Title
Development of Oligonucleotide Tether Platform for Thermally Activated Release of Growth Factor

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Development of Oligonucleotide Tether Platform for Thermally Activated Release of Growth Factor

A thesis submitted in partial satisfaction

of the requirements for the degree Master of Science

in Bioengineering

by

Kevin Sung

2015

ABSTRACT OF THE THESIS

Development of Oligonucleotide Tether Platform for

Thermally Activated Release of Growth Factor

by

Kevin Sung

Masters of Science in Bioengineering

University of California, Los Angeles, 2015

Professor Benjamin M. Wu, Chair

Chronic wounds affect approximate 6.5 million individuals in the United States alone. These types of wounds are usually locked in a permanent cycle between tissue inflammation, remodeling, and breakdown without appreciable progress towards complete healing. Current methods of treatment include consistent delivery of growth factors in hopes of easing the soft tissue through the proper stages of repair. While there is evidence that dose delivery of growth factors is more desirable than continuous dosing, there are no current on-demand delivery platforms for such growth factors. Our studies show the feasibility of a duplex oligonucleotide tethering system to create a heat-sensitive linker that can release a growth factor on-demand. Our system has the ability to last for a minimum of five days and has the potential to be incorporated into other biodegradable implants for delivery.

The thesis of Kevin Sung is approved.

James Dunn

Min Lee

Benjamin M. Wu, Committee Chair

University of California, Los Angeles

2015

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I would like to acknowledge the National Institutes of Health (NIH) and National Institute of Arthritis and Musculoskeletal and Skin Diseases (NIAMS) for providing the funding for this project. I would also like to acknowledge Chase Linsley for the help with work with the peptide sequences and Garrett Moseley for help with streamlining the radiolabelling protocol.

Introduction

Chronic wounds, by definition are wounds that have failed to have progressed through the natural process of healing, and is unable to generate stable, functional tissue[1]. The majority of chronic wounds fall into three main classes: venous ulcers, pressure ulcers, and diabetic ulcers[1]. Chronic wounds typically manifest with the raised, non-advancing wound margin. The local tissue areas respond poorly to factors that normally stimulate fibroblasts to proliferate and differentiate into mature phenotypes[1-2]. Furthermore, the wound area produces a large amount of inflammatory factors that inhibit the development of extracellular matrix, a key part in the tissue remodeling process[3].

Chronic wounds affect approximately 6.5 million individuals in the United States alone[4]. Treatments for these afflictions are extremely expensive, and place an enormous burden on not only patients but the health care providers as well.

As the population in the United States grow older, the prevalence of chronic wounds has steadily increased. In addition, diseases such as obesity and diabetes also predispose individuals towards the development of chronic wounds[4]. Currently in the market, the only approved therapeutic for diabetic ulcers is sold under the name Regranex®[5].

While the treatment methods for chronic wounds differ depending on the individual case, current approaches primarily revolve around a combination of addressing the underlying causes as well as dealing with the damaged tissue itself. In terms of managing the damaged tissue, hormones and growth factors are the main avenues to facilitate tissue progress through healing[3,4,6]. One of the common growth

factors used is platelet-derived growth factor (PDGF), a mitogenic protein that also plays a role in angiogenesis. This growth factor promotes the proliferation of cells such as fibroblasts and smooth muscle cells, and can ultimately induce the layering of extracellular matrix that is critical for wound healing[7,8].

PDGF comes in different isoforms that has the potential to bind to an alpha or beta PDGF receptor (PDGFR). Of the known isoforms, only PDGF-BB is known to both types of PDGF receptors, and this is the growth factor explored in these studies[9].

One proposed solution is to incorporate growth factors into fibrin gels as a potential biocompatible scaffold to stimulate healing in chronic wounds. The benefits of fibrin scaffolds are many. It is a well characterized material that naturally forms in the body as part of the wound healing cascade, is readily biodegradable into nontoxic components in the body, and relatively inexpensive to produce[10,11]. Furthermore, the enzymes that catalyze the cross-linking of fibrin such as Factor XIII, can be harnessed to help functionalize the fibrin structure itself[12].

In functionalizing the fibrin gel, we want to create a light-sensitive oligonucleotide growth factor tether system, as shown in Figure 1. We can do this by incorporating a light-sensitive component that readily releases thermal energy when exposed to light. One such method has been characterized in prior art[13]. The oligonucleotide growth factor tether will keep the growth factor bound until the introduction of thermal energy, which will cause the duplex strand to separate, releasing the growth factor[13]. Having this type of controlled dose release have potential benefits. For example, studies have shown the use of continual dosing clinical Regranex®, for extended periods of time to be linked increased risk of cancer and higher morbidity[5]. Using new methods of

controlling delivery can reduce the possibility of harmful complications as reduce the risk of reaching therapeutic toxicity levels[14].

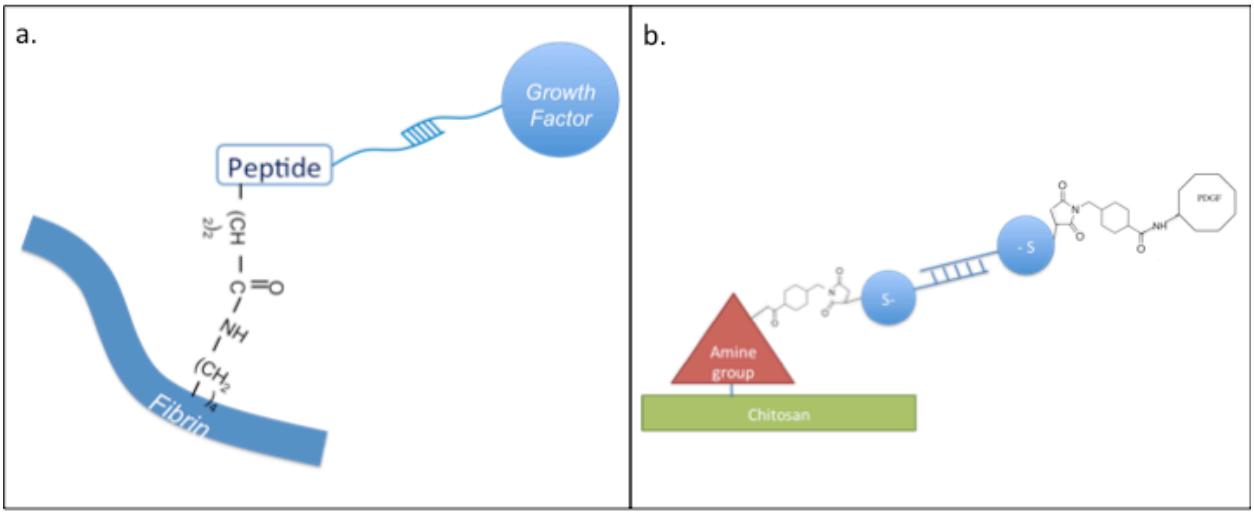


Figure 1 (a) The Proposed Functionalized Fibrin Schematic. A light-sensitive peptide can be conjugated to the individual strands of fibrin in the fibrin gel and generate heat when exposed to a specific wavelength of light. A duplex oligonucleotide tethering system conjugated to a therapeutic will be then conjugated to the peptide, allowing the therapeutic to be released upon exposure to the heat generated by the light sensitive peptide. (b) Conjugation setup for the PDGF release studies conducted. A chitosan-coated surface provides the amine groups necessary for the conjugation of the oligonucleotide duplex system that is then linked to a growth factor. In this case, the factor used is PDGF.

Part I

I. Oligonucleotide Conjugation to Growth Factor

We sought to functionalize the fibrin using a double stranded oligonucleotide tether concept, shown in Figure 1b. The primary strand would be chemically conjugated to a surface, which will eventually be a fibrin gel or some other biocompatible scaffold. A

complement strand with the growth factor conjugated to it will be annealed to the primary strand. Once enough heat is applied, the energy will break the hydrogen bonds holding together the two strands of oligonucleotides, and the complement strand with the growth factor will be released. Theoretically, this platform allows for on-demand release of the growth factor to the wound site[15]. Previous groups have utilized conjugation schemes that functionalized oligonucleotides by conjugating small molecules or peptide to it[16–19]. A very stable and specific functional group used was the thiol group, which reacts relatively specifically to maleimide groups at pH 6.5-7.2, a level mild enough for proteins to survive[20]. Hetero-bifunctional linkers have been identified as well that can react with the amines of a peptide or protein and a thiolated oligonucleotide[20]. Derfus et al has shown previously the feasibility of the oligonucleotide tethering mechanism through magnetic beads, and introducing heat through pulses of magnetic field[21]. These studies gave inspiration to the conjugation scheme that was developed for creating an oligonucleotide tether system to PDGF.

II. Materials and Methods:

1. Well-Plate Conjugation with Pre-labelled Complement Strand

a. Chitosan Coating

A 96 well plate (Corning Costar) was coated with 200 uL of 2% w/v chitosan (Sigma) dissolved in 1M acetic acid (Fischer). The well plate is left overnight in the fume hood and placed in a desiccator until completely dry. This leaves a dry chitosan coating on the well plate surface. Prior to conjugation, the surface was washed 3x with 200 uL of 1N sodium hydroxide (Sigma). The surface was washed again three times with 1x

phosphate buffer solution (PBS, Fischer) solution before the surface is ready for conjugation.

b. Oligonucleotide Annealing

Custom made oligonucleotides (Primary strand: GAA GTG CGG TTA GTC GGC TTG AAT CAG CGA, Complement strand: TGA TTC AAG CCG ACT AAC, Sigma) were deprotected with 100mM DTT (Sigma) at pH 8. The deprotection step was carried out for 3 hours at room temperature. Column chromatography with a NAP-10 (GE LifeSciences) was performed to isolate the oligonucleotides. A 1:1 molar ratio of primary and complementary oligonucleotide strands was heated to 70^{o}C for 10 minutes, cooled to room temperature, and left to anneal overnight.

c. Conjugation

The annealed oligonucleotides are then conjugated to the chitosan coating through the linker sulfosuccinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (sulfo-SMCC, Sigma). A 1 mg/mL sulfo-SMCC solution was prepared by dissolving solid sulfo-SMCC with 10 uL dimethyl sulfoxide (DMSO, Fischer) and dilute with 1x PBS. The chitosan surface was reacted with 200 uL sulfo-SMCC solution for 2-4 hours at 25^{o}C. Post reaction, the surface was rinsed 3x with 1x PBS. The annealed oligonucleotide is then reacted with the surface with the maleimide groups of the sulfo-SMCC overnight at 25^{o}C. Finally, the surface is rinsed again 3x with 1x PBS.

d. Release Study

A release study was conducted, where the plate was heated to 45^{o}C for 20 minute intervals before cooling it to room temperature. The supernatant was collected and replaced with 1x PBS. The supernatant was collected at 20 minute intervals for 1 hour after the heating interval. Texas red fluorescent readings of both the conjugated surface and supernatant were taken with the Infinite F200 plate reader (TECAN) after each supernatant collection step.

2. Radiolabelling PDGF Release Studies

a. Radiolabelling protocol

Platelet derived growth factor-BB (PDGF-BB, Sigma) was first reacted with 1 mg/mL sulfo-SMCC linker for 2 hours. The PDGF-BB-linker solution was then reacted with I-125 (MPBio) in a 50 mM HEPES (Sigma) buffer for 15 minutes. The sample was then isolated with column chromatography. Elutions were checked for radioactivity with a Cobra Auto-Gamma Counter Model B5002 (Packard).

b. Oligo First Method

In the Oligo First method shown in Figure 2a, the primary and complementary oligonucleotides were annealed first. The oligos were then conjugated to the chitosan surface pre-treated with sulfo-SMCC by incubating at 25^{o}C overnight. Both the oligo annealing step and sulfo-SMCC treatment steps are described in section 1. Afterwards, the surface was washed 3x with 1x PBS.

The PDGF-BB-linker was then added to the surface and incubated overnight in 50 mM HEPES at 25^{o}C.

c. PDGF-Oligo First Method

In the PDGF-Oligo First method shown in Figure 2b, the complementary oligonucleotide strand was first reacted with the PDGF-BB-linker overnight in 50 mM HEPES at 25^{o}C, and then annealed with the primary strand using the procedure described in section 1.

The PDGF-Oligo complex was then reacted to the chitosan surface at 25^{o}C overnight.

d. Radiolabeling Release Study

The test tube with the conjugated surface was washed overnight in 1x PBS to remove the unconjugated PDGF. The test tubes were then heated to 45^{o}C to stimulate release of PDGF from the test tube. The supernatant was collected 5 minutes after the heating interval to ensure all of the dissociated PDGF had come off the surface. The tube surface and supernatant were measured for radioactive activity with a Cobra Auto-Gamma Counter Model B5002 (Packard). Tubes were let sit at room temperature for 24 hours when another time point was taken, followed by another heating interval. This was repeated until the surface has been exposed to 5 heating intervals.

The first two heating intervals were 5 minutes long, and the subsequent were all 10 minutes.

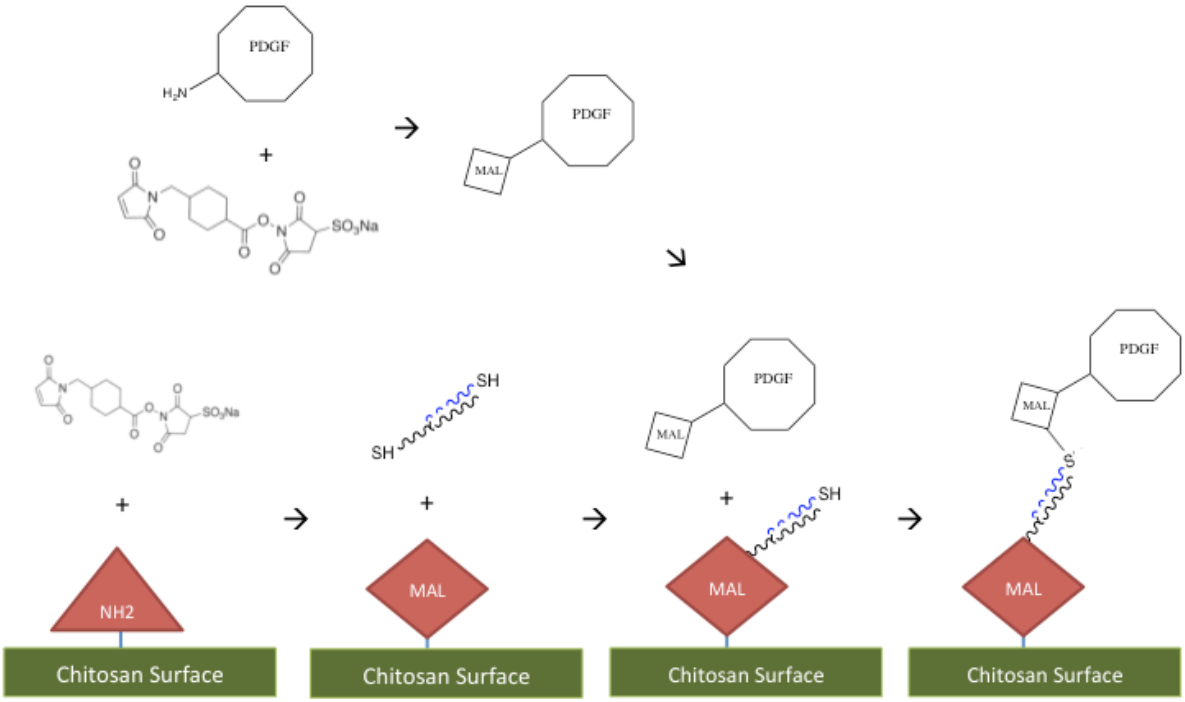


Figure 2a. Oligo-First Conjugation Scheme. The annealed oligonucleotide is first conjugated to the sulfo-SMCC activated chitosan surface. The sulfo-SMCC activated PDGF is then reacted to the oligonucleotide labelled chitosan surface.

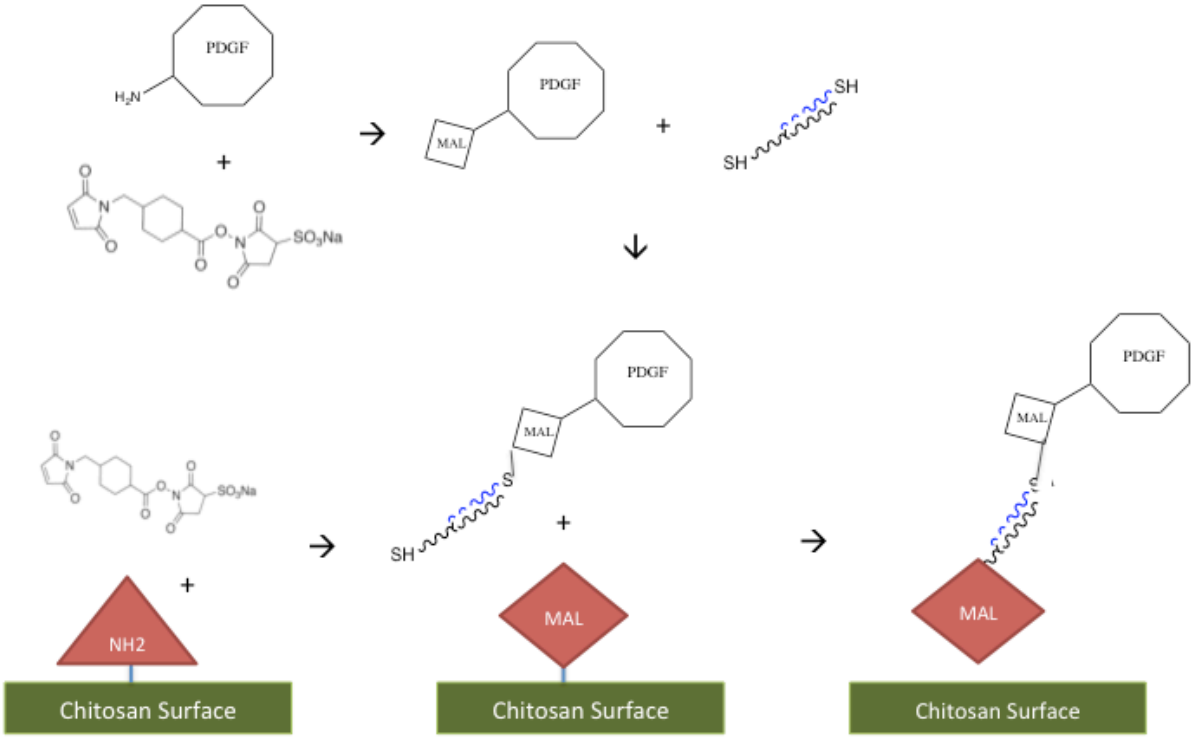
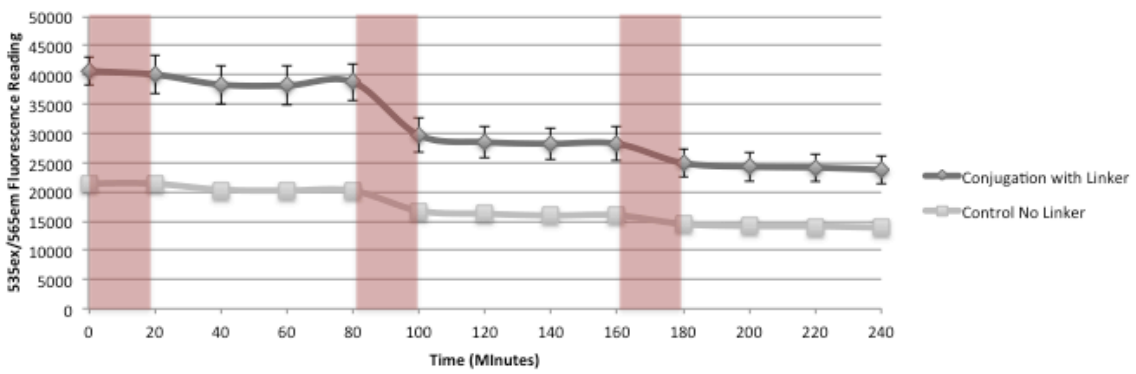


Figure 2b. PDGF-Oligo First Conjugation Scheme. The annealed oligonucleotides are first conjugated onto the PDGF protein before conjugated to the sulfo-SMCC activated chitosan surface.

IV. Data

a.



b.

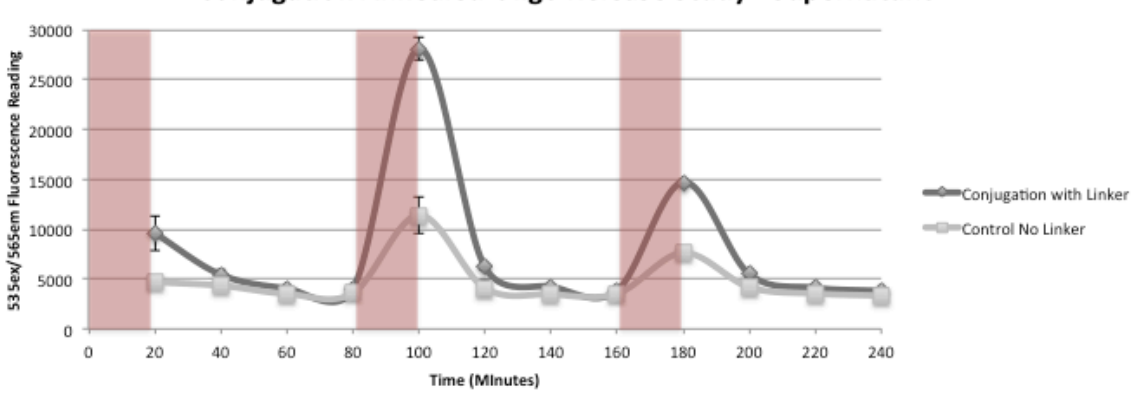
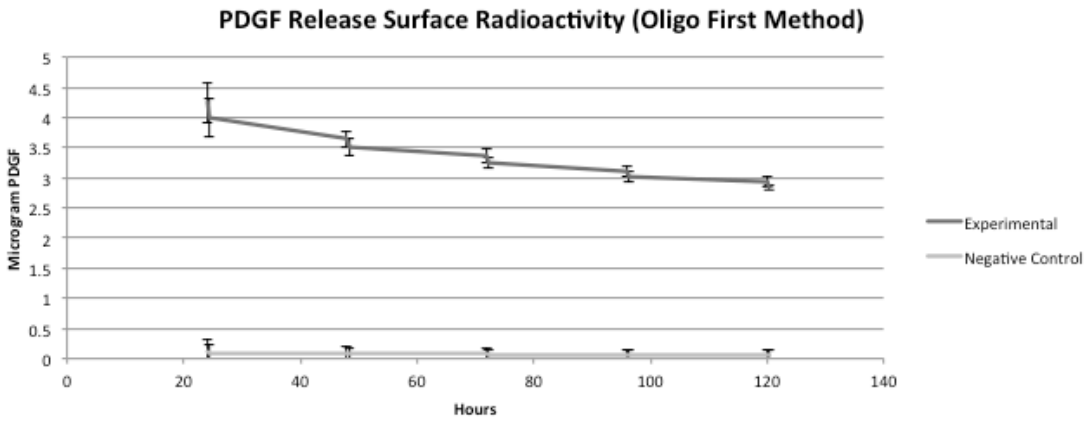


Figure 3. TxRed Conjugation tether release study (a) A release profile showing the TxRed fluorescence of the conjugated surface over the course of 4 hours (b) TxRed fluorescence levels in the collected supernatant. We can see the heat sensitive response of the oligo-tether conjugation system. In the hour after the heat is removed, there is an immediate drop-off in amount of TxRed being released from the surface into the supernatant

a.



b.

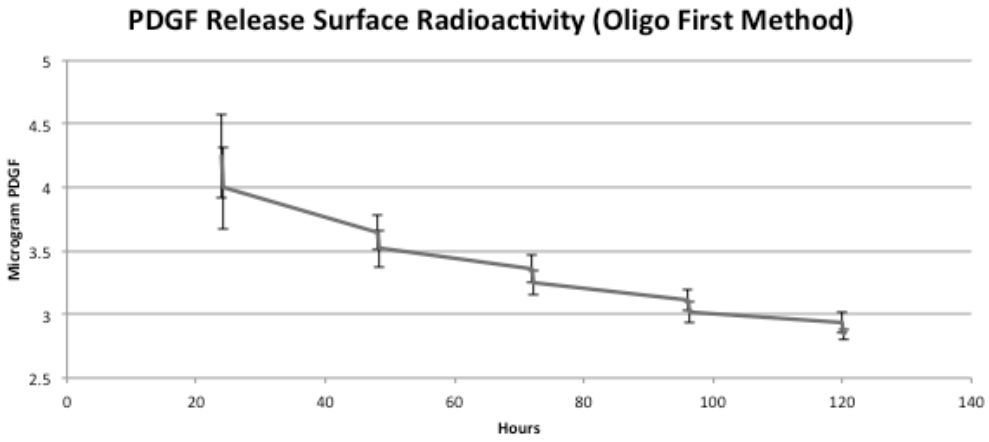
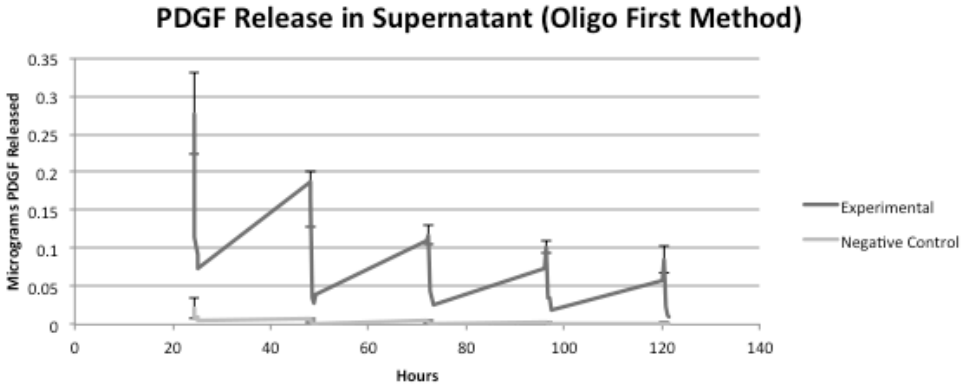


Figure 4. PDGF release profile from the conjugated chitosan surface using the Oligo First Method. Release studies start at the 24 hour mark due to an overnight wash to remove as much nonspecific PDGF as possible. An average of 4.25 micrograms of PDGF is conjugated onto the surface, which is a yield of 34.1% (a) Experimental conjugation versus the negative control release profile. The negative control retains almost no PDGF on the conjugated surface. (b) Focusing only on the experimental conjugation release, we can see appreciable release of PDGF after the heating intervals. However, there is also a large amount of PDGF released during the 24 hours after the first heating session.

c.



d.

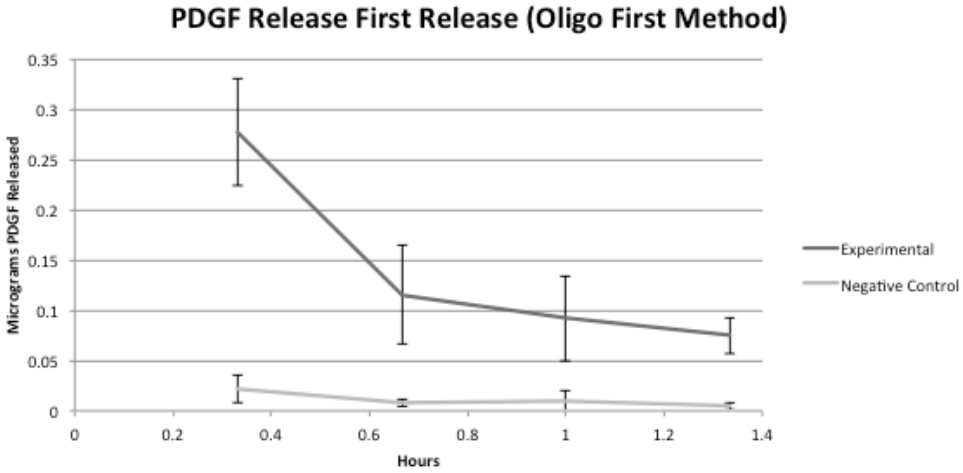
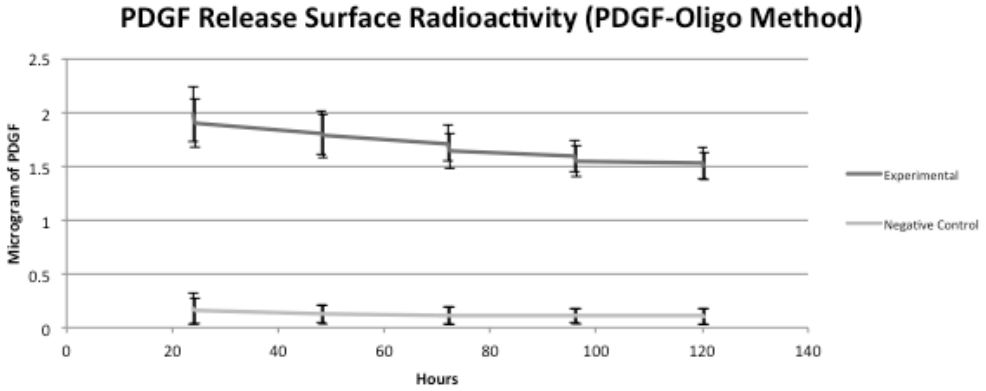


Figure 4 (c) Supernatant of the release study, showing the amount of PDGF released at each time point. (d) Focusing only on the first release of PDGF and 1 hour after, we see a significant increase in PDGF release immediately after the presence of heat. This release drops immediately afterwards ($p < 0.01$). Furthermore, compared to the negative control, the conjugated surface releases a substantial amount of PDGF.

a.



b.

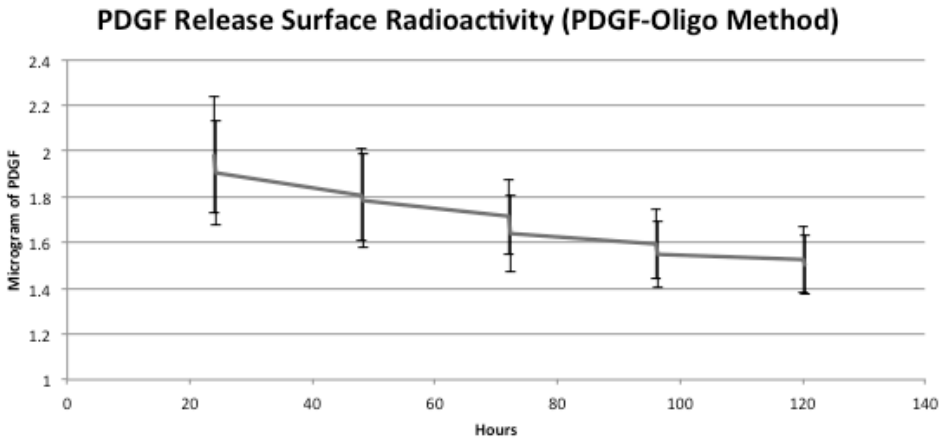
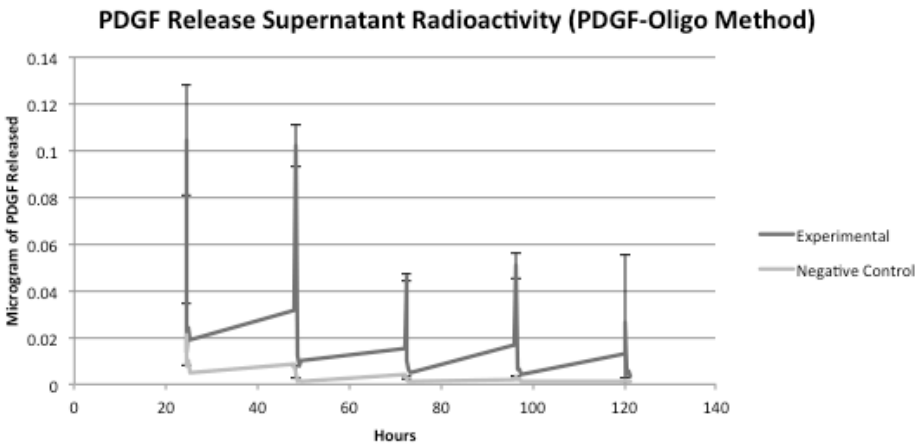


Figure 5. PDGF release profile from the conjugated chitosan surface using the PDGF-Oligo First Method. Release studies start at the 24 hour mark due to an overnight wash to remove as much nonspecific PDGF as possible. An average of 1.98 micrograms of PDGF is conjugated onto the surface, which is a yield of 16.5% (a) Experimental conjugation versus the negative control release profile. The negative control retains almost no PDGF on the conjugated surface. (b) Focusing only on the experimental conjugation release, we can see appreciable release of PDGF after the heating intervals. Compared to the Oligo First Method, the PDGF-Oligo Method release is far more controlled during the overnight incubations at room temperature, suggesting a more stable conjugation scheme.

c.



d.

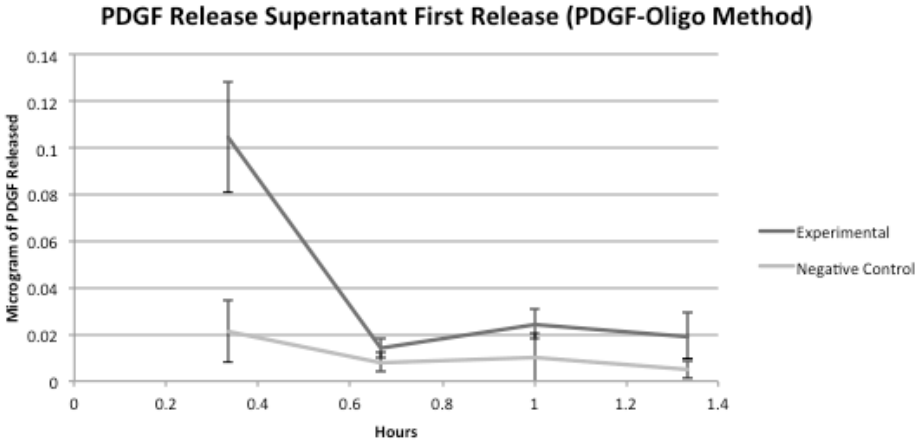


Figure 5 (c) Supernatant of the release study, showing the amount of PDGF released at each time point. While the amount of PDGF released at each time point is lower than that of the Oligo First Method, the amount released is far more consistent. (d) Focusing only on the first release of PDGF and 1 hour after, we see a significant increase in PDGF release immediately after the presence of heat. This release drops immediately afterwards ($p < 0.01$). Furthermore, compared to the negative control, the conjugated surface releases a substantial amount of PDGF. These results are very similar to that of the Oligo First Method.

V. Discussion and Future Directions

The initial release study with the oligonucleotide conjugation setup showed promising results. Heating of the conjugated surface produced an appreciable release of Texas-Red fluorescence signal into the collected supernatant as shown in Figure 3. Sharp drops in the fluorescent signal from the conjugated surface mirror this as well. Furthermore, lowering the conjugated surface back to room temperature quickly lowers the amount of Texas-Red released from the surface, and by the one hour, had been reduced to baseline levels of release.

The no-linker control negative control also showed small amount heat sensitivity in terms of Texas-Red release. However, this is likely due to the increased thermal energy allowing more nonspecifically bound molecules to be released from the chitosan surface. Compared to the surface treated with the linker, the release is significantly smaller.

Through the Texas-Red Oligonucleotide release study, we were able to demonstrate the feasibility of the conjugation scheme on a biomaterial coating with free amine groups. Past literature have shown the behavior of the oligonucleotide tether release by immobilizing the primary strand onto magnetic beads[21]. The results demonstrated shown here are consistent with those studies as well. The importance of proving this conjugation can take place on a surface coating of chitosan shows that there is great promise in incorporating this oligonucleotide tethering system into another surface or peptide with free amine groups[16,20]. With this conjugation setup, we can theoretically not only incorporate this system into biodegradable implants such as a

fibrin gel, but to any biomaterial based small molecule delivery platform with an appreciable number of free amines.

Once we had determined the feasibility of the oligonucleotide tether system, we proceeded to develop a model where we can replace the tethered Texas-Red molecule with a potential therapeutic protein, in this case, PDGF. Having to conjugate the PDGF protein to the complementary oligonucleotide strand presented an additional step to the conjugation scheme. This step could have been incorporated in two separate methods, described as “Oligo First” and the “PDGF-Oligo First” in the methods section. While the “Oligo-First” method allowed for better PDGF conjugation to the chitosan surface and was an easier conjugation scheme to perform, the “PDGF-Oligo” conjugation showed a much more controlled release of the PDGF from the system. Looking at the supernatant release in Figure 4c, there is a similar amount of PDGF released to the supernatant in the overnight incubation at room temperature compared to the heated dose release afterwards. There is a much lower release of PDGF in the “PDGF-Oligo” conjugation scheme in the absence of heat, as shown in the supernatant release graph in Figure 5c.

In both situations, the control used was radiolabelled PDGF without the use of sulfo-SMCC to attempt to link the growth factor onto the surface. The control was to test the amount of non-specific binding of the growth factor to the chitosan surface. From both Figure 4a and Figure 5a, we can see that very little of the PDGF binds nonspecifically to the chitosan surface.

There are several potential reasons for the higher apparent conjugation efficiency of the “Oligo-First” scheme as opposed to “PDGF-Oligo First”. In the “Oligo-First” setup, the PDGF with sulfo-SMCC linkers become PDGF-MAL molecules with multiple free

maleimide groups to react with the thiolated oligonucleotides. However, due to the number of amines outnumbering that of the oligonucleotides, there are free amines on the chitosan surface even after the treatment with the oligonucleotides. These free amines can react with the PDGF-MAL, forming a stable bond attaching the PDGF directly onto the chitosan surface[22]. A second reason for the lowered efficiency of the "PDGF-Oligo First" conjugation scheme is that all of the PDGF-Oligo conjugation process leads to some PDGF-Oligo along with some free oligo as well. This competition between the two leads to a lower overall efficiency of thermally-releasable PDGF. One potential method for improving the efficiency of the "PDGF-Oligo First" method is to remove the excess oligos from the PDGF-Oligo complex using column chromatography. The tradeoff would be a dilution of the PDGF-Oligo complex in solution.

One thing to note is that approximately 75% of the samples remained in the chitosan surface even after 120 hours of the release study. This can be a combination of several reasons. One can be that there is a higher amount of oligonucleotides in the conjugation setup. If this is the case, it is possible that several oligo tethers were conjugated to the growth factor. In this scenario, the growth factor would be more tightly bound to the surface. Some preliminary work have been done, showing that introducing a heating session of about 1 hour will continue to cause significant release of the radiolabelled PDGF. Another reason is that in preparing the PDGF for conjugation to the complementary strand, the treatment with sulfo-SMCC has allowed multiple linker molecules to be attached to a single PDGF molecule. It is possible that the un-used linkers on the PDGF growth factor allowed the maleimide group of the linker to directly conjugate onto the amines of the chitosan surface. This results in a more permanent

linkage of PDGF to the chitosan surface, which can be another reason for the difficult release of the PDGF. These situations can be reduced through optimizing the conjugation scheme and introducing additional purification steps through chromatography.

In future work, the bioactivity of the growth factor released from the system needs to be verified. This can be done by culturing the human foreskin fibroblasts (HFF) with the collected supernatant from a non-radiolabelled PDGF conjugated surface[2]. Methods of measuring cellular response to the PDGF will be outlined further in Chapter 2.

Another parameter of interest is controlling the melting temperature Tm of the annealed oligonucleotide. The Tm of a double stranded oligonucleotide is the temperature at which half of the nucleic acid strands are separated. This parameter would effectively determine how much heat is required to release the PDGF growth factor from the system. With the current system, a temperature of 45^{o}C is required for the release of the oligonucleotide. This is true for both the Texas Red release as well as the radiolabelling release experiments. Because the oligonucleotide annealing process is due to the formation of hydrogen bonds between the nucleotides of the nucleic acid strands, it follows that the total number of hydrogen bonds that can form between the primary and complement oligonucleotide strands will play a key role in determining the Tm[23].

We can control the Tm in one of three ways. First is by controlling the length of the oligonucleotides. A longer oligonucleotide strands will lead to more base pair interactions, requiring a higher Tm[24]. Second is by controlling the salt concentration of the environment. Having a higher cation concentration will stabilize the duplexing of

oligonucleotides because more cations can bind to the annealed oligos rather than separated strands. Thus, a higher salt concentration tend to increase Tm[25]. Finally, the G-C ratio of the oligonucleotide also affects Tm because of the difference in number of hydrogen bonds that form between guanine and cytosine (3 hydrogen bonds) versus thymine and adenine (2 hydrogen bonds). A higher G-C ratio effectively means there are more hydrogen bonds forming in a duplex oligo, and will result in a higher Tm[26]. The way these three factors change the salt-adjusted melting temperature were calculated with a Oligonucleotide Properties Calculator provided by Northwestern University and shown in Figure 6[27]. Given the Na^+ concentration in human blood is approximately 135-145 mmolar, we can see that the melting temperature with the current setup would be much higher than the target 45^oC[28]. In order to do so, we will need to consider both shorter and lower GC content oligonucleotides. Looking at Figure 6, this is feasible because the changes in melting temperature vary much more quickly with oligo length and GC content than cation concentration.

In future work, it would be important to determine the Tm of the oligonucleotides of interest in a salt concentration more consistent with that of an in-vivo or in-situ environment. We also are looking to conjugate this system into the free amines of light-responsive peptides in a fibrin gel as described in Figure 1. The Tm may also need to be adjusted depending on an implant depth and heat transfer efficiency of the light-activated system that we eventually wish to incorporate into the growth factor delivery platform[13].

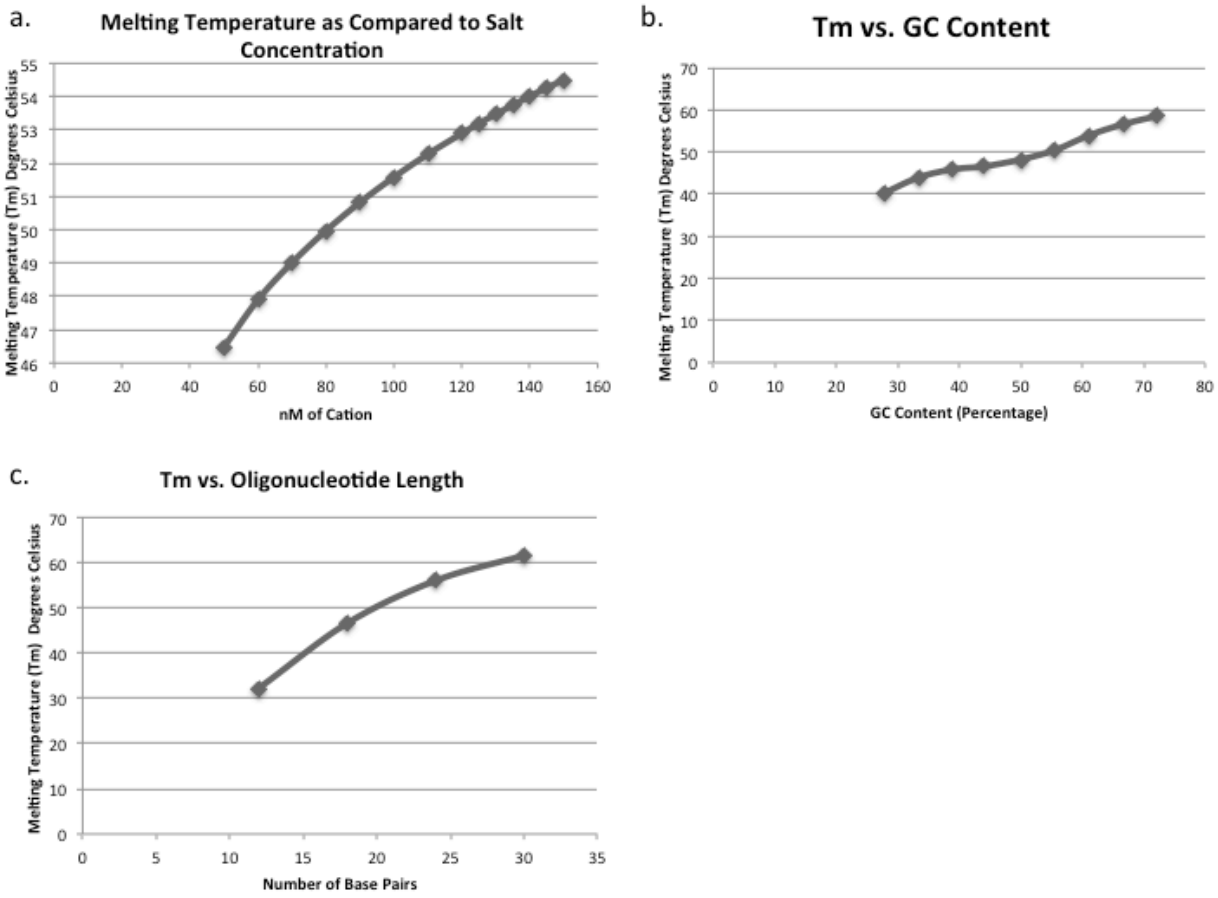


Figure 6. Comparisons of calculated melting temperatures versus different parameter. (a) Melting temperature vs. Salt Concentration. It is noticeable that under the experimental parameters, the Tm is just higher than 46^{o}C, which makes sense given we used 45^{o}C to generate the oligonucleotide release. The body normal Na^{+} concentration is around 135-145 nM. (b) Melting temperature vs. GC Content. Given GC content varies by several percentages by swapping a A or T base by C or G base, we can see that this is the easiest variable to change to generate the largest change in melting temperature. The current used parameters has a GC content of 44%, which yields a 46.6^{o}C. (c) Melting temperature vs. Oligonucleotide length. Changing the Oligonucleotide length also has a wide range of different melting temperatures. One thing to note is the oligonucleotides were added and subtracted in increments of 2 codons (or 6 base pairs). This is to ensure that the GC ratio added or subtracted remained constant at 50% to minimize variation in GC content in this comparison.

Part II

I. Use of Protein Peptide Mimics to Simulate Growth Factor Effects In-vitro

While growth factors and hormones have been shown to have positive results in treating chronic wounds, they are still a very expensive treatment option[1]. The drive for lowering costs incentivized the search for potential alternatives to stimulating the same biological effect.

In previously published research, there is promise that proteins can be replaced using small peptide fragments containing the amino acid sequence corresponding to protein sections that bind to their respective receptors[29]. Modeling the areas of PDGF that binds to the receptors, we hypothesized that the following amino acid sequences may induce a similar activation of PDGF receptors.

II. Materials and methods:

1. In-vitro Cellular Response to PDGF and Peptide Mimics

Human Foreskin Fibroblasts (HFF, ATCC) were cultured in serum deficient Dulbecco's Modified Eagle Medium (DMEM, Corning) at 10,000 cells per well in a 24 well plate (Corning). Cells were incubated in media spiked with PDGF (Sigma) at 10 ng/mL for 15 minutes, 30 minutes, and 1 hour. Cells were also exposed to media spiked with one of peptides A,C, R, and N (Peptide A - AECK, Peptide C - CNNRNV, Peptide R – RKIE, Peptide N – NPEQTPVL, CPC) at 10 ng/mL for 30 minutes. The HFFs were immediately fixed with 10% Buffered Formalin (Fischer) and stored in 1x Phosphate Buffer Solution (PBS, Fischer).

2. Phalloidin stain and Imaging

Cells were fixed for 30 minute in 10% phosphate buffered formalin (Fischer) and stained with a 1:100 dilution of Alexa Fluor Phalloidin (Thermo Fischer). The cells were then imaged with the TRITC filter of an Olympus IX71 inverted microscope (Olympus).

III. Data

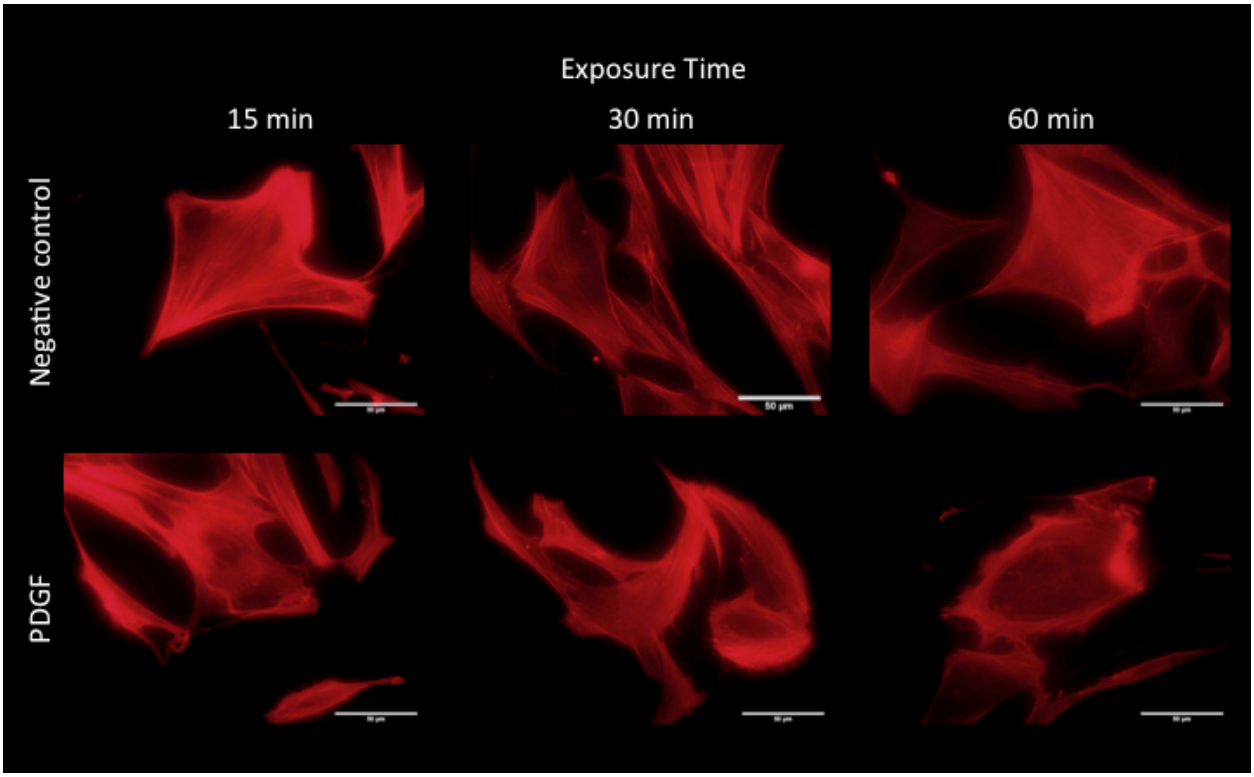


Figure 7. Phalloidin stain of Human Foreskin Fibroblasts responding to PDGF and exhibiting the ruffling phenomenon. The ruffling effect begins in as little as 15 minutes, and starts to wane after 60 minutes. This signals a very transient response of the cells to PDGF. In the negative control, where the cells are cultured only in serum-deficient media, we see the cells spread out and flat.

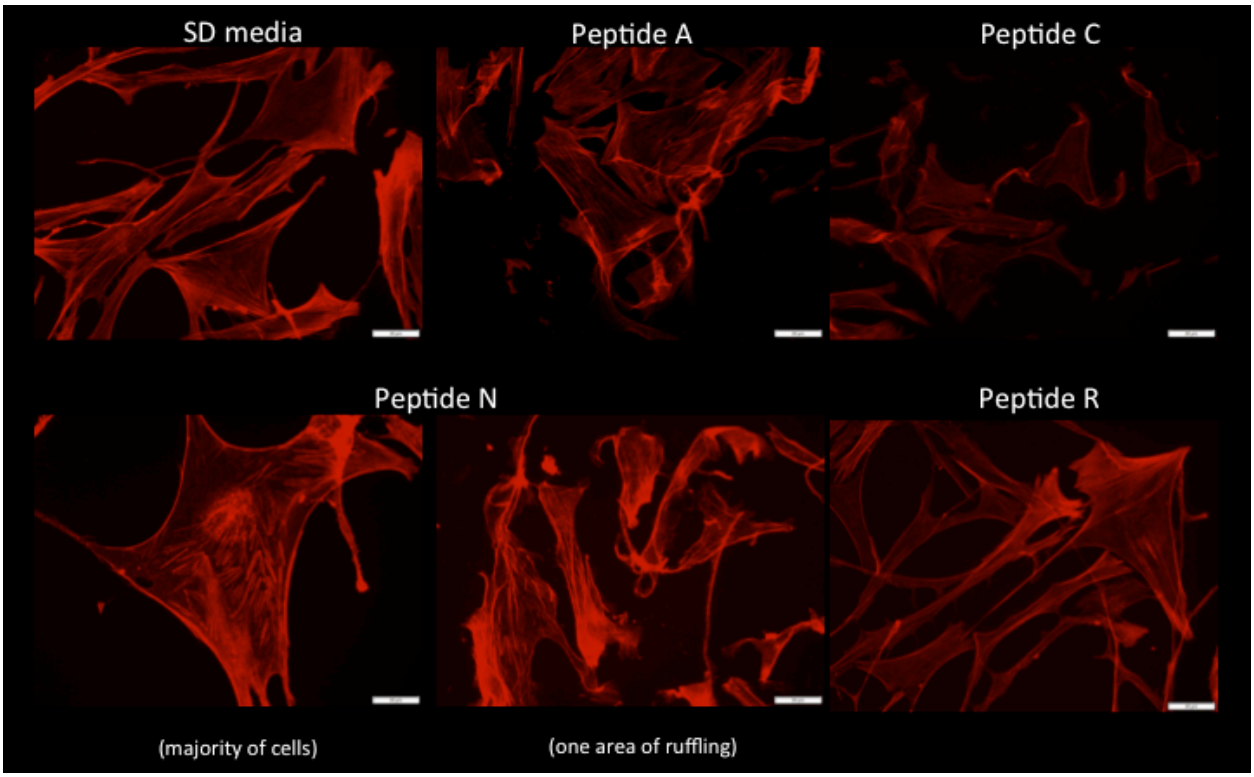


Figure 8. Phalloidin stain of Human Foreskin Fibroblasts responding to different peptide sequences found to be in the binding area of the PDGFR as well as a peptide of random sequence. All cells were exposed to respective media for 30 minutes. The ruffling effect is most apparent in Peptide A and Peptide C. Peptide N exhibits only one small area of folding whereas the rest of the cells look flat similar to that of serum-deficient media. Peptide R also does not induce appreciable amounts of cell ruffling. Peptide A and Peptide C look most promising to induce an affect similar to that of PDGF.

IV. Discussion and Future Directions

It has been shown that HFF responds qualitatively to the PDGF by displaying a ruffling phenomenon[30–32]. This is shown in the Figure (7), where you can see the folding of the HFF after exposure to media spiked with PDGF at 10 ng/mL for as little as 15 minutes. Extending the exposure time of cells to PDGF to 30 minutes and further until 60 minutes, we can still see the folding of the HFFs. However, it is important to note that by 60 minutes, the cells are exhibiting less of this phenomenon than either 15 minute or 30 minute exposures. Other results measuring the in-vitro response of cells to PDGF also expose the cells to the mitogen for less than 30 minutes[33,34]. These results suggest the cellular response to PDGF is very transient, and the PDGF is consumed very quickly as well.

Due to the relatively high cost of full growth factors such as PDGF, there is incentive to use potential replacement molecules that can stimulate a similar effect in cells. Researchers have identified several peptide sequences that correspond with the active sites of the PDGF-receptors (PDGFR). Three peptide sequences identified were Peptide A, Peptide C, and Peptide R, whose full peptide sequences are described in the methods section[35]. Looking at the exposure of HFF to Peptide A and Peptide C, we see a similar ruffling effect as shown in Figure (8) as in the PDGF exposure in Figure (7). Peptide N also displays some degree of ruffling, but there are only limited areas where this can be seen. Most of the cells exposed to Peptide N do not show this effect.

For future directions, we want to show quantitatively the effects of the Peptide A and Peptide C and compare them to the effects of PDGF. There are a couple well-characterized means of quantifying cellular response to PDGF. One method is to use a

radiolabelled phosphates to measure the amount of phosphorylation has occurred due to the binding of the PDGF to the PDGFR[33,36]. A second method that does not require radioactive material is to use immune-precipitation and Western blotting in order to separate out phosphotyrosine activated PDGF receptors[37]. A final method to confirm the specificity of the PDGF and peptide interaction with the cells is to introduce a silencing RNA that can stop the HFF expression of PDGFR. Both phosphorylation and transcription factor production in the presence of PDGF as well as Peptide A and Peptide C should drop compared to cells that weren't treated with the PDGFR silencing gene.

Going back to the oligonucleotide tethering platform described in chapter 1, we also need to check the bioactivity of the tethered growth factors released after heating intervals. These studies can be conducted by mixing the supernatant with serum deficient media for culturing the HFFs. We can then examine the cells for ruffling and quantify the degree of PDGF expression through any of the methods described earlier as well.

Conclusion

In conclusion, we have developed a conjugation scheme that adequately tethers growth factor to an oligonucleotide tethering system. It is able to produce appreciable release of the target molecule of interest after short exposure time to heat, and also minimizes the release of the molecule when the heat is absent. Furthermore, this is the first time that appreciable release has been to remain over the course of several days.

The next steps of the studies are to further test the bioactivity of the molecule released, as well as to incorporate the platform from a coated surface into a fibrin gel.

Furthermore, the preliminary studies have shown that there is promise in the ability to use short peptide sequences that match the active site of the PDGFR, thus eliminating the need for utilizing whole growth factors in this system.

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