspended in distilled water and analyzed for particle size in polystyrene cuvettes at 25°C and 37°C. Temperature sweeps were performed by varying temperature from 20°C to 50°C to 20°C in 1° increments with measurement of particle size with each change in degree. Disposable Malvern ζ-potential cuvettes were used to acquire ζ-potential measurements at 25°C and 37°C. Note after making any change in temperature, nanoparticle samples were allowed to equilibrate for five minutes before any sizing or zeta measurements were made.
3.3.4 Peptide Synthesis and Purification

The collagen type II binding peptide single amino acid sequence consisting of WYRGRLGC was identified from the literature.\textsuperscript{[24]} It was synthesized at a 0.4mmol scale on Knorr-amide resin (Synbiosci Corp.) using standard FMOC (9-fluorenylmethyloxycarbonyl) chemistry. Two different chemistries were used to couple each amino acid (Synbiosci Corp). The first coupling reagents consisted of N-hydroxybenzotriazole (HOBt; Synbiosci) and N,N’ diisopropylcarbodiimide (DIC; Sigma-Aldrich) and the second coupling reagents were O-Benzotriazole-N,N,N’,N’-tetramethyl-uronium-hexafluoro-phosphate (HBTU; Synbiosci) and lutidine (Sigma-Aldrich). Following synthesis, the peptide was cleaved from the resin with 95% trifluoroacetic acid (Sigma-Aldrich), 2.5% water, 1.25% triisopropylsilane (Sigma-Aldrich), and 1.25% ethanedithiol (Sigma-Aldrich), precipitated in cold ether, and recovered by centrifugation. It was then purified with an acetonitrile gradient on an AKTA Explorer FPLC (GE Healthcare) equipped with a 22/250 C18 reversed phase column (Grace Davidson). Molecular weight was confirmed by time of flight MALDI mass spectrometry using a 4800 Plus MALDI TOF/TOF Analyzer (Applied Biosystems). Theoretical molecular weight of WYRGRLGC was calculated to be 1009.1 while the actual molecular weight was found to be 1009.58. A biotinylated version of the peptide (biotin-WYRGRLC) was purchased from Genscript and its theoretical molecular weight was calculated to be 1179.42 while its actual molecular weight was found to be 1179.8.
3.3.5 Modifying Core + Shell Nanoparticle with Targeting Moiety

Nanoparticle targeting was achieved through the addition of a collagen type II binding peptide to the AAc groups on our core + shell nanoparticles using a heterobifunctional crosslinker (Figure 3.1). Briefly, 0.4mg of 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC; Thermo-Scientific) and 1.1mg of N-hydroxysulfosuccinimide (sulfo-NHS; Thermo-Scientific) were added to 1mg of core + shell nanoparticles for 15 minutes in activation buffer (0.1M 2-(N-morpholino)ethanesulfonic acid (MES; Amresco, pH 6.0). Excess EDC and sulfo-NHS was removed by a centrifuge wash. The heterobifunctional crosslinker, N-[β-maleimidopropionic acid] hydrazide (BMPH; Thermo-Scientific) was added to the activated nanoparticles (0.1mg for 1 mol% AAc nanoparticles or 0.3mg for 5 mol% AAc nanoparticles) for 2 hours in coupling buffer (0.1M MES, pH 7.2). Excess BMPH was removed using gel filtration chromatography through an ÄKTA Purifier FPLC (GE Healthcare) with Bio-Scale Mini Bio-Gel columns packed with polyacrylamide beads (Bio-Rad Laboratories). The collagen type II binding peptide (15% biotinylated) was added to the nanoparticles for 2 hours in coupling buffer. Excess peptide was removed via gel filtration chromatography. Confirmation of peptide addition was performed using a flouraldehyde assay (Pierce), which reacts with free amines, and a streptavidin color development assay, which confirmed the presence of the biotinylated peptide on the nanoparticle surface (data not shown).
3.3.6 Collagen Type II Binding Assay

Modified nanoparticles were tested for their ability to bind to collagen type II. A 96-well plate (Greiner) was coated with collagen type II from chicken sternum (Sigma) in 0.25% acetic acid at a concentration of 0.5mg/ml overnight. Following three washes, the plate was blocked with 1% bovine serum albumin (BSA; SeraCare Life Systems) for 1 hour. After three more washes, the collagen type II binding peptide modified core + shell nanoparticles and unmodified controls were incubated in the collagen type II coated plate for 1 hour. Following three more washes, streptavidin (R&D Systems) was diluted 200X in 1% BSA and incubated for 20 minutes in the plate. After more washing to remove unbound streptavidin, a color solution (R&D Systems) was added for 20 minutes. Sulfuric acid (Mallinckrodt Chemicals) was then used to stop the reaction and absorbance was read at 450nm with a correction at 540nm.

3.3.7 Cell Culture

Immortalized human monocytes (THP1, ATCC) were grown in RPMI 1640 with L-glutamine (Mediatech Inc) supplemented with 0.05mM mercaptoethanol (Sigma-Aldrich), 10mM HEPES (Mediatech Inc), 1mM sodium pyruvate (Mediatech Inc), 10% fetal bovine serum (Hyclone), and 1% penicillin/streptomycin (Mediatech Inc). Cells were used between passage number 4 and 12 for all assays and maintained at 37°C with 5% CO₂.
3.3.8 Nanoparticle Biocompatibility

The biocompatibility of the nanoparticles was assessed by measuring toxicity and inflammation in THP1 cells. Cells were seeded at a density of 250,000 cells/mL in 96-well plates (Corning) and treated with 10ng/mL phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich) for 48 hours to induce differentiation, which was confirmed by the monocytes becoming adherent. Following a change of media, cells were treated with various concentrations of core + shell nanoparticles. Control samples received PBS (negative control) or 50ng/mL lipopolysaccharide (LPS, Sigma-Aldrich) (positive control). After 24 hours, the media was collected for cytokine analysis and an MTT-based assay was performed to determine cell toxicity using the Aqueous One Proliferation Kit (Promega) according to manufacturer’s instructions. Briefly, 20µL of reagent was added directly to 100µL of cells and media. After two hours of incubation in the cell culture incubator, the absorbance was read at 490nm with a correction at 650nm.

The ability of the particles to cause an inflammatory response was determined by running conditioned cell media on a TNF-α ELISA (PeproTech) according to manufacturer instructions. Briefly, Nunc MaxiSorp 96-well plates were coated with capture antibody overnight. After blocking for one hour with 1% bovine serum albumin (Sera Lifesciences) in PBS, samples and standards were incubated for two hours with gentle rotation. Following incubation with a detection antibody and an avidin-horse radish peroxidase conjugate, the samples were developed with the addition of 2,2’-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) liquid substrate (Sigma-Aldrich) and monitored at 405nm with a correction at 650nm.
3.3.9 Statistical Analysis
Data was analyzed for differences using a single factor ANOVA with a Tukey post-hoc test. A value of $\alpha = 0.05$ was used for all analyses. Graphs are depicted as mean ± standard deviation.

3.4 Results and Discussion

3.4.1 Physical Characterization of the Core + Shell Nanoparticles
Synthesis of the core + shell nanoparticles occurred in two steps: synthesis of the PLGA cores followed by the addition of the pNIPAM shell (Figure 3.1). Verification of successful encapsulation of the PLGA cores with the pNIPAM shells was initially achieved using dynamic light scattering to measure the size of the nanoparticles before and after the addition of the shell to determine the change in nanoparticle diameter. The diameter of the core + shell nanoparticles (Table 3.1) increased post addition of the pNIPAM shell, suggesting successful encapsulation. pNIPAM shell thickness ranged from ~100nm to ~200nm at 25°C, where an increase in shell thickness corresponded to an increase in the mole percent of acrylic acid, and therefore charge density. This phenomenon has been shown previously in the literature.$^{[19]}$
Additional verification of successful encapsulation of the pNIPAM shell was provided by confirming a phase transition when the temperature was raised above its LCST. Because pNIPAM is a temperature sensitive polymer, we expected to see a difference in nanoparticle size, with the nanoparticle diameter being smaller at 37°C (above pNIPAM LCST) compared to 25°C (below pNIPAM LCST). All core + shell nanoparticles showed reduced diameters at 37°C compared to 25°C (Table 3.1). This trend is most pronounced with shell thicknesses that at 37°C range only from ~10nm to ~100nm with an increase in shell thickness again corresponding to an increase in the mole percent of acrylic acid incorporated into the pNIPAM.
Table 3.1 Characterization of Various Nanoparticle Samples

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>Lyophilization?</th>
<th>Temperature (°C)</th>
<th>Particle Diameter (nm)</th>
<th>Zeta Potential (mV)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Shell Thickness (nm)&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLGA Core</td>
<td>No</td>
<td>25</td>
<td>392.3±13.9</td>
<td>-32.1±2.0</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>37</td>
<td>377.2±20.2</td>
<td>-22.5±3.4</td>
<td>NA</td>
</tr>
<tr>
<td>PLGA Core + pNIPAM Shell 0 mol% AAc</td>
<td>No</td>
<td>25</td>
<td>605.3±10.8</td>
<td>-28.3±4.4</td>
<td>106.5±5.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>37</td>
<td>403.8±7.1</td>
<td>-25.0±2.5</td>
<td>13.3±3.6</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>25</td>
<td>610.2±14.2</td>
<td>-32.1±0.9</td>
<td>109.0±7.1</td>
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<tr>
<td></td>
<td></td>
<td>37</td>
<td>414.5±7.9</td>
<td>-24.3±1.8</td>
<td>18.6±3.9</td>
</tr>
<tr>
<td>PLGA Core + pNIPAM Shell 1 mol% AAc</td>
<td>No</td>
<td>25</td>
<td>659.1±6.7</td>
<td>-28.0±6.0</td>
<td>133.4±3.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>37</td>
<td>428.0±9.5</td>
<td>-22.7±2.7</td>
<td>25.4±4.7</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
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<td>-30.8±5.1</td>
<td>144.7±10.4</td>
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<tr>
<td>PLGA Core + pNIPAM Shell 5 mol% AAc</td>
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<td>78.4±7.4</td>
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<td></td>
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<td>37</td>
<td>575.9±16.6</td>
<td>-25.2±2.1</td>
<td>99.4±8.3</td>
</tr>
</tbody>
</table>

<sup>a</sup> pH of all samples was between 5 and 6.

<sup>b</sup> Determined by subtracting corresponding core from core+shell sample & dividing by 2.

To assess colloidal stability of our core + shell nanoparticles above and below the phase transition temperature, we measured their ζ-potentials. According to the literature, nanoparticle ζ-potentials above 30mV or below -30mV are considered stable. As shown in Table 3.1, all nanoparticles, are near -30mV indicating that they are stable.

3.4.2 Effects of Lyophilization on Core + Shell Nanoparticles

Lyophilization is an effective way to prevent the release of therapeutics loaded into PLGA nanoparticles during long-term storage. However, if not performed correctly, lyophilization can result in aggregation of the PLGA nanoparticles that prevents
resolubilization of the clumped nanoparticles (Figure 3.2).\textsuperscript{[10, 11]} We used TEM imaging to visually confirm that well-defined spherical PLGA and PLGA core + pNIPAM shell nanoparticles were successfully synthesized pre-lyophilization (Figure 3.2). However, as also observed by others,\textsuperscript{[10, 11]} TEM imaging showed that lyophilization caused the PLGA nanoparticles to form aggregates (Figure 3.2). This aggregation resulted in an inability to measure diameter and ζ-potential of post-lyophilized PLGA nanoparticles. In contrast, encapsulation of PLGA nanoparticles with pNIPAM shells prevented aggregation of the PLGA nanoparticles following lyophilization, further confirming that the pNIPAM shell fully encapsulated the PLGA core. Additionally, we found that lyophilization of the core + shell nanoparticles does not affect their size or ζ-potential at 25°C or 37°C (Table 3.1).
3.4.3 Responses of C+S nPs to Dynamic Environmental Stimuli

To further assess the response of our core + shell nanoparticles to temperature-based environmental stimuli, we used dynamic light scattering to measure the diameter of our core + shell nanoparticles as they were exposed to a dynamic range of temperatures from 20°C to 50°C to 20°C. We found that the core + shell nanoparticles readily respond within this temperature range with all core + shell nanoparticle types decreasing in diameter as the temperature was raised above their LCST (Figure 3.3). This response was
reversible, as the nanoparticles returned to their original diameter when the temperature was lowered back below their LCST. Additionally, the LCST of the pNIPAM shell was tuned by modifying the amount of acrylic acid that was incorporated as a co-monomer. As more acrylic acid was incorporated, the LCST of pNIPAM increased (Figure 3.3). The core + shell nanoparticles with 0 mol% acrylic acid exhibited an LCST at ~31-32°C, while the 1 mol% acrylic acid had an LCST at ~33-34°C, and the core + shell nanoparticles with 5 mol% acrylic acid had an LCST at ~35°C (Figure 3.3). This trend has previously been established in the literature.\textsuperscript{[19, 21]} In the future, these core + shell nanoparticles could be engineered to respond to different environmentally based stimuli in addition to temperature by changing the co-monomer composition of the pNIPAM.\textsuperscript{[14]}

3.4.4 Core + Shell Nanoparticle Biocompatibility

The biocompatibility of the core + shell nanoparticles was assessed by evaluating the toxicity and inflammatory response in an immortalized human monocyte cell line. This cell line was chosen because, in our experience, it shows increased sensitivity to toxicity compared to other cell lines. Additionally, monocytes have a significant role in the perpetuation of osteo- and rheumatoid arthritis,\textsuperscript{22, 23} an inflammatory disease model that could be used to evaluate the ability of these core + shell nanoparticles to deliver desired
therapeutics in the future. The core + shell nanoparticles were not toxic at any of the concentrations tested, as we saw no significant reduction in THP1 cell number (Figure 3.4). However, the core + shell nanoparticles with 5 mol% acrylic acid induced a significant increase in proliferation of the THP1 cells at concentrations of 2.5 and 5mg/mL compared to all other treatments (p<0.05; One-way ANOVA + Tukey post-hoc test). Next, the ability of the core + shell nanoparticles to elicit an inflammatory response was determined by measuring TNF-α production by THP1 cells using an ELISA. A significant inflammatory response, characterized by an increase in TNF-α production, is not desirable and would indicate non-biocompatibility. Similar to the PBS control, the core + shell nanoparticles did not elicit TNF-α production (Figure 3.5; p>0.05; One-way ANOVA + Tukey post-hoc test), suggesting these nanoparticles are biocompatible though further testing will be conducted in the future to verify this preliminary work. The positive control, THP1 cells treated with lipopolysacharide, did induce TNF-α production as expected.
Figure 3.4 Toxicity of the core + shell nanoparticles in human monocytes. φ represents p<0.05 compared to all other treatments (One-way ANOVA + Tukey post-hoc test). Data is presented as mean ± standard deviation (n=4).

Figure 3.5 TNF-α production by human monocytes treated with core + shell nanoparticles. φ represents statistical significance from all other data points (p<0.05, One-way ANOVA + Tukey Post-hoc test). Data presented as mean ± standard deviation (n=4).
3.4.5 Targeting Core + Shell Nanoparticles

In order to increase the targeting capabilities of our core + shell nanoparticles beyond the passive EPR effect,\cite{6,7} we modified them with a model active targeting moiety – a peptide that binds collagen type II (Figure 3.1).\cite{24} This model targeting moiety could be used to deliver therapeutics in an arthritic-based disease model seeing as collagen type II is readily found in the cartilage of the joints.\cite{25} However, this chemistry could easily be applied to attach other targeting moieties that contain a free thiol functional group.

To assess whether incorporation of the collagen type II binding peptide allowed the core + shell nanoparticles to bind to collagen type II, we utilized a microplate assay. This assay involved coating a 96-well plate with collagen type II, incubating it with collagen type II targeting enabled core + shell nanoparticles, and then probing for the presence of the biotin labeled collagen type II peptide. The results from this assay indicate that core + shell nanoparticles modified with collagen type II binding peptide bound collagen type II as compared to unmodified core + shell nanoparticle controls (Figure 3.6; p<0.05, One-way ANOVA + Tukey post hoc test). Furthermore, the number of collagen type II binding peptide modified core + shell nanoparticles able to bind to collagen type II was directly related to the concentration of acrylic acid that was incorporated into the pNIPAM shell.
Figure 3.6 Collagen type II binding assay for core + shell nanoparticles. 1% & 5% AAc modified NPs are statistically significant from each other and the controls as indicated by the different Greek letters (p<0.05; One-way ANOVA + Tukey Post-hoc test). Data presented as mean ± standard deviation (n=4).

3.5 Conclusions

In closing, we successfully encapsulated static PLGA core nanoparticles with a dynamic pNIPAM shell. The addition of this pNIPAM shell conferred many benefits including the ability to protect the PLGA cores from aggregating during lyophilization, the ability to modify the nanoparticles with active targeting moieties in a concentration-dependent manner, and the ability to change size based on external environmental-based stimuli. These core + shell nanoparticles are non-toxic and do not illicit inflammation in THP1 cells suggesting biocompatibility. All of these abilities will prove important in future in vivo studies focused on examining the ability of these nanoparticles to deliver therapeutics in various disease models.
3.6 List of References


CHAPTER 4. CONTROLLED RELEASE OF A CORTICOSTEROID FROM HYDROGEL TEMPLATED PLGA MICROPARTICLES FOR USE IN TREATING VOCAL FOLD SCARRING

4.1 Abstract

Vocal fold scarring is caused by improper regeneration of the extracellular matrix (ECM) post injury resulting in impaired phonation. Alas, current treatments for vocal fold scarring only relieve the effects of the scar on phonation in the short term. Long-term solutions, however, remain elusive. Researchers have developed therapeutics to help treat vocal fold scarring, but barriers to the efficacy of these agents remain. What is needed is an effective means by which to control administration of said therapeutics over the long term. We looked to address this issue by loading dexamethasone (dM), an anti-fibrotic and anti-inflammatory therapeutic, into a poly-lactic-co-glycolic acid (PLGA) microparticle (mP) delivery vehicle. Release studies showed successful loading and release profiles of dM from three types of PLGA microparticles whose polymer chain terminating moiety, acid or ester, and molecular weight, low or high, were varied. Acid low molecular weight PLGA mPs released ~100% dM the fastest at ~48hrs. Ester low molecular weight PLGA mPs were slightly slower at ~72hrs while ester high molecular weight PLGA mPs only released ~40% dM by the conclusion of the study. To ascertain dM’s anti-fibrotic and anti-inflammatory effectiveness when released from PLGA mPs over the long term, they were exposed to scarred human vocal fold fibroblasts in vitro.
Real-time PCR showed a marked down regulation in COL3A1 & COL1A2 in scarred fibroblasts treated with dM loaded mPs only, indicating dM maintained its anti-fibrotic activity over the long term. While ELISAs illustrated that of the four pro-inflammatory cytokines tested dM’s anti-inflammatory affects over the long term were only maintained for one, interleukin-6. Further work needs to be done, but our data show initial promise in using a PLGA microparticle based delivery system to control the release of a therapeutic relevant in the treatment of vocal fold scarring over the long term.

4.2 Introduction

Vocal fold scarring is an affliction that results in the formation of a disorganized and stiff ECM with abnormal ECM component densities & structures, including a significant increase in collagen deposition.[1-3] It is caused by improper healing post injury and results in profound changes in the biomechanical properties of the vocal folds.[3] Thereby, impairing their ability to generate a normal mucosal wave during phonation.[3]

Finding an effective treatment for vocal fold scarring has been challenging. Currently, treatments seek temporary solutions that correct glottal incompetence and reduce stiffness caused by the scar through the augmentation of the vocal folds using various injectable materials, such as hyaluronic acid or calcium hydroxylapatite.[4,5] Unfortunately, these solutions do not treat the actual scar, but only relieve its effects over the short term seeing as current materials used in the clinic readily degrade.[1]

To solve this problem researchers have been testing the impact various therapeutics have on helping prevent or regress vocal fold scarring. An example of such a therapeutic, and
the one used in this study, is the corticosteroid dM which has shown significant anti-fibrotic, in its suppression of collagen production, and anti-inflammatory attributes.\textsuperscript{[6,7]} However, realizing the full potential of such a therapeutic has not been fully achieved due to many barriers, including the fact that these therapeutics are usually most effective when delivered directly to the site of scarring.\textsuperscript{[6]} This can be problematic seeing as, although one can inject them directly into scarred vocal folds, they usually are cleared in a short period of time. This necessitates the need to re-inject the therapeutic multiple times in order to maintain its efficacy. Though this is possible, issues of patient safety and tolerance quickly become significant problems one must deal with if one were to pursue such a rigorous treatment method.

Here we seek to address both prolonging the residency of dM within a target area in addition to showing that this therapeutic will remain effective over the long term. To achieve this, a delivery system was used in which dM was loaded into mPs composed of PLGA. This polymer was chosen due to several beneficial attributes including its high biocompatibility, approval for use in humans by the US Food and Drug Administration, and proven ability at loading and releasing many different types of therapeutics.\textsuperscript{[8-11]} To guarantee the controlled delivery of an effective therapeutic dose we sought to synthesize micron sized particles whose larger size would allow more drug to be loaded and subsequently released over the long term. These dM-loaded microparticles were synthesized using a hydrogel template based method that yields a highly uniform production of microparticles.\textsuperscript{[12]} A release study was done to elucidate the release profile of dM from microparticles composed of different types of PLGA. Then based on this
data, dM loaded ester terminated LMW PLGA microparticles were selected and a long-term dM release effectiveness study was conducted on scarred immortalized human vocal fold fibroblasts (I-HVFFs). Long-term dM effectiveness was verified using real-time or quantitative PCR (qPCR) to ascertain collagen gene expression. Pro-inflammatory cytokine levels were also probed due to the significant role inflammation plays in scar formation.[13]

4.3 Materials and Methods

4.3.1 Microparticle Fabrication

Microparticles used here were synthesized using a hydrogel template method described in detail in the work by Acharya, et al.[12] Briefly, a polydimethylsiloxane (PDMS) template was generated from a pre-fabricated silicon wafer with circular posts 50um in diameter and 30um in height. The patterned side of this PDMS template was then covered in a thin film of clear melted (50-55°C) porcine gelatin (30% w/v; Sigma-Aldrich) and then allowed to cool at 4°C for 5 min. Post cooling the gelatin solidified and was gently peeled away from the PDMS template resulting in the formation of a 50um in diameter by 30um deep welled gelatin template.

Wells of this gelatin template were then filled with a 9:1 dichloromethane (Sigma-Aldrich) to methanol (Sigma-Aldrich) solution in which 1% w/v dexamethasone (dM; Alfa-Aesar) and either 40% w/v low molecular weight (LMW; ~6700Da) acid terminated poly-lactic-co-glycolic acid (PLGA; Lactel), 40% w/v LMW (~6700Da) ester terminated
PLGA (Lactel), or 10% w/v high molecular weight (HMW; ~111,500Da) ester terminated PLGA (Lactel) had been dissolved. This was achieved by transferring 50uL of one of the dM + PLGA solutions to the patterned surface and then evenly spreading it over the entire gelatin template using a razor blade. The solvents were then left to evaporate at room temperature for 5-10 min. Each template underwent three 50uL swipes with one of the dM + PLGA solutions.

The filled gelatin templates were then dissolved using 60°C deionized water in order to melt the gelatin thereby releasing the dM + PLGA microparticles into solution. The dM + PLGA microparticles were then separated from the gelatin utilizing centrifugation based washing. Briefly, the microparticles were pelleted at 4000g for 5 min. The melted gelatin solution was then removed and fresh deionized water was added and the pelleted dM + PLGA microparticles were resuspended in it. This constituted one wash. Overall, the dM + PLGA microparticles were washed three to four times. After the final wash, the dM + PLGA microparticles were pelleted and the supernatant was removed. The pelleted dM + PLGA microparticles were then dried for 12-24 hrs in a lyophilizer, and finally stored at -20°C for later use.

4.3.2 Dexamethasone Release Study

Into low-bind Eppendorf tubes 2mg of one of the three different types of PLGA microparticles, either dM loaded or not, were weighted out to give each an N=3. Next 100uL of PBS pH 7.2 was added to each tube and then all tubes were placed on an orbital shaker at 400rpm at 37°C. At various time intervals, the microparticles were pelleted at
4000g for 5min. The supernatant was then completely removed and stored in a fresh labeled tube for each sample. Next, the pelleted microparticles were resuspended in 100uL of fresh PBS pH 7.2. Finally, the tubes were put back on the orbital shaker at 400rpm at 37°C until the next time point was reached at which point this procedure was repeated.

The absorbance at 240nm, the peak absorbance of dM, was read for all samples at every time point. This data was then compared to a dM + PBS pH 7.2 standard curve in order to ascertain the concentration of dM in each sample. Finally, percent cumulative dM release was determined by comparing the amount of dM released from each PLGA type over time to the total amount of dM loaded in 2mg of fresh dM loaded PLGA microparticles of that corresponding PLGA type.

4.3.3 Cell Culture

An immortalized human vocal fold fibroblast (I-HVFF) cell line was obtained from Dr. Susan Thibeault from the University of Wisconsin, Madison\textsuperscript{[14]} These I-HVFFs were grown in a normal unscarred state in Dulbecco’s Modified Eagles Medium (Sigma-Aldrich) supplemented with 10% FBS (Sigma-Aldrich), 1% penicillin/streptomycin (Sigma-Aldrich), 1% MEM non-essential amino acids (Sigma-Aldrich), and 200ug geneticin (G418; Teknova) per mL of media. Unscarred I-HVFFs were induced into a scarred phenotypic state by changing to an induction media. This induction media includes all of the same aforementioned components of unscarred media except FBS. It also contains 10ng/mL of transforming growth factor-β1 (TGF-β1; Biosource).
4.3.4 RT-qPCR

I-HVFFs were initially seeded at 2000 cells/well in a 96-well plate and left to attach overnight at 37°C and 5% CO₂. Then all wells were rinsed with 200uL of Dulbecco’s PBS pH 7.4. Next a set of control wells were left unscarred while the rest were induced into a scarred phenotype by incubating them for 48hrs at 37°C and 5% CO₂ in induction media. Post scarring wells were rinsed with Dulbecco’s PBS pH 7.4 again and then either exposed to normal unscarred media, induction media + no dM, induction media + 50ng/mL free dM, induction media + 0.01mg per well dM loaded mPs, or induction media + 0.01mg per well PLGA no dM mPs. These various solutions were left on the I-HVFFs for 24hrs at 37°C and 5% CO₂. After this time had elapsed, the cells were spun at 2000g for 10min to remove the mPs from suspension in the media solutions as a way to mimic in vivo entrapment in tissue. The supernatants from each well were then removed to mimic the clearance of drug as seen in in vivo systems, and fresh either unscarred media for the control wells or scarred induction media for all other wells was added. The I-HVFFs were then left to grow at 37°C and 5% CO₂ for 72hrs more.

At this point the media was removed and saved for later pro-inflammatory cytokine analysis, while the RNA was purified from the fibroblasts in each of the different I-HVFF treatment samples using a Nucleospin total RNA isolation kit (Clontech). Once purified the RNA yield was quantified using a NanoDrop 2000 spectrophotometer (Thermo). Next the RNA was reverse transcribed into cDNA using the High-Capacity cDNA reverse transcription kit (Life Technologies). Real-time or quantitative PCR reactions were then conducted using TaqMan gene expression master mix (Life Technologies)
combined with three gene-specific probes (Life Technologies) including β-actin, COL1A2, or COL3A1 and template cDNA from the different samples. These reactions were then run on an Applied Biosystems 7500 real-time PCR machine under the following conditions: 50°C for 2min, 95°C for 10min, and 40 cycles of 95°C for 15sec, and 60°C for 1min. The endogenous control was human β-actin. Average cycle threshold (Ct) from each sample was then used for the calculation of COL1A2 or COL3A1 expression levels using the relative comparative Ct method.

4.3.5 Cytokine Analysis

Four human pro-inflammatory cytokines were analyzed including interferon-gamma, IL-1beta, IL-6, and TNF-alpha utilizing a sandwich electrochemiluminescent based ELISA. To start a multi-spot human cytokine tissue culture assay kit was purchased from Meso Scale Discovery (MSD). The kit contained a 96-well 4 spot human proinflammatory-4 I plate pre-coated with capture antibodies to the aforementioned cytokines. The analysis was conducted according to company instructions. Briefly, wells were blocked with 1% w/v Blocker B in PBS pH 7.2 for 1hr. Wells were then washed 3 times with PBS + 0.05% Tween20. Next either 25uL of standards or samples were added to each well, and the plate was sealed and vigorously shaken for 2hrs at 700rpm at room temperature. Addition of 25uL of detection antibody solution was then immediately added and the plate was resealed and shook at 700rpm at room temperature for 2hrs. Finally, the wells were washed 3 times with PBS pH 7.2 + 0.05% Tween20 and then 2x Read Buffer T was added to each well before the plate was read on a MSD Sector Imager 2400.
4.4 Results and Discussion

Production of the dM loaded microparticles using three different PLGAs including acid terminated LMW, ester terminated LMW, and ester terminated HMW microparticles was successful using the hydrogel template based method.\textsuperscript{[12]} All three types of PLGA yielded the same relatively uniform microparticle structure as shown in Figure 4.1.

![Figure 4.1 Example images of fluorescein loaded hydrogel templated 50um x 30um ester terminated LMW PLGA microparticles. A = brightfield image, B = fluorescent image. Scale bars = 50 um.](image)

A 168hr or 7 day release study was then conducted in order to ascertain the release profiles of the dM from the various types of PLGA microparticles (Figure 4.2). An initial 12hr burst release of dM was observed with all three types of PLGA. The release profiles of the three types of PLGA then diverged with the acid terminated LMW PLGA
microparticles releasing most of their dM pay load by the 48hr mark (Figure 4.2). The ester terminated LMW PLGA microparticles took slightly longer with most dM released within 72hrs (Figure 4.2). The ester terminated HMW PLGA microparticles on the other hand released dM at a much slower rate post burst release with only about 40% of the loaded dM released by the end of the 168-hour study (Figure 4.2). Based on this data, dM loaded ester LMW microparticles were chosen for our long-term cell studies as they gave the most desirable long-term dM release profile. Furthermore, this data agreed with the literature in that our acid terminated PLGA microparticles released dM slightly faster than our corresponding ester terminated PLGA microparticles of the same MW.\[15\] Our LMW PLGA released dM significantly faster than the HMW PLGA with the same functional group termination moiety.\[15\]
Figure 4.2 Comparative release profiles of percent cumulative dexamethasone (dM) release over a 168-hour or 7-day period of time from acid LMW, ester LMW, or ester HMW PLGA microparticles.

To assess the maintenance of dM’s anti-fibrotic effects over the long term in scarred I-HVFFs, we used qPCR and looked at its impact on collagen gene expression, specifically COL1A2 & COL3A1 genes. The literature has shown that collagen gene expression is up-regulated in vocal fold scarring due in part to the presence of TGF-β1.\textsuperscript{[3,16,17]} We sought to model vocal fold scarring \textit{in vitro} by exposing I-HVFFs to exogenous TGF-β1 in order to induce our normally unscarred I-HVFFs into a scarred phenotype. This was a
success and a ~2.5-fold increase was seen in COL1A2 and ~3.5-fold increase for
COL3A1 was observed in our scarred I-HVFFs versus unscarred I-HVFFs. Upon
administering both free dM and dM loaded ester terminated microparticles to our scarred
I-HVFFs we saw a marked decrease in COL1A2 and COL3A1 expression (data not
shown) early on confirming what had been seen in the literature.\[7\] However, by the end
of the long-term study at day 4 free dM no longer maintained its anti-fibrotic efficacy as
COL1A2 and COL3A1 expression returned to normal scar levels (Figure 4.3). Meanwhile, dM loaded ester terminated PLGA microparticles at day 4 maintained dM’s
anti-fibrotic efficacy by continuing to depress COL1A2 and COL3A1 expression levels
(Figure 4.3). Note we also observed that PLGA only microparticles did not elicit a pro-
fibrotic response. Instead, it seems they elicit an anti-fibrotic behavior in scarred I-
HVFFs with a slight reduction in COL1A2 and COL3A1 expression (Figure 4.3).
Figure 4.3 Illustrates that dexamethasone (dM) effectively knocks down COL1A2 and COL3A1 gene expression over the long term only in scarred immortalized human vocal fold fibroblasts exposed to dM loaded mPs. Those treatments within each collagen type with non-matching symbols are statistically different from each other according to a one-way ANOVA + Tukey post-hoc test; p < 0.05.

Finally, we sought to investigate the impact dM had on affecting the inflammatory responses of our I-HVFFs. Inflammation plays a major role in the formation of a scar with fibroblasts playing a role in this process.\cite{13,18} Using our in vitro vocal fold fibroblast scar model we sought to ascertain whether or not dM’s anti-inflammatory capabilities would remain effective over the long term. To assess the inflammatory responses of the I-HVFFs we looked at the deposition of 4 pro-inflammatory cytokines,
including interferon-gamma (IFN-g), interleukin-1β (IL-1b), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-a), using a sandwich electrochemiluminescent based ELISA. We found unscarred I-HVFFs released the following levels of all four cytokines; ~110pg/mL for IL-6 (Figure 4.4), ~0.5pg/mL for IL-1b, ~15pg/mL IFN-g, and ~2pg/mL TNF-a. However, cytokine levels significantly fell when unscarred I-HVFFs were induced into a scarred phenotype using TGF-β1 with IL-6 falling to ~45pg/mL (Figure 4.4), IL-1b to ~0pg/mL, IFN-g to ~2pg/mL, and TNF-a to ~0pg/mL. This shows that though TGF-β1, a cytokine itself,[19] acts as a pro-fibrotic agent by up-regulating collagen expression in our I-HVFFs (Figure 4.3), in the context of inflammation, it acts as an anti-inflammatory agent. This has been seen in other cell lines,[19, 20] but to our knowledge never before in vocal fold fibroblasts. When dexamethasone was added to our TGF-β1 scarred I-HVFFs it had no significant impact beyond the one already seen by TGF-β1 on IL-1b, IFN-g, or TNF-a implying no synergistic effect between the two for these three pro-inflammatory cytokines. However, it did show a significant impact in further decreasing the pro-inflammatory cytokine IL-6 (Figure 4.4), which has been seen in other cell lines.[21-23] We also observed that even at the end of the study on day 4 free dM maintained its anti-inflammatory effect on IL-6 only rising slightly above the level of the scarred I-HVFFs exposed to the dM loaded mPs (Figure 4.4). This prolonged anti-inflammatory effect was not expected as free dM was not able to have the same impact in pro-longing its anti-fibrotic affects without the aid of the microparticles. Finally, we also observed that I-HVFFs treated with PLGA only microparticles elicited the same level of inflammatory response for the 4 pro-inflammatory cytokines tested as those treated with TGF-β1 indicating they themselves do not impact the inflammatory response.
Of the 4 cytokines tested (IFN-γ, IL-1β, TNF-α, and IL-6) dexamethasone only indicated a synergistic anti-inflammatory effect with TGF-β1 on IL-6. Both free dM and dM loaded mPs were able to maintain dexamethasone’s additive anti-inflammatory effect on IL-6 over the long term. Treatments with non-matching symbols are statistically different from each other according to a one-way ANOVA + Tukey post-hoc test; p < 0.05.

In closing, treating vocal fold scarring is a challenging task due to the complex nature of this affliction. However, we show that a route to overcoming this problem could be through the controlled release of an anti-scarring therapeutic like dM. Such a method provides prolonged residency of dM thereby increasing its anti-fibrotic efficacy and helping to maintain its anti-inflammatory capabilities. Our future work will look at using
this microparticle based delivery system to control the delivery of other important anti-
fibrotic agents. We also would like to investigate the ability of the microparticles to
maintain their residency at their initial injection site based on their increased size in
addition to studying the impact of long-term dM release in an *in vivo* animal model.
4.5 List of References


CHAPTER 5. SUMMARY OF MAJOR FINDINGS AND FUTURE WORK

There exist limited clinical options to treat vocal fold scarring, and these treatments only seek to relieve the effects over the short term. Thus, the underlying vocal fold scar itself remains causing long-term problems to an afflicted patient’s ability to phonate. Therapeutics have been developed to treat scar tissue, but the full potential of such therapeutics has yet to be achieved in part due to challenges in delivering and maintaining these therapeutics at the site of scarring.

The overall goal of my dissertation research was to address this issue of effectively delivering therapeutics to an area of vocal fold scarring to facilitate healthy unscarred tissue regeneration. To accomplish this goal the following aims were proposed and completed.

In the first aim, chapter 2, we characterized our in vitro unscarred and scarred culture systems. This was accomplished by elucidating the impact RGD integrin binding site concentration and cell phenotype had on directing extracellular matrix gene expression in vocal fold fibroblasts, and found that both had an effect. With changes in fibroblast phenotype from unscarred to scarred resulting in ECM gene up-regulation for all genes
tested, except for HAS2 and DCN. Meanwhile, variations in RGD integrin concentration impacted ECM gene expression only in specific phenotypes. Unscarred I-HVFFs were unaffected by changes in RGD integrin concentration for all six ECM genes tested except HAS2 which saw down-regulation with addition of any RGD. Scarred I-HVFFs were affected, with ELN gene expression being up-regulated as the concentration of RGD increased. Similar results, albeit of smaller magnitude, were obtained for COL3A1 gene expression as well. HAS2 expression was also impacted with a further down regulation in its expression at the highest RGD concentration in scarred I-HVFFs. DCN, FN, & COL1A2 remained unchanged in scarred I-HVFFs with respect to changes in RGD concentration. This knowledge is critical to have when developing bioactive materials in which healthy ECM component ratios and morphologies are regenerated through variation in biological cues, like RGD.

In aim 2, chapter 3, we focused on developing an initial proof of concept nanoparticle based system that would provide multiple avenues for controlling release of a loaded therapeutic. To do this a novel poly-lactic-co-glycolic acid (PLGA) core + poly(N-isopropylacrylamide (pNIPAM)-co-acrylic acid (AAc) shell nanoparticle system was synthesized and characterized. A critical characteristic of this system is the ability of the pNIPAM-co-AAc shell to prevent the aggregation of the PLGA cores during lyophilization without the need of cryo-protectants. Secondly, the addition of the pNIPAM-co-AAc shell imbues the overall PLGA core + pNIPAM shell nanoparticle system with more dynamic properties including the ability to respond to environmental stimuli. Thirdly, the addition of the acrylic acid monomer to the pNIPAM shell polymer
provides a functional handle used to append targeting ligands in an acrylic acid monomer concentration dependent manner. Finally, results from *in vitro* experiments demonstrated the biocompatibility of these PLGA core + pNIPAM-co-AAc nanoparticles with cells.

Finally, the third aim, chapter 4, sought to develop a controlled release therapeutic system capable of delivering an effective concentration of dexamethasone for a sustained period of time, in order to return scarred I-HVFFs back to a healthy unscarred state. To accomplish this, hydrogel templated poly-lactic-co-glycolic acid (PLGA) microparticles were loaded with the corticosteroid dexamethasone. Larger microparticles were used to guarantee a controlled delivery of an effective therapeutic dose. Real-time PCR showed that only dexamethasone loaded microparticles proved effective at maintaining down regulation in the expression of COL3A1 and COL1A2, which are normally highly up-regulated in scarred fibroblasts, over the long term. While an ELISA showed that dexamethasone decreased the deposition of the inflammatory cytokine interleukin-6 over the long term, but had no impact on the other three cytokines tested.

Based on the aforementioned findings the goal to develop an effective controlled delivery system for administering therapeutics for treating vocal fold scarring has been accomplished. However, more work needs to be done before the delivery systems developed here are ready to make the leap from bench to bedside.

In looking forward, it would potentially be highly advantageous to add a pNIPAM-co-acrylic acid shell, from aim 2, to the PLGA microparticle delivery system, from aim 3.
This would combine the advantages of the larger microparticle size, with its ability to more readily deliver an effective dose of therapeutic, with the many beneficial attributes, like targeting and protection during lyophilization based storage, of the pNIPAM-co-acrylic acid shell. Furthermore, with the addition of a shell another level of control over therapeutic release is available. What also should be explored is whether the larger microparticle size anchors the particles within the tissue due to ECM entrapment. If the larger size of the microparticles does not facilitate increased residence time of the drug loaded microparticles at a site of injection, then the targeting ability of the pNIPAM-co-acrylic acid shell could facilitate anchoring at the site of injection. The targeting ligands that could be used in this case are a collagen type I or collagen type III binding peptide or one could also explore using RGD as a targeting ligand.

If the RGD targeting ligand option was explored, then it would also be interesting to see if any of the changes seen in ECM gene expression due to RGD concentration changes on a glass surface, from aim 1, would also be present when the RGD concentration was varied on a particle surface. Furthermore, we hypothesize that an RGD targeting ligand could reduce the stress perception of I-HVFFs thereby helping facilitate the modulation of phenotype of scarred I-HVFFs back to an unscarred state. This would be achieved as a result of the scarred I-HVFFs binding to the RGD ligands of the free floating, softer, polymer-based particles thereby reducing their perception of the stiff glass or plastic they grow on in vitro or stiff scarred ECM they grow on in vivo.
Finally, one could look at assessing the impact free dexamethasone and dexamethasone loaded microparticles, from aim 3, have on the expression of collagen in the *in vitro* scarred and unscarred culture systems described in aim 1. Furthermore, one could also look at using these culture systems to investigate other ECM components, besides collagen, that play a role in scarring, like decorin. Yet another way the culture systems could be used is to screen other anti-scarring agents, like pirfenidone, in both their free and PLGA microparticle or other drug delivery system loaded forms.
VITA
VITA

Aaron Michael Kosinski was born in 1982 in Jackson, Michigan. He graduated from Jackson Northwest High School in 2000. He obtained his bachelors of science from Michigan State University in 2004 in Biochemistry & Molecular Biology/Biotechnology. While at Michigan State University he also had chances to gain experience in many branches of the life sciences. His first formal laboratory experience was with Dr. Zachary Burton in the Biochemistry department studying RNA polymerase II and elucidating how transcription worked. He then moved on to conducting research with Dr. Jeff Landgraf in MSU’s Genomics Technology Support Facility doing high throughput replication of a LION rat clone set. Finally, he moved on to working with Dr. Matt O’Neal developing a non-conventional way to label crop insect pests using IgG and ELISAs for use in capture and release studies. In addition to school and research, Aaron also served as a Spartan Marching Band member for all four years of his undergraduate career.

After successfully completing his B.S. degree, he entered the work force as a research technologist in the lab of Dr. Janet Partridge in the Biochemistry department of St. Jude Children’s Research Hospital in Memphis, Tennessee. In this lab, he worked to ascertain the role proteins like Chp1, Tas3, & Ago1 in concert with siRNAs played in the
establishment and maintenance of centromeric heterochromatin utilizing fission yeast and *S. pombe* as model organisms. Then in 2006 he decided to pursue work doing more applied research as opposed to the basic research in epigenetics he had been doing. He therefore moved on to employment in the lab of Dr. R. Kiplin Guy in the, at the time, brand new Chemical Biology and Therapeutics department at St. Jude Children’s Research Hospital. Here he was promoted to a senior research technologist and did extensive work in the production, both small and large-scale, of pure recombinant proteins from various model organisms for use by himself, other Guy lab members, and Guy lab collaborators. These purified recombinant proteins were used to conduct various experiments, like high-throughput screening of chemical libraries for discovery & development of novel therapeutics. He also worked to establish the CBT Welcoming Committee and lead them in developing the CBT Chaperonine program. This program was a standardized department wide new employee orientation program covering hospital regulations as well as departmental specific rules and safety instructions.

Though successful at St. Jude, Aaron felt that in order to advance his career he needed to obtain a doctoral degree. So in 2008 he left St. Jude and began the pursuit of his Ph.D at Purdue University in the Biomedical Sciences working initially under Dr. Brandon Seal in the Weldon School of Biomedical Engineering where he did drug delivery research. Then in 2010 when Dr. Seal left Purdue he moved to Dr. Albena Ivanisevic’s lab and began work in collaboration with Dr. M. Preeti Sivasankar on how to regenerate damaged or diseased vocal folds. His work in the Ivanisevic lab continued until she left Purdue in 2011 at which point Aaron changed labs again joining Dr. Alyssa Panitch’s lab. Here he
continued his research with Dr. Panitch in continued collaboration with Dr. Sivasankar into vocal folds focusing in on the development of a drug delivery system for treating vocal fold scarring.

He is expected to conclude his graduate student career at Purdue University and obtain his Ph.D in the Biomedical Sciences through the Weldon School of Biomedical Engineering by defending his thesis August 22, 2013 with an official graduation date of December, 2013. He will then obtain employment in industry in either a management track position in a biotech/pharmaceutical company, in a life sciences/healthcare consulting position, as a life sciences/healthcare venture capitalist, or working on commercialization efforts for a life sciences/biotech research institution.