UTILIZATION OF COLLAGEN REMODELING PATHWAYS TO ACHIEVE EFFICIENT, CONTROLLED GENE DELIVERY IN CHRONIC WOUND REPAIR

by

Morgan A. Urello

A dissertation submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Chemical Engineering

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Morgan A. Urello

Approved: _____
Abraham M. Lenhoff, Ph.D.
Chair of the Department of Chemical and Biomolecular Engineering

Approved: _____
Babatunde A. Ogunnaike, Ph.D.
Dean of the College of Engineering

Approved: _____
Ann L. Ardis, Ph.D.
Senior Vice Provost for Graduate and Professional Education

I certify that I have read this dissertation and that in my opinion it meets the academic and professional standard required by the University as a dissertation for the degree of Doctor of Philosophy.

Signed: _____

Millicent O. Sullivan, Ph.D.
Professor in charge of dissertation

I certify that I have read this dissertation and that in my opinion it meets the academic and professional standard required by the University as a dissertation for the degree of Doctor of Philosophy.

Signed: _____

Kristi L. Kiick, Ph.D.
Member of dissertation committee

I certify that I have read this dissertation and that in my opinion it meets the academic and professional standard required by the University as a dissertation for the degree of Doctor of Philosophy.

Signed: _____

April M. Kloxin, Ph.D.
Member of dissertation committee

I certify that I have read this dissertation and that in my opinion it meets the academic and professional standard required by the University as a dissertation for the degree of Doctor of Philosophy.

Signed: _____

Sujata K. Bhatia, M.D., Ph.D.
Member of dissertation committee

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ABSTRACT

Chronic, non-healing wounds represent a growing burden to patients, medical professionals, and the healthcare system. Over 7 million Americans suffer from these wounds and the total patient care costs $25 billion per year. Even after treatment, a large percent (~50%) of chronic wounds never completely heal, leaving patients susceptible to elevated rates of infection, amputation, and even death, demonstrating a grave need for new approaches to restore wound bed health and address causative deficiencies in growth factor(GF) stability accessibility, and activity. The application of GF gene activated matrices offer many compelling advantages over GF protein delivery within the aberrant, protease-rich wound bed. GF gene-approaches better mimic endogenous repair by utilizing host cells to orchestrate sustained GF expression, activity, and microlocalization which are crucial in management of chronic wounds due to extended healing over months, spatiotemporal heterogeneity, and elevated protease activation. However, clinical translation of gene-based therapies has been largely hindered by off-target responses, and inefficient gene transfer.

In this dissertation, a novel peptide-based approach for achieving efficient, controlled gene delivery through leveraging naturally elevated wound bed protease activity and subsequent collagen remodeling is presented. Specifically, collagen-mimetic peptides (CMPs)-collagen affinity was used to engineer DNA collagen

matrices with tailored release profiles and improved activity. Variation in CMP-display on non-viral DNA carriers known as polyplex were demonstrated to increase retention/release time from collagen from 20 days to over a month, and in cell studies, bound polyplex exhibited enhanced stability in the presence of serum-containing media over a 2-week period as well as altered intracellular trafficking resulting in improved gene transfer efficiency. Moreover, transgene expression in CMP/polyplex/collagens was determined to be directly dependent on matrix metalloprotease(MMP)-stimulation, and fluorescent microscopy studies established the co-endocytosis and co-internalization of CMP-modified polyplex with collagen fragments, strongly suggesting CMP-modification can be used to harness collagen remodeling pathways to mediate controlled release and efficient cellular uptake, two major obstacles in gene delivery.

Furthermore, CMP/polyplex/collagens have been used to successfully tailor the expression of vital GFs platelet derived growth factor-BB (PDGF-BB) and keratinocyte growth factor (KGF), and decrease wound closure times through PDGF-BB expression when applied to an *in vitro* "wound" model. Experiments with an *in vivo* ECM depot model also demonstrated the capacity of CMP-modification to tailor the duration and extent of transgene expression in a more complex system, mediating expression for 10-fold longer time periods with average expression levels up to 2-orders of magnitude higher relative to samples with unmodified polyplexes. While the focus of this dissertation is application in chronic wound repair, this approach has enormous potential in improving delivery in other locations characterized by elevated

collagen turnover, including tumor and joints. Its targeting of released ECM fragments to facilitate delivery also marks a significant deviation from typical gene delivery approaches which has already shown promise.

Chapter 1

PROTEIN AND GENE-DELIVERY SYSTEMS IN CHRONIC WOUND REPAIR

1.1 Motivation

Chronic wounds, such as diabetic foot ulcers (DFUs), pressure ulcers, and venous ulcers, represent an enormous worldwide medical, social, and economic burden that critically impact the patients' standard of life. In the United States alone, DFUs afflict 2.5-4.5 million patients and cost an estimated 25 billion dollars annually.[1-7] Ulcers typically last 12-13 month and reoccur in up 70% of patients often resulting in loss of function, amputation, and even death.[8] The mortality rate of DFU patients surpasses that of even cancers' as a group at 10-20% within a year and 40-60% by year five, and demonstrates the critical need for new treatment approaches.[9]

Standard chronic wound care regimens consist of a recurring routine of wound bed cleansing, debridement, and dressing designed to prevent infection and preserve a moist healing environment.[8-10] However, treatments regularly fail to promote healing due to the aberrant nature of the chronic wound bed and failure to account for critical reductions in vital healing factors within the wound environment (Figure 1.1).[8,9,11] While non-healing wounds have different origins, chronic wound beds are characterized by uncoordinated healing and locally protracted, overly pronounced inflammatory phases. A vital part of repair, the primary purpose of the inflammatory phase (a.k.a. the defensive phase) is to recruit specialized immune cells, such as neutrophils and macrophages, to eradicate bacteria and remove devitalized necrotic

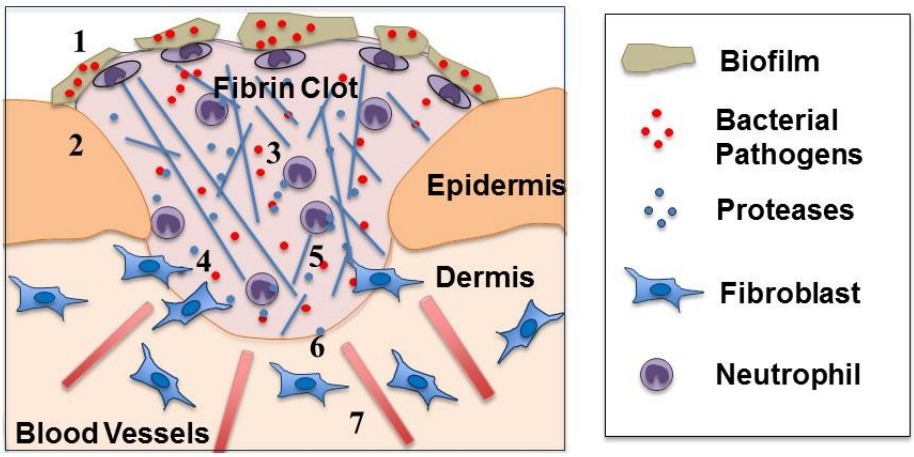


Figure 1.1 Chronic, Non-healing Wounds. Abnormalities in chronic wounds include infection/biofilms (1), hyperproliferative epidermis/stalled re-epithelialization (2), persistent inflammation (3), fibroblast senescence (4), impaired angiogenesis (5), fibrin cuffs (inhibit to oxygen) (6), and elevated MMPs (7).

tissue, essentially preparing the wound bed for new tissue growth; however, excessive recruitment of activated inflammatory cells delays wound healing. Inflammatory cell accumulation in the wound bed leads to the overproduction of various reactive oxygen species (ROS) that damage components of the extracellular matrix (ECM) and cell membranes resulting in premature cell senescence. It also causes the overstimulation of proteases (such as matrix metalloproteases (MMPs)) that degrade and inactivate elements of the ECM and growth factors (GFs) required for normal cell function. Altered cellular phenotypes and an aberrant extracellular environment comprised of fibrin, necrotic debris, and bacteria cause critical reductions in GFs production, stability, accessibility, and activity. Furthermore, inactivation of protease inhibitors by proteolytic degradation exasperate this self-perpetuating process[1,5,8,10,11]. The prevalence of infection in chronic wounds, as both an extrinsic factor that both causes and prolongs protracted inflammation, further complicates wound healing by

producing additional proteases and forming polymicrobial biofilms that increase bacterial survival and production of virulent factors, further alter cell phenotypes, and contribute to decrease GF mobility[1,3,8-10].

To overcome cellular and molecular abnormalities in the chronic wound treatment, the establishment of a functional ECM and salubrious physical and biochemical environment is vital. Understandably, the application of ECM-based scaffolds (e.g. collagen) has already gained widespread acceptance in advanced wound repair owing their inherent ability to act as a biocompatible, bioactive scaffold with the capacity to encourage cellular ingrowth and proliferation.[2,3,10,12] The scaffolds have also been shown to act as a sacrificial substrate reducing elevated protease activity. Numerous ECM-based skin substitutes have been FDA-approved for the treatment of acute wounds, surgical wounds, and burns, yet few have achieved clinical success in chronic wound repair owing the aberrant wound bed environment causing excessive ECM instability and turnover.[9,13] For instance, Apligraf (Organogenesis) and Dermagraft (Advanced Biohealing), two of the three devices FDA-approved for DFU treatment, are believed to promote healing by increasing ECM deposition, enhancing GF availability, and improving stem cell recruitment; however incidence of complete DFU closure post-treatment with engineered skins remains close to only 50%.[14]

It has become increasingly evident that additional bioactive cues are vital to encourage progression from the inflammatory phase and healing in most chronic wounds. Accordingly, many techniques have been explored in both research and clinical phases for delivering GF proteins and the genes that encode them based on the well-known roles GFs play in native wound repair and recognition of their compromised activity in the chronic wound bed.[3,4,9,11,13,15-22] Motivated by knowledge

of native delivery, ECM-inspired delivery systems have successfully been used to deliver and preserve the activity of GFs within the wound bed; however, the inherent instability of GF in the protease-rich environment and safety concerns associated with the supra-physiological GF dosages in turn required for efficacy, has led to increasing interest in GF gene delivery[7].

GF gene delivery systems offers many compelling advantages for improved GF delivery due to benefits in protein stability/bioactivity, sustained release, and cost, as well as relative ease of delivering multiple genes and spatial control through use of tissue-specific promoters.[23-29] In this work, a new peptide-based, ECM-inspired strategy for overcoming gene delivery obstacles through leveraging the naturally elevated levels of protease-activity and subsequent ECM turnover within the wound bed is presented.[30,31] Specifically, the potential to utilize collagen-mimetic peptides-collagen affinity to create stable, tunable links between collagen, a key ECM component, and active DNA carriers (i.e. polyplex) will be demonstrated with focus on the ability to (1) achieve tailorable, localized protein expression,[30] (2) utilize ECM turnover to overcome extra- and intracellular delivery obstacles,[31] and (3) attain multi-protein expression.

1.2 Therapeutic delivery systems for chronic wound repair

1.2.1 Growth factor delivery

GF-based treatments have garnered considerable interest in chronic wound repair due to their well-known roles in endogenous wound repair and compromised activity in chronic wound beds.[4,7,11,19,32,33] These signaling proteins stimulate various processes essential in tissue repair including cellular proliferation and differentiation,

and facilitate communication between the various cell types involved in orderly wound repair including fibroblast, endothelial cells, keratinocytes, and immune cells.[1,34] Accordingly, various preclinical studies and industry-sponsored studies have evaluated the utility of the key GFs involved in the wound healing process including platelet-derived growth factor (PDGF),[35,36] epidermal growth factor (EGF), fibroblast growth factor (FGF), insulin-like growth factor (IGF_1, IGF_2), vascular endothelial growth factor (VEGF), transforming growth factor (TGF-β), and keratinocyte growth factor (KGF) in chronic wounds repair via both topical and sustained release treatments.[37-39] However, only PDGF-BB has received FDA approval in chronic wound repair, and even though PDGF-BB treatment Becaplermin (Regranex®) is commercially available, the treatment was issued a boxed warning from the FDA and approval was withdrawn in Europe due to safety concerns.[7,38] In fact, the supra-physiological doses required to achieve therapeutic effect correlated with a five-times increased risk of cancer and only improved the number of healed DFU patients by <10% based four phase III trials.[7] Furthermore, GF therapies typically do not account for the physiological or synergetic interactions between GFs in the repair process which suggest clinical failure is in part due to a lack of approaches for delivering multiple GF at controlled dosages. The complications encountered with PDGF-BB highlight the most challenging obstacle in the clinical translation of protein-based chronic wounds treatments, achieving sustained, controlled therapeutic delivery within an irregular, protease-rich environment.

Attempts to address these obstacles have been made through the development of polymer-based (i.e. biomaterial) controlled release delivery systems. The main objectives of these systems being to preserve the stability of the GFs, minimize

systematic absorption, and inhibit immune responses. Already commonplace in wound repair, ECM-inspired materials are natural candidates for regulating the spatio-temporal release of therapeutics.[40] Besides providing cellular adhesion sites, one of the ECM's most vital functions is serving as a reservoir for GFs enabling rapid responses. Many GFs have innate affinities for specific ECM components meaning the release of ECM-bound GF is dependent on their respective binding affinities and local protease activity/sensitivity.[34,41,42] Numerous delivery systems have sought to recapitulate this aspect of endogenous delivery to similarly achieve on-demand release of therapeutics with locally tailorable kinetics through isolating GF-binding domains from ECM molecules. For instance, several GFs bind to heparin sulfate proteoglycans of the ECM.[43] Consequently, heparin and heparin sulfate-mimetic molecules have been incorporated into various biomaterials and used to regulate delivery of heparin-binding GFs. For example, a synthetic hydrogel gel cross-linked with heparin and derivatives of chondroitin sulfate successfully regulated delivery of FGF-2 and accelerated dermis formation and vascularization in a full thickness excisional wound in a db/db diabetic murine model.[44] GFs' affinities for other ECM components such as fibrin(ogen) [20,41,45,46] and vitronectin [47] have also been similarly exploited to achieve enhanced healing in diabetic murine models via stable GF delivery.[40]

Alternatively, GFs have been covalently bound to biomaterials via chemical and enzymatic reactions or engineered to have enhanced affinity for biomaterials via recombinant engineering. In one study, a heparin/fibrin-binding sequence isolated from PIGF-2 was incorporated into VEGF-A and PDGF-BB as a fusion resulting in significantly faster wound closure in a diabetic murine wound model compared to the wild-type GF treatments when delivered via fibrin or topically.[48] Additionally protein

engineering has been used to create GF variants of enhanced stability within the body (e.g. KGF[49] and FGF-1[50]). While protein engineering approaches show promise, the techniques are difficult to implement without potentially compromising GF activity and offer little ability for achieving tailorable delivery of multiple GFs, which has been found to be increasingly important in tissue repair.

Moreover, ECM -inspired delivery of GF proteins has been shown to promote healing through improving GF stability and retention[40,44,48], yet supra-physiological dosages are still required to account for elevated-protease activity and a lack of GF mobility in the chronic wound bed. These obstacles stress the fundamental incompatibility of GF protein delivery in the chronic wound bed as well as the inability of topical GF applications to truly mimic native cellular GF release mechanisms.[11,32,36]

1.2.2 Growth factor gene delivery

GF gene therapies offer many unique benefits over GF protein delivery in wound repair, especially in chronic wound healing. A key advantage is the capacity to prolong factor availability overtime for therapeutic proteins with short half-lives (on the order of hours), as transgene encoding proteins are expressed for days post-transfection.[11,26,27,51,52] Moreover, gene delivery better mimics endogenous repair utilizing host cells to orchestrate GF production, micro-localization, and activity to achieve *in situ,* on-demand release of bioactive GFs bearing post-translational modifications. These features have a proven capacity to provide orders-of-magnitude (~2000 fold) dose reduction compared to topical GF application in wound repair [53] and are particularly crucial in chronic wound repair due to their extended healing over month and spatiotemporal heterogeneity. [3,10,37] Additionally, gene manipulations are

low cost and can be utilized to delivery multiple genes and improve spatio-temporal control through use of tissue-specific promoters.

As such, the potential of gene activated matrix (GAM) in chronic wound repair has garnered widespread interest. Natural and synthetic GAMs have been successfully designed to extend vector retention and mediate delivery typically through diffusion and/or degradation controlled release of gene constructs retained via physical entrapment and/or electrostatic interactions. In several cases, hydrolytically- and enzymatically- degradable GAMs have achieved detectable protein expression for weeks *in vivo* [27,54,55] and enhanced wound repair via increased granulation tissue formation, vascularization, and epithelialization[54];however existing GAM technologies often fail to retain gene constructs within the delivery site and the gradual release is often problematic.[56-58] For example, collagen scaffolds containing PDGF-B-encoding adenoviruses increased the formulation of granulation tissue and maturation in wound models, yet escaped vector was detected in the axillary lymph nodes resulting in anti-Ad IgG titers 1000-fold above base levels.[36,54,59] Vector immunization was also made apparent by attenuated healing in pre-immunized rats highlighting another potential pitfall specifically for viral DNA delivery. [36,54,59]

Safety and efficacy concerns with both viral vectors and off-target delivery, has increased interest in improved non-viral GAM technologies. Improved spatial and temporal control over the delivery of non-viral DNA from GAMs has been achieved through the immobilization of DNA vectors onto scaffolds through better defined interactions, such as biotin-avidin[60] or antigen-antibody binding[22]. Specific binding has been used to achieve higher gene vectors density and longer retention times relative to the non-specific binding within various bioactive materials including

hyaluronic acid, fibrin, fibronectin, and collagen-based scaffolds. For instance, biotinylation of DNA carriers known as polyplexes was demonstrated to increase retention of DNA on avidin-modified HA-hydrogels and collagen scaffolds, enhancing retention by approximately 30% and promoting a 2-fold increase in transfection efficiency on the collagen scaffolds.[60] However, continued concerns of off-target and immune responses, as well as inefficiencies in gene transfer efficacy in protein/serum-rich environments have prevented clinical translation. Approaches for addressing intracellular and extracellular barriers of non-viral gene delivery are commonly addressed through different techniques, making design of non-viral vectors complex. Moreover, the majority of existing GAM technologies are unfit for many tissue repair applications due to the complexity of the healing process, which can involve extended healing periods over months and multiple out-of-phase healing cascades occurring simultaneously within repair sites. An approach for achieving prolonged DNA delivery with release kinetics conducive of local cell behavior and efficient cellular uptake is vital.

1.3 The importance of extracellular matrix dynamics in native repair and regenerative medicine

1.3.1 The ECM and ECM remodeling

The ECM is the non-cellular component within all tissues and organs which provides structure and support for cellular constituents as well as vital biochemical and biomechanical cues required for the most basic of cell behaviors, including cell proliferation, differentiation, polarity, and migration. Its chief components are fibrous proteins (e.g. collagens, laminins, fibronectin, vitronectin, and elastin), specialized proteins (e.g. GF, small matricellular proteins, and small integrin-binding

glycoproteins) and proteoglycans, however its exact composition is tissue dependent.[40,61,62] The versatile nature of ECM function is reliant on its highly dynamic structure and its remodeling -i.e. assembly and degradation - as an effective mechanism for regulating diverse cellular behaviors. The ECM is subject to a sustained remodeling process mediated by reciprocal interactions between the ECM and local cellular component. Remodeling rates a particularly high during development and wound repair, and can occur in response to signals transmitted from ECM receptors such as integrins or ECM-modifying proteins such as matrix metalloproteinases (MMPs).[42,61,63] For example, proteases have the capacity to degrade the ECM and control the release kinetic of ECM-bound GF as well as convert structural molecules to signaling molecules through release of small bioactive peptides. ECM dynamics in healthy tissues are securely regulated by redundant mechanisms that control the expression and function of ECM modifying enzymes (such as MMPs) to maintain ECM homeostasis via delicate balance between ECM synthesis, deposition, and degradation.[42,63] Understandably, poorly regulated protease activity is commonly associated with diseased states, such as chronic wounds. In fact, the persistence of chronic wounds, typically induced by aberrant levels of MMPs and compromised ECM stability, vividly illustrates the consequence of altered ECM dynamics.[9,34]

1.3.2 Collagen remodeling

Collagens are a family of triple helical proteins that provide the ECM both mechanical strength and structural integrity, while also playing an essential role in the regulation of key processes underlying tissue development and regeneration. They also act as a cell-responsive reservoir for a wide array of essential, highly potent GFs and other signaling molecules which allows for rapid, on-demand, and highly localized cellular responses without de novo synthesis. [64-66]

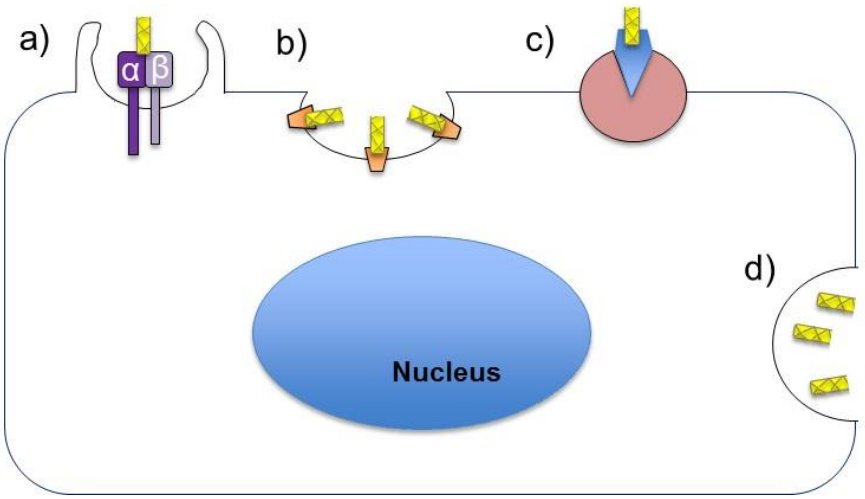


Figure 1.2 Collagen fragment cellular uptake is mediated by a) phagocytosis (integrin $\alpha_2\beta_1$), receptor-mediated endocytosis (UPARAP/endo180, mannose receptor), bridging molecules (Mfge8), and micropinocytosis. Many more cell surface receptors are recognized to bind collagen, however most just facilitate cell-ECM attachment or transmit signals from the ECM to the intracellular compartment. The receptors summarized above have been shown to mediate uptake of bound collagen fragments and in some cases, viral uptake as well.

Understandably, collagen turnover/remodeling is an integral part of ECM remodeling and similarly elevated during tissue repair.[12,42,63] It is essentially a two-step process: extracellular protease- mediated degradation and subsequent cellular uptake and recycling. Intact fibrillar collagen is initially cleaved by collagenolytic enzymes including a subset of MMPs (e.g. MMP-1) and cathepsin K.[67] The resulting fragments of the fibrillar collagen are either rapidly uptaken by the surrounding macrophage and fibroblast or further cleaved by gelatinasases (e.g. MMP-2, MMP-9) and cleared from the body.[67] Collagen cellular uptake is mediated by a myriad of macropinocytic, phagocytic, and endocytic pathways initiated by recognition of specific cell surface receptors **(**Figure 1.2**).** For example, collagen phagocytosis is dependent on integrin $\alpha_2\beta_1$ as demonstrated through uptake studies with collagen-coated beads and native fibrillar collagen. Fibroblasts expressing this integrin receptor, bind and internalize collagen fragments through a Gelsolin- and Rac-depedent pathway. [67] Alternatively, receptor-mediated endocytic pathways of collagen fragment uptake have been discovered for transmembrane receptors uPARAP/Endo180 and mannose receptor/CD206, and the significance of the uPARAP/endo180 collagen-uptake pathway is vividly illustrated by the accumulation of collagen nearby malignant areas and increased pulmonary fibrosis post treatment with bleomycinin in uPARAP/endo180-knockout mice. [67] The extracellular glycoprotein Mfge8 has also been demonstrated to regulate collagen internalization through binding and initiating macrophage collagen uptake and degradation and its *in vivo* importance has been similarly demonstrated in murine knockout studies demonstrating impaired collagen uptake and increased fibrosis post bleomycin-induced injury.[67] While the majority of these pathways are believed to culminate with lysosomal collagen degradation and

amino acid recycling, recent studies suggest the existence of non-lysosomal pathways mediated by integrin $\alpha_2\beta_1$ and caveolin-1. Furthermore, certain viruses such as echovirus 1 appear to hijack this non-recycling pathway to obtain efficient cellular uptake while numerous additional pathogens are known to target collagen[68,69] and ECM-specific integrins and theorized to utilize similar mechanisms (e.g. adenoviruses use vitronectin binding integrins $\alpha_v\beta_3$ and $\alpha_V\beta_5$) to facilitate virus cellular internalization.[70]

1.3.3 Extracellular matrix remodeling-stimulated viral delivery

As discussed earlier, the ECM serves as a natural reservoir for GFs and other signaling molecules. In turn, the release of bound GFs from the ECM is mediated by GF-ECM affinity and ECM degradation, in respect to the ECM component, which allows for cell-triggered, microlocalization of release kinetics of different GF. Like GFs, many viruses, such as the gammaretrovirus and human papillomavirus, have evolved mechanisms to bind to the ECM to enhance their cellular availability, preserve their stability, and increase the chance of internalization via interaction with specific virus receptors.[69-71] In turn, bound viral release is dependent on local cellular invasion and proteolytic degradation.

In addition to mediating release, ECM remodeling pathways are commonly used to facilitate cellular uptake of viruses via integrin-dependent pathways.[69-71] For instance, the collagen-binding integrins such as $\alpha_2\beta_1$ are utilized to regulate efficient internalization of echoviruses and rotaviruses. In some special cases, the ECM component is used as a bridge to facilitate binding and initiate internalization. For instance, ECM component fibronectin has been demonstrated to bind gammaretrovirus and assist in cellular uptake through colocalization studies displaying their co-

internalization via caveolar endocytosis and direct correlation between fibronectin turnover and gammaretrovirus virus cellular entry.[71]

1.3.4 Utilization of ECM remodeling in regenerative medicine

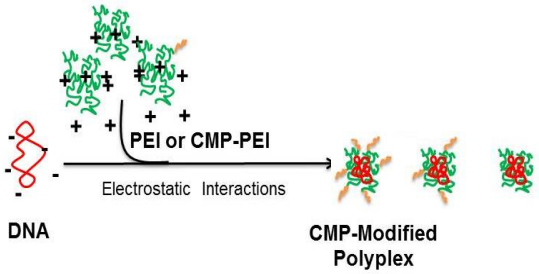
Regenerative medicine GAMs have been designed to similarly harness ECM remodeling to synchronize therapeutic delivery with tissue repair cascades, typically through application of cell-responsive, enzymatically degradable biomaterials. Therapeutic DNA has been incorporated into scaffolds with various degrees of protease-sensitivity, such as collagen with different degrees of crosslinking[19,72,73] or polymer-based hydrogels crosslinked with different MMP-labile peptides[74,75], to enable cell-triggered, localized gene delivery resulting in transgene expression over periods ranging from days to weeks. For instance, polyethylenimine (PEI) polyplexes encapsulated in PEG hydrogels crosslinked with MMP-degradable peptides promoted prolonged expression periods of up to 21-days directly related to protease-driven cell migration.[74] Furthermore, GF-encoding DNA has been incorporated into both protease-sensitive polymer- and protein-based scaffolds and used to promote essential reparative processes where no healing or minimal healing were previously observed.[76,77] For instance, the implantation of FGF-2 and FGF-6 pDNA encapsulated in degradable collagen–gelatin scaffolds at injured muscle sites in mice induced angiogenesis and remodeling that promoted artery formation. Significant numbers of transfected cells were only observed in injured opposed to healthy muscles suggesting the inflammation and enhanced proliferation initiated by the injury increased delivery.[78] Similarly, collagen-embedded PDGF-B DNA was applied to rabbit dermal ulcer wounds and promoted a 52% increase in new granulation tissue, 24% increase in epithelialization, and a 3-fold decrease in wound closure times compared to empty

collagen controls. [32] Similar results were achieved when expressing PDGF-A.[32] Enhanced spatiotemporal control has also been achieved by immobilizing DNA constructs on protease-labile scaffolds through better defined interactions, including antigen–antibody and biotin–avidin binding [60] and the incorporation of biomaterial (e.g. collagen) binding domains[79]. Alternatively, ECM integrin binding sites (such as GFOGER, GEKGER[30,31], and RGD) [80,81] have been incorporated into non-viral vectors to facilitate cellular targeting, binding, and entry, exploiting well-conserved, integrin mediated pathways into the cell in the same manner as many bacterial, viral, and eukaryotic pathogens. However, while non-viral GAM technologies have been developed to utilize natural ECM remodeling as a trigger for release and target ECM integrins via the addition of adhesion molecules/binding sequences[80,81], the ability to exploit the second part of ECM remodeling, rapid ECM fragment cellular uptake, to similarly achieve efficient cellular uptake has been largely unexplored. Our work demonstrates a novel approach with has the potential to enable viral-like exploitation of ECM remodeling, in particularly collagen turnover, to integrate non-viral gene delivery and uptake with tissue repair cascades.

1.4 Dissertation Synopsis

In this work, a novel strategy for enhancing control over the dynamics and location of GF-delivery through harnessing ECM turnover-stimulated non-viral gene delivery is presented. The focus will be on enhancing delivery from collagen-based devices due to their innate biocompatibility, bioactivity, and the key structural roles of collagens in various tissues as well as their current widespread application in regenerative medicine.[3,64] Specifically, collagen-mimetic peptide (CMP) have been designed to engineer DNA polyplex-modified collagen scaffolds with the potential to

a)



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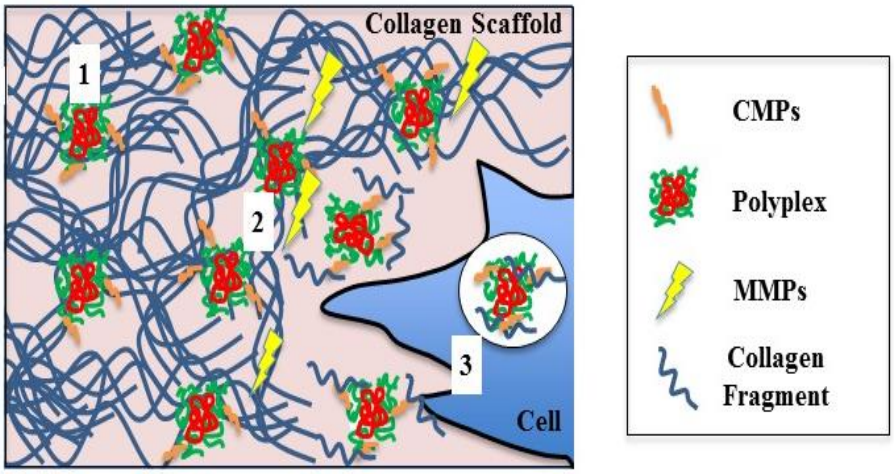

Figure 1.3. Collagen mimetic peptide (CMP)-mediated gene transfer. a) Non-viral DNA carriers are prepared via electrostatically condensing DNA with mixtures of polyethylenimine (PEI) and CMP-PEI conjugate. B) The purposed process of CMP-linked gene transfer includes: (1) Stable integration of CMP-modified polyplex into collagen scaffolds via CMP-collagen affinity, (2) Cell-mediated proteolytic release of collagen-linked polyplexes, and (3) Internalization by invading cells via attachment to endocytic collagen fragments.

induce localized, high efficiency GF expression coordinated with tissue repair kinetics (Figure 1.3).[30,31] CMPs have a unique affinity for collagen driven by a strand-invasion process which may be carefully tailored via CMP sequence design. Our groups as well as others have demonstrated CMP-collagen interaction can be utilized to robustly

modify collagens with various nanoscale cargoes both *in vitro* and *in vivo* and provide stable integration with collagen over prolonged periods of a month or longer as well as selectively target pathologic tissue with elevated ECM remodeling (e.g. prostate tumor xenografts; skeleton in murine Marfan syndrome models; joints; articular cartilage [82-89]) including wound beds. [90] These emerging results illustrate the capacity for short synthetic CMPs to selectively and stability modify collagen-based biomaterials as well as target and/or retain therapeutics in pathologic tissues such as chronic wound beds. Furthermore, cell-penetrating peptides and integrin binding sequences have been incorporated with relative ease into CMPs increasing their capacity to engage[65,81,91,92] and be up taken by cells.[93]

Our work demonstrates CMPs have a proven capacity to provide stable, tailorable links between DNA polyplexes and collagen, enabling localized and highly efficient gene expression, even after prolonged serum exposure. As shown in Figure 1.3, this dissertation will detail how CMPs can be utilized to exploit cell-mediated turnover of the ECM in a three part, viral-like process: (1) integration of stable gene-encoding DNA within collagen-based biomaterials such that DNA polyplex activity is preserved, (2) "on-demand" release inherently linked to tissue/ECM remodeling via cell-mediated proteolytic activity, (3) promotion of a "Trojan horse"-type strategy to encourage enhanced stability, biocompatibility, and activity via linking DNA polyplex to endocytic collagen fragments. Chapter 2 will discuss the optimization of CMP/polyplex-linked collagens for application in wound repair through achieving prolonged matrix retention and tailorable gene expression through CMP design/display in both *in vitro* and *in vivo* models. Chapter 3 will detail the purposed mechanistic steps underlying CMP/polyplex-mediated release and cellular

internalization. Furthermore, the expression and activity of functional GF genes expressed via CMP-driven activity, including highly promising results in an *in vitro* wound model, will be presented in Chapter 4 and followed by discussion in Chapter 5 of the potential of CMP-mediated delivery to promote controlled multi-gene expression where favorable expression profiles may be customized per individual GF requirement. Finally, Chapter 6 will include future suggestions for the direction of this project.

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Chapter 2

COLLAGEN-MIMETIC PEPTIDE-BASED METHOD FOR TAILORABLE, CELL-MEDIATED GENE DELIVERY FROM COLLAGEN BIOMATERIALS

The use of collagen-based biomaterials in regenerative medicine has rapidly increased over the past decade. The unique structural and biochemical properties of collagen make it a particularly promising material for delivering both protein and DNA-based therapeutics. Although many collagen modification techniques have been developed, the majority of them require multi-step chemical treatments that can damage the natural favorable properties of collagen. We have developed a promising biomimetic modification technique employing collagen-mimetic peptides (CMP)s to control the retention and delivery of DNA polyplexes from collagen structures, including both monomeric 2-D collagen films and fibrous, 3-D gels. Variations in the concentration of CMPs displayed on polyplexes enabled tuning of polyplex retention *vs.* release over periods of at least 2 weeks on films and a month on gels. Retention of CMP-modified polyplexes (20 days) was substantially improved compared to unmodified polyplexes, which were retained for only 2 days. The activity of bound polyplex in collagen gels was shown, through a series of transfection studies, to be maintained in the presence of serum for a minimum of 2 weeks. Only matrix metalloproteinase (MMP)-stimulated cells exhibited significant levels of transfection suggesting that cell mobility within the gel was vital and that collagen remodeling played a role in stimulating gene release and expression. To our knowledge, this study

is the first to deliver genes with CMP-modified polyplexes and to examine the effects of CMP display on DNA release. Moreover, experiments in an *in vivo* repair model showed that CMP modification enabled transgene expression for 10-fold longer time periods with average expression levels that were up to 2-orders of magnitude higher. The results suggest that this technique may be used more broadly to create tuneable, collagen-based delivery systems.

2.1 Introduction

Collagen is the principal component of all connective tissues, providing strength and a framework for cellular components and vital signaling molecules.[1,2] As the most abundant mammalian protein, both its function and abundance has made it an extremely valuable biomaterial in regenerative medicine applications with its earliest uses as an absorbable suture dating back to the early second century A.D.[3,4]

Over the past two decades, collagen-based biomaterials have become increasingly widespread in both research and medicine owing the abundance, functional versatility, and the structural tunability of collagen.[1,5] Collagen-based materials have been engineered with chemotactic and/or structural gradients among other features to facilitate desirable cell behaviors such as migration and differentiation;[1,6,7] however, while collagens and other ECM-inspired scaffolds commonly improve the regeneration of many tissues such as skin and bone, the incidence of complete healing remains low. In the case of chronic wounds, where the environment is particularly aberrant and unconducive to healing, efficacy is even lower. For instance, only half of diabetic foot ulcers fully closed during a therapeutic trial employing artificial skin.[8-10]

Clinical failure has led to increased interest in combining the delivery of biomaterials/artificial skins with additional therapeutic elements namely GFs. As detailed in Chapter 1, the topical and sustained delivery of numerous GFs have been examined for many applications, however the extraphysiological, repetitive dosage regimens required for even modest therapeutic effect has led to valid safety concerns with oncogenic and off-target effects.[11,12] For instance, FDA-approved REGRANEX® Gel, a treatment containing PDGF-BB increased the number of healed DFU patients by almost 10% in four phase III trials,[13] yet associated with a 5-fold increased risk of cancer.[14] Inefficient GF delivery and instability in the protease-rich delivery sites commonly treated in regenerative medicine have also hindered development.

GF gene-based approaches have the potential to address these obstacles through facilitating the sustained, localized production of fresh, bioactive GF. Gene-based approaches to deliver PDGF, VEGF, and other factors can promote enhanced healing at greatly reduced doses as compared to topical GF,[15-17] yet delivery obstacles, namely inefficient gene transfer and off-target effects, have severely limited GAM clinical translation.[11,12,15-21] Biomaterial-mediated delivery of genes or GAMS provide an opportunity to combine the benefits of the structural/bioactive properties of the biomaterial with increased spatio-temporal control over gene delivery. Moreover, delivery from a substrate better mimics natural mechanisms of GF and viral delivery and often enhances stability and prevents immune recognition.[22-25]

Understandably, numerous collagen-based GAMs have been tested with the majority utilizing electrostatic or physical interactions to immobilize DNA and/or DNA constructs with release driven by cell-mediated degradation. DNA, viruses (e.g. adenovirus), DNA constructs such as polyplex and lipoplex encapsulated in collagen

have successfully been used to express a plethora of GFs and improved therapeutic efficacy when compared to bolus delivery,[24-27] however insufficiencies in promoting strictly local delivery and tuneable release, have prevented clinical translation and led to continued safety concerns.[11,28] Chemical modification, such as the chemical cross-linking of collagen containing therapeutic, or the covalent modification of collagen to increase affinity for a therapeutic molecule (i.e. biotinylation[29] or cationization through modification with amino groups or cationic polymers like polylysine, or polyethylenimine [24-26,30]), has a proven capacity to prolong and achieve greater control over release, allowing the local delivery of DNA encoding factors such as bFGF, BMP, and PDGF and improved regenerative properties.[23,31-33] Despite these compelling results, many chemical modifications techniques damage the bioactive compounds, and the complexity of collagen often makes covalent modification problematic and inefficient.[34] Furthermore, current GAM technologies do not typically enable tailored delivery of multiple factors with the micro-localized release kinetics ideal for the individual factors.[35,36]

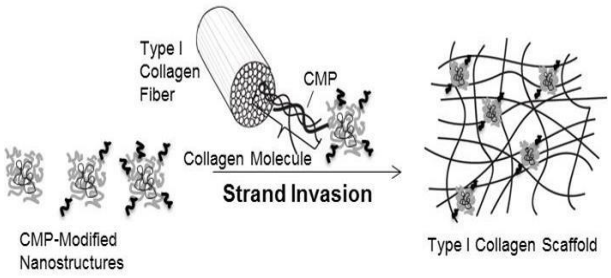

Figure 2.1. Tailorable CMP-based approach for producing DNA polyplex – modified collagen scaffolds. CMP-modified polyplexes are bound to collagen via thermally induced annealing that induces CMP strand invasion and CMP-collagen triple helical hybridization. The versatility of the CMP allows it to perform as both a reversible tether and an integrin-binding ligand. Controlled retention and release may be achieved through variation of CMP display on the nanostructures.

A recently developed collagen-modification approach, based on the affinity of CMPs for native collagen, has enormous potential in GAM development. CMPs are relatively short peptides comprised primarily of collagen-like $(GXY)_n$ triplets capable of thermally annealing with collagens and integrating into the native collagen triple helix via strand exchange.[2,37-51] The melting behavior of the CMP-collagen assemblies is highly tailorable via manipulation of CMP amino acid composition and molecular weight.[37,45,47,48] Both i*n vitro* and *in vivo* studies have established that CMPs can selectively detect minute quantities of collagen (e.g. 5 ng), target pathogenic tissues with increased collagen turnover, such as tumors, joints[40,43,47], and wounds[5,52], and significantly improve retention of nano-scale cargoes including gold nanoparticles, peptides, non-fouling polymers[40,47], and GFs on collagen-based scaffolds[37]. Furthermore, CMPs engineered with bioactive sequences, such as the integrin-binding sites like GEKGER or cell-penetrating peptides, can be used to modulate cell behavior,[45,46,49,51] such as the adhesive/migratory responses in human MSCs and

fibroblast or increase cellular uptake.[51] For instance, the capacity of proline-rich CMPs to aid in bolus transport of plasmid DNA into cells has been demonstrated, however the benefits of using CMPs to mediate gene delivery from collagen have been exclusively studied by our groups.

In this chapter, the capacity of our novel, CMP-based strategy for engineering DNA polyplex-modified collagens with tailorable release profiles/gene expression and improved gene transfer, is discussed (Figure 2.1). The CMPs used in this work were specifically designed to perform as both adjustable tethers to regulate collagen-polyplexes affinity, as well as adhesive/endocytic ligands, as these proline-rich CMPs contained the amino acid sequence GEKGER that was previously shown to engage $\alpha_1\beta_2$ integrin;[50,51] however the focus of Chapter 2 will be on tailoring gene release and expression via CMP display while a more in depth discussion of the mechanisms underlying CMP-enhanced gene transfer is in Chapter 3.

To study CMP-tailored gene delivery, CMPs were synthesized using solid-phase peptide synthesis and subsequently used to modify DNA polyplexes *via* Michael-type addition chemistry. Prolonged retention/release of CMP-modified polyplex on 2-D and 3-D collagen scaffolds demonstrated a direct dependence between polyplex retention and CMP incorporation, where polyplex were retained for days to over a month based on the extent of CMP modification and CMP sequence. Subsequent fibroblast transfection studies indicated gene expression was highly reliant on protease-activity suggesting CMPs facilitate stable integration of active polyplex into collagen and cell-triggered release ideal for achieving localized delivery. CMP-modified polyplex incorporated into collagen scaffolds also exhibited significantly enhanced stability and activity compared to released or electrostatically sequestered

polyplex, even after prolonged exposure to serum, indicating potential benefits over existing GAMs.[53] Furthermore, favorable results translated well *in vivo*, where CMP-modification was utilized tailor the extent and duration of transgene expression in murine ECM depot models for similar time periods (~days to month).[54] The ability to tailor release/retention of nanostructures from collagen for prolonged periods via physical modification coupled with the capacity to provide "on-demand" cell-triggered release and collagen-mediated uptake, has widespread potential in regenerative medicine and makes the study of CMP-modification of great interest.

2.2 Materials and Methods

2.2.1 Materials

Fmoc-protected amino acids were purchased from Anaspec (Fremont, CA) and H-Rink amide ChemMatrix® resin was purchased from PCAS Biomatrix (Quebec, Canada). O-Benzotriazole- N,N,N0,N0-tetramethyl-uronium-hexafluoro-phosphate (HBTU) and the rink amide 4-methylbenzhydrylamine (MBHA) resin were purchased from Novabiochem (San Diego, CA). Highperformance liquid chromatography (HPLC)-grade N,Ndimethyl formamide (DMF), acetonitrile, trifluoroacetic acid (TFA), and cell culture reagents, including Dulbecco's modified Eagle's medium (DMEM), Dulbecco's phosphate buffered saline (PBS), and tryspin were purchased from Fisher Scientific (Fairlawn, NJ). Piperidine, 4-methylmorpholine, all cleavage cocktail components, and branched polyethylenimine (PEI, 25 kDa) were purchased from Sigma-Aldrich (St. Louis, MO). Type I bovine collagen was purchased from Advanced BioMatrix (San Diego, CA) and AlexaFluor555 was purchased from Life Technologies (Carlsbad, CA). pCMV-GLuc plasmid was purchased from New

England Biolabs (Ipswich, MA). The plasmid was amplified in NEB 5-a electrocompetent E. coli purchased from New England Biolabs and purified from bacterial culture using a Qiagen Megaprep Kit (Valencia, CA), per the manufacturer's protocols. Growth factor reduced (GFR) phenol red free Matrigel® was purchased from Corning (Corning, NY), BMP-2 was purchased from GenScript (Piscataway, NJ), and coelenterazine was purchased from Clontech and GoldBio (Yorba Linda, CA).

2.2.2 Methods

2.2.2.1 Collagen-mimetic peptide synthesis

Two CMP sequences (GPP and GPO) and a scrambled CMP (GPO^S) were synthesized via automated Fmoc solid-phase peptide synthesis (SPPS) with a PS3 or Tribute™ peptide synthesizer (Protein Technologies Inc., Tuscon, AZ). The CMP sequences used in this work are summarized in Table 2. The GPP sequence was synthesized on a Rink amide MBHA resin as previously described[50,51,53,54], and the GPO and GPO^S sequences were synthesized in a similar manner on a Rink amide ChemMatrix® resin. For all synthesizes, amino acid residues were activated for coupling with HBTU in 0.4 M methylmorpholine in DMF and de-protected with 20% piperidine in DMF for 10 min. For the GPP sequence, 1 h coupling times were used

Sequence Name	Peptide Sequence	Melting Temperature (T_M)
GPP	$(GPP)_3GPRGEKGERGPR(GPP)_3GPCCG$	43°C
GPO	*$(GPO)_4GEKGER(GPO)_4GGCG$*	45 °C
GPO^S	*GGGPCPEGGOPOPPGPEPOGKGOOPOGGROGGOG*	N/A

Table 2.1 Summary of CMP sequences and melting temperatures.

and all residues after the 15th residue were double coupled. For the GPO and GPO^S sequences, 2 h coupling times were used and all amino acids after the 10^{th} residue were double coupled. The GPP sequence was cleaved from the resin using a cocktail of 94 : 1 : 2.5 : 2.5 TFA/triisopropylsilane (TIS)/water/1,2-ethanedithiol (EDT) for 6 h, whereas the GPO and GPO^S sequences were cleaved from the resin in the same cocktail for 3 h. After cleavage, the cocktail was evaporated and the cleaved peptides were precipitated in ethyl ether, dissolved in water, and lyophilized. Fluorescently labeled CMPs were produced by using carboxyl-amine chemistry to label the N-terminus of the CMPs with Alexa Fluor 555 on resin.

Crude peptides were purified using reverse phase-high performance liquid chromatography (HPLC) on a Prominence chromatography instrument (Shimadzu, Inc., Columbia, MD) equipped with a Viva C18 (4.2 mm _ 50 mm, 5 mm particle diameter) column from Restek (Lancaster, PA). Water with 0.1% TFA (Solvent A) and acetonitrile with 0.1% TFA (Solvent B) were employed as HPLC solvents with a gradient of solvent B from 25–35% over 30 minutes. The eluent absorbance was monitored at l ¼ 210 nm. Electrospray ionization mass spectrometry (ESIMS) was used to confirm the product, using a Thermo Fisher Scientific LCQ Mass Spectrometer. For GPP: m/z ¼ 1610.6 [(M + 2H)2+ ¼ 1610.85]; for GPO and GPOS: m/z ¼ 1543.6 and 1543.4, from 25-35% over 30 minutes. The eluent absorbance was monitored at λ=210 nm. Electrospray ionization mass spectrometry (ESI-MS) was used to confirm the product, using a Thermo Fisher Scientific LCQ Mass Spectrometer. For GPP: m/z =1610.6 [$(M + 2H)^{2+}$= 1610.85]; for GPO and GPO^S: m/z =1543.6 and 1543.4, respectively [$(M + 2H)^{2+}$= 1543.6] (Figure A1 (Appendix)).

Circular dichroism studies were used to confirm the triple helical structure and melting temperatures (T_m)s of the CMPs (Table 2, Figure A2), as previously described. [56]

2.2.2.2 Collagen-mimetic peptide retention on collagen films

To generate stable collagen films, 100 μL of 5 mg/mL bovine collagen type I solution was added to each well of an 8-well cell culture plate and allowed to air-dry. This process was repeated a total of four times. Subsequently, the resulting films were rinsed with distilled water, neutralized with cell-culture grade Dulbecco's phosphate buffered saline (PBS) solution (pH=7.4) and allowed to air-dry after additional distilled water washes to remove salts. To monitor CMP hybridization to the collagen films, preheated solutions of Alexa Fluor 555-labeled CMPs (2 mM in PBS) were added to the dry collagen films. The films were incubated with the CMP solutions for 3 h at room temperature, allowed to air-dry, and then thoroughly washed with PBS solution and distilled water until free peptide was no longer detected in washes as measured using a GloMax®-Multi Detection System (Promega). A standard curve was generated using CMP solutions of known concentration to enable calculation of the amount of CMP that was initially bound to the films *vs.* the unbound peptide that was detected in the washes. Subsequently, to monitor release *vs.* retention over time, films were incubated in PBS at a range of temperatures and CMP release was monitored through the detection of Alexa Fluor 555 or Alexa Fluor 350 in the washes.

2.2.2.3 Collagen-mimetic peptide-modified-polyplex formation

GPP-PEI conjugate was synthesized using a sulfo-succinimidyl-4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate (SMCC) crosslinker. A solution of SMCC in dimethyl sulfoxide (DMSO) was added to a solution of PEI dissolved in

PBS such that the SMCC:PEI molar ratio was 1:1 and the final concentrations of SMCC and PEI were 1 mM. These conditions were designed to allow the crosslinker NHS esters to react with PEI primary amines and form stable amide bonds as described by the manufacturer. After an hour, GPP was added to the solution at a molar ratio (PEI:GPP) of 1:3 and the mixture was incubated at room temperature for 24 h to facilitate Michael-type addition of the thiol-containing peptide to the maleimide-functionalized PEI. The product was purified with an Amicon Ultra-0.5 centrifugal filter device and formation of the product was confirmed with gel permeation chromatography (GPC) as shown in Figure A2 (Appendix).

CMP-modified polyplexes were formed by self-assembling GLuc plasmid with mixtures of unmodified PEI and the GPP-PEI conjugate, according to established, although slightly modified, protocols.[58] Briefly, equal volume solutions of plasmid and PEI were prepared in 20 mM HEPES, pH 6.0, and the PEI solutions were added drop-wise to the DNA solutions so that the final DNA concentration was 20 μg/mL. The PEI concentrations in the mixture were varied such that the N:P ratio, defined as the ratio of the number of amines (N) in the polymer to the number of phosphates (P) in the plasmid, would be as specified. CMP incorporation was varied by varying the ratio of GPP-PEI to total PEI in the PEI solution. The polyplex solutions were incubated for 10 minutes at room temperature to allow polyplex formation to occur. To confirm CMP incorporation into the polyplexes, polyplexes were formed with AF555-labeled GPP-PEI. The relative amount of fluorescence in the polyplexes was determined using a GloMax®-Multi Detection System (Promega; Madison, WI).

2.2.2.4 Polyplex characterization

To confirm that GPP-PEI was incorporated into the polyplexes, polyplexes were created using different amounts of AF555-labeled GPP-PEI. Subsequently, the polyplex solutions (N:P=10; 20 μg/mL DNA in 10 mM HEPES buffer, pH 6.0) were purified by centrifuging the solutions through a Centricon-100 membrane (Millipore, Eschborn, Germany) for 10 minutes at 500 g and diluting the retentate in HEPES buffer. This process was repeated five times. Following purification, the fluorescence of the DNA and GPP-PEI in the retentate solution containing the polyplexes was quantified using a standard curve with a Quant-iT™ PicoGreen® dsDNA Assay Kit (Invitrogen; Carlsbad, CA) and detection of the AF555 label, respectively.

DNA condensation efficiency and polyplex size were analyzed using agarose gel electrophoresis and dynamic light scattering (DLS), respectively. For electrophoresis assays, polyplexes were made with 0.2 μg of DNA, and either the N:P ratio or the fraction of GPP-PEI:total PEI was varied. The polyplexes were analyzed in 1% agarose gels containing 0.5 μg ethidium bromide per mL of tris/borate/ethylenediaminetetraacetic acid (EDTA) (TBE) buffer. Twenty microliters of each polyplex solution was added to 4 μL of gel loading buffer, and each well of the gel was loaded with 20 μL of the polyplex mixture. The gels were run at 100 V for 1 h and imaged with a BioRad Gel Doc XR (Hercules, CA). To determine the hydrodynamic radii of the polyplexes, a Brookhaven Instruments (Brookhaven, CT) ZETAPals with the 90Plus addition was used. Experiments were conducted using a 658 nm wavelength solid-state laser at an angle of 90° and a temperature of 25 °C. A second order cumulant fit was used to obtain the average hydrodynamic diameters of the polyplexes.

2.2.2.5 Polyplex retention on collagen films

To quantify the amount of polyplex that initially bound to the collagen films, and to determine the amount of polyplex that was retained on the films over time, polyplex retention studies were conducted after addition of different amounts of GPP-PEI polyplex or PEI polyplex to pre-made collagen films. To melt the CMPs and thereby enable efficient hybridization of the GPP-modified polyplexes to collagen, the GPP-PEI polyplexes were pre-incubated at 55 ^{o}C for 30 min. The control – produced with GPP-free PEI polyplexes – was treated similarly to ensure that any differences in collagen binding were caused by interactions of the GPP with collagen. Electrophoresis assays and DLS were used to confirm polyplex integrity after incubation. Subsequently, the films were incubated with the polyplexes for 3 h at room temperature, allowed to air dry, and thoroughly washed with a total of 5 mL of distilled water and PBS until free polyplex was no longer detected in the washes. To calculate the amount of polyplex that was initially bound to the films, the amount of polyplex in these initial washes was quantified by fluorescence-based analysis of DNA recovered in the washes. Polyplexes in the washes were disassembled through incubation with 20 mM heparin for 30 minutes. The amount of recovered DNA was quantified using a Quant-iT™ PicoGreen® dsDNA Assay Kit and the result was used to back-calculate the amount of DNA/polyplex retained on the film. Subsequently, to monitor release *vs.* retention over time, the polyplex-modified films were incubated in PBS at 37 ^{o}C. Polyplexes released into the washes were quantified as a function of time using the same fluorescence-based analyses to detect and quantify DNA.

2.2.2.6 Polyplex retention in collagen gels

Collagen gels with GPP-immobilized or GPP-free (physically encapsulated) polyplexes were constructed using acid-soluble, type I bovine collagen according to a modification of the manufacturer's procedures. GPP-PEI polyplex solutions or PEI polyplex solutions (20 μg/mL DNA) containing 2 mM of sucrose were preincubated at 50 ^{o}C for 30 minutes to melt the GPP. The polyplexes were then flash frozen using liquid nitrogen and lyophilized, as these procedures were previously shown to allow for dehydration while preventing aggregation and thereby preserving polyplex activity.[66] To formulate polyplex-containing collagen gels, the lyophilized polyplex was re-suspended in chilled DMEM at a concentration of 0.2 μg/μL of DNA in DMEM. Slowly, 100 μL of this chilled polyplex solution was added to 800 μL of chilled collagen solution while gently vortexing. The pH was then adjusted to 7.4 using sterile 0.1 M NaOH and the final volume was adjusted to 1.0 mL with sterile water, resulting in a solution containing collagen at a concentration of 4.0 mg/mL and DNA at a concentration of 20 μg/mL. To create each gel, 500 μL of neutralized polyplex-collagen solution was added to each well of an 8-well cell culture plate, and the plates were incubated at 4^{o}C for 3 h to allow for CMP-collagen hybridization and subsequently overnight at 37 ^{o}C. Gelation was confirmed visually, and subsequently, the gels were thoroughly washed with 7 mL of PBS and water, after which polyplex was no longer detectable in the washes. Initial retention of polyplex as well as polyplex retention over time were quantified using fluorescence-based DNA quantification assays, as described for the polyplex/collagen film retention studies.

2.2.2.7 Cell culture and collagen film transfection studies

NIH/3T3 cells (ATCC, Manassas, VA) were cultured at 37 °C and 5% CO_2 in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin−streptomycin (P/S), according to ATCC protocols. For collagen film transfection studies, cells were plated at a density of 8,000 cells/cm^2 on films modified with polyplexes containing the pCMV-GLuc plasmid. For comparison, cells were also plated at the same density on unmodified collagen films and transfected by the addition of non-CMP-linked polyplexes to the media, using either standard bolus polyplex dosing conditions (2.0 μg DNA/cm^2)[55,56] or dosing conditions that matched the maximum amount of DNA retained on any of the films (~0.2 μg DNA/cm^2). Transfection was monitored daily by quantifying luminescence in the media and cells were cultured under the same conditions. For these analyses, 10 μL of the media was analysed with a BioLux® Gaussia Luciferase Assay (NE Biolab®; Ipswich, MA), according to the manufacturer's protocol. MTT assays (Millipore; Billerica, MA) were used to assess cell viability and a Pierce BCA assay kit was used to determine total cell protein concentration at 1 and 4 days post-plating according to standard manufacturer-recommended protocols.

2.2.2.8 Collagen gel stability/transfection studies

To determine if cells could be transfected with polyplexes immobilized or encapsulated within collagen gels, GPP-PEI polyplex-modified collagen gels or PEI polyplex-encapsulating collagen gels were prepared with pCMV-GLuc plasmid. These gels were incubated in complete media maintained at physiological temperature and pH (DMEM with 10% FBS and 1% P/S at 37 °C and 5% CO_2) for a specified period of time ranging from 0 to 7 days. Subsequent to the incubation, NIH/3T3 cells

were plated on the gels at a density of 20,000 cells/cm^2. Cells were incubated either in the presence or absence of 10 ng/mL tumor necrosis factor-alpha (TNF-α), a well-known stimulator of MMP production,[57] and gene expression was monitored over several days. MMP activity was confirmed *via* a SensoLyte® 520 Generic MMP Assay Kit *Fluorimetric*, using the manufacturer's protocol, and gene expression was detected by analysing the luminescence of GLuc secreted into the media, using the BioLux® Gaussia Luciferase Assay.

2.2.2.9 *In vivo* gene delivery experiments

Institutional Animal Care and Use Committee-approved protocol was used for all animal studies. In all studies, male, 8 week-old (CD-1) white mice (Harlan Sprague Dawley, Inc., Indianapolis, IN) were used. Lyophilized polyplex solutions were prepared as previously described using pCMV-MetLuc-mem. pCMV-MetLuc-mem encodes for a membrane-bound luciferase (MetLuc-mem) that displays on the cell surface, making it readily accessible to substrate and available for repeated *in vivo* imaging. To prepare the polyplexes for injection, the lyophilized polyplexes were re-suspended in Matrigel® solution supplemented with BMP-2 (100 μg DNA/mL and 5 μg BMP-2/mL). The mixtures were vortexed and allowed to incubate on ice for approximately 1.5 h to allow bubbles formed during vortexing to disperse and for strand invasion to occur. A 1 mL sterile syringe was then used to draw up the solutions and the filled syringes were kept on ice to prevent gelation. Mice were then anesthetized using isoflurane and once under anesthesia, the abdomen of the mice were shaven and disinfected with isopropanol. Immediately before injection, the syringes containing the Matrigel® solution were briefly warmed at room temperature and mice were injected subcutaneously with 300 μL of solution into each of four

different locations on the abdomen using an 18-gauge needle. A visible pellet immediately formed at each site of injection. Each polyplex-containing pellet contained equal concentrations of Matrigel®, BMP-2, and polyplex, while control solutions included only Matrigel® and BMP-2. To visualize MetLuc-mem expression, images of the mice were obtained using a Caliper *In vivo* Imaging System Lumina (IVIS®) (Perkin Elmer, Waltham, Massachusetts), after the mice were injected subcutaneously in the vicinity of each pellet with 50 μL of luciferase substrate (coelenterazine). To prepare the substrate solutions, lyophilized coelenterazine was suspended in ethanol (5 mg/mL), and the substrate stock solution was diluted into PBS to a concentration of 0.5 μg/μL immediately before injection. Images of the mice were obtained every minute following substrate injection over a period of 45 minutes until MetLuc-mem expression was no longer detectable. The images obtained when luminescence plateaued at a maximum value were analyzed using the Living Image® software region-of-interest tool. The study was replicated in a total of 4 mice.

2.3 Results

2.3.1 Collagen-mimetic peptide design effects retention/release for collagen films.

In preparation for the work with CMP-modified polyplex, CMP retention/release studies from 2-D collagen films were conducted. The optimal hybridization conditions to maximize GPP and GPO retention on collagen films were determined by adding pre-heated solutions of AF555-labeled GPP, GPO, or GPO^S at a range of temperatures to pre-made collagen films and allowing hybridization at room temperature. As shown in Figure 2.2a, initial retention of GPP and GPO on the films increased as a function of increasing pre-incubation temperature up to a temperature of

65 ^{o}C, at which point GPP and GPO retention decreased with additional increases in temperature. For instance, approximately a 50% decrease in initial retention was observed for both peptides at preincubation temperatures of 80^{o}C relative to 65^{o}C. In contrast, the scrambled GPO sequence, shown not form a triple helix, was not significantly retained at any preincubation temperature, suggesting retention due to strand invasion opposed to non-specific interactions.

Extended CMP retention/release studies were subsequently conducted via fluoresce detection. As shown in Figure 2.2b, GPP and GPO sequences achieved sustained release for approximately 12 and 14 days at 25 ^{o}C, and for 8 and 10 days at 37 ^{o}C, respectively. The release rate was essentially constant for both CMPs at 25^{o}C, however two release profiles appeared to exist at an incubation temperature of 37 ^{o}C. Specifically, both GPO and GPP were released at a faster rate for the first five days (~75% of GPO and 85% of GPP were released), at which point the rate of release slowed by over a factor of 4. Additionally, GPO was consistently retained for longer periods than GPP at both incubation temperatures.

GPP peptide retention at elevated temperatures was also examined to gain insight on collagen-CMP complex melting behavior. As shown in Figure 2.2c, GPP was retained for approximately 2 weeks when incubated at room temperature, but for only 4 days at 45 ^{o}C. When incubated at 45 ^{o}C, approximately 87% of the initially retained GPP was released within 1 day of incubation which is almost equivalent to the release from films incubated at 37 ^{o}C over the initial 5 days. Sustained release was then maintained for the remainder of the study.

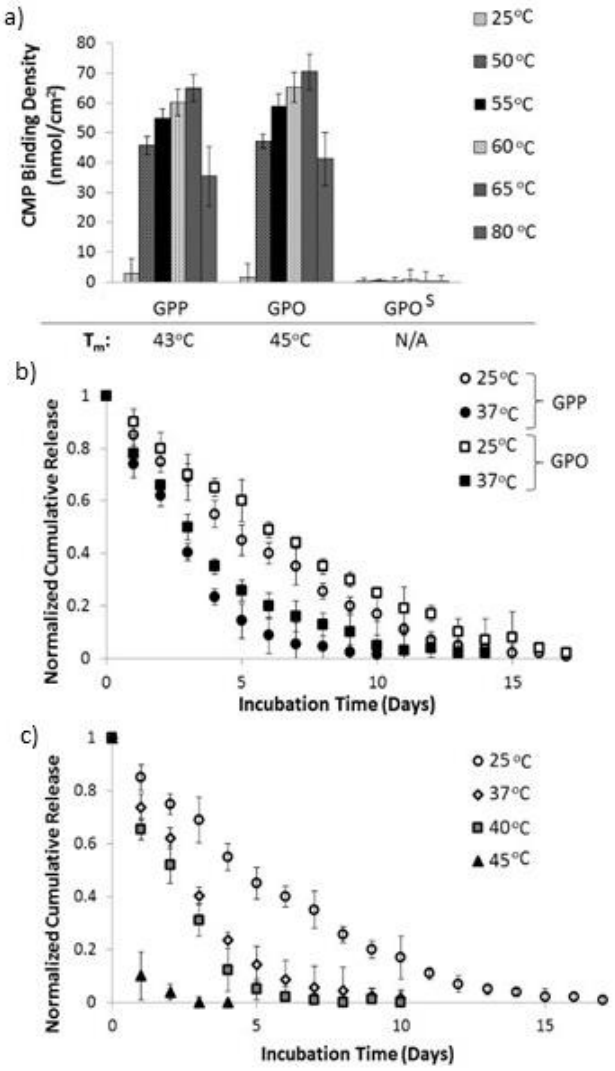

Figure 2.2 GPP/GPO Collagen Film Binding Studies. a) The optimal preheating temperature for maximizing CMP binding was determined through initial retention studies. b) The retention vs. release kinetics of GPP (circles) and GPO (squares) were compared at 25 °C (white) and 37 °C (black). (c) Retention vs. release of GPP as a function of temperature (25 °C (white diamonds); 37 °C (grey diamonds); 40 °C (grey squares); 45 °C (black triangles)). Each data point represents the mean ± standard deviation for a total of three separately prepared and analyzed samples.

2.3.2 Collagen-mimetic peptide can be incorporated into active PEI polyplex with varied display.

A highly versatile yet relatively simple method to incorporate CMPs in DNA polyplexes was developed. A GPP-PEI conjugate was synthesized using an SMCC crosslinker, purified *via* treatment with an Amicon Ultra-0.5 centrifugal filter device, and confirmed by GPC as shown in Figure A2 (Appendix). To modify polyplexes with GPP, a specified percentage of the PEI typically used to condense DNA was replaced with the GPP-PEI conjugate and polyplex formation was conducted by standard self-assembly procedures.[58] To quantify GPP incorporated into the polyplex, AF555-tagged GPP-PEI conjugates were synthesized and used to prepare polyplexes, and following removal of free GPP-PEI from the polyplex solutions *via* ultrafiltration, the fluorescence intensity in the polyplex solutions was quantified. As shown in Figure 2.3a**,** the 10% GPP-PEI sample fluorescence readings were about half that of the 20% GPP-PEI sample and approximately one fifth that of the 50% GPP-PEI sample. The data strongly suggests that manipulating the percent of GPP-PEI/PEI used during polyplex preparation could be used to directly manipulate the amount of CMP incorporated into the polyplex.

Modification of PEI with peptides or polymers lowers the charge density and can affect complexation,[58, 63-66] so the binding stability and structure of the GPP-modified polyplexes were further characterized. To assess the maximal effects on stability/structure imparted by large amounts of GPP, polyplexes containing 50% GPP-PEI were analysed. Polyplexes were prepared at a range of N:P ratios and the resulting structures were assessed by agarose gel electrophoresis and ethidium bromide staining. In these studies, polyplex formation is detected as a reduction in

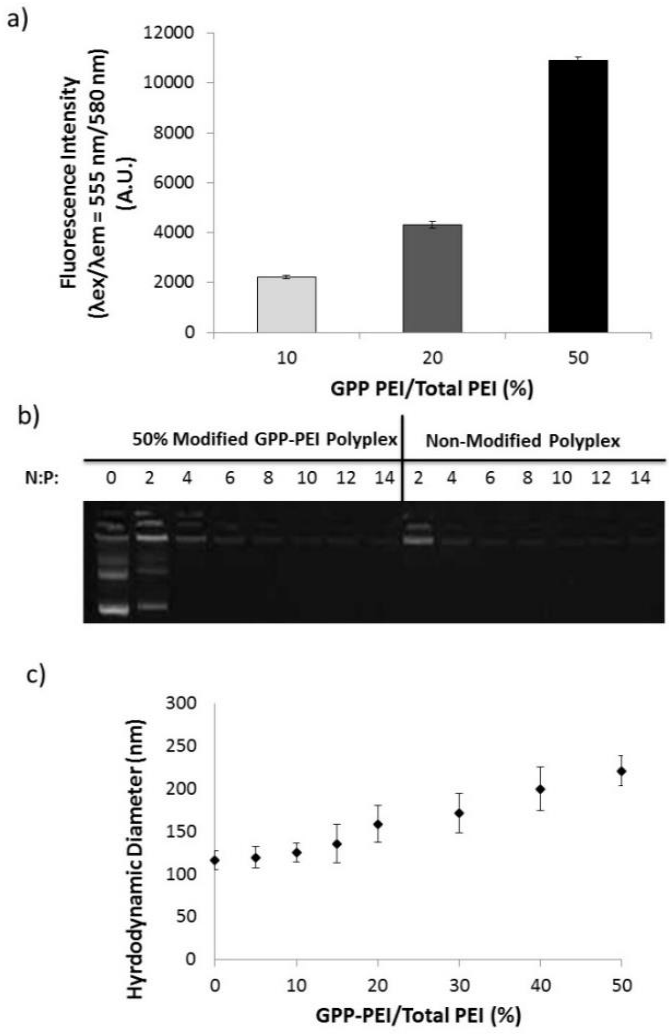

Figure 2.3. Polyplex Characterization. a) The relative fluorescence of AF555-labeled CMP in purified polyplexes as a function of the percent GPP included in the polyplex formulation. Each data point represents the mean ± standard deviation for a total of four separately prepared and analysed samples. b) Agarose gel electrophoresis and ethidium bromide staining analysis of DNA binding efficiency by 50% GPP-PEI polyplexes and unmodified polyplexes. c) DLS analysis of the hydrodynamic diameters of the polyplexes as a function of the percent of GPP-PEI. The data represent the mean ± standard deviation for nine separately prepared and analysed samples.

DNA mobility, and when DNA is sufficiently condensed, binding by the intercalating dye ethidium bromide is reduced. The electrophoretic assay showed that fluorescence in the well noticeably faded as the N:P ratio was increased, and fluorescence was effectively absent at N:P=10 (Figure 2.3b), an presence of the peptide may have caused a modest amount of polyplex aggregation.[38,58, 64-66] However, the size increases were relatively minimal and the polyplex diameters remained within a size range that permits endocytic internalization.

2.3.3 Collagen-mimetic peptide-modification of polyplexes directly impacts initial DNA retention efficiency and retention/release on 2-D collagen scaffolds.

To determine whether the amount of immobilized polyplex could be tuned through variations in the fraction of GPP-PEI in the polyplex, GPP-polyplex binding and retention studies were conducted on collagen films using polyplexes that contained various amounts of GPP. The polyplexes were preheated and incubated with pre-made collagen films at room temperature, and initial polyplex binding was quantified by using fluorescence measurements. The retention of GPP-PEI polyplex *vs.* PEI polyplex is shown in Figure 2.4a. Consistent with previously reported studies,[24-26,58,59] a small amount of PEI polyplexes were retained on the collagen films, even in the absence of the GPP modification. This non-specific retention is likely due to electrostatic interactions between the positively charged polyplexes and collagen (pI < 8).[60] By comparison, when 10% GPP-PEI was used, the amount of DNA initially retained increased by almost an order of magnitude (9-fold greater than the retention of unmodified PEI polyplex). When larger amounts of GPP were incorporated (e.g. 20% or 50% GPP-PEI), the amount of initial retention also increased, by 11- and 15-fold, respectively.

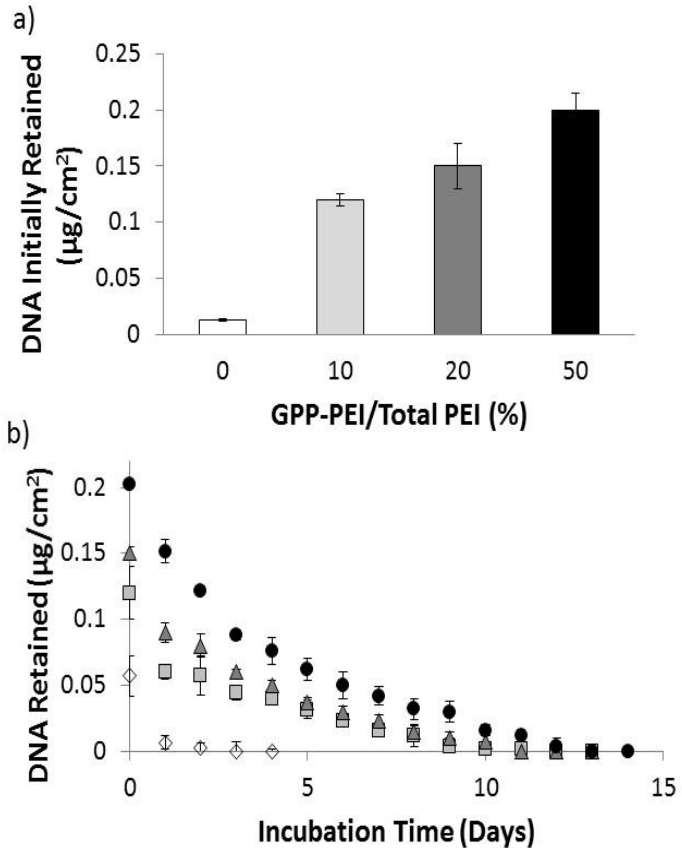

Figure 2.4. Polyplex Film Retention Studies. a) Initial retention studies to quantify the amount of DNA retained on collagen films as a function of the percent GPP-PEI in the polyplex. b) Retention of DNA on collagen films with time, as a function of the percent GPP-PEI within the polyplex including 0% (white diamond), 10% (grey square), 20% (dark grey triangle), and 50% (black circle). Each data point represents the mean ± standard deviation for a total of four separately prepared and analysed samples.

Polyplex retention at 37 ^{o}C was monitored over a two-week period *via* similar fluorescence-based approaches to detect DNA released into the supernatant as a function of time. As shown in Figure 2.4b, polyplexes modified with 10%, 20%, or 50% GPP-PEI were retained on collagen films for 8, 9, and 12 days, respectively, hereas unmodified polyplexes were only retained for 2 days. The GPP-polyplexes

were released at a significantly slower, sustained rate as compared with the unmodified polyplexes, as evidenced by the slopes of the retention curves. The release rates of the GPP-PEI polyplexes were relatively constant after an initial large release during the first day of incubation. Eventually, the rate of release became similar for each GPP-PEI polyplex as shown by the nearly identical slopes of the retention curves after 4 days. The initial burst release after 1 day of incubation likely consisted primarily of polyplexes that were interacting non- specifically with the collagen. Unlike when the collagen films were modified with GPP alone, the retention curves for the GPP-PEI polyplexes on collagen films did not have two distinct slopes. To further examine the release kinetics and distinguish these two possibilities, the polyplex retention data were normalized by the initial amount of DNA that was retained and a normalized retention curve was created (Figure A3). No significant difference was noted in the normalized slopes of polyplex retention *vs.* time after 4 days, suggesting that on a per polyplex basis, the release rates were similar, regardless of the amount of GPP incorporation.

2.3.4 Collagen-mimetic peptide-modification of polyplexes directly impacts initial DNA retention efficiency and retention/release on 3-D collagen scaffolds.

Given that many applications in regenerative medicine require 3-D scaffolds, retention studies were conducted using collagen gels in addition to collagen films. The goal of these studies was to determine whether increased levels of GPP-PEI polyplex functionalization would translate into increased probability of binding and/or avidity to collagen in the 3-D format. Polyplexes were mixed into neutralized collagen solutions, and after gelation, the initial polyplex retention was quantified after heparin was used to disassociate the non-retained polyplex in solution and a Quant-

it™ PicoGreen® Kit was employed to detect the liberated DNA. The gels were then incubated at 37°C in PBS and retention over time was monitored in a similar fashion.

As shown in Figure 2.5a, at least 7 μg/cm^2 of DNA was initially retained when GPP modification was employed, corresponding to 3.5 μg DNA per mg of collagen. The levels of initial retention on all GPP-PEI-modified polyplex gels were higher than the level of initial retention on gels with unmodified PEI polyplex, with 16-28% more retention depending on the amount of GPP modification. As was observed on the films, increasing the number of the GPPs in the polyplexes enhanced the probability of the GPP-collagen interaction, and thereby increased the initial polyplex retention. However, while the 3-D findings were similar to the trends observed on films, notably, much larger amounts of unmodified PEI polyplex were retained in the gels as compared with the films. This is consistent with the entrapment of unmodified polyplexes in the collagen gels, as has been demonstrated in past studies.[24,27]

Polyplex retention over time was also monitored by using fluorescence approaches to quantify DNA in the supernatant. As shown in Figure 2.5b, DNA was detected in the supernatants of both the 10% and 20% GPP-PEI polyplex-modified gels for approximately 10 days longer than it was detected in the supernatant of the unmodified polyplex gels. The 50% GPP-PEI polyplex was retained for 15 days longer than the unmodified polyplex. The same data were used to generate normalized retention curves, as presented in Figure 2.5c. The rate of release, as represented by the slopes of the curves, was significantly affected by GPP modification. Comparisons of the slopes suggest that the release of the 10%, 20%, and 50% GPP-polyplexes was reduced ~1.7, 2.3, and 3.1-fold, respectively. As was previously observed in the 2-D

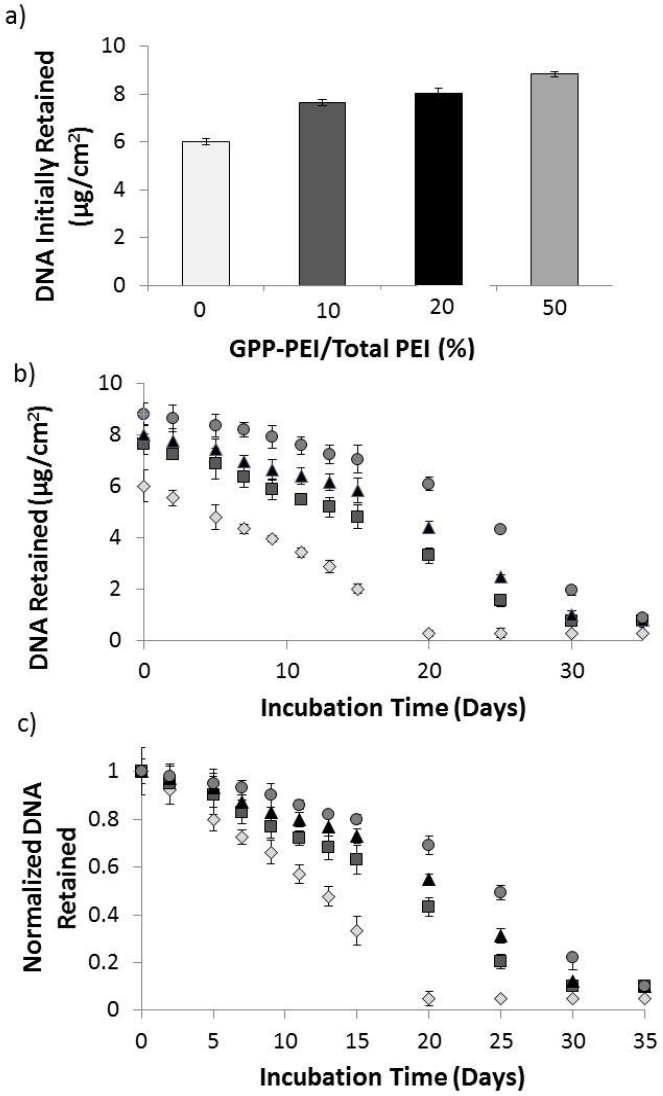

Figure 2.5 . Polyplex Gel Retention Studies. a) Initial retention studies to quantify the amount of DNA retained in collagen gels as a function of the percent GPP-PEI in the polyplex. b) Retention over time of DNA in collagen gels as a function of the percent GPP-PEI in the polyplex including 0% (white diamond), 10% (grey square), 20% (dark grey triangle), and 50% (black circle). c) Normalized retention data showing release kinetics on a per polyplex basis. Each data point represents the mean ± standard deviation for a total of four separately prepared and analysed samples.

modification with GPP still enabled the more stable modification of collagen.

2.3.5 Collagen-mimetic peptides modification of polyplexes preserves polyplex activity and promotes sustained release from 2-D collagen scaffolds under physiological-like conditions.

A series of transfection studies were conducted to determine whether the polyplexes retained their activity after immobilization, and whether the inclusion of the integrin-binding GPP linkage altered gene expression efficiency and/or kinetics. NIH/3T3 cells were plated on films that were freshly modified with polyplexes containing a Gluc-encoding plasmid. Luciferase expression was monitored daily, and expression in cells on the modified films was compared to expression in cells transfected by bolus delivery of polyplex. BCA protein assays were used to ensure that

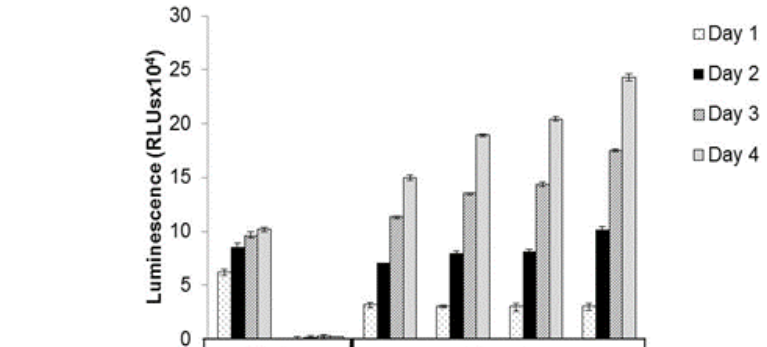

Figure 2.6. Film Transfection Experiments. Cells were transfected with either bolus or bound polyplexes and the levels of luciferase expression were monitored by luminometry analyses. Each bar represents the mean ± standard deviation for a total of six separately prepared and analyzed samples.

similar numbers of cells were present in each sample for accurate comparison of gene transfer efficiency.

As shown in Figure 2.6, the largest amount of luminescence at 1-2 days post-transfection was detected in the sample transfected using typical conditions and dosing for bolus delivery (2 μg/cm^2).[55,56] In contrast, by 3 days post-transfection, the luminescence in the samples transfected by the polyplex-modified collagens (0%, 10%, 20% or 50% GPP-PEI modified) was 18%, 41%, 49%, and 83% greater, respectively, than the luminescence detected in the 2 μg/cm^2 bolus delivery sample. Luminescence increased significantly, on a daily basis, in samples transfected with the bound polyplex, whereas luminescence in the bolus delivered polyplex samples was essentially constant after day 2; transfections were halted after 4 days due to high cell confluence, and accordingly reduced cell viability in all samples. Negligible luminescence was detected in the media extracted from the sample that received a bolus delivery of 0.2 μg/cm^2, a dose corresponding to the largest quantity of DNA delivered from the modified films. These results indicated that bound polyplex remained viable on the collagen films, and that active polyplexes were released even after multiple days of exposure to the culture conditions, in contrast to the bolus transfection induced only in the initial days. This is consistent with the literature showing that polyplexes are not stable, even for short time periods (<1 h), in media containing serum.[61,62] Hence, retention on collagen appeared to stabilize the polyplexes. The connections to collagen may prevent the polyplexes from aggregating with one another, in addition to slowing their release from the films.

The effect of the level of GPP-PEI modification was also examined. At 1 day post-transfection, no significant difference was detected in the luminescence readings

from the films containing different types of immobilized polyplex. However, after day 1, luminescence readings for samples drawn from the GPP-PEI modified films increased more rapidly when the polyplexes contained greater quantities of GPP-PEI; for example, between days 1 and 2, the luminescence readings increased by 2.2, 2.5, 2.7, and 3.4-fold in the 0%, 10%, 20%, and 50%-modified samples, respectively. The readings continued to increase in a similar manner for the entire monitoring period, with greater transgene expression at each day on films modified with polyplexes containing higher percentages of GPP-PEI. Notably, while the amount of DNA initially retained on the films increased as a function of the amount of GPP-PEI, transfection was initially similar on all the films, likely because GPP-modified polyplexes were released from collagen at a slower rate when more GPPs were present; therefore, less DNA would have been available at the earlier time points. Additionally, because the films that were modified with GPP-PEI polyplexes exhibited slower release rates, larger amounts of polyplex were available at later times; hence, by 3 days post-transfection, larger increases in luminescence were observed. These results indicated that transgene expression kinetics could be controlled through altering GPP display on polyplexes.

2.3.6 Collagen-mimetic peptides modification of polyplexes preserves polyplex activity for prolonged incubation periods under physiological-like conditions and facilitates MMP-dependent transgene expression.

In many tissue repair environments, the ability to maintain stable and localized gene constructs over long periods is essential, yet standard methods for forming gene-activated matrices can cause losses in activity and/or escape of polyplexes.[70-74] Ideally,

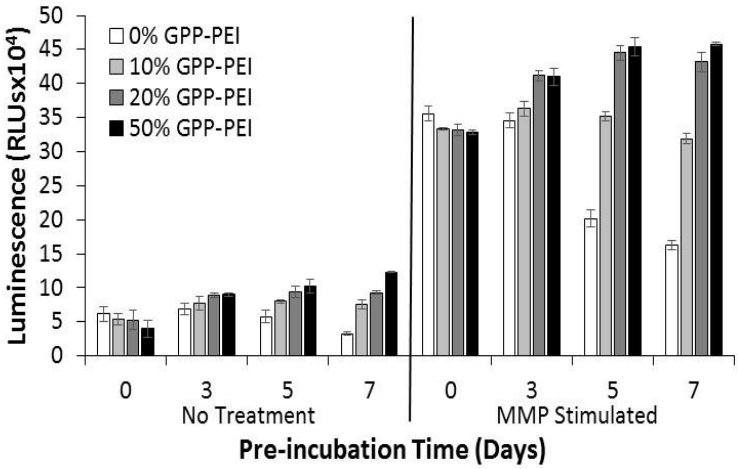

Figure 2.7. Gel Transfection Experiments. NIH/3T3 cells were plated on fresh polyplex-modified gels or polyplex-modified gels that were pre-incubated under physiological conditions for up to a week. Cells were treated with TNF-α to stimulate MMP expression as specified. The data represent the luminescence in the media due to luciferase expression by the cells after 4 days on the gels. Each data point represents the mean ± standard deviation for a total of eight separately prepared and analyzed samples.

gene-containing depots can maintain gene functionality and localization until cellular invasion activates the depots. Hence, to test transfection under conditions that mimic *in vivo* cell invasion, cells were plated on CMP-polyplex-modified collagen gels that had been pre-incubated in serum-containing media. Cells were cultured on the gels in the presence or absence of TNF-α, a well-known stimulator of MMP production.[59] The production of active MMP was confirmed *via* a SensoLyte® 520 Generic MMP Assay Kit *Fluorimetric*.

GPP-polyplex-modified collagen gels induced significantly reduced transgene expression levels in the absence of active MMP, whereas expression was robust in TNF-α stimulated cells (Figure 2.7). Specifically, while the trends in transgene expression were similar with and without MMP induction, the levels of transgene expression in all TNF-α stimulated cell samples were approximately 3-fold larger than

the levels in the corresponding samples in the absence of TNF-α stimulation. When TNF-α stimulated cells were plated on gels after short pre-incubation periods (0-3 days), the cells exhibited similar luminescence regardless of the percentage GPP-PEI. In contrast, luminescence in cells plated on gels pre-incubated for longer periods exhibited large differences as a function of the percentage of GPP-PEI. After 5 days pre-incubation, the luminescence induced by the unmodified polyplex gels was 43% lower than the luminescence on this gel after 3 days pre-incubation, whereas the luminescence induced by the 10%, 20%, and 50% GPP-PEI polyplex gels remained constant or increased between 3 and 5 days pre-incubation. Even when the GPP-PEI polyplex gels were pre-incubated for 7 days, transfection remained constant (10% gel) or increased (20% gel and 50% gel). In contrast, luciferase induction activity decreased to less than half of the original (0 day) level after a 7 day incubation of the unmodified polyplex gels. Hence, incorporating GPP-PEI into the polyplexes increased the amount of active polyplex accessible to cells for periods of over a week.

2.3.7 The extent and duration of expression may be tailored via collagen-mimetic peptide-modification in murine ECM depot models

A simple *in vivo* model was used to test the efficacy of the CMP-based gene transfer approach during tissue repair, using subcutaneous ECM depots to mimic the wound environment. The lack of an excision simplified the model while allowing for the examination of gene expression in a similar environment, containing the same cell types/tissues, and conceivably a minor inflammatory response triggered by the foreign body injection (unmeasured). Matrigel® was employed as the delivery substrate due to its prior successful implementation in both wound and bone healing studies and its potential to hybridize with CMPs due to its high collagen content [63]

As observed in Figure 2.8, insignificant levels of luminescence were detected, at each of the time points, in the pellets devoid of polyplex. Meanwhile, in the samples containing unmodified polyplex, only short periods (2-3 d) of low-level luminescence were detected at 2 to 4 days post-injection. Prolonged, localized expression was observed only in pellets containing GPP-modified polyplex. Expression was observed within the pellets for periods of 16 d and 20 d in the 10% and 50% GPP-modified polyplex samples, respectively. Furthermore, the initial area over which expression was observed at 4 days post-injection was significantly lower in the 50% GPP-PEI relative to the 10% GPP-PEI modified samples. For instance, within the representative mouse shown in Figure 2.8b and c, the initial area of expression is approximately 70% smaller in the 50% versus the 10% GPP-PEI polyplex sample. In addition to being smaller, the expression in the 50% GPP-PEI sample remained consistently localized over the entire time period over which expression was detectable. While the observed expression appeared markedly more localized and of longer duration in the 50% GPP-PEI polyplex samples as compared to the 10% samples, the overall expression levels were significantly higher in the 10% GPP-PEI polyplex. For instance, in the representative mouse shown in Figure 2.8b and Figure 2.8c, expression was first detected in the 10% GPP-PEI sample on the same day the maximum level of expression was detected, e.g. 4 days post-injection. A week after injection, the levels of expression in the 10% GPP-PEI samples had decreased by more than 75% and expression levels became insignificant by day 16. In the 50% GPP-PEI samples, expression was overall more consistent from day to day. Expression was first detected on day 3 and remained relatively consistent until day 10 before reaching a maximum level of expression on day 11 and then decreasing. The

maximum luminescence detected in the 50% GPP-PEI sample was approximately half that detected in the 10% GPP-PEI sample.

In Figure 2.8d, the replicates are reported as a median value in order to clearly demonstrate the spread of replicates, while highlighting the consistent capacity of CMP-modification to achieve tailorable, sustained expression within a collagen-based scaffold. Maximum transgene expression was similarly detected within the 10% GPP-PEI polyplex samples within the first week post injection and determined to be approximately 80% that detected in the 50% GPP-PEI polyplex sample. Expression in the rapidly decreased within the 10% sample after a week period, decreasing by nearly 70% by day 14, while maximum expression was not achieved in the 50% sample until day 12. Additionally, the area in which expression was observed (Figure 2.8 d), was determined to be overall smaller and more consistent in the 50% GPP modified polyplex samples versus the 10% modified polyplex over the duration of the work. For instance, within the representative mouse, the maximum area of expression in the 50% sample was over 3.1 fold greater than that observed in the 10% sample. The 0% areas of expression were not included due to their much lower values relative to the GPP modified samples.

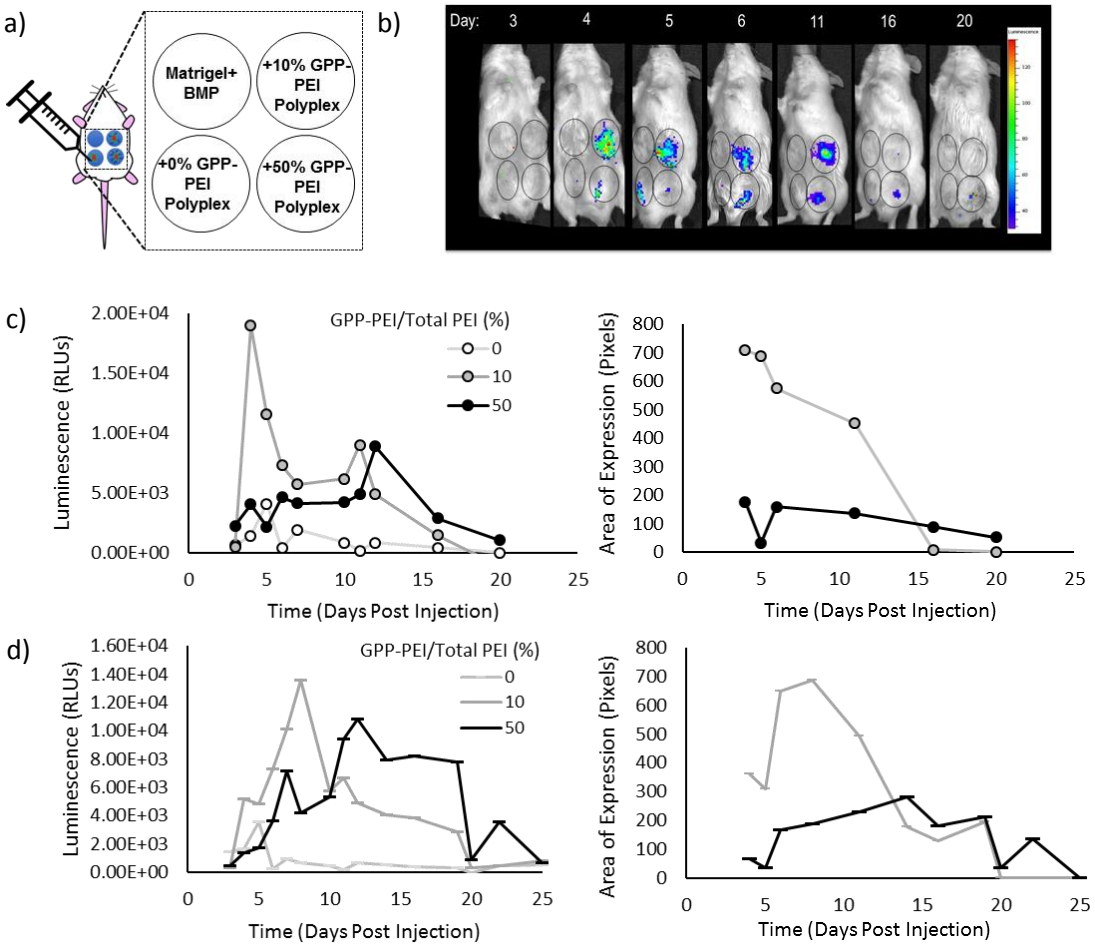

Figure 2.8. *In vivo* application of CMP-modified polyplex. a) Schematic indicating the location and contents of each subcutaneous pellet on the abdomens of CD-1 mice. Each solution contained Matrigel™, BMP-2, and polyplex where indicated, and the solutions formed visible pellets immediately after injection. DNA encoded for a membrane bound form of Metridia Luciferase to permit in vivo imaging. b) In vivo images of a representative mouse at various time points post injection, taken using an IVIS (Exposure: 5 s, Binning: Large, f/stop: 1) indicating luminescence over a maximum period of 20 d. c) Quantification of transgene expression and the area over which expression was identified in representative mouse. d) The median value of transgene expression and the area over which expression is detected within replicates. This study was replicated in four mice (SI).

2.4 Discussion

The ability to facilitate tailorable, stable release of DNA and subsequent gene expression is vital in GAM design. In this chapter, the ability of CMPs to act as tailorable tethers to mediate extended delivery from different bioactive collagen scaffolds was demonstrated. For this purpose, CMP design was vital. The GPP-based sequence predominant in this work was designed based on the principles outlined in work by Krishna *et al*. $(GPP)_3$ triplets were included due to their ability to promote triple helix formation and propensity to act as an efficient folding nucleus.[56] GPR triplets, shown previously to impart similar stability to triple helices as GPO triplets, were incorporated to flank an $\alpha_2\beta_1$ integrin-binding sequence, GEKGER,[56] as sequence motifs such as GXKGEX have also been shown to electrostatically stabilize triple helices. Additionally, the sequence was designed to form a type III collagen-mimetic cysteine knot at its C terminus. Cysteine knots have been shown to function as triple helix nucleation sites and to confer stability *via* covalently cross-linking the peptide strands of the triple helix.[38-40,42-46,49-51,64,65] The addition of reactive cysteine residues also provided a site for chemical conjugation in subsequent studies. Hydroxyproline was omitted so the sequence could potentially be mass produced *via E. coli* expression. The traditional GPO sequence was designed to contain standard GPO triplets known to initiate and form stable triple helices. This sequence also incorporated the same $\alpha_2\beta_1$ *integrin*-binding sequence, GEKGER.[50,51]

Collagen-binding of triple-helix forming CMPs, GPP and GPO, was initially confirmed through retention/release experiments conducted at 37ºC to confirm retention at physiological temperatures as well as a range of temperatures to better define collagen-CMP melting behavior. The optimal temperature of the peptide solution for binding both peptides to collagen films was found to 65ºC even though

GPO and GPP have melting temperatures of 45 ºC and 43 ºC respectively. The discovery that CMP retention continued to increase with temperature at pre-incubation temperatures above the T_M values for the two CMPs suggests that pre-heating the CMP solutions does more than ensure CMPs are single stranded and available for strand invasion. As suggested by earlier studies, it is possible solutions at elevated temperatures destabilized the native collagen triple helices making them more susceptible to strand invasion; meanwhile, above a certain threshold temperature, film denaturation disrupts or destroys natural collagen triple helical structure reducing the possibility of strand invasion.[53,54] In contrast, the GPO scramble sequence, designed to not form a triple helix, was not retained at any conditions suggesting CMP retention is because of strand invasion and not non-specific interactions.

Long term retention/release experiments confirmed retention of both peptides over a week at physiological temperatures and 25 ºC with GPO having longer release/retention time periods than GPP as expected based on the slightly higher triple helical stability of GPO (T_M=45ºC) compared to GPP (T_M=43ºC), and because hydroxyproline residues impart increased stability by enabling the formation of additional stabilizing hydrogen bonds through their γ-hydroxyl group.[60-62] However, because GPP-based sequences are cheaper to produce and may be mass produced via *E. coli*, the remainder of the work in this chapter utilizes GPP and a more in-depth release/retention study was conducted with this peptide as shown in Figure 2.3c. The pronounced increase in release upon incubation at 45 ºC relative to 40 ºC, 87% versus 36%, after 1 day of incubation, suggests that the approximate CMP-collagen complex melting temperature is within this range and supports GPP-collagen interaction is thermally sensitive and likely triple helical in nature.

Polyplex retention studies also confirmed CMP display, specifically GPP on DNA polyplexes, not only have the capacity to mediate attachment of nano-scale cargoes to 2-D and 3-D collagen scaffolds and improve retention efficiency, but manipulations in display can tailor release. For instance, on 2-D collagen films, increases in initial DNA retention were directly related to the amount of GPP incorporated with a 9-, 11- and 15-fold increase in initial DNA retention upon application of 10%, 20%, and 50% GPP-PEI polyplex relative to unmodified polyplex respectively. Increased retention is likely because of the larger amounts of GPP displayed on the polyplex increasing the likelihood of the GPP-collagen interaction, or because the incorporation of GPP increased the avidity of the polyplex for collagen. In fact, the total amount of polyplex retained on the modified films was very similar to that retained on covalently-modified substrates that were used successfully to achieve high levels of transfection in past studies.[27,66] The lack of perfect linearity in the retention may result from the heterogeneities in the distribution of GPPs on the polyplex, such that 2-D surfaces like collagen films may not be able access all of the GPPs displayed. Studies examining the retention of biotinylated polyplexes on a streptavidin-modified surface noted a similar dependence of initial retention on biotin display.[27,66]

GPP-display on polyplexes was also observed to expand release/release time periods on 2-D collagen films from 2 to 12 days, where release rates were significantly slower and consistent compared to unmodified polyplex release (Figure 4b). Initially the release rate of GPP-modified polyplex appeared dependent on the percent of GPP-PEI incorporation; however, after 4 days, the release rates for GPP-PEI polyplex were observed to be nearly identical (Figure 3c). Compared to CMP release/retention, many

factors contributed to sustained release of the polyplex including GPP-collagen interactions, different degrees of GPP availability on each polyplex surface, and variability in polyplex size and charge. The direct dependence between the amount of GPP on the polyplex and retention was consistent with expectations, given that, as in the peptide studies above, the GPP-modified polyplexes should integrate into the natural collagen structure through specific, physical interactions previously shown to be stable at 37^{o}C. Thus, the GPP-modified polyplexes were expected to be retained longer as compared with the unmodified polyplexes. Meanwhile, the increased retention as a function of increased GPP functionalization level was presumed, as before, to be caused either by the increased probability of binding of a single GPP chain in the more highly functionalized polyplexes, or by the increased avidity imparted by multiple GPP chains interacting simultaneously with collagen.

Furthermore, the lack in a significant difference in the normalized sloped of polyplex retention vs. time after 4 days, suggests that the release rates per polyplex were similar regardless of GPP incorporation. This result indicated that the primary effect of incorporating multiple GPP chains per polyplex was to increase the likelihood of GPP-collagen binding but not the number of GPP chains bound within a given polyplex on the 2-D collagen films. The fact that the normalized 50% GPP-PEI modified film release rate was faster for 4 days before slowing and becoming identical to the 10% and 20% GPP-PEI modified release rates suggested that incorporating large amounts of GPP increased the amount of unstable, partial GPP-collagen binding. Such an occurrence is likely the result of the 2-D architecture, as films in this geometry may not have access to all of the GPPs displayed on the spherical polyplex. This finding is contrary to what was found for the release of biotin-modified

polyplexes from streptavidin-coated surfaces as a function of biotinylation level. [27,29,66] The difference is likely because the GPP-collagen interaction is more reversible and the fact that biotin can be displayed more densely on the surface of a polyplex due to its smaller size.

The effect of CMP-modification was even more complex in the 3-D collagen gels, where high levels of initial DNA retention and sustained release for almost two weeks was observed for unmodified polyplex; however, a direct relationship between the level of GPP incorporation and both initial DNA retention efficiency (16-28% increase relative to unmodified polyplex) and prolonged retention times from days to over a month were still observed. The findings suggest encapsulation of polyplex enables sustained release through non-specific interactions, but modification with GPP enables more stable modification and tailoring of release/retention.

Importantly, transfection studies confirmed GPP-mediated attachments preserve polyplex activity on both 2-D and 3-D collagen scaffolds. Delivering the polyplex *via* collagen films as opposed to bolus delivery induced significantly higher transfection levels, despite the use of an order of magnitude less DNA. The magnitude of the improvements in transfection efficiency *via* substrate-mediated delivery were higher in the studies here than in those observed for other substrate-mediated delivery systems in the literature, particularly in the presence of serum.[59,67-69] For instance, in a citric acid-based polyester elastomer system that achieved sustained transgene expression through controlling biomaterial degradation, bound polyplexes achieved higher levels of transfection than samples receiving bolus delivery at 3 days post-transfection. High levels of transgene expression were also noted at 12 days post-transfection, while samples that received a bolus delivery of polyplex achieved high

levels for only 7 days. While similar to our results, the study used reduced serum media making the environment more stable for the polyplex.[69]

Additionally, previous studies have shown that polyplexes can be stabilized on the order of serval weeks in 3-D scaffolds periods *via* absorption onto polymer scaffolds [69] and chemical-modification of collagen,[23,29] but using GPPs, we obtained prolonged association with collagen using a physical modification technique and a natural substrate. Stable, tailorable integration of polyplex into collagen gels was achieved with this this highly versatile method without compromising the capacity of collagen to release DNA in a cell-triggered manner vital for localized delivery as made evident by the negligible gene expression in the absence of TNF-α stimulation of MMPs. Moreover, as will be elucidated in Chapter 3, improved activity was theorized to be the result of extended release/retention kinetics, enhanced stability, and interactions with collagen fragments post release. Previous studies have shown coating polyplexes with gelatin (denatured collagen) improves transfection efficiency through enhancing stability[70] and improving cellular uptake,[71] it is possible that the GPP modification of polyplexes improved stability/activity by mediating encapsulation of the polyplex within a stable layer of collagen

Other studies have also successfully demonstrated that substrate-modification can be used to obtain sustained gene delivery, but in previous studies, DNA retention and release were generally coupled, as both were typically controlled by DNA-substrate affinity. Hence, in most examples, increasing the quantity of DNA retained decreased the release rate and availability of the DNA to cells.[23,28,66,67,72,73] In contrast, the GPP-collagen interaction allowed stable polyplex retention for over a month under physiological conditions, and increased retention (mediated by increased GPP

functionalization levels) did not negatively affect the rate of MMP-mediated release. A related approach to employ MMP activity to stimulate gene release from polyplexes encapsulated in an enzymatically degradable hydrogel similarly found proteolytic degradation allows cellular infiltration, promotes cell growth and allows for long term delivery of DNA, which is essential for the regeneration of functional tissues.[66,74] The use of CMPs such as GPP instead of encapsulation alone should allow for increased control over retention *vs.* release, and should impart a higher degree of tailorability as compared with physical modification techniques that rely on electrostatics and nonspecific interactions. CMPs can also provide additional functionality by acting as both adjustable tethers to control the affinity between the collagen and the polyplexes, as well as adhesive/endocytic ligands or cell penetrating peptides.

Furthermore, i*n vitro* studies proved to be accurate predictors of the system's efficacy *in vivo*. Animal models have demonstrated that CMPs can be used to detect areas of excessive remodeling, such as tumors and joints[40,42-44,47], and CMPs can anchor cytoactive factors and collagen in wound beds to improve wound closure and granulation[5,52]. Utilizing a mouse model, gene expression in polyplex encapsulating or GPP-PEI polyplex-modified subcutaneous Matrigel® pellets was monitored. Matrigel® is a widely used, commercially available substrate that consists of basement membrane ECM components such as type IV collagen. [75] While type IV collagen has a lower triple helical content than type I collagen [76], significant CMP-type IV collagen interactions have been documented, albeit with lower efficiency than CMP-type I collagen interactions [41]. Type IV collagen has also been successfully employed in wound and bone in past studies [63,75,77]. Through GPP modification of the polyplex, we demonstrated the ability to enhance, extend, and localize expression,

even from the type IV collagen-rich pellets. Levels of expression in the pellets with GPP-PEI polyplex were higher and the duration of expression was directly dependent on the amount of GPP-PEI/Total PEI in the polyplex. This relationship was likely due to a difference in release kinetics, stability, and cellular internalization and is supported by the observation that level of expression in the 50% GPP-PEI/Total PEI pellet was generally lower than that observed in the 10% GPP-PEI/Total PEI pellet, but was more localized and occurred over a longer duration. Our work, as well as other works with CMPs, have demonstrated that the incorporation of multiple CMPs onto a nanostructure/polymers dramatically affects mobility in collagen-based scaffolds due to the affinity CMPs have, particularly for remodeled collagen [44]. The reversible, serum-stable reaction prevents CMP-modified materials from leaving the delivery site, and as demonstrated here, can be used to both control and localize release

2.5 Conclusions

Collagen is an abundant, versatile material with intrinsic properties that make it a promising biomaterial for use in regenerative medicine. While a greater understanding of both its physical and biochemical properties has aided in creating a myriad of medical technologies already in use, most modification techniques require multi-step chemical treatments which prevent the full capitalization of collagen's regenerative potential. In this work, we have developed a promising new biomimetic, collagen modification technique and are the first to our knowledge to demonstrate its efficient application in gene delivery. By mimicking collagen's natural structure, collagen can be modified with DNA through physical modification to allow delivery of genes *via* collagen.

The CMP-based delivery method was shown to be highly tuneable and versatile, as changes in the number of CMPs in the polyplex readily altered collagen binding *vs.* release, and alterations in the CMP sequence offer a readily accessible platform to change CMP-collagen affinity or incorporate integrin-binding functionality. As a prototypical CMP, GPP could be used to incorporate DNA polyplexes into collagen structures including both 2-D collagen films and fibrous 3-D gels for approximately 2 weeks and a month respectively. The incorporation of GPPs increased the efficiency of initial polyplex retention by an order magnitude on films and by over 25% in gels, with increases in retention of at least 50%. CMP linkage retained polyplexes on collagen films for a 5-fold longer period than polyplexes lacking CMPs, and CMP linkage retained polyplexes within collagen matrices for over a month. This is a markedly longer period than the vast majority of simple collagen-based systems that rely solely on physical modifications, in which retention is typically on the order of a few days.[79] Polyplex activity was also consistently and fully maintained in the presence of serum for at least a week, whereas most bolus and substrate-mediated gene delivery approaches report rapid and significant serum-induced reductions in activity within hours to a few days. Additionally, transfection in the gels was found to be cell-mediated and to require the presence of MMPs, suggesting that the system will be ideal for coordinating cellular invasion processes with localized, on-demand gene delivery and expression of healing factors during tissue regeneration. The robustness and stability of the modification as well as its versatility suggests that this technique may be used more broadly to create tuneable, collagen-based delivery systems for regenerative medicine applications requiring

highly localized gene retention, infiltration/adhesion of a diversity of cells, and on-demand gene expression over different periods of time.

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Chapter 3

ECM TURNOVER-STIMULATED GENE DELIVERY THROUGH CMP-PLASMID INTEGRATION IN COLLAGEN

A biomimetic, peptide-based approach was employed to modify collagen with DNA in order to exploit the natural process of collagen remodeling for high efficiency non-viral gene expression. The affinity of collagen-mimetic peptides (CMPs) for collagen permitted tailored delivery and improved activity. Specifically, incorporation of CMPs into polyethylenimine (PEI) polyplexes preserved serum-exposed polyplex collagen activity over a period of 14 days, versus less than a week in unmodified polyplex collagen. Agarose gel electrophoresis demonstrated a 4 orders-of-magnitude increase in intact DNA collected from CMP-modified polyplex collagen relative to unmodified polyplex collagen after a 10-day incubation under cell culture conditions. CMP-modification also altered endocytic uptake, as indicated by a nearly 20% decrease in transgene expression in response to caveolin-1 silencing. Furthermore, cellular internalization studies demonstrated polyplex-collagen association persisted within cells, suggesting that CMPs may regulate intracellular transport. Overall, these findings suggest that CMPs improved gene retention, altered release kinetics, improved serum-stability, and enhanced endocytic uptake, and provide useful mechanistic insight on the previously observed enhancements in GPP-PEI polyplex v. PEI polyplex activity described in Chapter 2. This versatile technique has great potential for multiple applications in regenerative medicine.

3.1 Introduction

Regenerative medicine has the potential to restore function to damaged tissues or organs, and its promise has prompted the development of a wide range of biomaterials and drug delivery approaches [1-8]. To guide complicated remodeling and repair processes, biomaterial-based scaffolds are commonly employed to provide a framework for cellular growth and tissue deposition [9-12]. While traditional scaffolds have been engineered with emphasis on providing biocompatible, structural support, focus in recent years has shifted towards materials designs with the capacity to guide more complex aspects of reparative processes [11-15]. To serve as good analogues of the extracellular matrix (ECM), scaffolds should act as cell-responsive structures capable of dynamically interacting with cells during cellular ingrowth, proliferation, and phenotypic commitment. For this purpose, the stable incorporation and controllable delivery of bioactive cues, such as therapeutic proteins or DNA, are essential [10,12,16,17].

The ECM serves as a natural reservoir for bioactive cues such as growth factors (GFs), whose release and activity are controlled by both ECM affinity and ECM turnover. ECM turnover is a tightly-regulated process that consists of two primary steps: protease-mediated ECM degradation and endocytic uptake of the resulting ECM fragments. The ECM undergoes continuous remodeling; however turnover rates are elevated during development and regeneration, and can also occur in response to signals transmitted from ECM receptors or ECM-modifying proteins such as matrix metalloproteinases (MMPs) [18-20]. In turn, cell-triggered, localized delivery of ECM-

bound cues (e.g. GFs), with tightly regulated and distinct release kinetics, is achieved in direct coordination with other reparative processes [21-24]. Similar mechanisms that exploit ECM remodeling have evolved to enable entry of other moieties and signals into cells with high efficiency. Viruses bind to ECM to enhance their cellular availability, preserve their stability, and increase the chance of internalization via interaction with specific cellular receptors [21,25,26], and ECM endocytic pathways are also commonly used to facilitate cellular uptake of viruses via integrin-dependent pathways [21,25,26], especially via collagen-binding integrins such as $\alpha_2\beta_1$ [26,27]. ECM components have even been found to act as bridges that directly facilitate binding and initiate viral internalization [21].

The ability to harness ECM turnover would also have key benefits for synthetic materials engineered to control therapeutic delivery. Enzymatically degradable polymer matrices of both synthetic [13,14] and natural origin [10,16,17] have been designed to confer proteolytic sensitivity such that tissue remodeling and scaffold degradation are synchronized, and these approaches have been shown to dramatically improve therapeutic stability and activity. For instance, matrix metalloproteinase (MMP) degradable polymers [14,28] and tethers [16,17] have been used to coordinate the delivery of stabilized GFs such as vascular endothelial growth factor (VEGF) and bone morphogenetic protein 2 (BMP-2) with cellular remodeling during diabetic ulcer healing [1,16,29-32] and bone regeneration [10,33], respectively. Additionally, scaffolds have also been designed to achieve improved, localized gene delivery and thereby provide a

compelling alternative for creating GF-rich environments; gene manipulations are less expensive, more stable, and have a proven capacity to elicit improved therapeutic effects with orders of magnitude (~2000-fold) dose reductions compared to those necessary for topical administration of GFs [34]. Furthermore, gene delivery mimics endogenous repair, using host cells to coordinate localized, sustained GF production and *in situ,* on-demand delivery of nascent proteins with authentic post-translational modifications [35]. These characteristics are of particular value in tissues such as chronic wounds due to their extended healing over months and spatiotemporal heterogeneity [35]. For instance, *in vivo* studies have demonstrated that incorporation of GF-encoding DNA into both polymer-based [36] and protein-based [37] scaffolds can promote transgene expression over periods ranging from days to weeks, leading to essential reparative processes where no healing or minimal healing was previously observed.

Despite the potential benefits of scaffold-mediated gene transfer, existing materials have been unable to provide sufficient healing activity due to their lack of sufficient *in vivo* stability and prolonged delivery. Improved delivery strategies thus are vital for clinical translation. Currently, DNA is most commonly incorporated into polymer- and protein-based scaffolds through either simple encapsulation approaches or adsorption methods employing non-covalent interactions between the substrate and the DNA or DNA carrier (e.g. electrostatic, hydrophobic, or van der Waals interactions). Such methods offer simplicity and have the capacity, as noted above, to achieve cell-triggered release through use of protease-degradable materials [11,13,28,38].

However, existing methods for incorporating DNA within biomaterials often fail to retain gene carriers in the delivery site for prolonged periods *in vivo* and off-target delivery is problematic. For instance, collagen scaffolds containing electrostatically entrapped, PDGF-B-encoding adenovirus accelerated healing in experimental rat wound models, yet vector escape and immunogenicity were apparent and hindered clinical translation [39]. While the use of non-viral gene delivery can potentially minimize immunogenicity concerns, non-viral approaches often fail to induce complete repair in preclinical models [40], and insufficient gene transfer efficacy has been identified as the major limiting factor. Furthermore, most existing technologies for sustained therapeutic delivery are not suitable for many tissue repair applications because of the complexity of the healing process, which can include extended healing periods over months and multiple out-of-phase healing cascades within repair sites. A reliable strategy to retain active gene carriers until cells initiate repair and facilitate efficient gene transfer is essential.

In this work, our goal was to demonstrate that the anchoring of polyplexes via specific interactions with collagen could be used to achieve stable gene expression *in vivo* by harnessing collagen turnover as a driver for not only gene release, but also enhanced activity. Our studies employ collagen-mimetic peptides (CMPs), which comprise primarily a collagen-like $(GXY)_n$ motif and have the unique capacity to stably integrate within native collagen through a reversible strand-exchange process [41-49]. Our prior work was the first to demonstrate that CMP-based integration of

polyplexes could be used to retain stabilized DNA on collagen with improved control over the timing and extent of gene delivery for periods of a week to over a month (2-fold longer than that of unmodified polyplexes), and the direct correlation of transgene expression with MMP concentrations. In this work, we evaluated the persistence of the collagen-polyplex interaction and the capacity to drive gene delivery via endocytic collagen turnover. CMP-modified PEI polyplexes were incorporated into collagen gels [50-52] and long-term transfection studies and DNA integrity assays were employed to monitor polyplex efficacy and stability. Collagen-polyplex co-internalization studies and gene silencing experiments were used to assess mechanisms of cellular uptake, particularly via caveolar mechanisms [53,54]. Additionally, extended studies in murine models were used to determine the feasibility of CMP-based mechanisms for improving gene transfer *in vivo*, and its potential application in regenerative medicine.

3.2 Materials and Methods

3.2.1 Materials

Fmoc-protected amino acids were purchased from Anaspec (Fremont, CA). H-Rink amide ChemMatrix® resin was purchased from PCAS Biomatrix (Quebec, Canada). O-Benzotriazole- N,N,N',N'-tetramethyl-uronium-hexafluoro-phosphate (HBTU) was purchased from Novabiochem (San Diego, CA). High performance liquid chromatography (HPLC)-grade N,N-dimethyl formamide (DMF), acetonitrile, trifluoroacetic acid (TFA), Lipofectamine® RNAiMAX Reagent, and cell culture reagents, including Dulbecco's modified Eagle's medium (DMEM), Opti-MEM® I

Reduced Serum Media (Opti-MEM), Dulbecco's phosphate buffered saline (PBS), and trypsin were purchased from Fisher Scientific (Fairlawn, NJ). Fetal bovine serum (FBS) was purchased from Corning (Manassas, MA). Piperidine, 4-methylmorpholine, all cleavage cocktail components, branched PEI (25 kDa), collagen type I-fluorescein isothiocyanate (FITC), and syringes were purchased from Sigma-Aldrich (St. Louis, MO). Type I bovine collagen was purchased from Advanced BioMatrix (San Diego, CA). DNA gel loading dye and Alexa Fluor 350 NHS ester were purchased from Thermo Fisher Scientific (Waltham, MA). pCMV-GLuc plasmid was purchased from New England Biolabs (Ipswich, MA) and pCMV-MetLuc-mem plasmid was purchased from Clontech (Mountain View, CA), and both plasmids were amplified in NEB 5-α electrocompetent *E. coli* purchased from New England Biolabs. The plasmids were purified from bacterial culture using a Qiagen Megaprep Kit (Valencia, CA), following the manufacturer's protocols. Caveolin-1 siRNA and caveolin-1 Antibody (N-20) was purchased from Santa Cruz Biotechnology (Dallas, Texas) and Goat Anti-Rabbit IgG (Horseradish Peroxidase (HRP)) was purchased from Abcam (Cambridge, MA). Collagenase I was purchased from Worthington Biochemical Corp (Lakewoord, NJ).

3.2.2 Methods

3.2.2.1 Preparation of modified collagen gels

The GPP-based CMP [GPP: $(GPP)_3GPRGEKGERGPR(GPP)_3$] GPP was synthesized via Fmoc solid phase peptide synthesis, conjugated to PEI using Michael-type addition chemistry, and subsequently, the GPP-PEI was used to prepare GPP-modified polyplexes as described in [52]. Using a variation of well-established polyplex

formation protocols [53,54], equivolumetric solutions of PEI and DNA suspended in 20 mM HEPES buffer (pH 6.0) were combined to yield a final solution with an amine to phosphate ratio (N:P) ratio of 10. To incorporate the GPP, a specified percent of PEI was replaced with the GPP-PEI conjugate after preincubation at 50ºC for 30 minutes to prevent triple-helical hybridization of the GPP. Collagen gels with GPP-immobilized or encapsulated unmodified polyplexes were constructed by re-suspending dehydrated polyplex in neutralized type I bovine collagen-solution (4 mg/mL, pH 7.4) as described [52]. 250 µL of this solution was added to each well of an 8-well plate (0.8 cm^2 surface area/well) and the solutions were incubated for 3 h at 4ºC to enable CMP-collagen hybridization and subsequently overnight at 37ºC to allow for gelation. After visually confirming gelation, the gels were thoroughly washed with PBS and water.

3.2.2.2 Polyplex-serum activity studies

To determine whether GPP-mediated immobilization enhanced the serum stability of the polyplexes, GPP-PEI polyplex-modified collagen gels and GPP-free PEI polyplex-encapsulating collagen gels were prepared with pCMV-Gluc plasmid. GPP-PEI polyplex-modified gels were created with polyplex containing different amounts of GPP-PEI (10, 20, or 50% GPP-PEI/Total PEI) whereas GPP-free polyplex-encapsulating gels were created similarly but with polyplex containing 0% GPP-PEI/Total PEI. These gels were preincubated under physiological conditions (37ºC, 5% CO^2) in media containing a range of serum concentrations (0.25, 10, 20, or 50% (v/v) FBS and 1% penicillin-streptomycin (P/S) for up to 2 weeks. After the specified gel preincubation period, the gels were washed 3 times with PBS and 3 times with DMEM supplemented with 1% P/S and 10% FBS. Subsequently, NIH/3T3 cells

were plated at a density of 20,000 cells/cm^2 in complete media (DMEM supplemented with 1% P/S and 10% FBS). The cells were allowed to adhere over a period of 6 h before treatment with tumor necrosis factor-alpha (TNF-α) (10 ng/mL), a well-known stimulator of MMP production [55]; gene expression subsequently was monitored over several days. As previously reported, elevated MMP activity was confirmed using a SensoLyte® 520 Generic MMP Assay Kit Fluorimetric, following the manufacturer's protocol, and gene expression was monitored via detection of the luminescence of GLuc secreted into the conditioned media, via the Bio-Lux® Gaussia Luciferase Assay.

3.2.2.3 DNA stability experiments

To assess the stability of the DNA incorporated into the collagen scaffolds, an ethidium bromide exclusion assay was performed. Polyplex-containing collagen gels were produced as previously described. The gels were incubated in media (DMEM supplemented with 1% P/S and 10% FBS) under physiological conditions (37ºC, 5% CO^2) for 2 weeks. During the incubation, media was collected from each sample 10, 12, and 14 d after starting the incubation. To examine the stability of the collected DNA, heparin was added to dissociate the polyplexes, as previously described [20]. Equal amounts of recovered DNA (0.1 μg, based on quantification using spectrophotometric analysis) were analyzed with 1% agarose gels stained with 0.5 μg ethidium bromide per mL of TBE [tris/borate/ethylenediaminetetraacetic acid (EDTA)] buffer. Forty μL of recovered DNA solution was added to 8 μL of 6x gel loading dye, and 40 μL of the mixture was added to each well. The gels were run at 100 V for 1 h and imaged with a BioRad Gel Doc XR (Brookhaven, CT). To analyze the gel, the ImageJ (National Institutes of Health) measurement tool was used.

3.2.2.4 Colocalization studies

To determine if polyplexes co-internalized with collagen fragments during collagen remodeling and polyplex uptake, fluorescence-based colocalization studies were conducted. FITC-labeled collagen was incorporated into the collagen gels (25% FITC-collagen (m/m)), and 25% of the PEI used to form the polyplexes was prelabeled with Alexa Fluor 350 via Michael-type addition chemistry, according to the manufacturer's protocols. Unreacted dye was removed via dialysis (MWCO: 1000 Da). Otherwise, polyplex-modified collagen gels were prepared as previously described and subsequently preincubated in media supplemented with 10% FBS for a specified period of time ranging up to 2 weeks. NIH/3T3 cells were seeded on the surface of the gels, and after a 3 d incubation, the gels were subjected to a 30 minute collagenase digestion (0.2 mg/mL in serum-free DMEM supplemented with 0.1% BSA) at 37^{o}C. Cells were recovered by centrifugation for 4 min at 400 x g. Recovered cells were replated into 24-well plates (5000 cells/cm^2). Six hours post-plating, the cells were fixed using 4% paraformaldehyde (PFA) for 15 min at room temperature. Additionally, the cells were treated with Trypan blue (10% v/v in PBS) to quench extracellular fluorescence[12]. The cells were imaged using a Leica DMI6000 B inverted microscope (Wetzlar, Germany). Image analysis tools in ImageJ were used to quantify intracellular colocalization of the polyplex and collagen via Mander's Coefficient analysis.

3.2.2.5 Caveolin-1 silencing studies

To determine whether CMP-modification encouraged cellular uptake via caveolin-1-mediated endocytosis, caveolin-1 silencing studies were conducted. Initially, NIH/3T3 cells were seeded at a density of 80,000 cells/well in 6-well plates

(Corning). After a 24 h recovery, cells were transfected with caveolin-1 siRNA complexes (1 μg siRNA/well) made using a commercial transfection agent (Lipofectamine® RNAiMAX Reagent), according to the manufacturer's protocols. The complexes were prepared by mixing equal volumes (100 μL each) of the transfection agent solution (6 μL of Lipofectamine RNAiMAX Reagent diluted in 94 μL Opti-MEM) and siRNA solution (1 μg suspended in Opti-MEM) and allowing complexation to occur for 15 minutes. During complexation, the preplated cells were washed with Opti-MEM and covered with a fresh layer of Opti-MEM (800 μL/well). 200 μL of the siRNA complex solution was subsequently added to each well and these solutions were allowed to incubate with the cells at 37°C for 6 h. After the incubation period, the cells were washed 3 times with PBS, and fresh DMEM (containing 1% P/S and 10% FBS) was subsequently added. After 24, 48, or 72 h, the extent of gene silencing was assessed by Western blotting using caveolin-1 antibody (N-20) (rabbit polyclonal IgG) and Goat Anti-Rabbit IgG (HRP). As a control, the same procedures were carried out using a non-coding siRNA sequence in place of the caveolin-1 siRNA.

After confirming the extent to which caveolin-1 was silenced, gene transfer studies employing caveolin-1 silencing were conducted. 24 h after transfection with the siRNA complexes, the cells were collected using standard cell recovery protocols involving treatment with trypsin (0.25% v/v). The cells were subsequently seeded onto collagen gels containing pCMV-GLuc and varying amounts of GPP-PEI, and the expression of Gaussia Luciferase was monitored over a 3 d period via detection of luminescence using a Glomax Multimodal Plate reader (Sigma), as described in [52].

3.3 Results

3.3.1 CMP-modification preserved polyplex activity under physiological conditions

A series of transfection experiments was conducted to determine whether using GPPs to immobilize polyplexes in collagen would impart stability under physiological conditions over extended periods of time. To simulate the *in vivo* wound environment, GPP-PEI polyplex-modified collagen gels and GPP-free PEI polyplex-encapsulating collagen gels were prepared and preincubated in media containing increasing amounts of serum for 1, 7, or 14 d. Subsequent to this preincubation, NIH/3T3 cells were plated on the modified collagen gels, treated with TNF-α to induce MMP expression, and allowed to grow for 3 d before reporter protein expression (GLuc) was monitored by analyzing luminescence in conditioned media. The luminescence detected 4 d post-plating is reported in Figure 1. Maximum expression was detected in samples containing unmodified polyplex (0% GPP-PEI/Total PEI) after a 1 d preincubation period with 0.25% (v/v) FBS. However, the level of expression in the 0% GPP-PEI samples rapidly decreased when the samples were preincubated with higher concentrations of FBS, with a 44% decrease in luminescence when the FBS concentration increased from 0.25% to 10%, an additional 58% decrease when the FBS was increased from 10% to 20%, and a further 75% decrease when the FBS was increased from 20% to 50%. Levels of expression in samples containing unmodified polyplex became undetectable at all preincubation times longer than 1 d.

Levels of expression in the GPP-PEI polyplex-modified gels were maintained more consistently in the presence of serum (FBS). In the samples preincubated for 1 d, all of the GPP-modified samples had higher expression levels than the corresponding 0% GPP-PEI/Total PEI samples, with the exception of the samples preincubated in

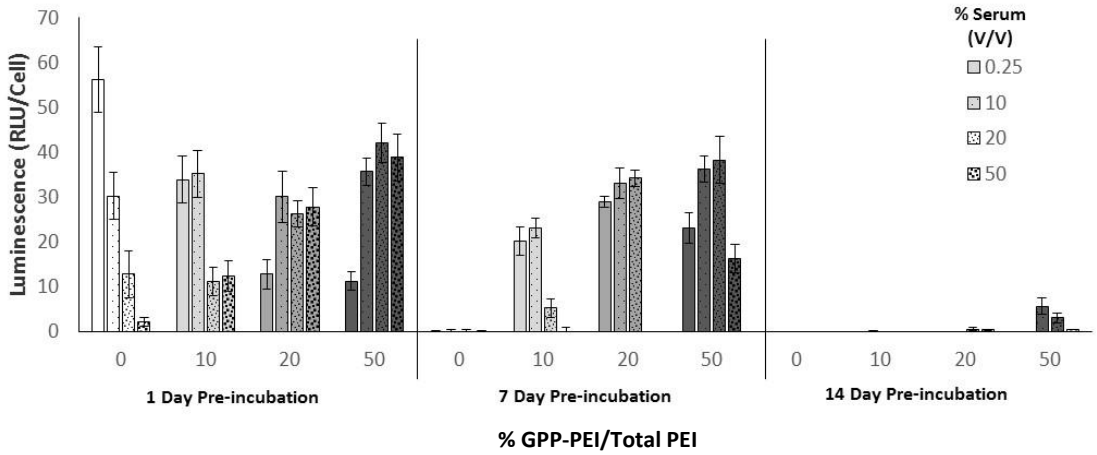

Figure 3.1. Polyplex activity studies. Polyplex-containing collagen gels were preincubated at 37°C in media containing a range of serum concentrations (0.25%-50% (v/v)) for up to 2 weeks. NIH/3T3 cells were plated onto the preincubated gels and treated with TNF-α to stimulate MMP expression. The data represent the luminescence in the media due to luciferase expression by the cells after 4 d on the gels. Each data point represents the mean +/- standard deviation for a total of eight separately prepared and analyzed samples.

0.25% FBS; under these conditions, expression in the 0% GPP-PEI samples was approximately 43% greater than the expression levels in any of the GPP-modified samples. After 1 d of preincubation, the 10% GPP-PEI/Total PEI polyplexes had the highest levels of expression when preincubated with either 0.25 or 10% FBS, and expression levels decreased by approximately 33% when these samples were preincubated in media containing either 20% or 50% FBS. The opposite trend was noted in the more extensively modified polyplexes, with the 20% and 50% GPP-PEI/Total PEI polyplexes exhibiting the lowest levels of expression after preincubation with 0.25% (v/v) FBS. The luminescence detected in the 20% and 50% GPP-PEI samples increased approximately 120% and 320%, respectively, when these

samples were preincubated with any of the higher concentrations of serum (10%, 20%, or 50%) versus preincubation with 0.25% (v/v) FBS.

After longer preincubation periods, the differences in the levels of expression in samples containing unmodified polyplex (0% GPP-PEI) *vs.* GPP-modified polyplex became more pronounced. When samples were preincubated for 1 week, expression levels in the unmodified samples were undetectable, whereas in the 10% GPP-PEI/Total PEI polyplex samples, the levels of luminescence remained detectable but decreased approximately 34%, 35%, 50%, and 85% for the 0.25%, 10%, 20%, and 50% FBS (v/v) samples, respectively, as compared with the luminescence in 1 d preincubated samples. In the 0.25% FBS samples modified with 20% and 50% GPP-PEI polyplexes, the levels of expression increased by approximately 51% and 65%, respectively, compared to the corresponding 1 d preincubated samples. Levels of expression were higher when the 20% and 50% GPP-PEI samples were preincubated for 1 week with 10% and 20% FBS, as compared with 0.25% or 50% FBS, and the overall level of expression significantly increased in the 20% GPP-PEI/Total PEI samples when these samples were incubated for 7 d *vs.* 1 d at these FBS concentrations. After a 14 d preincubation, significant expression was only detected in the 50% GPP-PEI/Total PEI modified gels that were preincubated with 10% and 20% FBS.

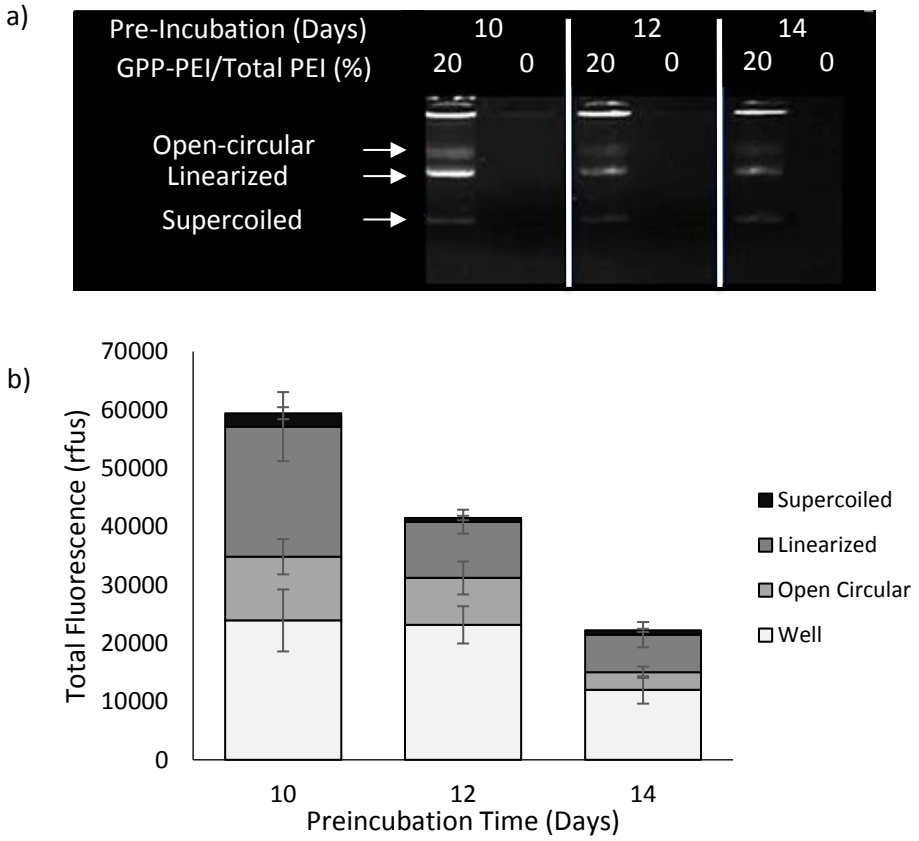

Figure 3.2. DNA stability analysis. a) Representative agarose gel showing the integrity of DNA recovered from 20% and 0% GPP-PEI/Total PEI polyplex-containing collagen gels after various preincubation time periods in media (DMEM supplemented with 10% FBS). b) Quantification of gel fluorescence observed in GPP-modified samples where the mean +/- standard deviation is for a total of three separately prepared and analyzed samples run within the same gel.

3.3.2 CMP-modification preserved DNA integrity under physiological conditions

To determine if the integrity of the DNA was preserved under physiologically relevant conditions that would be encountered *in vivo*, DNA was recovered from the

media collected from polyplex-containing collagen gels after various serum preincubation periods. The integrity of the DNA was assessed by agarose gel electrophoresis (Figure 3.2). The three bands characteristic of plasmid DNA (open circular, linearized, and supercoiled) were visible in each sample collected from the GPP-PEI polyplex-modified gels. These three bands notably faded in intensity when samples were collected from gels that were preincubated for longer time periods. For example, there was a 37% decrease in the plasmid band intensity observed in the 20% GPP-PEI polyplex-modified sample collected after a 12 d incubation, and a 53% decrease in the plasmid band intensity in the sample collected after 14 d, as compared to those in the 10 d sample. Fluorescence was also observed in the wells for each preincubated 20% GPP-PEI sample. In comparison, fluorescence from the three plasmid bands was difficult to detect with the naked eye in the samples collected from collagen gels containing 0% GPP-PEI polyplex, even though the same quantity of DNA was employed in the polyplexes as indicated by spectrophotometric analysis. Based upon image analysis of the bands, the 0% GPP-PEI sample band intensities exhibited decreases of 22% and 53%, respectively, for samples collected after 12 d and 14 d *vs.* 10 d. Fluorescence/DNA was also observed in the wells of samples collected from the GPP-PEI polyplex-modified gels. DNA immobilization in the wells suggested that complexation of DNA with PEI was still occurring despite the addition of heparin, a method previously shown to completely dissociate DNA polyplexes [52].

3.3.3 CMP-modification facilitated collagen-polyplex co-internalization

Cell-triggered release and efficient cell-uptake are essential for high efficiency expression *in vivo*. To determine the extent to which endocytic collagen remodeling was able to drive the gene delivery process in the GPP-PEI polyplex-modified

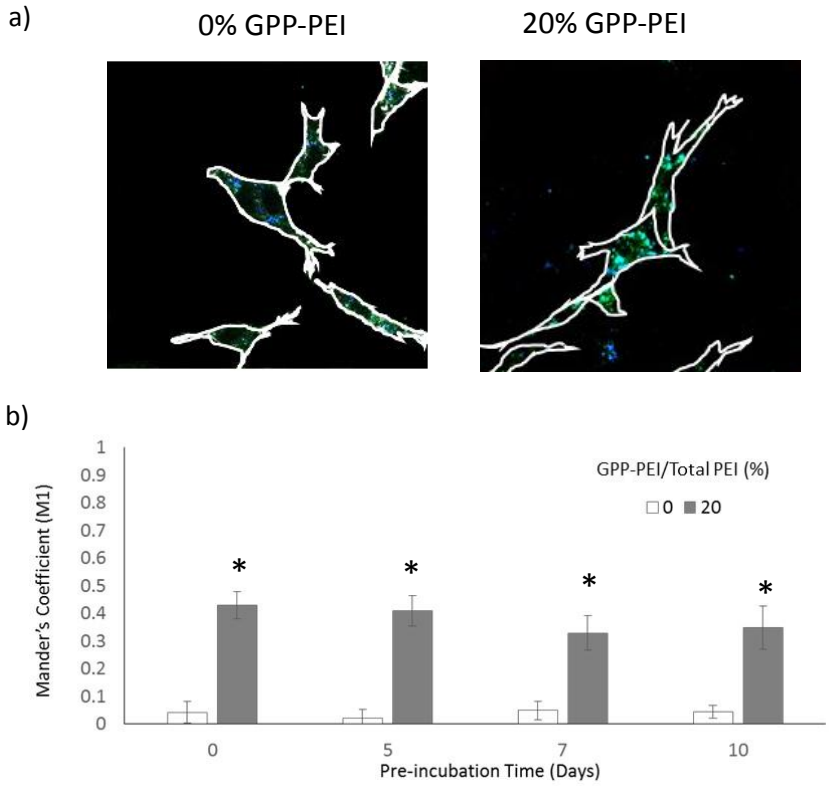

Figure 3.3. Collagen-polyplex colocalization study. a) Representative fluorescent microcopy images of cells collected from gels preincubated for 5 days. b) Quantitative analysis of intracellular collagen-polyplex association by calculation of Mander's coefficients for colocalization of FITC-collagen with Alexa Fluor 350-polyplexes in NIH/3T3 cells. The data for the 0, 5, and 7 day pre-incubations represent the mean +/-standard deviation of 10 separately analyzed cells. For the 10 day preincubation, 5 separately analyzed cells were analyzed. * denotes a statistically-significant difference ($p<0.05$) relative to the 0% GPP-PEI control.

collagen versus the GPP-free polyplex-encapsulated collagen, confocal microscopy was used to examine the cellular co-internalization of polyplex and collagen. In order to study co-localization of the two materials, FITC-labeled collagen was incorporated into the collagen gels (25% (m/m) FITC-collagen) and 25% of the PEI (m/m) used in the polyplexes was labeled with Alexa Fluor 350. Gels were preincubated in media

supplemented with 10% FBS for a specified period of time ranging from 5 day up to 2 weeks; subsequently, NIH/3T3 cells were plated on the preincubated gels, and after a 3 day incubation, the cells were recovered and replated for imaging. Intracellular colocalization of the polyplex and collagen was quantified via Mander's Coefficient analysis.

As shown in Figure 3, the Mander's Coefficient was an order of magnitude greater in samples modified with GPP as compared to the unmodified samples. In the samples containing unmodified polyplex, the Mander's coefficient indicated insignificant colocalization between the polyplex and collagen after each incubation period, whereas in the samples modified with 20% GPP-PEI polyplex, the Mander's Coefficient was approximately 0.4 after each incubation period. The Mander's Coefficient for each sample remained constant, independent of preincubation time. In Figure 3, representative images of the cells recovered from the collagen gels are presented. Notably, less polyplex uptake was detected in the GPP-modified versus unmodified samples particularly after longer preincubation periods. These findings are consistent with previous data confirming that the unmodified polyplexes were retained for shorter periods, and therefore less polyplex was available for uptake after longer preincubations.

3.3.4 Intracellular trafficking study

Collagen endocytosis occurs naturally during tissue remodeling through a mechanism involving MMP-mediated release of collagen fragments followed by $\alpha_2\beta_{Fig}$-integrin and caveolin-1-regulated uptake [56]. To examine whether GPP-modification and collagen association affected the endocytic uptake of polyplexes, transfection experiments were conducted as previously described, but using NIH/3T3

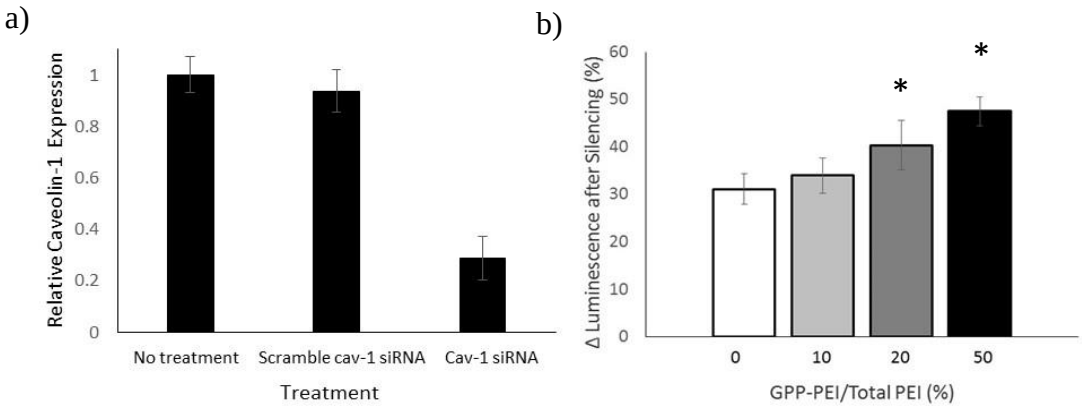

Figure 3.4. Cellular uptake of GPP-modified polyplex post caveolin-1 silencing. a) Caveolin-1 (cav-1) silencing efficiency was determined 2 days post-treatment via western blots. Expression levels relative to a non-treated control were also examined 1 and 3 days post treatment (Figure A5). b) Changes in polyplex endocytic pathways mediated by GPP-modification and continued association with collagen fragments were elucidated in caveolin-1 silencing experiments. Significantly decreased levels of gene expression were observed in each sample post caveolin-1 silencing but the impact of silencing was observed to be greater levels in samples modified with GPP-PEI polyplex. The decrease in luminescence was directly related to the level of polyplex modification. * denotes a statistically-significant difference ($p<0.05$) relative to the 0% GPP-PEI control.

cells with silenced caveolin-1 expression cultured in media supplemented with collagenase (Figure 4). Significant reductions in gene expression were observed following caveolin-1 suppression in all samples, suggesting that both the unmodified and GPP-modified polyplexes were internalized via caveolar endocytosis. Notably, the decrease in expression with caveolin-1 silencing was directly dependent upon the amount of GPP-PEI/total PEI incorporated into the polyplex. In both the 20% and 50% GPP-PEI polyplex samples, the changes in luminescence after silencing were significantly greater (30% and 53% greater, respectively) than that observed in the unmodified sample.

3.4 Discussions

The use of cationic polymers in non-viral gene delivery has many potential advantages by virtue of their low immunogenicity, ease of scaling, and versatile chemistry. Polymers such as PEI have been investigated extensively due to their DNA condensation capacity and ability to encourage cellular uptake. However, successful clinical application of these vectors has been largely inhibited by their relatively low transfection efficiency caused by their poor pharmacokinetics, lack of serum stability, and inefficient intracellular trafficking [13,57,58]. Safety concerns associated with off-target effects and immunogenicity also hinder the progression of these therapies [11,13,28,39,59,60]. In localized delivery applications, substrate-mediated delivery offers many advantages through its improved capacity to control retention/release of gene cargoes both spatially and temporally, as well as to increase serum-stability through steric inhibition [13,38]. Cell-mediated production of healing factors can synchronize the presentation these factors with natural tissue remodeling/healing. Furthermore, the properties of many native ECM proteins, such as collagen, make them attractive delivery substrates. However, substrate-mediated gene delivery approaches continue to face major obstacles when applied in healing tissues due to the exceptionally harsh, nuclease-rich environment and prolonged repair periods, which can extend over weeks to months. To address these issues, we sought to integrate gene cargoes *within* collagen fibrils to both abrogate DNA losses due to nuclease degradation, and directly harness native endocytic repair processes for improved gene localization, optimal release kinetics, and efficient intracellular trafficking.

Our previous experiments demonstrated the ability to tailor the release/retention of PEI polyplex from collagen, one of the most well-established, natural biomaterials, through the incorporation of different amounts of adjustable

CMP-linkers into the polyplex [52], and also showed enhancements in transfection efficiency of GPP-PEI polyplexes delivered from collagen. In this work, we sought to develop the CMP materials to optimally harness the tissue remodeling process to trigger gene stabilization and delivery, and our results indicated both the mechanisms controlling CMP-mediated gene delivery and the benefits of the CMP approach for producing enhanced, prolonged, and better localized gene expression in an *in vivo* model.

Collagen remodeling proceeds through processes regulated by MMP-1, $\alpha_2\beta_1$ integrin, and caveolin-1 [56]. Our prior studies showed that significant transfection was contingent on the stimulation of MMP secretion [52], providing the first evidence of cell-responsive delivery via collagen remodeling mechanisms. Furthermore, the improvement in transfection efficiency in the GPP-PEI polyplex-modified gels compared to the unmodified polyplex gels was vastly enhanced when gels were preincubated under physiological conditions in the presence of serum over long periods of time, indicating fundamental differences in the surface properties and stability of the polyplexes by virtue of their interactions with collagen. In particular, transfection studies with gels preincubated in serum concentrations of 0.25-50% (v/v) FBS over time periods ranging from 1 d up to 2 weeks demonstrated that GPP-PEI incorporation had a significant effect on gene expression as a function of both time and serum concentration (Figure 1). For example, unmodified polyplex had the highest levels of transfection when preincubated for the shortest time period examined (1 d) and with low serum concentrations (0.25% (v/v)). Expression levels rapidly decreased when the unmodified structures were incubated with higher serum concentrations and for longer periods, and for preincubation periods over a week, no significant

expression was detected. In contrast, significant levels of expression were observed in the GPP-PEI polyplex samples even after a week-long preincubation period when the polyplex contained 10%-50% GPP PEI, and the 50% GPP-PEI/Total PEI samples exhibited marked expression even after a 2 week long preincubation.

Differences in gene expression levels are likely due to differences in both polyplex retention/release kinetics as well as differences in gene stability/processing associated with collagen linkage. As previously observed, approximately 20% less DNA was initially retained in the unmodified polyplex samples as compared with the GPP-PEI polyplex samples, and the DNA that was retained was released approximately 1.63x, 2.10x, and 3.25x faster than the DNA in the 10, 20, and 50% GPP-PEI polyplexes, respectively, over 15 d in PBS [52]. While the release kinetics were likely not identical in the media containing serum, our study suggests that GPP modification and multivalency still improved polyplex retention over a range of serum concentrations for longer periods. Once released into the serum-supplemented media, the unmodified polyplex's high density of positive surface charges likely led to many interactions with negatively charged serum proteins such as albumin, leading to rapid aggregation and loss of viability within an hour [13,58]. Due to the faster release kinetics of the gel containing the unmodified polyplex, compounded by the fact it initially held less polyplex, the collagen's concentration of viable polyplex decreased at a significantly faster rate leading to lower levels of gene expression, which would also be exacerbated by the higher concentrations of serum expediting the aggregation of released polyplex. Differences in expression levels as a function of time were also observed for polyplex containing different amounts of GPP-PEI/Total PEI, with

samples containing higher amounts of GPP-PEI/Total PEI (10%, 20%, and 50%) exhibiting higher expression levels particularly after longer time periods.

The increase in expression observed in the more extensively modified polyplexes at higher serum concentrations was also likely due to a combination of effects stemming from scaffold and/or polyplex interactions with serum proteins. While collagen encourages cellular adhesion, serum gradients have been shown to encourage cellular in-growth, enhanced mobility, and increased proliferation [59,61]. In substrate-mediated delivery, cellular access to DNA, which is determined by cell mobility and DNA scaffold concentration, is one of the largest determinants of gene expression. Preincubation of the gels with serum likely leads to the absorption of serum proteins onto the scaffold, making the environment more hospitable to the cells. In unpublished observations, cells plated on these samples appeared more likely enter the scaffold and remain in the scaffold, unlike in the scaffolds preincubated in lower levels of serum, where it was observed cells were predominantly located in the top layers of the gels or adhered to the bottom of the polystyrene plate. We also theorize that increased association with collagen may have encouraged some favorable interaction between the polyplexes and serum proteins, leading to alterations in the cellular processing of the released gene-containing structures. FBS contains a myriad of proteins, including adhesion mediators as well as signaling and transport proteins. One possibility is pre-exposure to serum allowed the positively charged polyplexes to interact with negatively charged serum proteins while still remaining stably bound to collagen. This interaction would decrease the charge of the polyplex, diminishing the tendency for polyplexes to aggregate or be degraded post release. Pre-exposure of PEI

polyplex and other positively charged nanoparticles to serum has previously been documented to have this effect [62,63].

Integration in collagen and serum protein adsorption have also been shown to affect cellular uptake mechanisms, meaning that these interactions may also have led to greater endocytic uptake and/or more efficient intracellular trafficking to the nucleus [63,64]. In fact, our studies supported the existence of these effects in the CMP-polyplex materials, showing that the higher amounts of GPP peptide affected not only polyplex release kinetics, but the polyplex's final composition. In addition to mediating affinity with the collagen scaffold, the GPP peptide encouraged the polyplex to associate with the collagen fragments post-release, as shown in the colocalization study (Figure 3**)**. The consistent and significant differences between the Mander's coefficient for collagen association with the GPP polyplex, versus the unmodified polyplex, even after extended pre-incubation periods, indicated a continued GPP-mediated association of GPP-PEI polyplex with collagen following liberation and cellular uptake. The continued CMP-collagen interaction is consistent with previous studies that have shown that the serum-stability of the almost-neutral CMPs allows them to associate with remodeling collagen in highly complex environments including *in vivo* murine tumor, bone abnormality, and wound models. [45,47-49]. Continued association of the polyplex with collagen fragments has many potential benefits. It may further reduce the surface charge of the polyplex, reducing interaction with serum proteins and aggregation. In other works, neutral, hydrophilic polymers, such as poly(ethylene glycol) (PEG), or anionic polymers, such as alginate or hyaluorinic acid, are used to help shield the high density of positive charges of the PEI [13,57,58]. In our system, collagen fragments may play a similar role and afford the

released polyplex with both increased serum stability and resistance to aggregation and dissociation. Increased plasmid stability in GPP-PEI polyplex-modified gels was confirmed using gel electrophoresis. As shown in Figure 2, intact plasmid DNA was present in the media collected from all GPP-PEI polyplex-modified samples, even after a 14 d preincubation, indicating that the DNA was at least partially protected. After longer preincubation, the plasmid bands were reduced in intensity, indicating a degree of degradation, but the fluorescence intensity still remained significantly higher than the levels observed in the unmodified polyplex samples. Because equal amounts of DNA were loaded into each well, the result suggests that CMP-mediated DNA incorporation into the collagen gel protected the DNA more effectively than DNA retained through non-specific interactions. We hypothesize that GPP-PEI polyplexes were able to better preserve the integrity of the DNA because of their slower release kinetics, causing DNA to remain bound for longer periods, and because of the continued association of polyplex with collagen fragments post-release from the scaffold. The fluorescence observed in the wells of the samples collected from the GPP-PEI polyplex-modified gels suggests that complexes of the DNA with either PEI, a serum protein, and/or collagen, are present, and these structures were not dissembled with heparin as readily as those collected in our previous, serum-free study [52].

The collagen fragments may also impart other collagen-specific benefits. Collagen has been used to mediate gene delivery in various forms, ranging from macroscopic porous sponges to nanoparticles (100-300 nm in diameter) [9,65-67]. Atelocollagen-mediated delivery has been shown to increase cellular uptake and nuclease resistance when compared to typical polycation transfer methods and is capable of transferring genes into both dividing and non-dividing cells [65,66]. While the

majority of current studies utilize atelocollagen for localized delivery, previous works have shown atelocollagen-nucleic acid nanoparticles could be successfully implemented for systemic delivery, lengthening circulation time and increasing treatment efficiency [65,66,68]. The collagen fragments also have a series of integrin binding sites, which may aid in encouraging cellular internalization. To better understand the impact of the GPP peptide on polyplex uptake mechanisms, transfection experiments were conducted in which the caveolar endocytosis pathway was blocked. The caveolar endocytic pathway was chosen because previous studies with PEI polyplex have shown that this pathway initiates high efficiency intracellular trafficking to the nucleus and expression, and therefore targeting this pathway is desirable [53,54]. The GPP peptide, as well as endogenous collagen, contains an $\alpha_2\beta_1$-integrin binding site, which known to be initiate to caveolar endocytosis by collagen fragments. We discovered a direct correlation between the percent of GPP-PEI/Total PEI incorporated into the polyplex and the impact caveolin-1 silencing had on transfection. The decrease in transfection between the unmodified polyplex vs. the 20% and 50% GPP-PEI/Total PEI polyplexes (30% and 53%, respectively), was significant (p-value < 0.05). The results confirm that the GPP peptide acts not only as a tether to control release, but also as a ligand, providing a better-defined, efficient pathway way into the cells. The change in cellular uptake was likely due to the synergistic effects of the proline-rich CMP and collagen fragments. Further experimentation would be necessary to elucidate which effect was prevalent.

Other matrix-mediated gene delivery approaches have similar period of expression; however, our work differs substantially in that most approaches rely solely on scaffold degradation to mediate DNA delivery, limiting localized responsiveness

and enhancing the danger of off-target delivery. The application of protease-sensitive scaffolds has provided a means of engineering localized, cell-triggered gene transfer activity [11,12], but has not eliminated the danger of vector escape [39] or addressed potential issues associated with achieving gene delivery tailored to the irregular environments common in wound repair. CMP-modification of non-viral vectors has provided a method for tailoring release/retention from collagen, and additional studies strongly suggest the reversible nature of CMP/collagen hybridization synergistically prevent vector escape from the delivery site through re-association with the collagen scaffold and surrounding remodeled collagen [44,47]. Furthermore, unlike most matrix-mediated approaches, CMP-modification directly addresses obstacles associated with both the extra- and intracellular environments. CMP-modification has been demonstrated to not only enhance vector availability/stability in the delivery site, but also to provide a ligand promoting cellular uptake through endocytic pathways linked to high efficiency expression. Additional studies have demonstrated that the CMPs' unique triple helical structure is integral in achieving enhanced cellular binding and cell penetration [69]. For instance, CMPs were successfully used to enhance liposome targeting of melanoma cells through specific ligand/receptor interactions, whereas no binding to non-targeted cells was recorded [70]. Given the many obstacles that inhibit gene delivery, the introduction of a novel multifunctional peptide has enormous potential for accelerating vector optimization required for pragmatic/clinical application. While multifunctional peptide vectors have been developed, typically through the fusion of peptide moieties known to overcome a particular barriers (ex: DNA condensation, cellular internalization/targeting, nuclear localization), only a small fraction have demonstrated utility *in vivo*, suggesting that the practice of

studying the effects of these moieties individually is not sufficient [71]. The continued development of multifunctional CMPs and continued studies examining the impact of CMP-modification on different aspects of vector efficiency has enormous potential in advancing gene delivery applications.

3.5 Conclusion

Protease-sensitive biomaterials, including collagen, have been employed to synchronize the delivery of cues with tissue remodeling and the reparative process. In particular, the availability and biocompatibility of collagen have secured its place as a versatile biomaterial, both as a bioactive scaffold and as a cell-responsive reservoir for additional therapeutics. While many techniques for modifying collagen with therapeutics have been developed, the biomimetic approach utilizing CMPs has many advantages including its highly specific yet physical nature and its versatility in regards to tuning release/retention. In this work, CMP-modification was shown to modulate and enhance delivery of PEI polyplex from collagen-based scaffolds both *in vitro* and *in vivo*. Variation of the amount of GPP-PEI/total PEI in PEI polyplexes demonstrated the capacity to control release and stabilize polyplex, even in the presence of high concentrations of serum under physiological conditions for up to 2 weeks. The cell-responsive nature of the GPP-polyplex-modified collagen substrates was clearly indicated by the fact that significant transfection was only observed in the presence of MMPs/collagen remodeling. Furthermore, the colocalization of the GPP-PEI polyplex with collagen following polyplex release from the scaffold improved DNA structural integrity and enhanced the targeting of the polyplexes into a high efficiency endocytic pathway leading to gene expression. The unique properties of the GPP peptide allowed for its use as both an adjustable tether, via CMP-collagen

hybridization, and as an endocytic ligand. This method allowed for the “hijacking” of the natural process of collagen remodeling to overcome major obstacles in gene delivery, such as improving vector activity in the presence of serum and providing a well-defined intracellular pathway, in addition to serving as a cell-responsive trigger for release. As suggested by our *in vivo* work, these properties can translate to even more complex systems and have great potential in the treatment of sites in which excessive collagen remodeling occurs.

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Chapter 4

INTEGRATION OF GROWTH FACTOR GENE DELIVERY WITH COLLAGEN-TRIGGERED WOUND REPAIR CASCADES USING COLLAGEN-MIMETIC PEPTIDES

Growth factors (GFs) play vital roles in wound repair. Many GF therapies have reached clinical trials, but success has been hindered by safety concerns and a lack of efficacy. Previously, we presented an approach to produce protein factors in wound beds through localized gene delivery mediated by biomimetic peptides. Modification of polyethylenimine (PEI) DNA polyplexes with collagen-mimetic peptides (CMPs) enabled tailoring of polyplex release/retention and improved gene transfer activity in a cell-responsive manner. In this work, CMP-mediated delivery from collagen was shown to improve expression of platelet-derived growth factor–BB (PDGF-BB) and promote a diverse range of cellular processes associated with wound healing, including proliferation, extracellular matrix production, and chemotaxis. Collagens were pre-exposed to physiologically-simulating conditions (complete media, 37^{o}C) for days to weeks prior to cell seeding to simulate the environment within typical wound dressings. In cell proliferation studies, significant increases in cell counts were demonstrated in collagen gels containing CMP-modified polyplex vs. unmodified polyplex, and these effects became most pronounced following prolonged pre-incubation periods of greater than a week. Collagen containing CMP-modified polyplexes also induced a 2-fold increase in gel contraction as well as enhanced

directionality and migratory activity in response to cell-secreted PDGF-BB gradients. While these PDGF-BB-triggered behaviors were observed in collagens containing unmodified polyplexes, the responses withstood much longer preincubation periods in CMP-modified polyplex samples (10 d vs. <5 d). Furthermore, enhanced closure rates in an *in vitro* wound model suggested that CMP-based PDGF-BB delivery may have utility in actual wound repair and other regenerative medicine applications.

4.1 Introduction

Identification of the growth-promoting activities of the secreted, signaling proteins known as growth factors (GFs) has inspired much anticipation about their potential in tissue repair applications, particularly in refractory wounds and other hard-to-heal tissues. These multifunctional and potent proteins play fundamental roles in a range of regenerative activities including regulation of cellular proliferation, chemotaxis, and extracellular matrix synthesis, with their activity often recognizable in the picomolar range.[1,2] For example, altered cell phenotypes and an aberrant extracellular environment in chronic wounds are factors recognized to reduce GF production, stability, accessibility, and activity, further complicating the already intricate reparative processes.[3-7] Accordingly, multiple preclinical studies and industry sponsored trials have examined the efficacy of topical and sustained release GF formulations in chronic wounds.[6,8] In 1997, Becaplermin/Regranex (Systagenix; Skipton, UK), a topical platelet-derived growth factor-BB (PDBF-BB) gel, became the first successful, FDA-approved growth factor treatment for the treatment of diabetic foot ulcers (DFUs);[9] however, existing GF therapies exhibit only modest clinical utility overall. Based on clinical trials, the FDA concluded that topically-applied PDGF-BB increased the number of healed DFU patients by less than 10%,[6] and while

the application of PDGF-BB has been shown to augment wound repair in several human studies, many were never published due to a lack of efficacy.[9-12] Clinical failure has largely been blamed on incompatibilities of traditional GF therapies with the hostile, irregular chronic wound environment that limits GF penetration into the wound bed, causes rapid GF degradation due to elevated protease activation, and decreases cellular responses to GFs.[3,5,8] Accordingly, extraphysiological, repetitive doses are typically required to achieve therapeutic effects. These dosing regimens increase the danger of GF toxicity, elevate treatments costs, and elevate the risk of substantial off-target effects or even oncogenic responses.[3,9,13-15] New approaches for creating healthy, GF-rich wound beds are essential.

Based on the need for improved wound therapies, promising alternatives include GF gene delivery and the application of biocompatible matrices that can regulate multiple aspects of cell behavior through controlled presentation of extracellular cues. GF gene therapies offer exciting potential benefits for improved GF delivery due to their ability to foster localized, on-demand GF production within the wound bed. In particular, gene-based approaches to GF delivery better mimic endogenous repair responses by allowing host cells to orchestrate sustained GF expression, microlocalization, and activity, which are essential in chronic wound repair due to extended healing over months, spatiotemporal heterogeneity, and elevated protease activation. Because of these characteristics, GF gene therapies have exhibited increased efficacy in experimental wound models as compared with topical delivery approaches, with the capacity to achieve similar healing responses with orders of magnitude less GF (e.g. ~3 orders of magnitude reduction in dosage of GF expressed relative to a typical topically-applied dose).[16-23] These observed dosage

reductions coupled with the spatiotemporal control achievable via promoter choice/vector design [24-26] suggest that gene therapies may have exciting potential to create controllable, more effective, and less toxic approaches to deliver GFs. While clinical data on GF gene therapies are limited, localized gene therapy approaches show promise for improved safety and efficacy, and are amongst the most rapidly advancing gene therapies in clinical trials for diseases such as ocular disorders.[6,19,27]

In terms of delivery regimens, therapeutic DNA has been incorporated into biomaterial matrices designed to mediate, prolong, and enhance gene transfer while reducing potential off-target and/or immune responses.[8,19,28-36] In addition to providing enhanced gene stability and improved control over release, gene activated matrices (GAMs) provide a permissive environment that promotes cellular ingrowth, increases tissue deposition, triggers *in situ* production of GFs, and enhances cell health.[15,28,36] In fact, the application of collagen-based artificial skins, such as Apligraf (Organogenesis) and Dermagraft (Advanced Biohealing), has been shown to enhance chronic wound repair even in the absence of incorporated GFs; however, the incidence of complete closure after a therapeutic trial with engineered skin remained close to 50%, highlighting the need for improved bioactivity.[8] The synergistic effects in wound repair between biomaterials and GF-encoding genes have been demonstrated in numerous studies, such as when collagen-embedded *PDGF-B* DNA was shown to increase the formation of new granulation tissue by up to 52% and re-epithelization by up to 34%, as compared to collagen alone, in a dermal ulcer model in rabbits. The same materials stimulated a more than 4-fold increase in cell repopulation over a 10-day period in an *ex vivo* human gingival defect repair model. [8,37]

Additional studies demonstrate the clear advantages for natural and synthetic GAMs in controlling gene transfer efficacy, with some approaches reporting detectable gene expression *in vivo* over a few weeks via diffusion- and/or degradation-controlled retention/release of entrapped plasmids or polyplexes.[30,38-40] Furthermore, improvements in spatial and temporal control over the delivery of DNA from GAMs have been achieved through the immobilization of DNA onto scaffolds through better defined interactions, such as biotin-avidin or antigen-antibody binding.[32,41-44] However, while current gene-based therapeutics are very promising, they often have failed in translation due to continued concerns of off-target and immune responses, as well as inefficiencies in gene transfer efficacy in protein/serum-rich environments.[19,45-48] Moreover, the majority of existing GAM technologies are unfit for many tissue repair applications due to the complexity of the healing process, which can involve extended healing periods over months and multiple out-of-phase healing cascades occurring simultaneously within repair sites.

In our prior studies, a novel approach with the potential to overcome these issues through application of collagen-mimetic peptides (CMPs) in gene delivery was demonstrated. CMPs have a natural affinity for collagen driven by a reversible strand-invasion process that can be tailored with relative ease by altering CMP sequence and molecular weight. This unique ability has been exploited to modify extracted collagens *in vitro*,[34,49-53] as well as to target and bind remodeling collagens *in vivo*,[49,54,55] using various CMP-linked cargoes such as GFs. Our labs were the first to use CMPs to modify collagen with DNA. Specifically, CMP display on DNA-polyethylenimine (PEI) polyplexes was shown to have the capacity to improve control over both the extent and duration of gene expression. Through varying CMP display,

DNA release/retention was tailored for over a month, two times longer than the retention/release periods of unmodified polyplexes. CMP-modification also maintained polyplex activity in serum-supplemented media for up to 2 weeks, in contrast with most gene delivery approaches which report losses to nuclease degradation within hours.[34,56] Additionally, we demonstrated the novel ability to "hijack" collagen remodeling,[56] a process that occurs in excess in the protease-rich chronic wound environment.[3,8,9] Whereas previous studies have utilized proteolytically-sensitive materials to synchronize cell invasion with therapeutic release, the reversible, serum-stable nature of the CMP-collagen interaction allowed for continued association with collagen fragments, confirmed through colocalization microscopy studies. The alteration in polyplex composition resulted in enhanced polyplex activity linked to an increased capacity to preserve DNA integrity in the presence of serum and an increase in caveolar uptake, a pathway linked to high efficiency gene transfer. Furthermore, the benefits of using collagen remodeling as a driver for gene release and activity were confirmed in a more complicated, *in vivo* model in which transgene expression was localized and extended from 3 to over 20 days.[56] This versatile approach, which capitalizes on collagen remodeling, has the potential to efficiently augment any collagen-containing device with gene expression and has tremendous potential for overcoming non-viral gene delivery obstacles in multiple regenerative medicine applications.

In this work, our objective was to demonstrate the benefits of CMP gene delivery for GF expression during wound healing[57]. For this purpose, we chose PDGF-BB as a target GF due to its well established ability to effect a diverse range of cellular processes associated with wound healing, including proliferation, extracellular matrix

production, and chemotaxis.[8,19,37,43,58-60] Our studies demonstrated that CMP display on PEI polyplexes encoding *PDGF-B* significantly enhanced polyplex activity, even after prolonged exposure to physiologically-simulating conditions mimicking the wound environment. [57] PDGF-BB levels were up to 4-fold greater in CMP-modified samples, vs. unmodified samples, and maintained up to a 3-fold increase in expression even after the gene-modified collagen scaffolds were exposed to serum for 7 days at 37°C. Various desirable cell repair behaviors were also enhanced by CMP-modification. Cell counts were increased by up to 75% in CMP scaffolds, and CMP-induced differences in proliferation remained significant even after a 10-day serum preincubation. Collagen remodeling and cell migration were also enhanced by CMPs, as highlighted through collagen contraction assays and cell migration studies. Moreover, we demonstrated promising capacity for the CMP-modified DNA/collagen gels in wound scaffold applications, based on increased cell densities and accelerated migration in collagen-based, 3-D wound models. Defects treated with CMP-modified collagens reached approximately 90% wound closure after 10 days of treatment, whereas wound closure never exceeded 40% using scaffolds containing unmodified polyplex. Moreover, collagen scaffolds supplemented with recombinant PDGF-BB (rPDGF-BB) at levels comparable to the maximum expression levels exhibited no differences in cell invasion/proliferation from collagen alone; in fact, rPDGF-BB levels had to be increased by an order of magnitude to achieve similar bioactivity as in CMP-modified scaffolds. The increase in gene stability and improved expression associated with CMP-based gene delivery may translate *in vivo*, aiding in multiple aspects of wound repair. Furthermore, our findings stress the advantages of GF gene therapies for providing substantial reductions in GF exposure and thereby reducing

concerns with both cost and safety. These advantages highlight the potential of CMP-triggered gene delivery to enhance numerous collagen-based materials through improved non-viral gene delivery regimens.

4.2 Materials and Methods

4.2.1 Materials

Type I bovine collagen was purchased from Advanced BioMatrix (San Diego, CA) and pCMV-PDGF-B plasmid was purchased from Origene Technologies, Inc. (Rockville, MD). pDNA was amplified in NEB 5-α electrocompetent *E. coli* purchased from New England Biolabs and purified from bacterial culture using a Qiagen Megaprep Kit (Valencia, CA), following the manufacturer's protocols. The Mouse/Rat PDGF-BB Quantikine enzyme-linked immunosorbent assay (ELISA) kit was purchased from R&D Systems (Minneapolis, MN). Fmoc-protected amino acids were purchased from Anaspec (Fremont, CA). H-Rink amide ChemMatrix® resin was purchased from PCAS Biomatrix (Quebec, Canada). O-Benzotriazole- N,N,N',N'-tetramethyl-uronium-hexafluoro-phosphate (HBTU) was purchased from Novabiochem (San Diego, CA). High performance liquid chromatography (HPLC)-grade N,N-dimethyl formamide (DMF), acetonitrile, trifluoroacetic acid (TFA), CellTracker^{TM} Deep Red, and cell culture reagents, including Dulbecco's modified Eagle's medium (DMEM), Dulbecco's phosphate buffered saline (PBS), penicillin-streptomycin (P/S) ,and trypsin were purchased from Fisher Scientific (Fairlawn, NJ). Fetal bovine serum (FBS) was purchased from Corning (Manassas, MA). Collagenase I was purchased from Worthington Biochemical Corp (Lakewoord, NJ). Piperidine, 4-methylmorpholine, all cleavage cocktail components, and branched PEI (25 kDa)

were purchased from Sigma-Aldrich (St. Louis, MO). The Oris™ cell migration assay kit was purchased from Platypus Technologies (Madison, WI).

4.2.2 Preparation of modified collagen gels

The CMP [GPP: $(GPP)_3GPRGEKGERGPR(GPP)_3$] used in prior studies[34,61,62] was synthesized using Fmoc solid phase peptide synthesis and purified using reverse phase-HPLC. GPP was conjugated to PEI using Michael-type addition chemistry and the conjugate (GPP-PEI) was used to prepare GPP-modified polyplexes as previously described.[34] Using a variation of well-established polyplex formation protocols,[63,64] equivolumetric solutions of PEI and DNA in 20 mM HEPES buffer (pH 6.0) were mixed to produce a final solution with an amine to phosphate ratio (N:P) ratio of 10. To incorporate GPP, the GPP-PEI conjugate was preincubated at 50°C for 30 minutes to prevent triple-helical hybridization of GPP, and a specified percent of PEI used to create the polyplex was replaced with the GPP-PEI. Collagen gels with GPP-immobilized or encapsulated unmodified polyplexes were prepared by re-suspending dehydrated polyplex in neutralized type I bovine collagen-solution (4 mg/mL, pH 7.4) as previously described.[34] After a 3 h incubation at 4°C to enable GPP-collagen hybridization, the solution was allowed to gel overnight at 37°C.

4.2.3 Quantification of PDGF-BB expression in modified collagens

DNA/collagen gels were prepared with 500 µL of DNA/collagen solution in 8 well plates (0.8 cm^2 surface area/well). Gels were incubated at 37°C overnight to allow gelation, after which 200 µL of complete medium (DMEM with 10% FBS and 1% P/S) was added. The gels were then preincubated in complete medium at 37°C and 5% CO_2 for a specified time period ranging from 0 to 14 days to simulate

physiological conditions. MMPs and other wound-relevant proteases were purposefully excluded from the pre-treatment step based on wound environment studies that demonstrate localization of proteases in the expressing cells' microenvironment coinciding with the wound edge or adjacent tissues.[65] Subsequently, gels were washed with PBS and DMEM and 20,000 NIH/3T3 cells were seeded per well. Cells were cultured under the same conditions as the preincubation. After a 4-day culture, a sandwich ELISA and a direct ELISA were used to determine PDGF-BB concentrations in the conditioned media and in the gels, respectively. PDGF-BB remaining in the gels was recovered through a 72-hour incubation with 500 µL of extraction buffer (10 mg/ml heparin, 2% BSA, 2M sodium chloride, and 0.01% Triton-X in phosphate buffered saline) at 4^{o}C. Released PDGF-BB was quantified using a commercially available Quantikine ELISA kit in a 96-well plate format, according to manufacturer's instructions. A seven-point standard curve was used to quantify the concentrations, spanning a range from 0 to 2000 ng/mL PDGF-BB. Each sample was read twice with a Glomax Multimodal Plate reader (Sigma), and the average of the two readings was used to calculate the concentration of PDGF-BB in the sample. PDGF-BB still remaining in the gel was quantified through use of a direct ELISA. After removal of the extraction buffer, the gels were gently washed twice with PBST (PBS supplemented with 0.05% Tween-20, 5 minutes per wash) and incubated with the anti-mouse/rat PDGF-BB antibody conjugate supplied in the Quantikine ELISA kit (antibody specific for PDGF-BB conjugated to horseradish peroxidase [HRP]) for 2 h at room temperature. Following two 5-minute PBST washes, 300 µL of a 1:1 solution of color reagent A (hydrogen peroxide) and color reagent B (tetramethylbenzidine) were added. Colored solution was removed

from each gel after 30 min and a stop solution was added. The Glomax Multimodal Plate reader (Sigma) was then used as described above to determine PDBF-BB concentrations using solutions collected from an empty collagen control gel as a background measurement.

4.2.4 Quantification of proliferation

DNA/collagen gels were prepared and preincubated for periods ranging from 0 to 14 days as described above. Four days after seeding cells (20,000 NIH/3T3 cells per well), gels/cells were imaged using a Leica DMI6000 B inverted microscope (Wetzlar, Germany), and image analysis tools in ImageJ (National Institutes of Health) were used to count the number of cells per field of view. Cells were subsequently recovered from the gels through use of a collagenase (0.1 U/mL PBS)/dispase® (0.8 U/mL PBS) digestion solution, as well as gentle pipetting to break the gel apart. After a 45-minute digestion at 37^{o}C, the total cell count was determined using a hemocytometer and compared to the number of cells initially seeded. Samples were analyzed at 4 days to provide time for cellular invasion, transfection, and proliferation while excluding the effects of cellular confluency on cellular behavior.

4.2.5 Contraction Assay

DNA/collagen gels were prepared and preincubated as described above. After a 0- to 10-day preincubation, 20,000 cells were seeded onto the gels. Three days after seeding the cells, gel height was determined by analyzing images taken of the gels with a camera, using a ruler tool in ImageJ. To account for gel irregularities, the gel

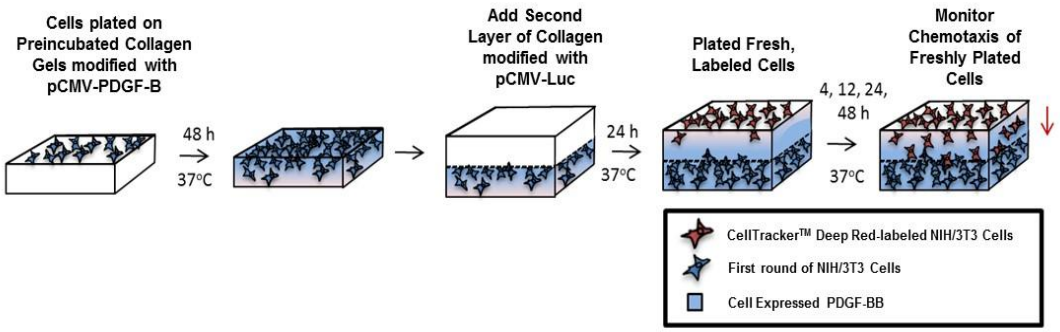

Figure 4.1. Schematic of collagen bi-layer migration study. NIH/3T3 cells were seeded onto a layer (layer 1) of collagen containing polyplex encoding for PDGF-BB. Layer 1 was preincubated in complete culture media containing 10% serum prior to cell plating. After a 48 h culture of the cells on layer 1, a second layer (layer 2) of collagen (containing pCMV-Luc) was added. Twenty-four hours after the addition of layer 2, additional NIH/3T3 cells were plated on top of layer 2 and migration in response to the cell-expressed PDGF-BB (in layer 1) was monitored.

height for a given gel was determined by averaging the heights measured at 5 set-points in the gel (one point at the center of gel; 2 points at the gel edges, and 2 points equidistant from the prior points). The average gel height reported for each sample was based upon the average of individual gel heights from 5 different samples.

4.2.6 Collagen bi-layer cellular migration study

Layered DNA/collagen gels containing a gradient in cell-expressed PDGF-BB were produced through a multi-step procedure (Figure 1). First, 200 µL DNA/collagen gels containing PDGF-B-encoding polyplexes were prepared and preincubated in complete media [(10% FBS, 1% P/S) at 37 ºC and 5% CO_2], as described, to simulate physiological conditions. After a 0- to 10-day preincubation

period, 20,000 NIH/3T3 cells were seeded onto the collagen gels and cultured for 2 days under the same conditions as the preincubation to allow cell invasion and transfection. Media was subsequently removed from the gels and a second layer of collagen containing luciferase-encoding polyplex (100 µL of collagen/DNA solution) was added atop each cellularized gel. Following gelation at 37 ºC for 2 h, complete media was added to the bi-layer collagen gels. After allowing an additional 24 h for a gradient in PDGF-BB expression to form, 5,000 fresh NIH/3T3 cells (labeled with CellTracker™ Deep Red) were plated onto each gel. These cells were pre-labeled with the CellTracker™ Deep Red dye via the manufacturer's protocol to enable rapid tracking of cell migration, and the cells were plated at a low density (~6,250 cells/cm^2) to minimize the effects of cell-induced contraction[66,67]. A 75% reduction in cell seeding densities lessened contraction in collagen gel height from approximately 16% to 5% over 48 h (data not shown) and migration was monitored using a Leica DMI6000 B inverted microscope (Wetzlar, Germany). All image analysis was performed using ImageJ.

4.2.7 *In vitro* wound model

3-D *in vitro* defect wounds were constructed using an Oris™ cell migration assay kit. A physical "stopper" barrier was placed in the center of each well of a 96-well plate. 20 µL of collagen solution containing 100,000 NIH/3T3 cells/mL were added to either side of the stopper (total of 40 µL per well) to ensure the collagen/cell mixture was spread evenly around the stopper. After allowing gelation at 37ºC for 1 h, the stopper was removed, creating uniform, cell-free defects at the center of each well (diameter = 2 mm). 20 µL of DNA/collagen solution encoding *PDGF-B*, prepared as described previously, was immediately added to the defect. In the 20%/50% GPP-

modified polyplex samples a ratio of 8:2 (mass of DNA in the 20% vs. 50% GPP-modified polyplex) was used. After allowing gelation to occur for 1 h at 37ºC, complete media was added atop each gel and cell invasion into each defect was monitored via Calcein AM staining (5 μM Calcein AM in Opti-MEM). Defect/wound "closure" was analyzed using the thresholding function in the ImageJ MRI Wound Healing Tool (NIH), with the new wound edge defined as the point when the average cell density/fluorescence in the defect matched the average cell density/fluorescence in the area surrounding the defect. Percent wound closure was defined as the fractional area of the defect that has the same cell density as the area surrounding the defect.

4.3 Results

4.3.1 PDGF-BB expression was detected in modified collagens after prolonged exposure to physiological-simulating conditions

To quantify cell-expressed PDGF-BB in the modified-collagens, both direct and indirect sandwich ELISA assays were used. No significant differences in the PDGF-BB concentrations were detected in conditioned media collected from any of the samples, based upon sandwich ELISA measurements; however, significant differences between samples were detected when the levels of collagen-bound PDGF-BB were analyzed by release from collagen via extraction buffer and direct ELISA (Figure 4.2). In particular, when samples were not preincubated, collagen-bound PDGF-BB levels were elevated in all samples containing polyplex encoding for *PDGF-B* relative to samples containing polyplex encoding for luciferase. In the non-preincubated samples, the highest levels of expression were observed in the *PDGF-B*-encoding samples with unmodified and 20% GPP-PEI polyplex, which exhibited over a 4-fold increase in expression relative to the luciferase controls. Levels of expression

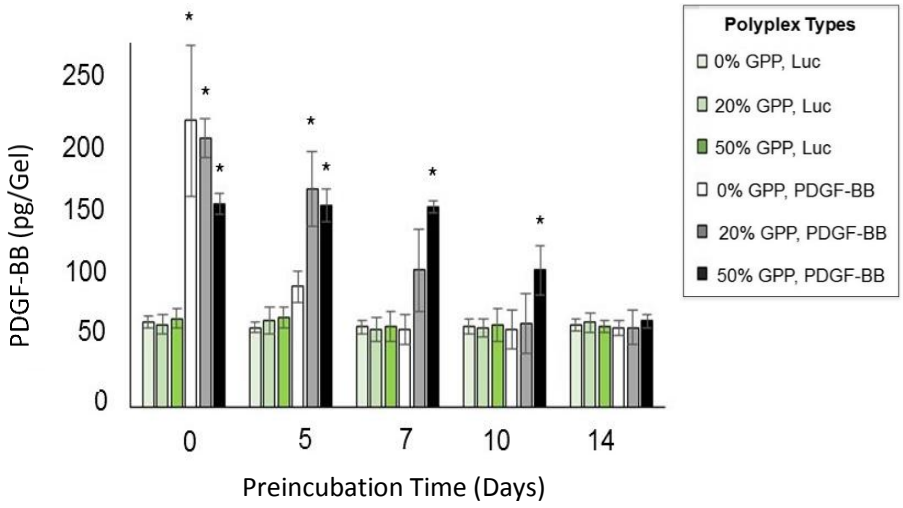

Figure 4.2. Cellular expression of PDGF-BB was analyzed 4 days after NIH/3T3 cells were plated onto collagen gels containing polyplexes. The data represent the amounts of PDGF-BB expressed in each gel, as assessed by ELISA. The data represent the mean +/-standard deviation in 4 separately prepared and analyzed samples. * denotes a statistically-significant difference ($p<0.05$) relative to the luciferase-encoding controls.

in the 50% GPP-PEI PDGF-BB sample were also elevated in these samples, with a nearly 3-fold increase in expression. In the unmodified polyplex samples, the level of expression rapidly decreased when the gels were exposed to wound-mimetic conditions, through preincubation of the gels with serum solutions at 37°C. PDGF-BB expression decreased by more than 60% after a 5-day serum preincubation in the unmodified polyplex samples, and at this time point, the expression levels ceased to be higher than those overserved in the luciferase controls. Expression in the GPP-modified samples also decreased as a function of preincubation time; however, the rate of decrease was substantially slower. After a 5-day preincubation, expression

decreased in the 20% GPP-PEI/total PEI samples by only 20%, and the expression levels decreased 55% after a 7-day preincubation, relative to the non-preincubated samples. PDGF-BB expression in the 20% GPP-PEI/total PEI samples ceased to be greater than the luciferase-encoding control samples after preincubation periods over 7 days. The 50% GPP-PEI/total PEI samples exhibited an increased longevity in gene transfer activity when exposed to physiologically-simulating conditions. These samples maintained consistent levels of expression that were approximately 3-fold greater than the luciferase-encoding controls for up to a 7-day preincubation period. The samples exhibited significantly elevated levels of PDGF-BB relative to the controls for up to a 10-day preincubation.

4.3.2 Quantification of PDGF-BB-mediated cellular proliferation

To assess if cell-mediated PDGF-BB expression impacted cellular behavior after prolonged pre-incubation, cell proliferation was quantified via cell recovery from the modified collagen gels after 4 days of culture. Samples were analyzed at 4 days to exclude the effect of cellular confluency on cell behavior while determining the maximum period over which polyplex activity could be preserved in the presence of physiologically-simulating conditions. As shown in Figure 3, the non-preincubated unmodified polyplex samples and 20% GPP-PEI/total PEI samples encoding *PDGF-B* exhibited elevated cell counts that were approximately 65% greater than the levels observed in the controls. After preincubation under physiologically-simulating conditions for 5 days or longer, no significant difference in cell count was found in the unmodified polyplex samples, whereas cellular proliferation in the 20% GPP-PEI/total PEI samples remained significantly elevated relative to the controls for up to a 7-day preincubation. Proliferation in the 50% GPP-PEI/total PEI samples remained elevated

relative to controls for the longest period, with a significant increase of about 50% in cell counts even after a 10-day preincubation. Additionally, the cell counts recorded in the 20% GPP-PEI/total PEI were approximately 56% greater than those in the 50% GPP-PEI/total PEI when the gels were not preincubated, but the count levels between the 20% and 50% samples were determined to be statically indistinguishable after either a 5-day or a 7-day preincubation. After a 10-day preincubation, proliferation in the 50% GPP-PEI/total PEI samples surpassed that recorded in the 20% GPP-PEI/total PEI samples by approximately 23%. The cell counts recorded in the 50% GPP-PEI/total PEI samples preincubated for 5, 7, or 10 days were statistically indistinguishable from one another. 14-day preincubation studies were also conducted,

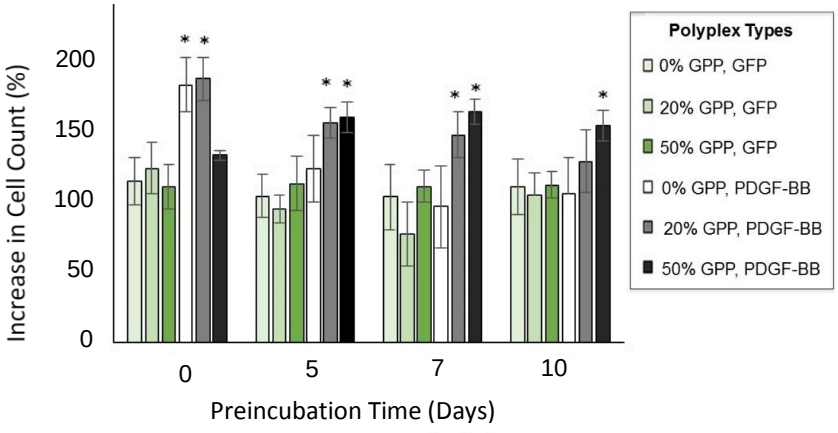

Figure 4.3. Cellular proliferation in preincubated collagen gels containing polyplexes, 4 days after NIH/3T3 cells were seeded onto the gels. Proliferation was analyzed to determine the mitogenic activity of cell-expressed PDGF-BB. The data represent the mean +/-standard deviation of the increase in cell count, relative to the number of cells initially seeded, as assessed with a hemocytometer after cell recovery from the gels, in 4 separately prepared and analyzed samples. * denotes a statistically-significant difference ($p<0.05$) relative to the GFP-encoding controls.

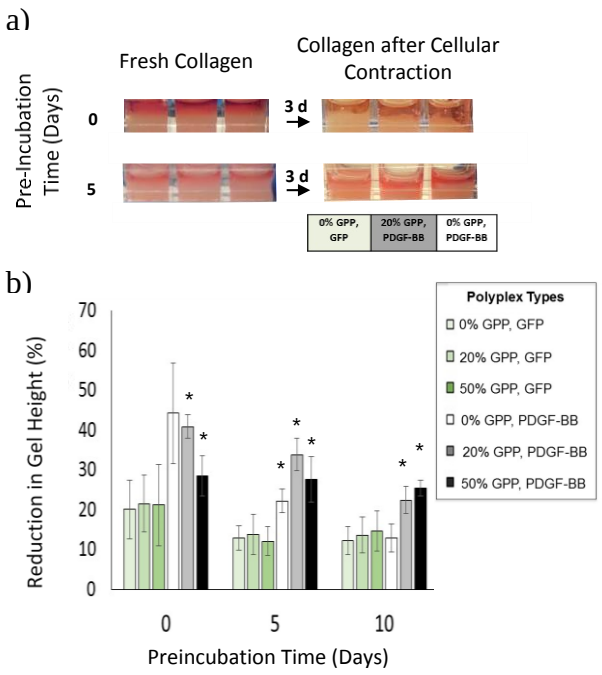

Figure 4.4. Collagen remodeling in the preincubated polyplex-containing collagen gels in response to cell-mediated expression of PDGF-BB. Remodeling was monitored by measurement of the reduction in gel height (z), relative to the initial height in each gel, using ImageJ to assess gel images, 3 d after NIH/3T3 cells were seeded onto the gels. The data represent the mean +/-standard deviation of 5 separately prepared and analyzed samples. * denotes a statistically-significant difference ($p<0.05$) relative to the GFP-encoding controls.

however the results were not included because the samples exhibited no statistical difference from the controls and thus did not add any substantive information to the figure.

4.3.3 Quantification of PDGF-BB-mediated ECM remodeling

A contraction assay was used to assess the level of ECM remodeling in response to cell-mediated PDGF-BB expression. As shown in Figure 4, gel heights shrank due to contraction at a significantly greater rate in all samples containing polyplex encoding for *PDGF-B*. Reflecting the PBGF-BB quantification and PDGF-

BB-mediated proliferation results, the non-preincubated unmodified polyplex samples and 20% GPP-PEI/total PEI samples exhibited the greatest reductions in gel height, deceasing approximately 20% more relative to the controls. After pre-incubating the samples, no significant differences in gel height were observed in the controls vs. the unmodified polyplex samples. However, the reduction in gel height remained significant in the 20% and 50% GPP-PEI/total PEI samples for up to a 10-day preincubation. Contraction was greatest in the 20% GPP-PEI/total PEI sample when the sample was not preincubated, and contraction levels in this sample were indistinguishable after preincubation for 5 vs. 10 days.

4.3.4 PDGF-BB-Triggered Migration in Layered Collagen Gels

To determine if cell-mediated PDGF-BB expression triggered chemotaxis, layered collagen gels were constructed. The first layer (layer 1) contained polyplex encoding for *PDGF-B* (or luciferase, as a control), and cellular ingrowth and gene transfection were allowed to occur within this layer for a period of 2 d. Subsequently, a second collagen layer (layer 2) was added, and additional cells containing a tracking dye were plated atop this fresh collagen (Figure 5a). The labeled cells in the second layer were allowed to migrate in response to PDGF-BB expressed by cells in the first layer, and the extent of cell migration towards the bottom layer was quantified via fluorescence microscopy. As shown in Figure 5, the length of time the first layer was preincubated directly impacted cellular migration. Specifically, in samples in which the first layer was preincubated for 3 d, all samples containing the *PDGF-B*-encoding polyplex exhibited accelerated migration as compared to controls in which luciferase-encoding polyplexes were used. In these samples, about 90% of the analyzed cells migrated 300 μm towards the bottom layer when the first layer contained *PDGF-B*-

encoding polyplexes, whereas only ~50% of the cells migrated as far with the luciferase-encoding polyplexes. When the first layer gel was preincubated for a 10-day period, approximately 44% of cells migrated to the bottom level in both the luciferase-encoding polyplex samples and the unmodified polyplex samples, whereas almost 75% of the analyzed cells had migrated to the bottom layer in the 20% GPP-PEI/total

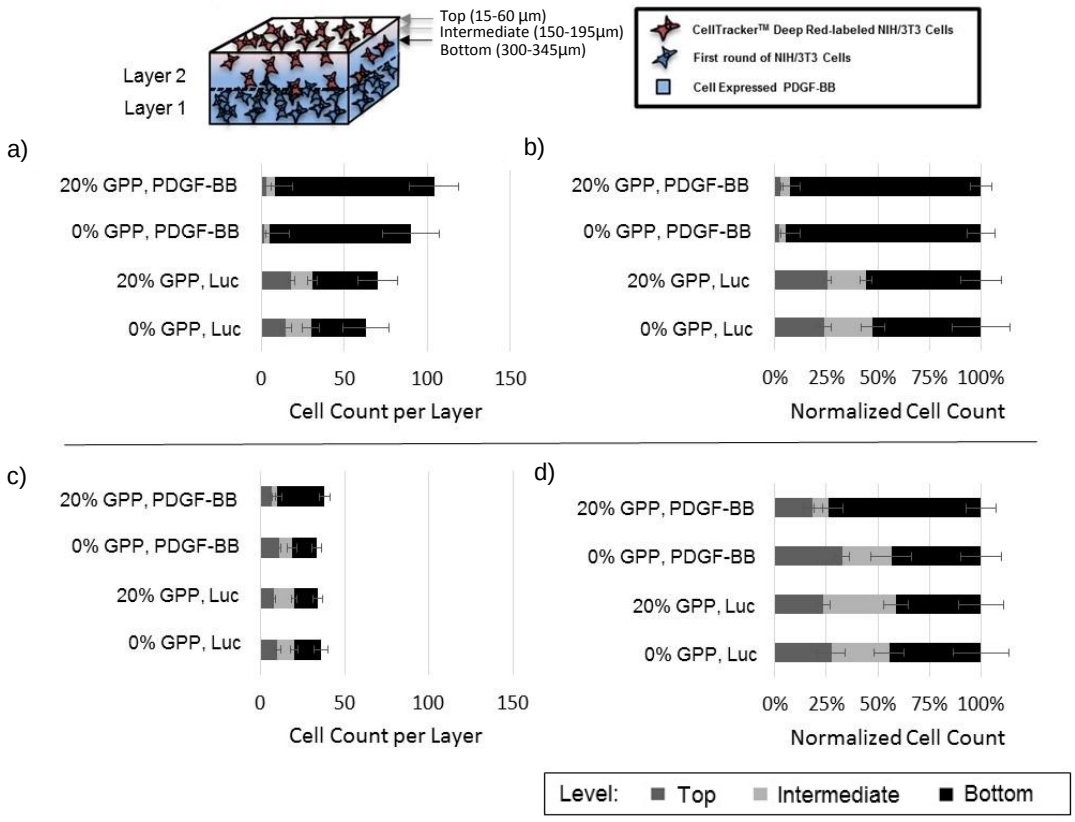

Figure 4.5. Cell migration in a collagen bi-layer model in response to cell-expressed PDGF-BB was tracked using microscopy. The initial collagen layer (bottom layer; layer 1) was preincubated for 3 (a,b) or 10 (c,d) days before the addition of the next layer (layer 2). The data represent the mean +/-standard deviation in cell counts (left) and cell counts normalized by the total number of analyzed cells per gel (right) of 4 separately prepared and analyzed samples. Cells were allowed to migrate for 48 h prior to analysis.

PEI *PDGF-B*-encoding samples. A significantly greater amount of cells were also observed in the intermediate level of the gel in the unmodified polyplex samples relative to the GPP-modified samples.

4.3.5 *In vitro* wound model

To culminate the studies, the ability to achieve enhanced wound closure via cellular responses triggered by cell-expressed PDGF-BB was examined in an *in vitro* wound model (Figure 6). Cellular invasion of uniform wounds created in collagen after application of DNA-modified collagen scaffolds or collagen embedded with rPDGF-BB was monitored via calcein AM staining. In the samples in which a scaffold containing polyplex encoding *PDGF-B* was administered, accelerated cellular invasion was noted, particularly in the GPP-modified samples. Specifically, the wound closure in the unmodified sample encoding for *PDGF-B* was only statistically different from closure in the luciferase-encoding polyplex control, whereas it was not statistically distinguishable from pure collagen or low rPDGF-BB-containing controls (1 ng/mL PDGF-BB). After 5 days, the 20% and 20%/50% GPP-modified samples achieved similar levels of wound closure (approximately 65% and 53%, respectively), but these samples did not achieve the same levels of closure as the high rPDGF-BB dosage control (10 ng/mL PDGF-BB). After an additional 5 days (10 days post-defect), a significant increase in percentage of wound closure was only recorded in the GPP-modified samples, with the 20%/50% GPP-modified sample achieving the same level of wound closure as the high dosage rPDGF-BB control. Studies with 50% GPP-modified samples were also initiated, but these samples were terminated early due to the low levels of observed wound closure within the monitoring period (Figures A6 and A7 (Appendix)). Additionally, a similar *in vitro* wound defect model employing

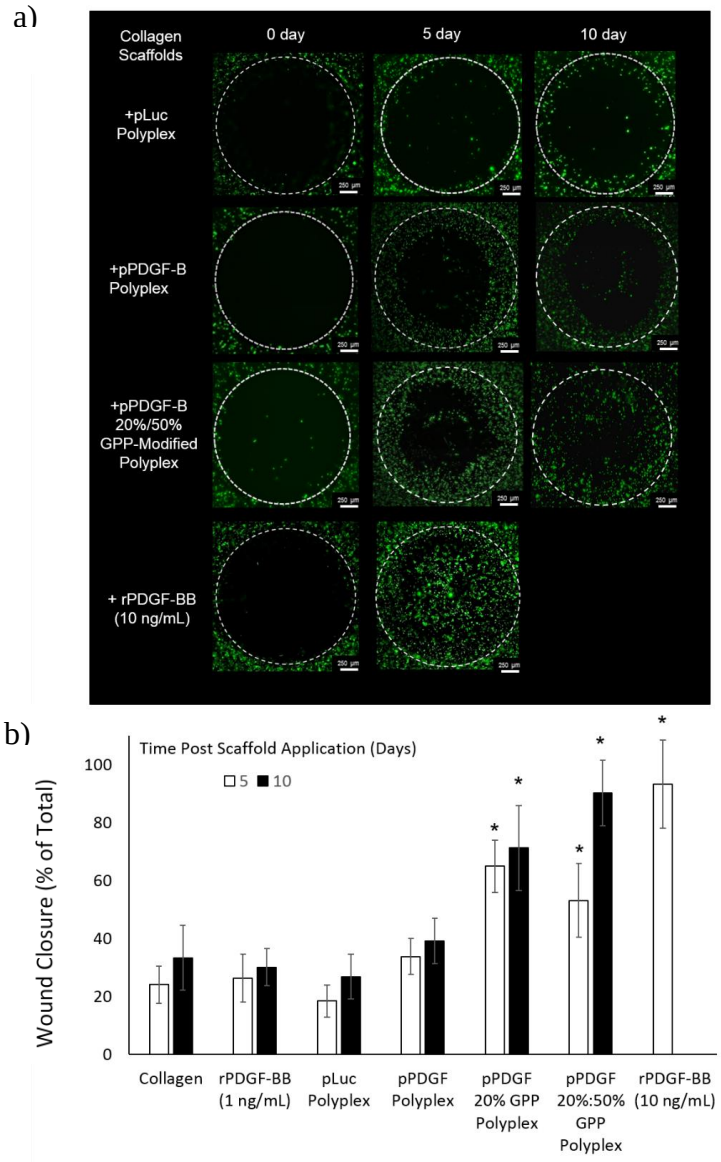

Figure 4.6. *In vitro* wound model. Defects in cell-seeded collagen gels were filled with collagen scaffolds modified with rPDGF-BB, polyplex encoding for luciferase, or polyplex encoding for *PDGF-B*, and subsequent defect invasion or "wound closure" was monitored via microcopy. Representative images (a) were analyzed via ImageJ to quantify wound closure (b). The data represent the mean +/-standard deviation of 3 separately prepared and analyzed samples. * denotes a statistically-significant difference ($p<0.05$) relative to the luciferase-encoding controls.

experimental demonstrated that wound closure did not occur in any samples during the initial 3 days (Figure A6 (Appendix)).

4.4 Discussion

The inherent complexity and hostility of the chronic wound environment has greatly complicated treatment, causing the failure of most monotherapies containing just GFs or biomaterial-based scaffolds. [3,37,60] Chronic wounds exhibit a self-sustaining, out-of-control inflammatory response in which excessive protease activation impairs cellular infiltration and causes the rapid degradation of GFs and other molecules essential for the reparative process.[3,5] The addition of GF through topical or sustained applications has been shown to enhance repair in several human studies, demonstrating its potential for modifying the wound environment; however, the modest degree of clinical success with these approaches underscores significant deficiencies in the way GFs are presented. GF therapies are reliant on repetitive, extraphysiological doses, increasing the likelihood of off-target effects and oncogenic responses. GF gene therapies, particularly when delivered via a biomaterial, exhibit benefits in protein stability, protein bioactivity, sustained release, and cost. The potential to deliver multiple genes with relative ease and the spatiotemporal control achievable though promoter choice/vector design are also extremely advantageous. Moreover, gene delivery better mimics endogenous repair, allowing host cells to orchestrate GF production, microlocalization, and activity, which are particularly crucial in mitigating the pathophysiology of a chronic wound.

In our work, we have shown that CMP-mediated gene delivery has compelling advantages for overcoming the obstacles that have prevented the translation of genetic therapeutics in wound repair. CMP-based gene transfer reduces the dangers of

therapeutic escape and abrogates concerns of low activity in protein/serum-rich environments. In particular, we demonstrated previously that GPP-modification of PEI polyplexes greatly enhanced control over both the extent and duration of transgene expression through utilization of the reversible, serum-stable affinity between CMPs and natural collagen. Enhanced control and activity were demonstrated both *in vitro* [34] and *in vivo*, using a murine subdermal repair model[56]. Additional studies concluded that these improvements were the result of superior serum-stability and endocytic trafficking driven by increases in polyplex affinity for collagen and the natural process of collagen remodeling. While other reports have utilized collagen remodeling/proteolytic sensitivity to achieve cell-triggered release, CMP-modification provided a tool for direct integration of both release and endocytic uptake with collagen remodeling, while providing enhanced serum-stability.

Having demonstrated the ability to integrate gene delivery with collagen remodeling, our objective in this work was to incorporate our DNA/collagen system into a wound environment and demonstrate its benefits for promoting key wound repair activities. Cell invasion, proliferation, and ECM remodeling are prerequisites for both wound healing and the initiation of gene transfer activity from the GAMs, and therefore PDGF-B, which is capable of stimulating these cellular responses, was a natural target. Through modification with a CMP containing an established, GPP-rich sequence, we were able to identify significant improvements in both PDGF-BB expression and activity. GPP modification substantially expanded the length of time the DNA/collagen gels could be exposed to physiological-simulating conditions (37^{o}C in serum-supplemented media), from 0 days to 10 days, and still observe enhanced PDGF-BB expression. While MMP concentrations are elevated in the wound

environment, no MMPs or wound-relevant proteases were added during the pre-treatment step, based on studies showing localization of MMPs in the cellular microenvironment at the wound edge or immediate tissues.[65] Utilizing preincubation to simulate the environment of an applied collagen-based dressing before cell invasion, this study suggests that GPP modification can be used to prolong the activity of DNA polyplexes during the process of cellular invasion, which often occurs over a period of weeks to months in chronic wounds. Varying the display of GPP on the polyplex from 20% GPP-PEI/total PEI to 50% GPP-PEI/total PEI expanded the period the gels could be exposed to physiological-simulating conditions from 5 days to 10 days, suggesting that GPP display could also be used to tailor the extent and period of PDGF-BB expression as required. Additional studies have also shown varying CMP sequence allows for further tailoring of retention/release, suggesting additional tuning could be achieved with relative ease. For example, it was demonstrated that lengthening the CMP sequence (GPO_7 vs. GPO_{10}) could be used to decrease the CMP-collagen initial dissociation index (IDI) by 1.7-fold and expand retention/release of a GPP-modified polymer from approximately 7 days to over 12 days.[68]

Our studies also highlighted the benefits of GF gene vs. GF protein delivery, and demonstrated in particular the mechanisms by which GPP-modification of gene vectors amplifies these benefits. For instance, we demonstrated the ability to trigger cellular responses similar to those observed in rPDGF-BB/collagen systems, yet with 3 orders-of-magnitude lower levels of local PDGF-BB expression than the levels of rPDGF-BB delivered in GF-collagen therapies.[6,8,37,43,59] DNA/collagen gels containing *PDGF-B*-encoding polyplex were able to trigger cellular behaviors not observed in controls with luciferase encoding polyplex. Cell proliferation was increased by up to

65%, gel contraction was increased by as much as 2-fold, and cell migration was triggered via cell-produced PDGF-BB gradients. When *PDGF-B*-encoding polyplexes were used in collagen bi-layer migration studies, as many as 90% of cells had migrated 300 μm into the gel within 48 h post cell seeding. Furthermore, cells were visible at this level (300 μm-345μm) within 24 h post cell seeding, and given that the typical speed of NIH/3T3 cell migration in collagen is less than one cell diameter per hour, the determined speed of these cells (12.5 μm/h) indicates both the directionality and consistency of their migration.[31] Our findings accentuate the importance of cell-mediated expression and micro-localization encouraged by efficient gene delivery, and show how gene delivery can be used to overcome the high costs and safety concerns associated with high doses of GFs. Consistent with the enhanced levels of PDGF-BB detected in samples containing GPP-modified, PDGF-expressing polyplex, GPP-modification was found to stabilize the systems even after prolonged periods of preincubation. For example, increases in proliferation were observed after a 10-day preincubation, whereas no increases were observed in any of the unmodified samples preincubated for this length of time. Collagen remodeling and chemotaxis were also enhanced for up to a 10-day preincubation in GPP-modified samples, vs. only a 5-day preincubation in unmodified samples. The extent of GPP-modification was also found to expand other observable cellular responses, underscoring the flexibility CMP-modification provides. For example, cell counts in 20% GPP-PEI/total PEI samples were increased by up to 65% relative to luciferase controls, and these levels remained statistically greater than the controls for up to a 7-day preincubation. Alternatively, cell counts in the 50% GPP-PEI/total PEI samples preincubated for 5 or 7 days were consistently about 50% greater than those in the luciferase controls, and these counts

were statistically indistinguishable from those recorded in the 20% GPP-PEI/total PEI samples. Cell counts remained significantly enhanced for up to a 10-day preincubation in the 50% GPP-PEI/total PEI samples. The fact that increased proliferation was not observed for the same times periods over which enhanced chemotaxis and remodeling were observed is consistent with the finding that higher concentrations (>5 ng/mL) are required to promoted mitosis, whereas lower concentrations (<1 ng/mL) are sufficient for migration.[2]

The benefits of GPP-mediated PDGF-BB gene delivery also translated in a 3-D wound model where wound closure was defined as the point when the average cell density in the defect matched the average cell density in the area surrounding the defect. Collagen-based scaffolds containing *PDGF-B*-encoding polyplex promoted increased invasion and elevated cell densities within the defects within 3 days (unpublished data). Mirroring the results observed in the preincubation studies, GPP-modification appeared to preserve the activity of the scaffolds in environments containing nucleases and serum proteins at physiological temperatures. Scaffolds with the 20% GPP-modified polyplex had wound closure rates 2.3-fold and 1.6-fold faster than those observed in the empty collagen scaffolds and in the scaffolds containing unmodified *PDGF-B*-encoding polyplex, respectively, over a 5-day closure period. By day 10, wounds containing the 20% GPP-modified polyplex scaffolds had induced nearly a 2.1-fold and 2-fold increase in wound closure compared to wounds in which empty collagen and unmodified polyplex scaffolds were applied. The relative rate of closure was comparable to that achieved using in other *in vitro* wound models[37] as well as *in vivo* models[8,28] of soft tissue wounds. For example, the application of *PDGF-B* encoded by plasmid or adenovirus DNA vector embedded in collagen matrix

induced a 2.5-fold increase in wound closure in 6-mm or 8-mm ischemic skin wounds in rabbits, relative to a negative control.[8,28] To achieve the same effect in our wound model with PDGF-BB, an order of magnitude increase in rPDGF-BB exposure was required. The greater dosage not only necessitates greater treatment costs, but also increases the danger of off-target responses, as PDGF-BB leakage from collagen-gels and sponges is a major problem in protein-based delivery.[69] Moreover, when rPDGF-BB was applied in the wound model at the same concentration as that expressed in the GPP-modified collagens (1 ng/mL), wound closure was indistinguishable from the empty collagen control, further supporting the fact that efficient gene delivery allowed dramatically reduced dosages. The identical levels of overall wound closure via gene delivery were achieved in this model through application of an 8:2 (m/m of DNA in the 20% vs. 50% GPP-modified polyplex) mixture of 20%/50% GPP-PEI/total PEI polyplex. Five days after application, the percent of wound closure was identical to that in the 20% GPP-PEI/total PEI sample, but while the 20% sample plateaued at approximately 75% wound closure, the combined polyplex sample achieved over 90% wound closure, the same as the high dosage rPDGF-BB control. These results highlight the versatility of CMP-modification, where both CMP sequence and display can be used to tailor not only release, but also the extent and duration of GF expression. While these outcomes have been demonstrated here in a simplified wound model, the results support the potential utility of localized gene delivery over protein delivery in the more general context.

4.5 Conclusion

GF gene therapies may have the potential to overcome the innate incompatibility many current GF therapies have with the wound bed, through

promoting on-demand GF production that better mimics endogenous repair responses by allowing host cells to orchestrate sustained GF expression, microlocalization, and activity. CMP-modification of DNA carriers, particularly PEI polyplexes, has been demonstrated by our labs and shown to enable the hijacking of not only the cellular machinery needed to express GFs (or any gene of interest), but also the cellular mechanisms inherent in the natural process of collagen remodeling to achieve cell-triggered delivery and enhanced endocytic uptake. In this work, GPP-modification was shown to enhance expression of functional GF (PDGF-BB) and trigger essential cell behaviors associated with wound repair. Furthermore, CMP-mediated gene delivery achieved similar levels of wound closure as the levels reached when an order of magnitude more rPDGF-BB was applied. The fact that a combination of 20% and 50% GPP-PEI/total PEI polyplexes could be used to tailor this response highlights the versatility of this approach, and identifies additional tuning factors which have yet to be fully explored. Both the tunablity of this method and its ability to hijack collagen remodeling, a process that already occurs in excess in wounds, make this an ideal method for achieving efficient GF delivery in the wound bed.

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Chapter 5

COLLAGEN-MIMETIC PEPTIDES AS A TOOLS FOR PROLONGED, MULTIGENE EXPRESSION CONDUICIVE FOR GF WOUND DELIVERY

Wound healing is a complex process governed by intricate cellular signaling mediated by numerous components including GFs. To promote healing in nonhealing, chronic wounds, in which healing has become uncoordinated and GF activity compromised, the topical and sustained delivery of vital GF proteins and GF genes has aided in promoting healing; however, clinical translation has been stagnated by delivery obstacles, validated safety concerns with oncogenic and off-target effects, and an overall lack of efficacy. Recent studies have suggested clinical failure may be due to the focus of most current delivery systems on unitary or dual GF protein/gene delivery without controlled release.[1-6] In this chapter, a CMP-based strategy for mimicking the endogenous healing via controlled delivery of multiple GFs is purposed. Specifically, the capacity to tailor the delivery of multiple proteins, both reporter and functional, within the same system was demonstrated over periods of nearly a month, a relevant time frame in chronic wound repair. The expression profiles of two GFs integral for different stages of healing, PDGF-BB and KGF, were individually tailored within the same collagen-based scaffolds and expressed at levels capable of triggering desirable cell behaviors.[5-19] Moreover, the observed capacity to alter expression of multiple genes, suggests the CMP/collagen system may be used to

tailor the expression of a plethora of additional GFs and signaling molecules for different regenerative medicine applications.

5.1 Introduction

Wound healing is a complex process consisting of several integrated stages, including inflammation, granulation/neoangiogenesis, re-epithelization, and tissue remodeling/maturation. Each stage is governed by intricate cellular signaling orchestrated by reciprocal ECM-cell interactions and a plethora of cytokines, chemokines, growth factors (GFs), and nuclear receptors facilitating promitogenic gene expression and cell division (Figure 5.1). Coordination of these processes is vital in wound repair and disruptions, most commonly the result of ischemia compromised cellular functions and compromised GF activity, result in chronic nonhealing wounds.

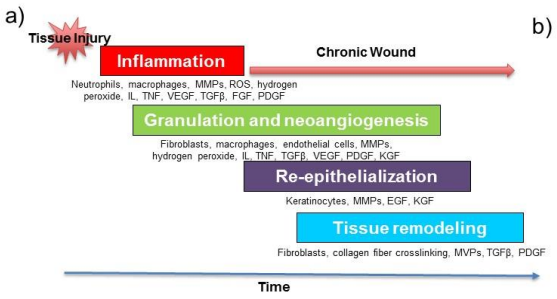
As summarized in Figure 5.1, a multitude of signaling molecules, cell types, and ECM components are required for each process underlying repair, such as ECM regeneration and angiogenesis, to facilitate complete healing. For instance, many GFs are required for different time periods during the stages of healing; Platelet-derived growth factor (PDGF), transforming growth factor (TGF), epidermal growth factor (EGF), and insulin-like growth factor (IGF) mitigate cell recruitment and activation during the early stages of wound repair, TGF, EGF, and PDGF are vital for matrix [5,6,16,19-26]synthesis throughout healing, and FDF, PDGF, and vascular endothelial growth factor (VEGF) mediate angiogenesis and tissue maturation. Understandably, recognition of the integral roles of GFs has led to the development of GF-based therapies in chronic wound repair. However, as previously discussed, clinical translation of both topical and sustained release therapies of GF proteins and GF genes has been limited. Clinical failure has largely been blamed on insufficient delivery and

oncogenic and immunogenic concerns, yet emerging results strongly suggested insufficiencies are exasperated by ignorance of the synergistic behaviors of GFs and focus on the delivery of a single factor or component opposed to combinations. For instance, as previously discussed, the only GF treatment approved for diabetic foot ulcers (DFUs) treatment, Becaplermin (Regranex®), consists of repetitive, extraphysiological doses of a single GF, PDGF-BB and boasts only modest improvements in the number the number of healed DFUs (<10%) due in part because of the PDGF-BB-stimulated hyper-granulation which impedes epithelization and progression of the reparative process[15,27]. The simultaneous or sequential delivery of additional GFs has the potential to trigger progression through the orderly reparative process.

Acknowledgement that the complex cellular processes of cell migration, differentiation, and proliferation require specific GFs with time-dependent and spatial distributions, has led to the development of multiple GF delivery strategies. Composite polymer systems have been engineered for spatio-temporal delivery of multiple GFs with specific release kinetics. For instance, the early stages of angiogenesis require VEGF, FGF, and angiopoietin-1 to disrupt the structure of existing blood vessels, followed by the release of angiopoietin-1 and PDBG-BB to stabilize newly formed blood vessels. In turn, the sequential and simultaneous delivery of these GFs have been demonstrated to enhance vascularization compared to when solely VEGF is delivered.[28,29] In a similar manner, proteolytic degradation mediates sequential delivery of BMP-2 and IGF-1 from a degradable layered structure, BMP-2 and TGF-β3 from degradable alginate hydrogels, and VEGF and BMP-2 from gelatin microparticles and demonstrated to promote enhanced healing compared to the

delivery of a single growth factor.[30] Additionally, the inherently different affinities VEGF, PDGF-BB and TGF-β1 have for alginate have been utilized to govern release kinetics and promote enhanced vascularization compared to when only basic fibroblast growth factor (bFGF) was delivered[30]. As outlined in Chapter 1, delivery systems that chemical immobilize or physically encapsulate GFs in delivery systems have been similarly employed to govern delivery; however, the obstacles that inhibit the delivery of single factors via these techniques, namely off-target delivery and oncogenic concerns, further complicate the delivery of multiple factors.

Multi-growth factor gene delivery strategies have also garnered interest due to the potential benefits of GF gene vs. GF protein therapies such as reduced costs and GF microlocalization and improved bioactivity. To mediate sequential delivery, polymer systems similar to those engineered for protein delivery, have been utilized. For instance, tailorable delivery of multiple genes, such as those encoding for BMP-2, VEGF, EGF-1, and TGF-β1, has been achieved through encapsulating genes into biodegradable polymeric microspheres with different degradation rates and subsequently bound to biomaterials.[30,31] Gene delivery is then dependent on a combination of polymeric delivery and DNA diffusion and sequential delivery could be tailored via choice of polymer, for instance, poly(L-lactic-glycolic acid) is a suitable candidate owing its degradation rate may be tailored via manipulating its molecular weight and copolymer ratios. As detailed in Chapter 1, additional strategies involving the encapsulation and chemical binding of viral and non-viral DNA have been attempted, however safety concerns with off-target delivery and inefficiencies with gene transfer inhibit their development.



b)

Action	Factors
Fibroblast Migration/Replication	PDGF, EGF, FGF, TGF-β, TNF, IL-1
Keratinocyte Replication	HB-EGF, FGF-7, HGF
Angiogenesis	VEGF, Angiopoietins FGF
Collagen Synthesis	TGF-β, PDGF
Collagenase Secretion	PDGF, FGF, TNF, TGF-β inhibits
Monocyte Chemotaxis	Chemokines, TNF, PDGF, TGF-β

Figure 5.1. Summary of Vital Healing Factors during the 4-Step Healing Process. a) A timeline outlining the vital healing factors (GFs, cytokines, healing components) involved in each step of wound repair and the processes b) they mediate. Notably, individual factors are required for differing time periods depending on their roles and the majority of processes underlying repair require multiple factors and many factors are integral for many processes.

In this work, we have introduced a new approach for achieving tailorable delivery through both mediating release and a viral-like uptake. Specifically, CMP-modification of polyplexes was demonstrated to stably integrate polyplexes into collagen scaffolds, the most common biomaterial, in a tailorable manner for periods of up to a month and preserve polyplex activity in the presence of serum for 2-weeks. Transfection studies demonstrated release to be protease-mediated and CMP-facilitated interactions with collagen fragments post-scaffold release, promoted the targeting of an endocytic uptake previously linked to high efficiency expression.[32] Furthermore, CMP/collagen delivery systems were observed to enable tailoring of the extent and length of transgene expression in murine ECM depot models directly dependent on CMP-modification and also promoted enhanced wound closure in *in vitro* wound models through mediating PDGF-BB expression. Notably, in order to promote healing in the wound model, collagens with mixtures of polyplex containing

different amounts of CMP were applied to expand the PDGF-BB expression time periods. Specifically, polyplexes with relatively fast and slow release kinetic (e.g. 10% GPP-PEI and 50% GPP-PEI respectively) were used to facilitate quicker and more sustained expression.

In this chapter, the ease of varying CMP display on polyplex and in turn, its release kinetics and activity, was utilized to manipulate the expression of multiple proteins within the same system. Mechanistic studies expressing reporter proteins GFP and GLuc demonstrated a high degree of tailorability and subsequent studies with genes encoding for PDGF-BB and KGF, demonstrated a similar capacity to tune expression via CMP modification of functional studies albeit in a less predictable manner due to GF-triggered cellular behaviors. Additionally, in this chapter, the capacity to further expand expression time periods via the establishment of highly stable collagen-based scaffolds through fibrin incorporation is discussed. The ability to encourage tailorable expression of multiple factors for prolonged periods via a method that also encourages improved gene transfer, has widespread potential in regenerative medicine applications.

5.2 Materials and Methods

5.2.1 Materials

Type I bovine collagen was purchased from Advanced BioMatrix (San Diego, CA) and pCMV-PDGF-B and pCMV-KGF plasmid was purchased from Origene Technologies, Inc. (Rockville, MD). pDNA was amplified in NEB 5-α electrocompetent *E. coli* purchased from New England Biolabs and purified from bacterial culture using a Qiagen Megaprep Kit (Valencia, CA), following the

manufacturer's protocols. The Mouse/Rat PDGF-BB and KGF Quantikine enzyme-linked immunosorbent assay (ELISA) kit was purchased from R&D Systems (Minneapolis, MN). Fmoc-protected amino acids were purchased from Anaspec (Fremont, CA). H-Rink amide ChemMatrix® resin was purchased from PCAS Biomatrix (Quebec, Canada). O-Benzotriazole- N,N,N',N'-tetramethyl-uronium-hexafluoro-phosphate (HBTU) was purchased from Novabiochem (San Diego, CA). High performance liquid chromatography (HPLC)-grade N,N-dimethyl formamide (DMF), acetonitrile, trifluoroacetic acid (TFA), CellTracker^{TM} Deep Red, and cell culture reagents, including Dulbecco's modified Eagle's medium (DMEM), Dulbecco's phosphate buffered saline (PBS), penicillin-streptomycin (P/S) ,and trypsin were purchased from Fisher Scientific (Fairlawn, NJ). Fetal bovine serum (FBS) was purchased from Corning (Manassas, MA). Collagenase I was purchased from Worthington Biochemical Corp (Lakewoord, NJ). Piperidine, 4-methylmorpholine, all cleavage cocktail components, branched PEI (25 kDa), calcium chloride, thrombin, and fibrinogen were purchased from Sigma-Aldrich (St. Louis, MO).

5.2.2 Methods

5.2.2.1 Multi-gene Modified Collagen/Fibrin Co-gel Preparation

The CMP [GPP: $(GPP)_3GPRGEKGERGPR(GPP)_3$] used in prior studies[33-35] was synthesized using Fmoc solid phase peptide synthesis and purified using reverse phase-HPLC. GPP was conjugated to PEI using Michael-type addition chemistry and the conjugate (GPP-PEI) was used to prepare GPP-modified polyplexes as previously described.[35] Using a variation of well-established polyplex formation protocols,[32,36] equivolumetric solutions of PEI and DNA in 20 mM HEPES buffer (pH 6.0) were

mixed to produce a final solution with an amine to phosphate ratio (N:P) ratio of 10. To incorporate GPP, the GPP-PEI conjugate was preincubated at 50ºC for 30 minutes to prevent triple-helical hybridization of GPP, and a specified percent of PEI used to create the polyplex was replaced with the GPP-PEI.

To prepare the collagen/fibrin co-gels, lyophilized polyplex are mixed with neutralized bovine collagen solution (8:1:1 = Nutragen®: 10x PBS: 0.1M NaOH; final collagen concentration: 4 mg/mL) in a pre-chilled container and subsequently placed on ice. A pre-chilled thrombin/Ca^{2+} solution (DMEM supplemented with thrombin and 0.2% 2N Ca^{2+}) and fibrinogen solution (in 20 mM HEPES buffered saline) are then added to the collagen/polyplex solution with thorough mixing in between each addition, resulting in a final solution of 2 mg/mL of collagen, 2.5 to 5 mg/mL fibrinogen, 2 U/mL of thrombin, and 50 µg/mL of DNA. Mixtures are then immediately plated and incubated overnight at 37ºC to allow gelation.

5.2.2.2 Detection of GLuc and GFP in Multigene Fibrin Co-gels

DNA/collagen/fibrin co-gels were prepared as described above with 250 µL of DNA/collagen solution in 8 well plates (0.8 cm^2 surface area/well). Gels were incubated at 37ºC overnight to allow gelation, after which gels were washed three times with 200 µL of PBS and complete medium (DMEM with 10% FBS and 1% P/S) and 200 µL of complete media was added 1 h post cell-plating to allow rehydration. 50,000 NIH/3T3 cells were seeded per well and cells were cultured under standard conditions (37ºC, 5% CO^2). The cells were allowed to adhere over a period of 6 h before treatment with tumor necrosis factor-alpha (TNF-α) (10 ng/mL), a well-known stimulator of MMP production. [37] To account for the long half-life of secreted protein GLuc of almost 7 days [38] and the need to replenish media to prevent cell death, kinetic

GLuc expression was analyzed in this study. Specifically, every 2 days, conditioned media was collected from the samples, stored at -20ºC, and subsequently replaced with fresh media after which samples continued to be cultured under standard conditions. GLuc expression was quantified through detection of luminescence in 10 µL of the media collected from each sample with a BioLux® Gaussia Luciferase Assay (NE Biolab®; Ipswich, MA), according to the manufacturer's protocol.

Alternatively, GFP is not secreted and has a half-life of about 26 h.[38] Levels of GFP expression were examined using fluorescence readings of the cell lysate, therefore the majority of fluorescence detected is the result of GFP expressed over the past 2 days. Specifically, to quantify GFP expression, cells were recovered from the gels incubated for 4, 8, 12, or 16 days through use of a collagenase (0.1 U/mL PBS)/dispase® (0.8 U/mL PBS)) digestion solution, accompanied with gentle pipetting to break the gel apart. After a 45-minute digestion at 37ºC, cells were pelleted via centrifugation at 2,000 g for 5 minutes. The cell pellet was re-suspended in 200 µl of non-denaturing lysis buffer (1% w/v Triton X-100; 50 mM TrisCl, pH=7.4, 300 mM NaCl, 5 mM EDTA, and protease inhibitor) for 5 minutes on ice and the cell lysate solution fluorescence was immediately quantified using a Glomax Multimodal Plate reader. To compare expression, luminescence and fluorescence readings were normalized by their respective highest readings.

5.2.2.3 Detection of functional protein expression

DNA/collagen/fibrin co-gels were prepared with 250 µL of DNA/collagen solution in 8 well plates (0.8 cm^2 surface area/well). Gels were incubated at 37ºC overnight to allow gelation, after which gels were washed with three 200 µL of PBS and complete medium (DMEM with 10% FBS and 1% P/S) and 200 µL of complete

media was added 1 h post cell-plating to allow rehydration. 50,000 NIH/3T3 cells were seeded per well and cells were cultured under standard conditions (37ºC, 5% CO^2). Every 4 days, 100 µL of cultured media was carefully collected from each sample and replaced with fresh media. Media samples were stores at -20ºC, until a sandwich ELISA was used to determine PDGF-BB and KGF concentrations in the conditioned media. PDGF-BB and KGF were both quantified using a commercially available Quantikine ELISA kit in a 96-well plate format, according to manufacturer's instructions. A seven-point standard curve was used to quantify the concentrations, spanning a range from 0 to 2000 ng/mL PDGF-BB or KGF. Each sample was read twice with a Glomax Multimodal Plate reader (Sigma), and the average of the two readings was used to calculate the concentration of PDGF-BB or KGF in the sample. PDGF-BB and KGF have similar half-lives of 8-13 h [18] and 12 h[8] in NIH/3T3 cell culture therefore the majority of detected proteins in both cases, were expressed in the past 24 h.

5.3 Results

5.3.1 The enhanced stability of collagen-fibrin co-gels enables extended CMP-mediated expression

To achieve the prolonged DNA delivery vital for regenerative medicine applications like chronic wound repair, a scaffold stable for the required duration is required. To expand the periods of expression previously observed in collagen gels, CMP-mediated gene expression and cell behavior in collagen/fibrin co-gels co-gels, previously shown to have enhanced stability relative to collagen gels, were

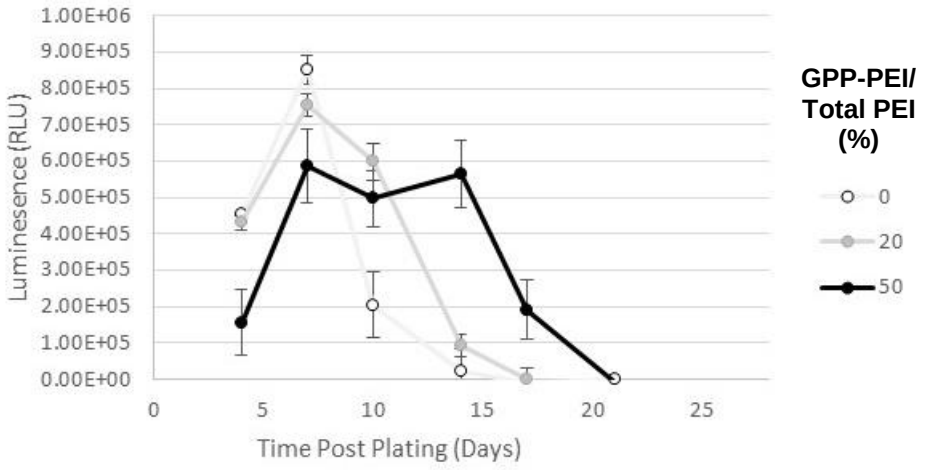


Figure 5.2. Kinetic Expression of Gluc in Collagen/Fibrin Co-gels. Kinetic expression of Gluc was accessed via detection of luminescence in conditioned media replaced at each time point. The reported luminescence therefore indicates the expression of fresh protein subsequent to the previous collection (3 or 4 days prior) and permits assessment of polyplex activity while negating the effects of reporter protein degradation kinetics. Each set of data points represents the mean ± standard deviation for a total of 3 separately prepared and analysed samples.

examined.[24,39-45] Polyplex retention experiments revealed a profound enhancement in initial DNA retention in the collagen/fibrin co-gels compared to the collagen gels. When 20-100 μg of complexed DNA was incorporated into gels comprised of 2 mg/mL of collagen and 5-10 mg/mL of fibrinogen, initial DNA retention efficiency was approximately 100% regardless of the level of CMP incorporation in the polyplex. On the other hand, the maximum DNA retention in co-gels containing 2 mg/mL of collagen and 2.5 mg/mL of fibrinogen was significantly reduced at 86 μg/mL relative to the compositions with high fibrinogen concentrations when gels were initially prepared with 100 μg of complexed DNA/mL, however initial DNA retention was still

greatly enhanced compared to collagen gels (5.8- and 4-fold for 0% and 50% GPP-PEI modified polyplex respectively) and similarly independent of CMP-modification. Moreover, long-term release studies conducted in PBS at 37^{o}C demonstrated that less than 12% of initial retained unmodified polyplex were released over a 7-day period as detected via picogreen assays of PBS collected from the samples daily (not shown).

In subsequent studies, co-gels comprised of 2 mg/mL of collagen, 2.5 mg/mL of fibrinogen, and 50 µg of complexed DNA. The composition was chosen based on preliminary cell behavioral studies which showed impaired cellular invasion of matrices with higher fibrinogen concentrations (5 and 10 mg/mL). Additionally, the 50 µg/mL of complexed DNA were incorporated into the co-gel because the addition of larger amounts (e.g. 100 µg/mL) of complexed DNA had observable effects on gelation which decreased overall collagen retention in the scaffold as shown via a retention/release study with pre-fluorescently labeled collagen (roughly 63% of collagen was released v. 26%). However, even at the lower concentrations of DNA, approximately 2.5-fold more was able to be encapsulated stably in the co-gels relative to collagen gels.

Gluc expression studies were subsequently conducted to confirm the expected expansion of gene expression and the effect of CMP-modification as shown in Figure 5.2. Gluc was accessed via detection of luminescence in conditioned media replaced at each time point, therefore the reported luminescence indicates the expression of fresh luciferase expressed subsequent to the previous collection (3 or 4 days prior) and permits assessment of polyplex activity while negating the effects of reporter protein degradation kinetics. Incorporation of fibrin into the collagen gels was confirmed to expanded the time period in which increases in GLuc expression were observed from

4 to 9 days post cell seeding for unmodified polyplex gels and 5 days to 17 days post cell seeding for 50% GPP-PEI modified polyplex gels. General overall expression was also approximately an order of magnitude greater compared to that observed in collagen gels. Additionally, CMP-modification has a direct impact on polyplex activity within the co-gel, indicated by significant luciferase expression for up to 17 days in the 50% GPP-PEI sample and 14 days for the 20% GPP-PEI compared to 10 days for the unmodified samples. Cumulative expression was also greater in both GPP-modified samples (approximately 19% and 30% greater in 20% and 50% GPP-PEI samples respectively) relative to the unmodified sample. Furthermore, 50% GPP modification promoted significantly more consistent expression over a period of about a week (7 to 14 days post cell seeding) relative to the other samples strongly suggesting sustained release is promoted by GPP-PEI modification.

5.3.2 CMPs display on polyplexes enables individual manipulations of reporter protein expression profiles.

The ability to achieve controlled multigene delivery has tremendous benefit in the intricate process of wound repair. Through incorporation of polyplex encoding GFP or GLuc and manipulations of CMP display, the ability to tailor expression within collagen/fibrin co-gels was examined. As shown in Figure 5.3, the activity of both reporter proteins was normalized by the maximum luminescence or fluorescence detected for GLuc and GFP studies respectively. The findings show that both proteins achieved max expression over approximately day 6-8 when both pGFP and pGLuc polyplex were unmodified, while max expression was expanded to day 14-16 for GLuc and day 10-12 for GFP when both has 50% GPP-PEI modification. In comparison, when pGFP polyplex was unmodified but pLuc has 50% GPP-PEI

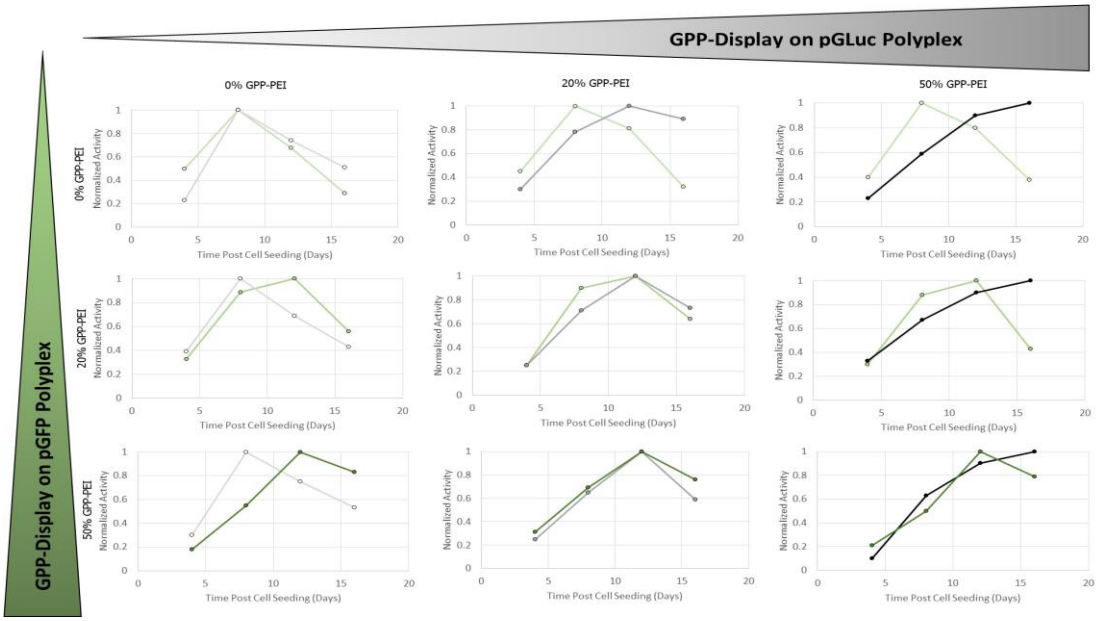


Figure 5.3. Tailored delivery of GFP and GLuc. The effect of different levels of GPP-modification on GLuc (Gray-scale) and GFP expression (Green-scale) was accessed via detection of luminescence and fluorescence respectively. To compare the expression, readings were normalized by the maximum luminescence or fluorescence readings. Each data point represents the mean ± standard deviation for a total of 2 separately prepared and analysed samples.

modification, maximum expression of GFP remained detected over day 6-8 while GLuc maximum expression was detection was over day 14-16. Furthermore, when unmodified pLuc polyplex and 50% GPP-PEI polyplex were employed, max expression of Gluc and GFP were detected over day 6- 8 and day 10-12 respectively. The other samples modified to various degrees, demonstrated similar trends where maximum expression times and GPP-modification were directly related.

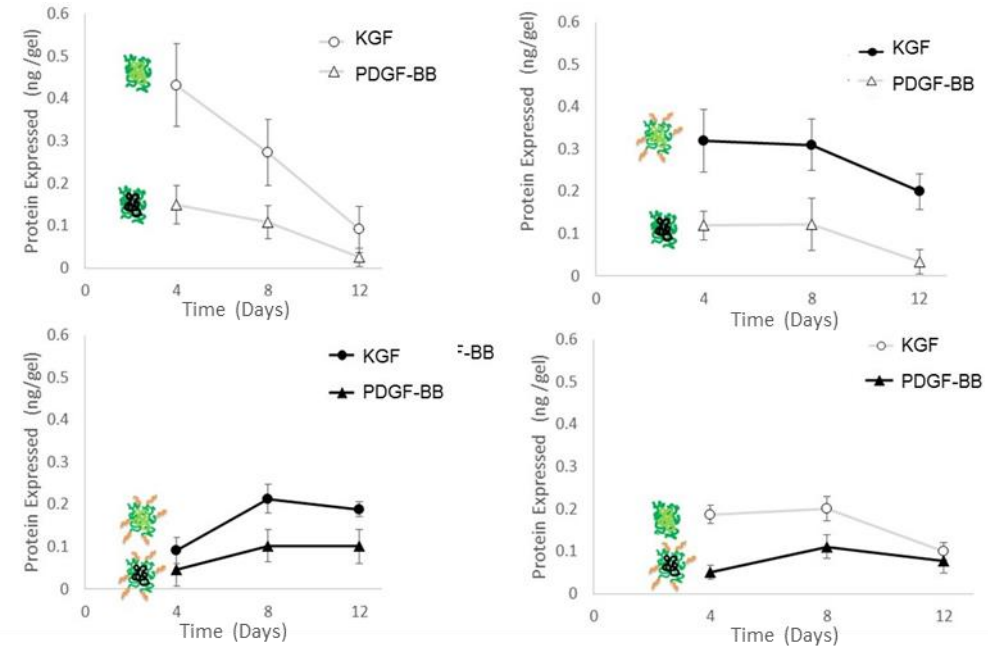


Figure 5.4. Tailoring the expression of GFs in collagen/fibrin co-gels via varying CMP display. The expression of PDGF-BB and KGF was monitored within the same samples via ELISA. Empty markers indicate the application of unmodified polyplex and black markers indicate the application of 50% GPP-PEI modified polyplex. Additionally, the illustrations denote polyplex modification and DNA where the following materials are: CMP, PEI, pPDGF-B, and pKGF. The data represent the mean +/-standard deviation of 3 separately prepared and analyzed samples.

5.3.2 Function Protein (GFs) expression profiles can be tailored via CMP display.

Given the therapeutic value of multi-protein delivery, the capacity of the CMP/DNA/collagen system to tailor the expression of GFs, PDGF-BB and KGF was explored through incorporation of polyplex encoding for the two GFs and modified with different amounts of GPP. As demonstrated in Figure 5.4, GPP-modification caused significant alterations in expression profiles. Max expression for both proteins

when expression was mediated by unmodified polyplex occurred over day 0-4 where KGF concentrations were 2.9-fold high then PDGF-BB concentrations. By day 8, PDGF-BB and KGF expression had decreased by 23% and 34% respectively, and by day 12, KGF expression has decreased a further 63% while levels of PDGF-BB levels were negligible. Alternatively, when both were 50% GPP-PEI modified, expression levels of both proteins were significantly low than in the unmodified sample (decreased by 88% and 31% for KGF and PDGF-BB respectively). Maximum expression was not achieved until over day 4-8 with a 2-fold increase from days 0-4, and protein expression remained relatively constant for the 12-day duration of the study. In samples in which pKGF polyplex was GPP-PEI modified and pPDGF-B polyplex was not, PDGF-BB expression remained almost identical to that observed in samples in which neither was modified; however KGF expression was decreased by approximately 28% over day 0-4 relative to the unmodified sample and overall expression was more KGF concentrations were more consistent. In samples in which only pPDGF-BB was GPP-modified, overall levels of expression for both proteins are muted when compared to the results when neither were modified.

5.4 Discussion

Prolonged multi-GF gene expression has been demonstrated be have utility in wound care. In this chapter, the ability to tailor the expression of reporter proteins, GFP and GLuc, and GF, PDGF-BB and KGF, was demonstrated. Sustained delivery requires the application of a stable scaffold, therefore the first part of this work explored the utility of incorporating fibrin in to our CMP/DNA/collagen system based on studies demonstrating enhanced stability of collagen/fibrin co-gels. [6,7,16,22,24,39-45] A

composition comprised of 2.5 mg/mL of fibrinogen and 2 mg/mL of collagen with 50 μg/mL of complexes DNA was chosen, based on the observation that higher fibrinogen concentrations greatly impede cellular invasion which is vital for transfection and higher DNA concentrations affects gelation and reduce the co-gels retention of collagen which is integral from CMP binding. Furthermore, the co-gel still has the capacity to incorporate approximately 2.5-fold more DNA then collagen-gels alone and longer expression periods (from < a week to 2 weeks).

The length of time polyplex activity was observed in the co-gel was similar to that achieved by another system where NIH/3T3 cells were co-encapsulated into fibrin with PEI polyplex encoding for reporter protein secreted alkaline phosphatase. The same concentration of complexed DNA was used (50 μg/mL) and a similar protein content (5 mg/mL fibrin v. 2.5 mg/mL of fibrin and 2 mg/mL of collagen) was employed,[43] and while the length of time gene expression occurred in this system and our co-gels were similar in the case of the unmodified polyplex (up to 11 in the fibrin system v. 9 days in the co-gel), GPP-PEI modification greatly expanded when increases in expression were observed to 17 days. Significantly, at this composition, the initial DNA retention efficiency and release/retention was not GPP dependent and overall very slow, however transfection studies suggest GPP-modification may aid in achieving sustained release in the presence of cellular components and proteolytic degradation. The results suggest the new scaffold may aid in the prevention of target off-target responses, while still providing a stable scaffold to mediate release. Additional studies in which fluorescently labeled polyplex release either during cell culture or exposure to cell cultured media, would provide more conclusive evidence of this ability.

The ability to tailor release of both GFP and GLuc and KGF and PDGF-BB was also expressed. While the mechanistic study with the reporter proteins proved solid evidence of the ability to tailor the expression of two proteins within the same system, the GF tailoring results are harder to predict. In summary, as expected, the levels of expression are highest at earlier time periods when unmodified polyplex are employed and quickly reduce at longer time points and KGF concentrations are dependent on PDGF-BB concentration. For example, KGF expression is delayed and reduced regardless of GPP-modification when co-delivered with 50% GPP-PEI pPDGF-BB polyplex. This finding is likely because PDGF-BB triggers NIH/3T3 proliferation and migration,[9,12] which are both behaviors known to promote increased polyplex uptake and in turn, transfection. On the other hand, KGF is not recognized to trigger any behaviors in NIH/3T3. In the future, as purposed in more depth in Chapter 6, co-culture experiments with cells responsive to KGF such as keratinocytes, may provide more insight on the potential of CMP/DNA/collagen systems to tailor both multi-gene expression and behaviors in various cell types relevant to wound repair applications including keratinocytes and macrophages.

5.5 Conclusions/Future work

CMP-mediated delivery in collagen/fibrin co-gels was demonstrated to enable the tailoring of both reporter proteins and GFs. The incorporation of fibrin greatly reduced non-cell mediated release of polyplexes independently of CMP-modification, however CMP-modification was observed to preserve polyplex activity. The finding suggests the fibrin/collagen composition could be used to prevent vector escape, while still providing a stable scaffold to facilitate CMP-mediated release. In the future, additional GF may be expressed and cellular responses monitored. Furthermore,

additional study of approaches for overcoming slowed cellular invasion of these co-gels relative to collagen gels, should continue. While high levels of transfection have been observed in the co-gels, expression is delayed (4-7 days) relative to that observed in collagen gels (2-3 days) and likely due in part because of slower matrix invasion. Studies with PEG-crosslinked gels via the addition of 0.5-1 mg/mL of SG-PEG-SG to the precursor solution of 2 mg/mL collagen and 2.5 mg/mL fibrinogen were found to both reduce gelation time via a simple "tilt test" in which samples are rapidly moved to confirm gelation and to also improve cellular invasion by 23%-14% by day 4 post plating; however current crosslinking strategies have been shown in preliminary studies to reduce gene expression and CMP-mediated enhancements in expression. This may be the result of PEI crosslinking to the scaffold via amine reactivity or alterations in scaffold degradation and in turn polyplex release. Overall, this approach shows great promise and a method for promoting prolonged expression and cellular invasion would be compelling in wound care.

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Chapter 6

CONCLUSIONS AND FUTURE RECOMMENDATIONS

6.1 Summary

Chronic non-healing wounds represent an enormous, worldwide burden, causing significant morbidity and mortality, especially in the diabetic, elderly, and obese. They affect approximately 6.7 million patients in the United States and the incidence is predicted to increase by 2% a year over the next decade.[1-4] Cellular and pathophysiological abnormalities within the wound bed have greatly limited healing incidences with standard therapies (e.g. 30% at 20 weeks [5,6]). Specifically, the chronic wound environment is characterized by uncoordinated healing and exaggerated inflammation. Altered cellular phenotypes and an aberrant extracellular environment comprised of fibrin, necrotic tissue, and bacteria, cause critical reductions in GF production, stability, accessibility, and activity.[1,2]

Accordingly, sustained release growth factor (GF) protein therapies have been developed; however, the extraphysiological dosages required to overcome the elevated protease activity and reduced cellular responsiveness in the wound bed has greatly hindered clinical translation and caused valid safety concerns.[7-9] For instance, the only GF protein therapy FDA approved for chronic wounds treatment has exhibited only clinical modest utility (10% increase in the number of patients healed) and linked to a 5-fold increase in the occurrence of cancer.[10] Biomaterial-mediated GF gene

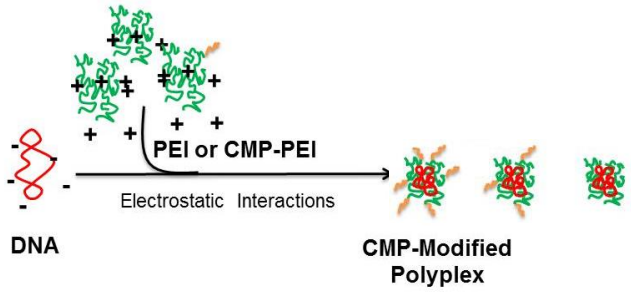

Figure 6.1. CMP-modified Polyplex Schematic. Polyplex are prepared via electrostatically condensing DNA with mixtures of PEI and CMP-PEI

therapies, also known as gene activated matrices (GAMs), offer potential benefits over GF protein delivery in protein stability and bioactivity, sustained release, and cost, yet concerns with off-target responses and poor gene transfer efficiencies have inhibit their development. [7,8,11-13]

In this work, a new peptide-based strategy for achieving high-efficiency gene delivery stimulated by the naturally elevated levels of protease-activity and subsequent ECM turnover within the wound bed is presented.[14,15] CMP-collagen affinity was utilized to create stable, tunable links between DNA carriers known as polyplexes and collagen, a major ECM component, and the effect of CMP modification and collagen attachment on gene delivery were monitored.

As discussed in Chapter 2, a GPP-based CMP (referred to as GPP) was synthesized using automated solid phase peptide synthesis and conjugated to cationic polymer polyethyleneimine (PEI) via Michael-type addition. CMP-modified polyplex were then prepared through electrostatically condensing DNA with mixtures of PEI and PEI-CMP conjugate (Figure 6.1). After characterization of the CMP-modified polyplex via gel electrophoresis and DLS, initial studies focused on the capacity of CMP display on polyplexes to promote stable integration into collagen scaffolds and

tailored release/retention as a function of CMP display density. As demonstrated in Chapter 2, the incorporation of GPP-PEI increased initial retention of DNA on 2-D collagen films in respect to the unmodified PEI polyplex by 9- to 15-fold and expanded for 10% and 50% GPP-PEI polyplexes respectively and expanded release/retention time periods from 2 days to 10 days. Similarly, CMP display increased initial DNA retention in 3-D collagen gels by as much as 20% and expanded release/retention periods by 10 to 15 days for 10% and 50% GPP-PEI polyplexes. Subsequent transfection studies on modified 2-D and 3-D collagen scaffolds revealed CMP-mediated attachments preserved polyplex activity and promoted sustained delivery. As shown in Figure 6.2, on the 3-D collagen scaffolds, polyplex activity was preserved even after prolonged periods of serum exposure (up to 7 days v. 5). Notably, gene expression was significantly enhanced (by nearly an order of magnitude) when

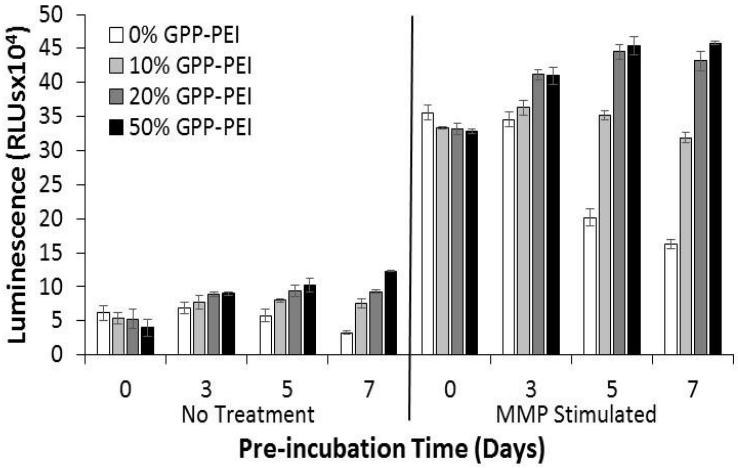

Figure 6.2. Gel Transfection Experiments. NIH/3T3 cells were plated on fresh polyplex-modified gels or polyplex-modified gels that were pre-incubated under physiological conditions for up to a week. Cells were treated with TNF-α to stimulate MMP expression as specified. The data represent the luminescence in the media due to luciferase expression by the cells after 4 days on the gels. Each data point represents the mean ± standard deviation for a total of eight separately prepared and analyzed samples.

elevated MMP-expression was stimulated, suggesting reliance of collagen remodeling to achieve elevated levels of transgene expression. Furthermore, as shown in Figure 6.3, CMP-mediated enhancements in polyplex stability and sustained release translated in a murine ECM depot model in which luciferase expression was observed for 10-fold longer a time period and reached maximum expression levels 2 orders of magnitude greater than the unmodified polyplex samples.

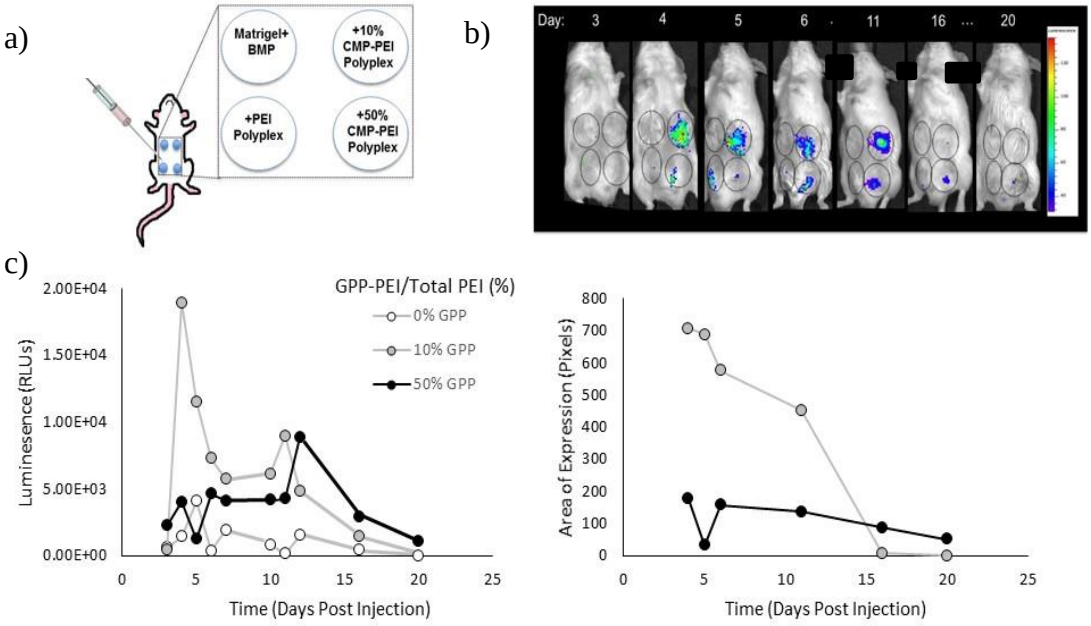

Figure 6.3. *In vivo* application of CMP-modified polyplex. a) Schematic indicating the location and contents of each subcutaneous pellet on the abdomens of CD-1 mice. Each solution contained Matrigel^{TM}, BMP-2, and polyplex where indicated, and the solutions formed visible pellets immediately after injection. DNA encoded for a membrane bound form of Metridia Luciferase to permit *in vivo* imaging. b) *In vivo* images of a representative mouse at various time points post injection, indicating luminescence over a maximum period of 20 d. c) Quantification of transgene expression and the area over which expression was identified. This study was replicated in four mice (Figure A4).

To access the impact of CMP-modification and the role of collagen remodeling in gene expression, mechanistic studies were conducted as presented in Chapter 3. The direct dependence of gene expression within the collagen/CMP/DNA system was again shown to be highly dependent MMP stimulation through transfection studies. Additionally, enhancements in the level and duration of gene expression facilitated by CMP-modification, were found to be in part due to the preservation of DNA integrity even after prolonged serum exposure through gel electrophoretic analysis and altered endocytic uptake by conducting transfection studies post caveolin-1 silencing. Significantly, as shown via fluorescent microscopy in Figure 6.3, CMP-modified polyplex were found to co-internalize with collagen into cells, strongly suggesting CMPs facilitate attachment to endocytic collagen fragments which in turn, impact uptake.

The expression of functional GFs PDGF-BB and KGF was also demonstrated in the CMP/DNA/collagen systems. As presented in Chapter 4, in the case of PDGF-BB, extensive studies on expression and the resulting cell behaviors, including cellular proliferation, migration, and collagen remodeling, were conducted. During these studies, CMP/DNA/collagen systems were preincubated under physiological-simulating conditions (37^{o}C in serum supplemented media) to mimic the environment experienced by a wound dressing before cellular invasion. Proteases were not included in the preincubation as proteolytic activity has been demonstrated to remain localized to the wound edges. CMP-modification of polyplexes resulted in significant levels of

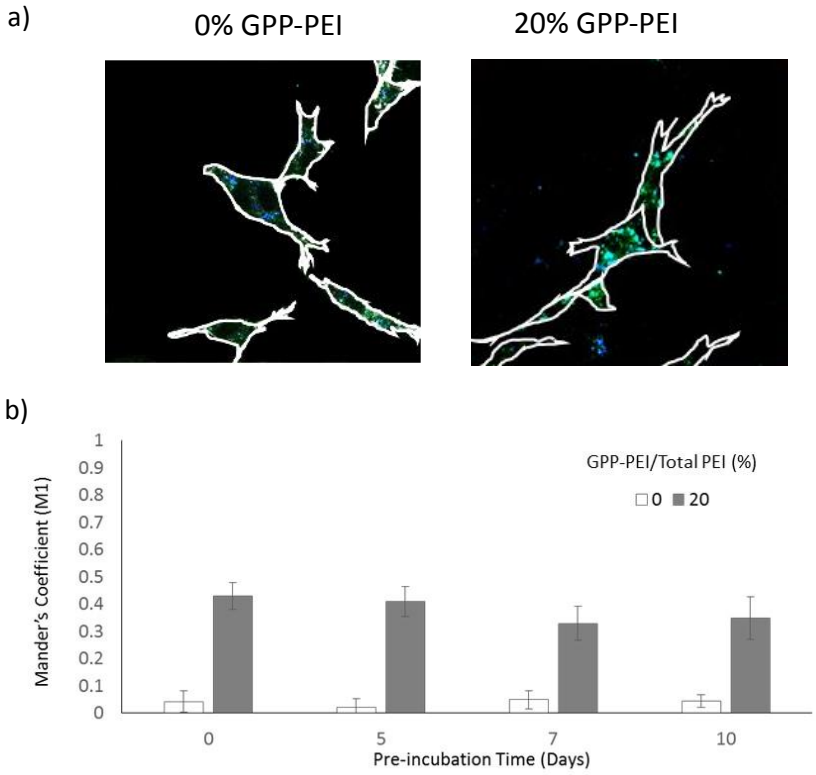

Figure 6.3. Collagen-polyplex colocalization study. a) Representative fluorescent microcopy images of cells collected from gels preincubated for 5 days. b) Quantitative analysis of intracellular collagen-polyplex association by calculation of Mander's coefficients for colocalization of FITC-collagen with Alexa Fluor 350-polyplexes in NIH/3T3 cells. The data for the 0, 5, and 7 day pre-incubations represent the mean +/-standard deviation of 10 separately analyzed cells. For the 10 day preincubation, 5 separately analyzed cells were analyzed.

PDGF-BB detection via ELISA and observance of PDGF-BB triggered cellular behaviors after 10 v. <5 day preincubation periods, confirming CMP-modification improves polyplex stability/activity and that functional proteins could be expressed at levels capable of inducing desirable cell responses. Furthermore, the CMP/DNA/ collagen scaffolds were applied to simple *in vitro* wound models through

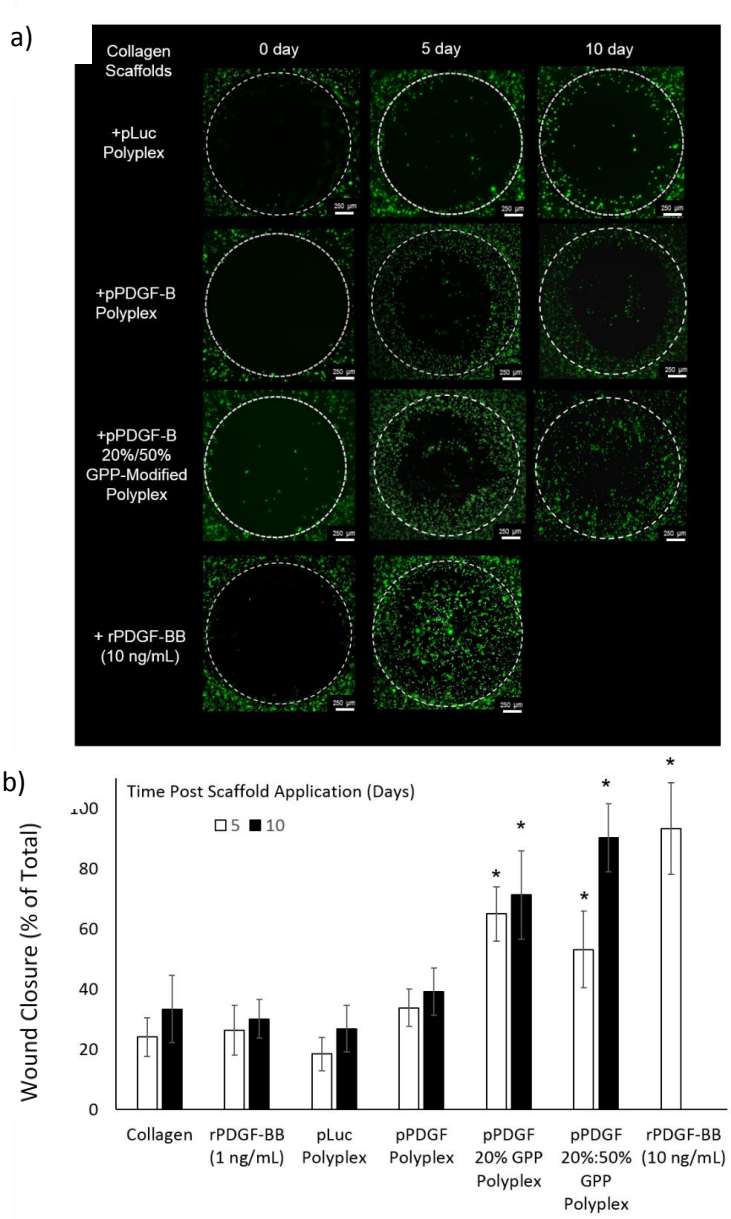

Figure 6.4. *In vitro* wound model. Defects in cell-seeded collagen gels were filled with collagen scaffolds modified with rPDGF-BB, polyplex encoding for luciferase, or polyplex encoding for *PDGF-B*, and subsequent defect invasion or "wound closure" was monitored via microcopy. Representative images (a) were analyzed via ImageJ to quantify wound closure (b). The data represent the mean +/-standard deviation of 3 separately prepared and analyzed samples. * denotes a statistically-significant difference ($p<0.05$) relative to the luciferase-encoding controls.

creating defects in cell-seeded collagen gels. As shown in Figure 6.4, CMP-modification improved wound closure rates, and when a mixture of polyplex with different degrees of CMP-modification were applied to expand PDGF-BB expression (e.g.10% GPP-PEI polyplexes are release/available upon initial cell invasion while the 50% GPP-PEI polyplex facilitate PDGF-BB expression at latter time points) wound closure levels eventually equaled those achieved with application of recombinant PDGF-BB at an order of magnitude higher dosage.

In addition to PDGF-BB, the ability to express KGF within CMP/DNA/collagen/fibrin scaffolds has been demonstrated. Specifically, the capacity to tailor the individual expression profiles of both GFs within the same scaffold over a period of almost 2 weeks, has been shown Figure 6.5 and detailed in Chapter 5 of this work. A similar capacity to individually tailor the expression of reporter protein expression GFP and GLuc was also demonstrated and easier predict compared to GF expression because of a lack of cell behaviors induced by the reporter proteins expression. Moreover, the capacity to expand the duration of expression through use of scaffolds with increased stability was explored. While work is on-going, the application of collagen-fibrin co-gels crosslinked, shows tremendous potential and has shown to have the capacity to expand expression periods 4-fold relative to CMP/DNA/collagen scaffolds with significantly faster gelation times more conducive to wound repair applications as an injectable gel.

In conclusion, CMP/DNA/collagen scaffolds have shown tremendous potential in overcoming both extracellular and intracellular non-viral gene delivery obstacles. CMP-collagen attachment has facilitated prolonged retention in several different collagen-based scaffolds including 2-D collagen films, 3-D collagen gels, Matrigel^{TM},

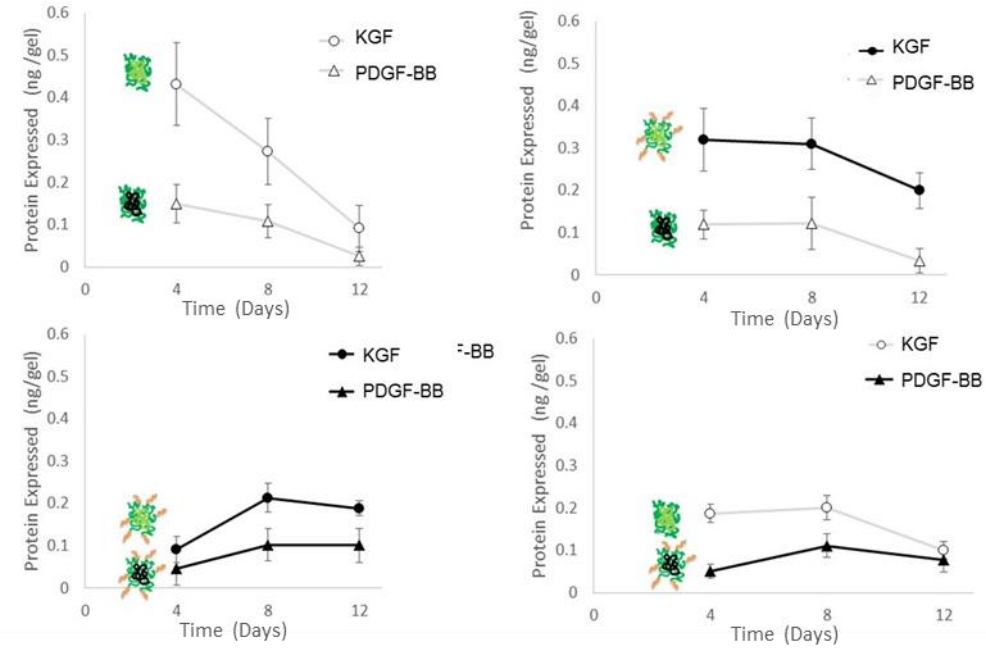

Figure 6.5. Tailoring the expression of GFs in collagen/fibrin co-gels via varying CMP display. The expression of PDGF-BB and KGF was monitored within the same samples via ELISA. Empty markers indicate the application of unmodified polyplex and black markers indicate the application of 50% GPP-PEI modified polyplex. Additionally, the illustrations denote polyplex modification and DNA where the following materials are: CMP (orange), PEI (green), pPDGF-B (light green), and pKGF (black). The data represent the mean +/-standard deviation of 3 separately prepared and analyzed samples.

and fibrin/collagen co-gels, enhanced polyplex stability, and altered endocytic trafficking targeting a pathway previously demonstrated to result in high efficiency gene expression. While the focus has been on its application in chronic wound repair, this approach has utility in any delivery site with elevated protease activity/collagen remodeling including tumors and joints.

6.2 Future Recommendations

6.2.1 Study the capacity to further expand and tailor gene delivery in CMP/Collagen systems through purposeful collagen-mimetic peptide design

The results of this work strongly suggest the significant utility of CMPs to impart polyplex retention and gene expression for extended periods in collagen-based scaffolds. Specifically, a simple GPP-based sequence, denoted GPP (Table 1.1), was shown capable of tailoring release/retention and gene expression for nearly a month through straightforward variations of GPP density incorporated into the polyplex. To further develop the CMP/collagen delivery system, the consequences of manipulation CMP sequence should be explored. Preliminary studies (not shown) conducted with polyplex modified with a GPO-based sequence, $(GPO)_4GEKGER(GPO)_4GGCG$, strongly suggested GPO-based sequences (or more generally, sequences with greater triple-helical melting temperatures) could be used to expand retention/release profiles of polyplex from collagen, increasing initial DNA retention on 2-D collagen films by 12% compared to GPP-PEI-modified polyplex; however, more exhaustive studies have not yet been conducted owing the minute differences in gene expression

CMP Sequence	Triple-Helix Melting Temperature*
CGGG$(GPP)_4$GPRGEKGERGPR$(GPP)_4$	~50 ^oC
CGGG$(GPO)_4$GEKGER$(GPO)_4$	~50 ^oC
CGGG$(GPO)_5$GEKGER$(GPO)_5$	~62 ^oC
CGGG$(GPO)_6$GEKGER$(GPO)_6$	~70 ^oC

Table 6.1. Proposed CMP sequences. The presented sequences are all predicted to have melting temperatures within a suitable range (37^{o}C-80^{o}C) (*pre-oxidation/cysteine knot formation) and designed to include an integrin binding sequence (blue) and cysteine for subsequent conjugation to PEI.

observed between GPO and GPP -polyplex modified 2-D collagen films. Based on subsequent studies with GPP-modified polyplex in the 3-D versus 2-D environment and work with more stable collagen-based systems (e.g. collagen/fibrin co-gels, Matrigel^{TM} etc.), moving forward with this sequence and additional CMPs with greater collagen affinities is sensible for preventing vector escape and further expanding/tailoring expression.

To create CMP displays that impart greater increased in stability in collagen and robust $\alpha_2\beta_1$ integrin engagement, polyplexes will be produced with CMPs (Table 6.1) that are longer and/or contain hydroxyproline, to promote more stable strand invasion with collagen scaffolds. All purposed CMPs should exhibit triple-helical melting temperatures between 37^{o}C-80^{o}C to enable stable CMP integration with collagen under physiological conditions without requiring temperatures that would denature collagen. Sequences should also include glycine linkers, as 3 to 4 glycines permit efficient association with collagen[16,17], and a cysteine should be incorporated to enable subsequent Michael-type addition of cysteine residues to maleimide-modified

PEI if the same chemistry demonstrated in this work is to be utilized. Additionally, a scramble sequence, comprised of the same amino acids within the CMP sequence arranged to not for a triple helix, should be synthesized to serve as an additional control (other than unmodified polyplex) in both collagen retention/release studies and transfection experiments that highlights the role of the triple helix in promoting the observed behaviors. Furthermore, CMPs should be designed with and without integrin binding sequences, such as GEKGER and GFOGER, to further elucidate whether altered trafficking is solely due to association with collagen or if the inclusion of the integrin biding sequence has significance. The results of bolus transfection experiments in which NIH/3T3 cells were transfected by unbound GPP-modified polyplex encoding for GFP and analyzed via flow cytometry, showed GPP-modification has an insignificant effect on the percent of cells transfected or mean fluorescence; however transfection studies in which polyplex delivery is facilitated by CMPs with and without the integrin binding sequence delivered via solution or collagen would provide more conclusive evidence of the role of CMPs in delivery other than association with collagen. Furthermore, immunohistochemical staining should be used to determine if uptake is facilitated by $\alpha_{2,}$ $\beta_{1,}$ and caveolin-1, as predicted, via polyplex co-localization transfection studies.

6.2.2 Application in more extensive animal wound models

The CMP/collagen gene delivery system has demonstrated efficacy in both *in vitro* wound models through the promotion of shorter wound closure times via of prolonged PDGF-BB expression and in *in vivo* murine ECM depots by tailoring the duration and extent of transgene expression and localized delivery. Application in animal wound models will determine whether the CMP/collagen gene delivery system

can promote GF expression at levels and timing that are sufficient to promote *in vivo* wound repair. It will also provide a well-controlled environment for optimization of system parameters including scaffold composition (e.g. concentration of collagen and fibrin) and DNA dosing. *In vivo* models will also provide information on potential toxicity and off-target gene expression.

In preparation for wound model studies, a preliminary study confirming gelation and transgene expression in type I collagen/fibrin co-gels in subcutaneous ECM depots is recommended. In a study identical in protocol to the *in vivo* study detailed in Chapter 1, mice should be injected at 4 different sites with (1) Matrigel^{TM}, BMP-2, and 50% GPP/PEI modified polyplex (100 μg DNA/mL)(+ control) and mixtures of fibrin (2.5 mg/mL) and type I collagen (2 mg/mL) containing either (2) no polyplex (- control), (3) 0% GPP/PEI polyplex (100 μg DNA/mL), or (4) 50% GPP/PEI polyplex (100 μg DNA/mL). Polyplex should encode for membrane-bound Metridia luciferase to allow for live quantification of expression through luminescence readings. On-going work has suggested a higher polyplex concentrations impairs gelation (Chapter 5); therefore, if gelation/pellet formation is not visible, low polyplex concentrations (e.g. 50 μg DNA/mL) may be required. Additional *in vitro* work to optimize scaffold co-gel composition is necessary to finalize the compositions, but those reported above represent a good start point as discussed in Chapter 5.

After confirmation of transgene expression within the delivery system in the *in vivo* model, the following animal wound models are proposed: murine splinted models and rabbit ear "ulcer" models. Murine splinted wounds have well-defined dimensions making them ideal for study of histological, morphometric, and biochemical aspects of repair. Additionally, splinted wounds provide a more accurate representation of human

wounds by inhibiting the rapid contractile response typical in loose-skinned animals characterized by decreased granulation tissue formation and epidermal migration. Different CMP/polyplex/collagen mixtures encoding for PDGF-BB with different dosages will be applied and the rate of wound closure as well as safety will be monitored. For instance, blood sampling can be used to observe systematic escape and experiments with mem-MetLuc reporter genes can be used to both visualize and quantify off-target delivery. Similar studies can be conducted in the rabbit ear "ulcer" model to ensure species-to-species translation and evaluate the system in a wound model more represented of a human chronic wound.

6.2.3 Study the capacity to trigger various cell-behaviors via CMP-mediated multi-gene expression

Given the complexity of the reparative process, there is great value in the ability to facilitate simultaneously or sequentially deliver multiple therapeutics. The capacity to express two GF, PDGF-BB and KGF, with individually tailorable expression profiles within the same collagen-based scaffold has been demonstrated in recent work through the manipulation of CMP display on polyplexes. In a similar manner to the PDGB-BB-stimulated cellular behavior studies discussed in Chapter 4, examination of cellular behaviors induced by the tunable GF concentrations would greatly complement the completed work. For KGF/PDGF-BB expressing systems, the behaviors of mouse keratinocytes (XB-2) and fibroblasts (NIH/3T3) can initially be assessed in co-culture studies in which the pre-labeled cells (via CellTracker Dye) are seeded onto the DNA/collagen systems. In turn, cellular invasion of the scaffold and proliferation can be monitored via microscopy in which proliferation/migration is initiated in keratinocyte by KGF and in fibroblast by PDGF-BB. Based on previous

publications, KGF and NIH-3T3-induced differentiation of the keratinocytes may also be observed via the stacking of the cells and the formation of a spinous layer. Differentiation may be quantified by accessing nuclear density via nuclear staining and microscopy or detection of markers K1/10 and involucrin through ELISA.[18] Alternatively, fibroblast may be encapsulated into the DNA/ collagen scaffold and keratinocytes seeded on top after gelation and incubation under standard culture conditions for a few days (4 d) to allow for transfection of the fibroblasts and GF expression. This set-up better mimics endogenous tissues and should more rapidly trigger KGF/PDGF-BB induced responses in keratinocytes. Furthermore, given the relative ease of changing the gene and in turn the protein expressed, the findings with the CMP/DNA/collagen delivery system strongly suggests its capacity to control the expression of various proteins. Similar studies with different GFs expressed should yield compelling results of general interest in the field of regenerative medicine.

6.3 Final perspective

In this work, a novel peptide-based strategy for achieving high-efficiency gene delivery through leveraging the naturally elevated levels of protease-activity and subsequent ECM turnover within the wound bed is presented.[14,15] Specifically, CMP-collagen affinity was utilized to create stable, tunable links between collagen, a key ECM component, and active DNA carriers known as polyplex and demonstrated to promote (1) tailorable, localized protein expression,[14] (2) utilization of ECM turnover to overcome extra- and intracellular delivery obstacles,[15] and (3) attain multi-protein expression. While the focus has been on application in chronic wound repair, our findings strongly suggest the highly versatile CMP/collagen gene delivery system has utility in many other applications in which delivery sites are characterized by

pronounced protease activity. Moreover, CMPs may be used to delivery additional cargoes including antimicrobial peptides and other materials required for the promotion of wound bed health.

Furthermore, our approach is unprecedented in its apparent ability to harness ECM remodeling to facilitate delivery and efficient cellular uptake. It marks a departure from other gene transfer strategies in that insoluble ECM fragments, released by remodeling, are used to facilitate gene transfer activities of the DNA constructs. While collagen remodeling was the focus of this work, similar abilities to harness the remodeling pathways of other ECM components like fibronectin offer unique, unexplored potential for the controlled delivery of additional therapeutics and the promotion of reparative processes.

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APPENDIX

SUPPLEMENTAL INFORMATION

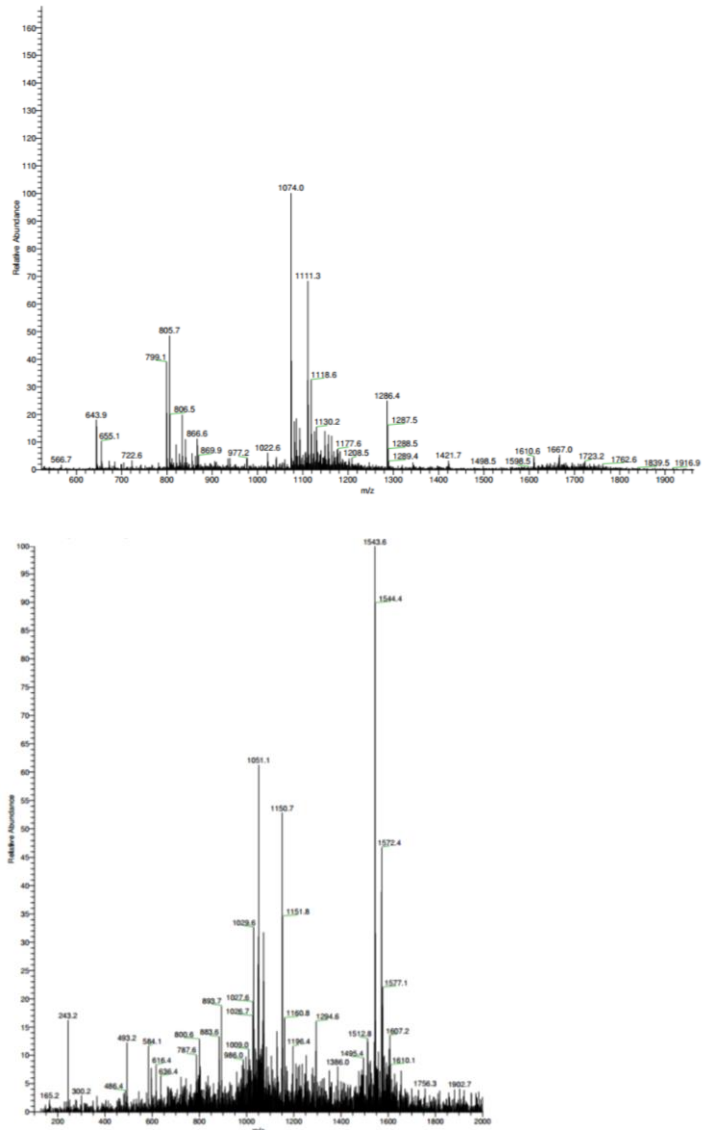

Figure A1. Peptide molecular weight was confirmed using electrospray ionization mass spectrometry. Examples: a) For GPP: *m*/*z* =1610.6 [(M + 2H)$^{2+}$= 1610.85] and *m*/*z* =1674.0 [(M + 3H)$^{3+}$= 1073.9]. b) For GPO: *m*/*z* =1543.6 [(M + 2H)$^{2+}$= 1543.6] and *m*/*z* =1051.1 [(M + 3H)$^{3+}$+Na= 1051.3].

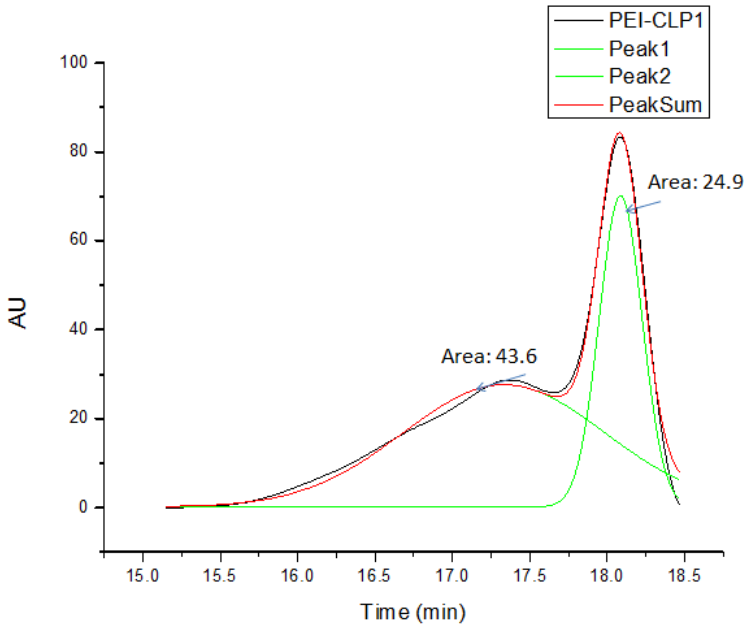

Figure A2. The GPP-PEI conjugation was confirmed with gel permeation chromatography (GPC) and functionality was determined through integrating the GPC refractive index data. The black curve represents the product after conjugation while the green curves are separated peaks for the GPP-PEI and excessive PEI respectively. The red curve is the summation of the green curves and it fits the collected data well. The relative area ratio of the two peaks suggest the functionality is approximately 0.64 GPP/PEI molecule.

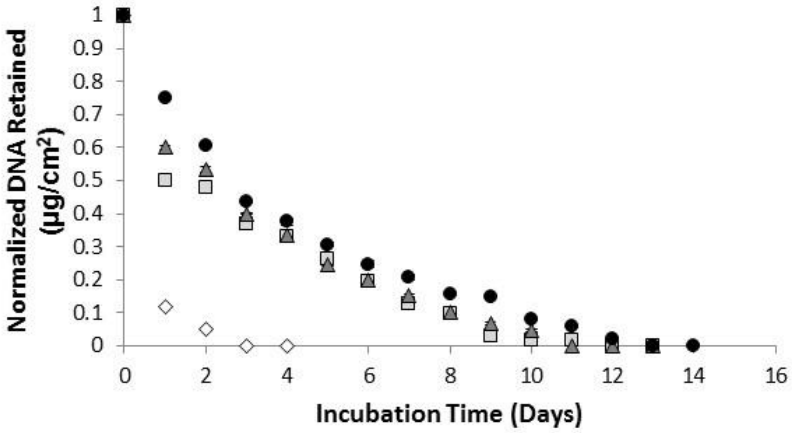

Figure A3. Normalized retention of DNA on collagen films with time, as a function of the percent GPP-PEI within the polyplex including 0% (white diamond), 10% (grey square), 20% (dark grey triangle), and 50% (black circle).

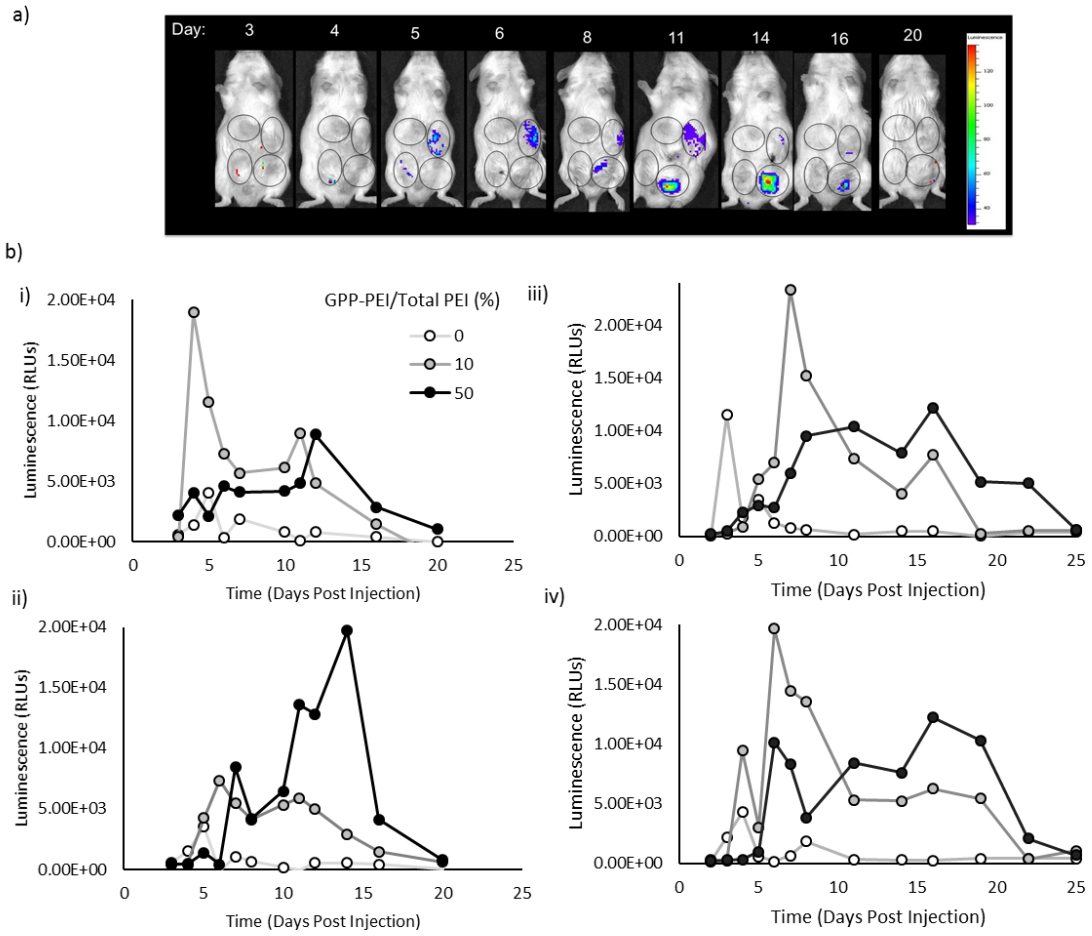

Figure A4. *In vivo* Expression Model. The murine ECM-depot model presented in the manuscript was replicated in multiple mice (n=4). Representative images of a second mouse (a) and additional quantitative analysis of luciferase expression in 3 additional mice (b) show the expression profiles in each pellet.

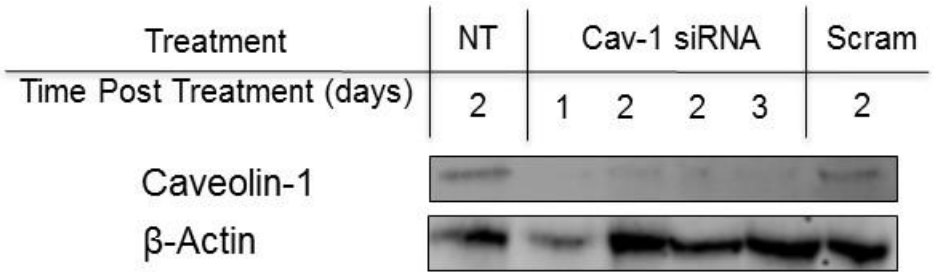

Figure A5. Caveolin-1 Silencing. The extent of caveolin-1 silencing post treatment was accessed using a Western blot at different time points.

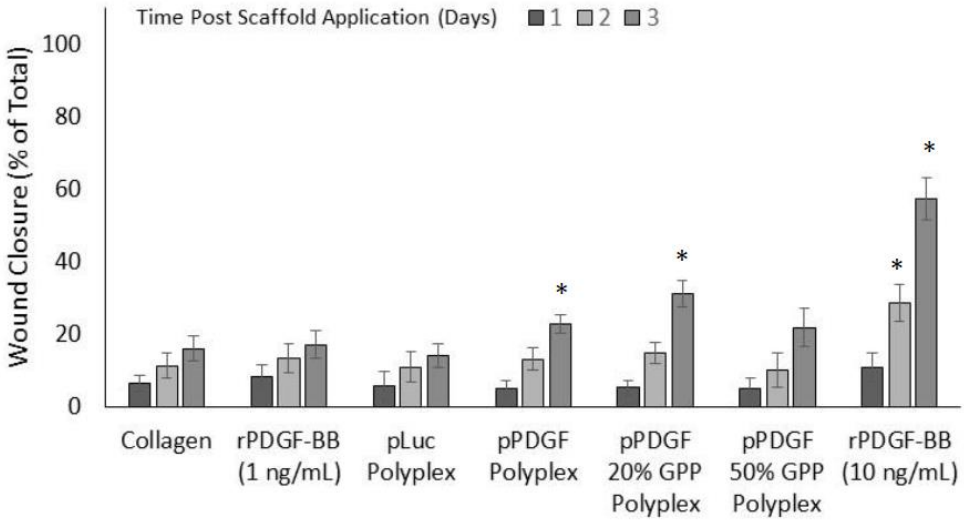

Figure A6. *In vitro* wound model wound closure at early time points. To evaluate initial closure, the same defect model was implemented with NIH/3T3 cells pre-labeled with CellTracker™ Deep Red. As previously described, defects in cell-seeded collagen gels were filled with collagen scaffolds modified with rPDGF-BB, polyplex encoding for luciferase, or polyplex encoding for *PDGF-B*, and initial defect invasion was monitored via microcopy. The data represent the mean +/-standard deviation of 3 separately prepared and analyzed samples. * denotes a statistically-significant difference ($p<0.05$) relative to the luciferase-encoding controls.

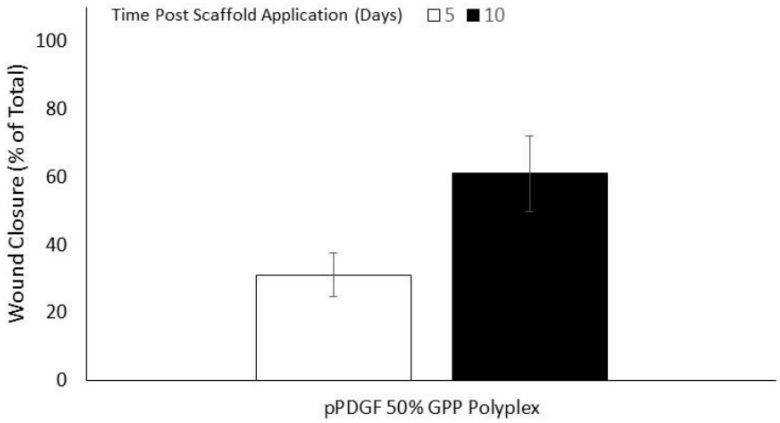

Figure A7. *In vitro* wound model. As previously described, defects in cell-seeded collagen gels were filled with collagen scaffolds modified with polyplex encoding for *PDGF-B*. Defect invasion was monitored via microcopy and the effect of GPP-modification was studied. The data represent the mean +/-standard deviation of 2 analyzed samples.

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IRB APPROVAL

Please see supplemental files.