Bioactive Sutures to Prevent Fibrosis in Injured Skeletal Muscle Tissue

Núria Codina Castillo

University of Colorado Boulder, nnuria.codina@gmail.com

Follow this and additional works at: https://scholar.colorado.edu/chbe_gradetds

Part of the Biological Engineering Commons, and the Chemical Engineering Commons

Recommended Citation
https://scholar.colorado.edu/chbe_gradetds/1

This Thesis is brought to you for free and open access by Chemical & Biological Engineering at CU Scholar. It has been accepted for inclusion in Chemical & Biological Engineering Graduate Theses & Dissertations by an authorized administrator of CU Scholar. For more information, please contact cuscholaradmin@colorado.edu.
BIOACTIVE SUTURES TO
PREVENT FIBROSIS IN INJURED
SKELETAL MUSCLE TISSUE

by

NÚRIA CODINA CASTILLO

B.A., University of Barcelona, 2010
M.S., University of Barcelona and Polytechnic University of Catalonia, 2012

Advisor: Joel L. Kaar

A thesis submitted to the
Faculty of the Graduate School of the
University of Colorado in partial fulfillment
of the requirement for the degree of

Master of Science Thesis
Department of Chemical and Biological Engineering
2014
This thesis entitled:

**Bioactive Sutures to prevent fibrosis in injured skeletal muscle tissue**

written by Núria Codina Castillo

has been approved for the Department of Chemical and Biological Engineering

---------------------------------------------
Joel Kaar

---------------------------------------------
Stephanie Bryant

---------------------------------------------
Mark Stoykovich

Date ________________

The final copy of this thesis has been examined by the signatories, and we find that both content and form meet acceptable presentation standards of scholarly work in above mentioned discipline.
Primitive animals and early mammalian embryos have the capacity to respond to injury by regenerating the affected tissue. Most tissues in the human body, however, follow an inferior method of wound repair that finishes with an accumulation of collagen, also known as scar tissue. Scar tissue blocks progenitor cells from infiltrating the site of injury, resulting in incomplete regeneration. Slowing the fibrotic response would shift the wound repair process towards regeneration and considerably improve human health.

The primary goal of this project is to develop novel bioactive sutures for preventing fibrosis in injured skeletal muscle. This will be achieved by immobilization of collagenase enzymes on polyethylene terephthalate (PET) sutures.

Carboxylic groups were introduced on PET sutures, which were later coupled via EDC and sulfo-NHS to primary amines on the collagenases forming an amide bond. We immobilized Clostridium histolyticum collagenases on the sutures, which have broad substrate specificity, being able to degrade water-insoluble collagens as well as soluble collagens, and making multiple cleavages within the triple helical region.

While trying to control the immobilization reaction, we observed that there was non-specific binding between the enzyme and the suture. Specifically, around 70% of the activity on the sutures was due to enzyme non-covalently attached to the suture, and only 30% was due to enzyme covalently attached to the suture. The hydrophobic adsorption of the enzyme to the suture was minimized by addition of 0.05% tween 20 (v/v) to the coupling and washing solutions.

We tested the stability of the collagenases immobilized on the sutures relative to the native collagenases at 37°C, and the enzymes on the sutures lost activity faster. After two hours, there was no enzyme activity on the suture, whereas the free enzyme in solution still retained more than 50% of the activity.
In order to obtain higher collagenolytic activity on the sutures, further improvement of the immobilization method is required. This can be achieved, for example, by introduction of non-natural amino acids on the enzyme that allow for site-specific conjugation of the enzyme on the suture.
DEDICATION

To my loving family, my supportive friends, and Cam
ACKNOWLEDGMENTS

First of all, I would like to thank all the members that served in the committee for the defense of this Master’s Thesis: Professor Mark Stoykovich, Professor Stephanie Bryant and Professor Joel Kaar. I am very grateful for their time and dedication in reviewing this thesis.

I would like to thank my advisor, Joel Kaar, for his mentoring and support during these two years, and for providing me with an excellent atmosphere for doing research.

I am particularly thankful to the Balsells Graduate Fellowship program for giving me the opportunity to come and pursue my studies at CU Boulder.

Last but not least, a heartfelt thanks to my family, friends and partner for their unconditional support and encouragement throughout this process.
LIST OF CONTENTS

Part I. Background

1. Clinical Burden of Fibrosis ..................................................... 1
2. Skeletal Muscle ................................................................. 3
   2.1. Anatomy of Skeletal Muscle ............................................ 3
   2.2. Biological Process of Skeletal Muscle Healing Following Injury ...... 5
   2.3. Current Treatments and Research Trends for the Prevention of Scarring .... 11
3. Collagen Degrading Enzymes ................................................ 17
   3.1. Matrix Metalloproteinases (MMPs) .................................... 17
   3.2. Clostridium Histolyticum Collagenases .............................. 18
4. Bioactive Sutures ............................................................... 26

Part II. Objective ................................................................. 28

Part III. Materials and Methods ............................................ 29

1. Materials and Chemical Agents ............................................ 29
2. Protocols and Methods ...................................................... 31
   2.1. Cloning, Expression and Purification of recombinant MMP-1 and CoLG .... 31
   2.2. Functionalization of polyethylene terephthalate (PET) sutures ............. 32
   2.3. Immobilization of the enzyme onto PET sutures ............................ 33
   2.4. Activity Assay with FALGPA – peptidolytic activity ....................... 36
2.5. Activity Assay with Gelatin (denatured collagen) .......................... 37
2.6. Collagen gel – collagenolytic activity ........................................... 38

Part IV. Results and Discussion

1. Produce and characterize recombinant collagenase active against collagen ............... 40
2. Covalently attach collagenase to PET sutures and control the immobilization ............ 44
   2.1. Characterization of PET surface treatment .................................... 44
   2.2. Immobilization of collagenase onto PET sutures ............................ 47
   2.3. Control the activity of collagenase-modified sutures ........................ 49
3. Characterize the stability of the collagenase-modified PET sutures ......................... 57
4. Characterize the degradation of type I collagen gels by collagenase-modified PET sutures 58

Part V. Conclusions and Future Work .................................................. 59

Bibliography ......................................................................................... 62
Part I. Background

1. Clinical Burden of Fibrosis

Repair of wounds is one of the most complex biological processes that occur during human life\(^1\).

Primitive animals respond to injury by mitosis of their cells, being capable of regenerating exact duplicates of the missing parts. This form of healing is known as regeneration. Early mammalian embryos are also able to regenerate injured tissues, replacing the damaged cells by the same cells that were there before and so, rebuilding the exact same tissue. Studies of skin wounds for different mammalian embryos (mice, rats, rabbits, sheep, pigs, marsupials, monkeys) show that if the injury is made during the first one-third to one-half of gestation, it heals perfectly\(^2\). Some organs in our body still retain this biological gift of regeneration too. As examples, small scratches of the skin heal by epidermal regeneration, hepatocytes in the liver are able to regrow after mild toxic conditions and some peripheral nerves are able to heal by axoplasm regeneration. However, this regenerating capacity is limited to minor injuries and healing under optimal conditions. Bigger wounds such as burns, cirrhosis, and large nerve gaps overwhelm our regenerating capacity.

Nature has provided us with another means of survival. It is an inferior method compared with regeneration, but it is the primary means of repair for all vertebrates\(^3\). Special cells in our body respond to injury by forming a collagenous "glue"\(^4\). This body glue is called scar tissue and consists of excess extracellular matrix components, predominantly collagen. Almost every tissue in the body follows the same wound repair process after an injury, despite the cause or the organ affected. Thus, the sequence of events that follows a myocardial infarction (heart attack), for example, is very similar to that after a spinal-cord injury, a burn, or a gunshot wound. At the end, the wound is bridged by scar tissue that leads to tissue dysfunction regardless of the injury site.

Healing by scarring instead of regeneration has evolved to quickly mend injuries encountered in the past, such as primitive bites, blows, or contusions. These wounds would typically involve widespread tissue damage and frequently be dirty. The evolutionary forces shaping healing focused on preventing infection, walling off foreign bodies, and rapidly restituting the missing tissue. Quickly restoring
suboptimal function was more important than full tissue regeneration. These are not the types of wound commonly encountered today. Nowadays, the most frequent wounds are made by a sharp object under clean and sterile conditions, and with its margins closed by sutures or bandages. This is a new wound condition, which has arisen only in the past 500 years or so and it has not been optimized by the evolutionary forces shaping wound healing mechanisms. Therefore, scar-free healing could be possible and more appropriate for modern injuries.

Fibrotic diseases are one of the largest groups of disorders for which there is no effective therapy and thus represent a major unmet medical need. Healing by fibrosis, instead of regeneration, places a huge burden on public health. In the case of a heart attack, the formation of scar tissue in the heart can result in congestive heart failure (the heart cannot supply the body with enough blood) and abnormal heart rhythms, which together account for nearly 100,000 deaths each year in the United States alone. The total economic cost that results from fibrosis is difficult to calculate precisely, because it also takes into account cirrhosis of the liver, fibrosis of the lungs, and lifelong disabilities caused by fibrosis, but it is estimated to be on the order of tens of billions of dollars.

Manipulating the wound repair process in mammals, so that it shifts towards regeneration, will require the ability to slow the rapid fibrotic response allowing the progenitor cells to regenerate the tissue. If fibrotic healing processes could be transformed into regenerative ones, in which the original tissues are restored, this would considerably improve human health.
2. Skeletal Muscle

Skeletal muscle pain is among the most common ailments treated by physicians in a general medical practice, and is specifically frequent with athletes and soldiers. Muscle injuries present a challenging problem in traumatology, as injured muscles heal very slowly and often with incomplete functional recovery. The frequency of re-injuries remains as great as 44%, and the risk of re-injury has been correlated with the amount of scar tissue formation in the muscle after injury.

2.1. Anatomy of Skeletal muscle

Skeletal Muscle is one of three major types of muscle, the other two being cardiac and smooth muscle. Skeletal muscle represents the largest tissue mass in the body, constituting 40% to 45% of total body weight. As its name suggests, the muscles are attached to the skeletal system. Muscles and bones are bound together by bundles of collagen, known as tendons. When a muscle contracts, it moves the bones that are attached to it allowing us to run, breath, lift, talk, type, etc. Skeletal muscles are voluntarily controlled by the somatic nervous system.

Skeletal muscle is composed of muscle cells called myofibers and connective tissue. Within the connective tissue there are blood vessels that supply the cells with oxygen and nutrients, and nerves that transmit the electric signal that will translate into movement. The connective tissue provides the framework that binds the individual muscle cells together and sums up their individual contractions to efficient locomotion. Figure 1 shows the anatomy of a skeletal muscle from the whole muscle belly to the molecular level.

The epimysium is the outside connective tissue layer that surrounds the entire muscle. It is a strong and thick sheath that protects the muscle and reduces its friction with the surroundings. Within the epimysium there is more connective tissue called the perimysium that divides fascicles or bundles (from some tens to a couple of hundreds) of myofibers. Inside the fascicle the connective tissue is called endomysium, or also basal membrane, and covers individual muscle cells. A single skeletal muscle cell is known as a myofiber. Myo refers to muscle and receives the name fiber because it is longer than it is wide. Myofibers can be as short as several hundred micrometers or as long as several centimeters. Because muscle cells are so long, they need multiple nuclei. Myofibers are multinucleated, which allows
for on-demand protein synthesis at sites where needed, instead of the synthesis of proteins from a single nucleus, requiring translocation of nascent proteins to other regions of the fiber. Skeletal muscle fibers are formed from the fusion of multiple myogenic precursor cells called myoblasts. Myoblasts fuse together to form the long, cylindrical and multinucleated immature myofibers with central nucleation. Eventually, the nuclei migrate to the periphery, under the plasma membrane, to form a mature myofiber.

![Figure 1. Anatomy of a skeletal muscle fiber](image)

The plasma membrane of the muscle cell is called sarcolemma. Inside the muscle cell there are more tubes called myofibrils and they are the major constituents of the myofiber. Under a light microscope this fibrils present striations, with units of repetition called sarcomeres. Sarcomeres are contractile units consisting of two filamentary proteins, actin and myosin. Every myofiber is contacted by one nerve terminal of a motor neuron. The single nerve axon and all of the myofibers that it contacts constitute a motor unit. When a motor neuron tells the muscle to contract there is an action potential that travels along the membrane in all directions and eventually goes into the cell. This causes the sarcoplasmic reticulum to momentarily release calcium, and the calcium causes the contractile proteins (actin and myosin) to interact and to generate force. When all the myofibrils contracting are added together enough force is generated to produce work.

During the fetal development a pool of undifferentiated reserve cells, called the satellite cells, are set aside between the basal lamina and the plasma membrane of each individual myofiber. In response to injury, they will start a regeneration process. First they will proliferate, then differentiate into myoblasts, and finally join with each other to form multinucleated myotubes and mature myofibers.
2.2. Biological Process of Skeletal Muscle Healing Following Injury

Muscle injuries occur through a variety of mechanisms, including direct trauma (e.g., lacerations, contusions, and strains) and indirect causes (e.g., ischemia and neurological dysfunction), but the general process of muscle damage and repair is similar in most cases, irrespective of the underlying cause\textsuperscript{6-8}. Once injured, muscles undergo interrelated, time-dependent processes, including: (1) degeneration and inflammation, (2) regeneration, and (3) fibrosis (Figure 2).

\textbf{Figure 2.} This illustration shows the steps of muscle healing: a) intensity of each phase with time, b) cartoon depiction of what happens in each phase at the cellular level, and c) skeletal muscle tissue under the microscope. Image (1) corresponds to the Degeneration and Inflammation phase and we can see macrophages cleaning and removing the damaged muscle fibers, and the formation of an early granulation tissue. Image (2) corresponds to the Regeneration phase where satellite cells become activated and start fusing together to regenerate the muscle fibers, and fibroblast start depositing collagen. Image (3) corresponds to the Fibrosis phase where a scar has been form as the end result of wound healing and regeneration could not take place\textsuperscript{6}. 
(1) Degeneration and Inflammation:

Muscle degeneration and inflammation occur in the first 1-3 days post-injury. The objective of this stage is to stop blood loss, to remove dead tissue and to prevent infection.

When a muscle is injured, the most common situation is that all the components within the muscle get damaged: myofibers, ECM, blood vessels and nerves. Mechanical trauma disrupts the plasma membrane of the myofiber leaving the cell wide open. The ruptured myofibers retract and a gap develops between the stumps. As the myofibers are very long, there is an imminent threat that the necrosis initiated at the site of injury will extend along the cell. A specific structure called the contraction band based on condensation of cytoskeletal material seals the plasma membrane by forming a protective barrier. This way, within hours after the injury, the necrosis has been limited to a local process.

The majority of tissues contain growth factors stored in an inactive form in their extracellular matrix (ECM) that become active when the tissue is damaged, starting to direct the repair process. A delicate interplay between infiltrating inflammatory cells, multiple growth factors, cytokines, and myogenic cells will take place.

The skeletal muscle has a rich vascular supply, with many capillaries throughout the muscle belly. These blood vessels will also be disrupted after an injury, giving direct access to the injury site to the blood borne inflammatory cells. Within the first few minutes after the injury, platelets aggregate to form a clot. Blood-derived fibrin and fibronectin cross-link to form granulation tissue, that acts as an ECM scaffold and anchorage site for the invading cells. The first cell to infiltrate the site of injury is the neutrophil, which begins within the first hour after injury and peaks at 24 hours. Attracted by proinflammatory cytokines, macrophages also migrate to the site of injury and arrive after the neutrophils. Macrophages have two functions: one, remove the necrotic myofibers by phagocytosis and two, together with fibroblasts, release chemotactic signals such as growth factors and cytokines. Macrophage phagocytosis is remarkably specific for the necrotic tissue, leaving intact the undamaged basal lamina around the necrotic debris.

The growth factors and cytokines released in the injured muscle perform a wide range of functions. Some substances influence the blood flow, the vascular permeability, and accelerate the inflammatory response; examples are cytokines such as interleukins (IL-8, IL-6, IL-1) and tumor necrosis
factor-α (TNF-α). Adhesion molecules are also secreted (P-selectin, L-selectin, and E-selectin). The other growth factors released during inflammation will have roles in muscle regeneration and fibrosis.

Once the cleaning phase has diminished, the actual repair of the injured muscle will begin. Two simultaneously supportive but competitive processes take place: the regeneration of the disrupted myofibers and the formation of a connective tissue scar between the stumps.

(2) Regeneration:

Muscle fibers have the ability to regenerate after a muscle injury. The regeneration phase begins about 1 week after injury, peaks during the second week, and then decreases at three to four weeks post-injury.

The key cell involved in regeneration of skeletal muscle is the satellite cell\(^9\). Normally satellite cells lie quiescent between the plasma membrane of the myofiber and the basal lamina. After a muscle injury, growth factors like: insulin-like growth factor 1 (IGF-1), basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), and hepatocyte growth factor (HGF) will activate the satellite cells. Satellite cells start proliferating, differentiating and fusing into multinucleated myotubes, with the nuclei located in the center. These newly formed multinucleated myotubes then will fuse with the part of the myofiber that has survived the initial trauma and the nucleus will migrate to the periphery. Some satellite cells are ready to begin differentiation to myoblasts immediately after the injury, but there are stem satellite cells, that first undergo cell divisions before differentiation, to replenish the reserve of satellite cells for the possible future demands.

The extremes of the damaged myofiber try to reconnect with each other from day 5 onwards. The ends of these regenerating myofibers have a growth cone appearance, with multiple branches, until about day 10-14. However, at the same time, a connective tissue scar has started to form between the surviving stumps of the myofiber. The regenerating stumps, after managing to extend only for a relatively short distance, begin to adhere to the connective tissue, forming mini-myotendinous junctions (MTJs) with the scar.
(3) Fibrosis:

The development of fibrosis begins two weeks after muscle injury and increases over time for up to four weeks post-injury. As we have seen, muscle tissue has the capacity to begin regeneration promptly after injury; however, this phase is often blocked by the appearance of the fibrosis phase.\textsuperscript{10}

Fibrosis, or scarring, is defined by the accumulation of excess extracellular matrix components. The name comes from the primary cell of scar production, the fibroblast. Fibroblasts respond to the signals sent by the macrophages and transform into cells with migratory ability. These migratory fibroblasts follow the fibrin meshwork created earlier and access all depths of the wound. Once in place, fibroblasts are ready to begin their synthesis of proteins of the extracellular matrix. First, they express fibronectin, and later, collagen (Figure 3). They express fiber-forming collagens, initially type III collagen, a weaker form that can be produced rapidly and later type I collagen, long-stranded and stronger, which remains activated for several weeks.\textsuperscript{11,12}

Collagen synthesis begins with fibroblasts expressing three polypeptide chains: two identical $\alpha_1$ chains and one $\alpha_2$ chain (that differs slightly in its chemical composition).\textsuperscript{13} The most common motifs in the amino acid sequence of collagen are: glycine-proline-X and glycine-X-hydroxyproline (where X is any amino acid other than glycine, proline or hydroxyproline). Each alpha peptide forms a left-handed helix. In the endoplasmic reticulum of fibroblasts, these three left-handed helices are twisted together into a right-handed triple helix, known as procollagen. Procollagen is stabilized by numerous hydrogen bonds formed between the oxygen of a proline C=O in one helix and the H of a glycine N-H in another helix. Procollagen is a precursor of collagen that is unable to assemble in larger structures, avoiding this of happening inside the cell. Procollagen is composed of a twisted portion in the center and two loose ends on either end, these ends are what prevents the procollagen molecules of assembling together. Procollagen is packaged into a secretory vesicle and exported to the extracellular space. Once outside the cell, membrane bound enzymes known as collagen peptidases remove the N- and C- terminal ends. What is left is known as tropocollagen or collagen molecule. Tropocollagen is approximately 300 nm long and 1.5 nm in diameter. Tropocollagens spontaneously associate in a staggered array to form a collagen fibril (it is a right-handed super-super-coil). These collagen filaments lay disorganized in the wound, still in a gelatinous state.
Figure 3. Collagen overview: a) space filling representation of the collagen triple helix, b) three left-handed helices coiled together into a right-handed triple helix, c) hydrogen bonds between the triple helix are formed between the oxygen of a proline C=O and the H of a glycine N-H, and d) collagen formation. In the ER the 3 alpha helices twist to form a triple helix, and once is outside the cell the N- and C- ends are cleaved and assembly of the collagens for form a fibril can take place\textsuperscript{14}.

The amount of collagen filaments formed does not build strength. Tensile strength increases as covalent bonds start to form. Lysyl oxidase, an extracellular enzyme, produces the final step in the collagen synthesis pathway. This enzyme acts on lysines and hydroxylysines producing covalent aldehyde bonds. The initial crosslinks are intramolecular, between a single tropocollagen molecule, and these bonds enhance the stability of the molecule but tropocollagen is still soluble. Intermolecular bonds between tropocollagen molecules are the forces that will provide tensile strength to the wound.

During the first three weeks of healing the collagen accumulates in the wound, resulting in a large mass of assembled collagen. The morphology is a bulky, rough, tender, and red scar. The formation of cross-links until this point allowed the wound to tolerate early loads, but the tensile strength of the
muscle is only about 15% of the strength before the injury. The quantity of collagen present at this time is an indication of the final outcome, the higher amount meaning a less functional outcome.

After the third week, the collagen content stabilizes and rearranges to try to fit the properties of the tissue it is healing. Collagen synthesis still continues at a high rate, but there is no increase in scar mass, meaning that simultaneous collagen synthesis and removal is happening in a balanced way. New collagen is formed as old collagen is broken down, and both rates are higher than pre-injury rates (Figure 4). This process continues until the remodeling phase ends at six months to a year post-injury. During this time, the collagen fibers undergo alignment and reorganization.\textsuperscript{15}

\textbf{Figure 4.} a) New collagen accumulation in excised wounds and control skin. The difference between curves represents scar collagen accumulation, showing that collagen accumulates during the first 3 weeks but the synthesis equals the normal skin synthesis rate after that, meaning there is no further accumulation. b) Rate of new collagen deposition in excised wounds and control skin, shows that the rate of collagen synthesis is still elevated even though there is no accumulation, implying that the collagen degradation rate is also high.\textsuperscript{15}

Initially, the physiological role of the collagenous scaffold is to transmit load across the defect so that the injured muscle can be used before the completion of the repair process. The problem is that in extensive muscle trauma, the lack of an endpoint for the fibrosis cascade leads to the proliferation of fibroblasts producing an overabundance of the extracellular matrix. The fibrotic tissue becomes a dense scar that impedes the infiltration of satellite cells to the site of injury. The muscle cannot regenerate efficiently across the scar tissue and the result is an incomplete recovery. Instead of the original (pre-injury) tendon-myofiber-tendon unit, the result is two successive tendon-myofiber-myotendinous junctions (MTJ) units separated by the scar. These two successive units still contract synchronously, as the same nerve innervates both of them.
Despite the high capacity for regeneration, the end result of muscle healing is the formation of a dense fibrotic scar between the normal muscle tissue. The scar tissue lacks the elasticity of the native muscle, thus, preventing the full recovery of function and increasing the likelihood of re-injury. Scar tissue is the end product of the muscle repair process and as long as it forms, complete regeneration of muscle tissue cannot occur.

### 2.3. Current Treatments and Research Trends for the Prevention and Reduction of Scarring

Optimal treatment methods for muscle injuries have not yet been determined. Clinically, muscle injuries are treated with conservative treatments that include RICE (rest, ice, compression, elevation), nonsteroidal antiinflammatory drugs (NSAIDs) and physical therapy. These traditional treatments usually do not achieve full functional recovery and the recurrence of injury is common, indicating that such treatments are likely ineffective at preventing the formation of permanent scar tissue at an injured site.

After a muscle injury, generally the RICE principle is applied\(^ {16} \). The aim of RICE is to minimize the haematoma of the injured muscle and, in the long term, the size of the connective tissue scar. The value of this treatment is not fully known, but most authors consider it as not harmful and maybe helpful to limit the bleeding in the muscles. During the first seven days after a muscle injury, rest should be taken, so that the scar tissue can gain strength. After that, physiotherapy can be started. Experimental studies have shown that early immobilization is needed to prevent rerupture of the early fragile scar. However, it should be followed by active mobilization to promote the restoration of tensile strength, because the mechanical stress applied to the regenerating muscle appears to reinforce adhesion of the regenerating myofibers to the ECM and reduce the final size of the scar. NSAIDs can be used after 48 hours. The rationale for using NSAIDs in these conditions is based on their anti-inflammatory properties. However, if used improper, NSAIDs may suppress an essential inflammatory phase in the healing of injured skeletal muscle.
As explained earlier, skeletal muscle is able to repair itself through regeneration; however, an injured muscle often does not fully recover its strength because regeneration is hindered by the development of fibrosis. On this basis, current research trends focus on enhancing muscle regeneration and minimizing fibrosis, so as to reduce the amount of scar tissue formed within the muscle\(^\text{17}\).

(1) Improving Muscle Regeneration

- **Use of human recombinant growth factors to improve muscle healing:**

  There is a balance between regeneration and fibrosis; enhancing the activation of satellite cells and accelerating the proliferation and fusion of myoblasts may tip the balance towards regeneration\(^\text{18}\). Growth factors are small peptides that bind to membrane receptors and influence various steps of the growth and development of cells through several signaling pathways. Some growth factors are potent stimulators of the proliferation and fusion of myoblasts, and they have been studied to elucidate their potential in muscle healing. Researchers have looked at: insulin-like growth factor-1 (IGF-1), fibroblast growth factor (bFGF), nerve growth factor (NGF), and platelet-derived growth factor. The direct injection of these recombinant growth factors has shown some beneficial effect on muscle healing, as an increase in the number and size of the regenerating myofibers; however, the recovery of function still remains incomplete.

  IGF-1 is the most promising growth factor, presenting the highest improvement of muscle healing after all of the muscle injuries tested. IGF-1 displays the greatest potential for satellite cell stimulation. It is highly mitogenic for myoblasts. As the following experiments show, it appears to be critical for growth of skeletal muscle. Transgenic mice overexpressing human IGF-1 exhibit muscle hypertrophy. In a study of healthy older men, the loss of muscle mass associated with aging was prevented when endogenous levels of IGF-1 were externally administered. In addition, delivery of IGF-1 in a mouse model at two, five and seven days post-injury showed a higher number of regenerating myofibers and the diameter of these fibers was bigger.

  However, in addition to stimulating satellite cells, IGF-1 also stimulates myofibroblasts to proliferate and deposit extracellular matrix. There is an increase in the production of matrix components, such as collagen, and a decrease in the expression of matrix degrading enzymes, such as collagenase. As
a result, development of fibrosis still takes place. In an experiment where immunodeficient mice were injected with adenovirus transferring the IGF-1 gene and IGF-1 expressing myoblasts, scar tissue still formed. Another possible reason for the formation of scar with the use of growth factors is that some myogenic cells, including myofibers, have been shown to differentiate into fibrotic cells during this phase. Consequently, increasing the number of myoblasts through growth factor stimulation also augments the number of cells that could potentially differentiate into fibrotic cells.

Direct injection of the growth factors: insulin-like growth factor-1, basic fibroblast growth factor, and nerve growth factor enhance muscle regeneration but the healing process remains incomplete. Treating the injured muscle with growth factors can improve muscle regeneration but cannot block the development of muscle fibrosis.

(2) Inhibition of Fibrosis

The result of the body’s natural wound-healing process is the formation of a scar, but this scar is a barrier for the regeneration of the injured skeletal muscle. The fibrotic tissue may be mediated by two different strategies: the first, involves prevention of fibrosis through blocking the transactivation of genes that trigger the fibrosis pathway, and the second, entails regeneration of functional muscle by the resolution of pre-existing scar tissue.

- Antifibrotic Therapy by Blocking Overexpression of TGF-β1:

TGF-β1 plays a key role in skeletal muscle fibrosis and it is probably the most important factor in wound healing. TGF-β1 promotes fibroblasts migration and proliferation, in consequence, increasing the deposition of collagens and the overgrowth of the extracellular matrix. Infiltrating cells such as lymphocytes, monocytes/macrophages, and platelets produce it. High levels of TGF-β1 are found in patients with Duchene muscular dystrophy, where a high accumulation of extracellular matrix is present. The same result is observed in the muscle biopsies of patients with dermatomyositis. Supporting these findings, a strong expression of TGF-β1 is also present in injured skeletal muscle in animal models.

Preventing fibrosis by neutralizing this key fibrotic stimulator could inhibit the formation of scar tissue. Several drugs that inhibit or down regulate the expression of TGF-β1 have been studied:
· Decorin: it is a human proteoglycan that antagonizes TGF-β1’s stimulatory effect on myofibroblasts. It has been shown to reduce fibrosis in kidney, liver and lung. In a mouse model, injured skeletal muscles injected with decorin showed an increase in the number and diameter of regenerating myofibers and an increase in strength\textsuperscript{20}.

· Suramin: inactivates the effect of TGF-β1 on myofibroblasts and has also been shown to break down collagen after its deposition. This product could be extremely useful for the elimination of scar tissue that is already present within healing skeletal muscle. Mouse injected with suramin also presented less scar tissue and greater fast-twitch and tetanus strength\textsuperscript{21}.

· Gamma-interferon: it is a cytokine that inhibits TGF-β1 signaling. It induces the expression of SMAD 7, which participates in a negative feedback loop in the TGF-β1 signal transduction pathway. The Food and Drug Administration have approved it and it is currently used to treat liver fibrosis. In a mouse laceration model, the area of fibrosis decreased when γ-interferon was injected at either 1 or 2 weeks after injury, but more importantly, it was found to improve muscle function in terms of both fast-twitch and tetanic strength too\textsuperscript{22}.

Studies demonstrate that the use TGF-β1 inhibitors are successful in achieving a near normal architecture and function of muscle after injury.

- **Antifibrotic Therapy by collagen degrading enzymes:**

Another approach that has been investigated is to degrade existing fibrosis by delivery of exogenous ECM degrading enzymes. One example of these enzymes is the family of matrix metalloproteinases (MMPs). In the next chapter, these enzymes will be studied in more detail.

The first member of this family, matrix metalloproteinase-1 (MMP-1), also known as interstitial collagenase, hydrolyzes types I, II and III collagens. Collagens type I and III are the major component of scar tissue, and thus, local delivery of MMP-1 into the zone of injury may improve muscle regeneration by breaking apart fibrotic tissue. MMPs are inhibited by the tissue inhibitors of metalloproteinases (TIMPs), which regulate their proteolytic activity by forming a complex with them. There is a delicate equilibrium between MMPs and TIMPs, and increasing the concentration of MMPs in the place of injury can help improve muscle healing.
Myoblasts require MMPs for migration, and it is known that satellite cells express gelatinases, MMP-2 and MMP-9, to cross the basal membrane. However, these gelatinases are ineffective against scar tissue. Thus, delivery of MMP-1, which specifically targets collagen I and III, may aid the regeneration process by removing the fibrotic tissue\textsuperscript{23}.

Bedair H. et al. demonstrated in a study that direct injection of proMMP-1 into fibrotic skeletal muscle in mice helps in the healing process\textsuperscript{24,25}. The MMP-treated limbs presented more regenerating myofibers than did the control limbs (MMP: 170 ± 96 fibers, control: 62 ± 51 fibers; P < 0.001) and less fibrous tissue (MMP: 24 ± 11%, control: 35 ± 15%; P < 0.01). These results suggest that the direct injection of MMP-1 into the zone of injury during fibrosis can enhance muscle regeneration by increasing the number of myofibers and decreasing the amount of fibrous tissue (Figure 5).

![Figure 5](image_url)

**Figure 5.** Effect of MMP-1 on skeletal muscle fibrosis after injury: a) % area of fibrosis in the injured muscle, b) number of regenerating myofibers and c) muscle physiology tests: peak and tetanic forces\textsuperscript{24}.

Although these approaches have met with partial clinical success, delivery of molecules to the injury site remains an obstacle. In vivo, high concentrations of the molecules are typically required to achieve a detectable enhancement. This is due mainly to two reasons: (1) the bloodstream rapidly clears the molecules, and (2) the molecule's relatively short biological half-lives, that are enzymatically degraded.

The common means of delivery of molecules is by direct injection. It is safe and easy, but multiple injections are required to achieve an effective concentration. Other possibilities of delivery are being investigated. One possibility is delivery through a viral vector (adenovirus, retrovirus, herpes simplex virus, and adeno-associated virus) or through a non-viral vector (plasmid DNA and liposomes).
Cell and gene therapy may prove to be an effective method for delivering stable high concentrations of the target molecule. Delivery of the target molecule will take place in the injured site, in a continuous way and with decreased secondary effects. More investigation is still required in the identification of a safe, effective vector through which to deliver efficacious amounts of growth factors to the injury site.

**3) Suturing as means to limit fibrosis**

Sutures are the most common means of wound closure. Recent studies have studied the healing of a lacerated muscle in mice and they have observed that suturing the muscle immediately after injury promotes healing and prevents the development of a deep scar, although a superficial scar was still observed. The functional outcome was also improved. They were surprised to see that suturing the muscle allowed the restoration of more than 80% of the intact muscle contractile properties at 1 month after injury. In contrast, immobilization of the muscle resulted in slower muscle regeneration and the development of a large scar.

Suturing allows a narrowing of the initial gap and brings the two ruptured myofiber extremities closer to each other. The distance between the damaged basal lamina is shortened, and because the newly formed myofibers mainly regenerate inside the old basal lamina, this reduced space facilitates the reconnection. The major goal of the suture is to reconstruct the framework of the muscle by enhancing the microenvironment in which the regenerating myofibers have to grow.

Based on these findings, the lacerated muscle should be sutured. Suturing the muscle resulted in better morphologic and functional healing than immobilization or the 'natural healing'.
3. Collagen Degrading Enzymes

Due to their triple helical conformation, collagens present a high resistance to proteolytic attack. They are almost totally resistant to nonspecific proteinases, thus only specific collagenases are able to tackle the highly complex task of collagen hydrolysis. In nature, two independent lines of collagenases have evolved to find a solution for collagen degradation. One are the members of the mammalian matrix metalloproteinases (MMPs), which produce an enzyme with the purpose of catalyzing their own collagen breakdown. The others are bacterial collagenases. These organisms do not produce collagen themselves, but secrete the enzyme to digest protein for nutritional purposes or use it to facilitate invasion into a collagen-containing host.

3.1. Matrix Metalloproteinases (MMPs)

Matrix Metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases responsible for tissue remodeling. Collectively, these enzymes can degrade all components of the extracellular matrix, influencing many important processes such as cell proliferation, differentiation, migration, death, and cell–cell interactions.

The first report about MMPs dates back to 1962, when Gross and Lapiere, while attempting to establish how a bullfrog tadpole loses its tail during metamorphosis, discovered the first member of this family. They gave it the name interstitial collagenase, also known as matrix-metalloproteinase-1 (MMP-1). It was the first vertebrate collagenase to be discovered (until that moment only the clostridial collagenases were known). Later in 1968, it was purified from human skin, and since then, MMPs have been found in invertebrates and plants. The MMP family has expanded and to date there are 23 human MMPs known, with MMP-28 as the last member to be discovered.

As their name suggests, MMPs were characterized initially as matrix-degrading proteases. Each member of this family has a specific affinity toward certain element of the ECM, making the most common classification of MMPs based on substrate classification and cellular localization. As examples, collagenases (include MMP-1, MMP-8, MMP-13, and MMP-18) have the ability to cleave interstitial collagen types I, II, and III, whereas gelatinases (including MMP-2 and MMP-9) degrade denatured collagen.
The MMPs have a common domain structure, based on three domains:

- Pro-peptide: MMPs are synthesized as inactive zymogens to maintain latency. A cysteine in the pro-peptide interacts with the zinc in the active site and prevents binding and cleavage of the substrate. To activate the enzyme this pro-peptide needs to be removed.
- Catalytic domain: there is a catalytically important Zn$^{2+}$ ion in the active site, bound to three histidines in the conserved sequence HExxHxxGxxH, and a fourth water molecule. There is a flexible hinge region linking the catalytic to the hemopexin-like domain.
- Hemopexin-like C-terminal domain: it is necessary for the enzyme to be able to cleave the three chains of the triple-helical collagen.

In this project we will focus on MMP-1 (Figure 6)\textsuperscript{27}. ProMMP-1 is 55 kDa and the active form of the enzyme is 43 kDa. It contains five metal ions, three Ca$^{2+}$ and two Zn$^{2+}$ (only one of them with catalytic role). MMP-1 breaks down the interstitial collagens, types I, II, and III. It cleaves the triple-helical collagen by making a single scission across all three alpha chains approximately ¾ away from the N-terminus of the substrate, resulting in ¾ and ¼ length fragments. These fragments are unstable at body temperature and undergo denaturation of the triple helix, rendering them susceptible to gelatinases and other proteinases that continue the collagenolysis task.

![Ribbon representation of the three-dimensional structure of human MMP-1](Image)

\textbf{Figure 6.} Ribbon representation of the three-dimensional structure of human MMP-1\textsuperscript{27}.
3.2. *Clostridium Histolyticum* Collagenases

Interestingly, the most efficient collagenases are those found in clostridial bacteria, which enable them to colonize and infiltrate host tissues. Bacterial collagenases differ from vertebrate collagenases in that they exhibit broader substrate specificity. They are unique because they can attack almost all collagen types (water-insoluble native collagens and water-soluble denatured ones) and they can make multiple scissions within the triple helical region, allowing them to complete the digestion of the fragments to a mixture of small peptides. This is their primary distinguishing factor: their ability to digest native, triple-helical types I, II, and III collagens into a mixture of small peptides.

The most studied collagenases are the ones produced by *Clostridium histolyticum*, an anaerobic Gram-positive bacterium, which causes gas gangrene\(^{28}\). The histotoxicity of Clostridia is primarily caused by specific toxins, but they also secrete proteinases and collagenases to help them spread the toxin. In fact, they produce a large quantity of collagenases that help them degrade the collagen in the connective tissue.

Much of what is presently known about the characteristics of clostridial collagenases comes from the pioneering studies in the 1950s by Mandl, Seifter, Harper and their associates, and the later classification work of Van Wart and Bond\(^{29}\). Initial studies were searching for the collagenase enzyme of *Clostridium histolyticum*, but to their surprise, they found seven different enzymes from the culture filtrate of the bacterium with collagenolytic activities. They denoted them by the letters α, β, γ, δ, ε, ξ, and η, according to the order in which they were purified. All the enzymes are single polypeptide chains that contain one catalytic zinc and require calcium ions for activity. The molecular weights range from 68 to 130 kDa (clostridial collagenases have unusually high molecular weights compared to other bacterial collagenases, which are closer to 35 kDa). The isoelectric points lie between pH 5.35 and 6.20. Due to the similarities in the isoelectric points and molecular weights, researchers had many difficulties separating the individual collagenases. Some of the physicochemical properties of the seven clostridial collagenases are summarized in Table I.
Table I. Properties of *Clostridium Histolyticum* collagenases.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Molecular Weight (kDa)</th>
<th>pI</th>
<th>FALGPA $K_{cat}/K_M$ (M$^{-1}$ min$^{-1}$)</th>
<th>Type I Collagen $K_{cat}/K_M$ (M$^{-1}$ min$^{-1}$)</th>
<th>$\varepsilon_{280}$ M$^{-1}$ cm$^{-1}$ (x 10$^{-3}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha$</td>
<td>68.000</td>
<td>5.85, 5.90</td>
<td>87</td>
<td>690</td>
<td>1.06</td>
</tr>
<tr>
<td>$\beta$</td>
<td>115.000</td>
<td>5.55, 5.60, 5.75</td>
<td>61</td>
<td>530</td>
<td>1.67</td>
</tr>
<tr>
<td>$\gamma$</td>
<td>79.000</td>
<td>6.10, 6.20</td>
<td>44</td>
<td>290</td>
<td>1.19</td>
</tr>
<tr>
<td>$\eta$</td>
<td>130.000</td>
<td>-</td>
<td>36</td>
<td>430</td>
<td>1.69</td>
</tr>
<tr>
<td>$\delta$</td>
<td>100.000</td>
<td>5.80</td>
<td>650</td>
<td>320</td>
<td>1.48</td>
</tr>
<tr>
<td>$\varepsilon$</td>
<td>110.000</td>
<td>5.90, 5.95</td>
<td>640</td>
<td>290</td>
<td>1.37</td>
</tr>
<tr>
<td>$\xi$</td>
<td>125.000</td>
<td>5.35</td>
<td>840</td>
<td>200</td>
<td>1.55</td>
</tr>
</tbody>
</table>

Assays were carried out in 50 mM Tricine, 0.2 M NaCl, 10 mM CaCl$_2$, pH 7.5 at 25°C for FALGPA and 30°C for type I collagen.

All seven enzymes share similarities. They all have a Zn$^{2+}$ in the active site tetrahedrally coordinated to two histidines in the sequence HEXXH, a glutamate, and a water molecule. They are all gluzincins because they use a glutamate as the third zinc binding ligand. Antibodies grown against one collagenase cross-react with the other collagenases, indicating that they all have portions of their 3-dimensional structure similar enough to constitute a common antigen. Lastly, they all hydrolyze native collagen, gelatin and synthetic peptides that contain amino acid sequences found in collagen.

When the activities toward collagen and gelatin are compared in assays in which the same mass of substrate is present, gelatin is hydrolyzed 5-10 times faster than native collagens. This difference is due to the ease with which the collagenases can hydrolyze the dissociated chains compared to the triple helix. Thus, while collagenases are unique in their capacity to attack triple-helical collagen, these clostridial collagenases can hydrolyze even more easily the unordered chains of gelatin.

In spite of the similarities listed above, there are differences between the collagenases. With each difference, the collagenases are divided into 2 classes: Class I ($\alpha$, $\beta$, $\gamma$, and $\eta$) and class II ($\delta$, $\varepsilon$, and $\xi$). These classes differ with respect to their activities, stabilities, and amino acid composition. Class I have higher collagenase and gelatinase activities but lower activities toward synthetic peptides with collagen-like sequences. They are more stable to freeze-thawing. Class II instead preferentially acts on short synthetic substrates and are less stable to freeze-thawing. While the amino acid compositions of all
seven enzymes are similar, there is extensive homology between all of the enzymes within the same class, and marked differences between the two classes.

These discoveries led to the prediction that the two classes of enzymes are encoded by different genes and that one class evolved from the other by gene duplication followed by divergent evolution to form a new class of enzymes with different substrate specificities. Nowadays we know that Clostridium histolyticum has two separate and distinct genes in its chromosome encoding two different collagenases: colG encodes class I collagenase (also known as ColG) and colH encodes class II collagenase (or ColH). They are both single copy genes, and each gene is transcribed into a single message\textsuperscript{30}.

ColG and ColH are large multidomain proteins, with the segmental structure shown in Figure 7\textsuperscript{31}. Their domain organization consists of\textsuperscript{32,33}:

- A pre-domain of variable length containing the export signal, which is clipped in the mature protein.
- An N-terminal catalytic domain containing the catalytic zinc.
- A variable number of polycystic kidney disease like (PKD-like) domains.
- One or more collagen binding domains (CBD). These domains contain calcium, which is required to maintain the appropriate structure to accept the triple helix structure of the collagen fibril.

![Figure 7. Domain organization of the collagenases of Clostridium Histolyticum\textsuperscript{33}.](image)

To know the relationship between the two collagenases (ColG and ColH) and the smaller gelatinases, researchers sequenced the N-terminal sequences of all the enzymes. The results showed that all the N-terminal sequences of the seven enzymes correspond to either \textit{colG} or \textit{colH}. These results suggest that all the gelatinases are produced from the respective full-length collagenase by the proteolytic removal of C-terminal fragments. Crude collagenase mixtures can therefore contain six to eight different molecular weight species ranging from 68 to 130 kDa.
Unlike vertebrate collagenases that cleave the triple helix collagen at a single site, clostridial collagenases are capable of rapidly hydrolyzing multiple sequences found in collagen. The mode of attack of these collagenases on triple helical type I, II and III collagens is very similar for the enzymes within each class, but different for the two classes. The class I enzymes first hydrolyze loci near the ends of the triple helical domains of these collagen molecules, while the class II enzymes make their initial cleavages in the interior (Figure 8). After these initial cleavages, small collagen fragments are broken down by both enzymes, giving rise to a pattern complex to analyze. The two classes of collagenases are complementary in their action and act synergistically to cleave the collagen molecule at different points. They are more effective at cleaving collagen when they work together than acting alone.

![Figure 8](image)

**Figure 8.** Schematic representation showing the sequence of cleavages of type I collagen by a) class I and b) class II collagenases. c) Cartoon representation of collagen's triple helix digestion^34^.

*Clostridium histolyticum* digest both the insoluble fibrous collagen as well as the soluble fragments that result when the triple helix is cleaved. The initial cleavages of the triple helical collagens are very specific, but instead, clostridial collagenases hydrolize gelatin very fast, rapidly forming a large number of products. This comparison shows that the structure of the native collagen marks the sites at which the initial cleavages will occur, to the extent that there are a limited number of hyperreactive sites in the triple helical collagen molecules. The hyperreactivity of these sites may be determined in large part by some local conformation that these enzymes recognize. Thus, we can say that collagen directs its own degradation.

*Clostridium histolyticum* collagenase does not require proteolytic cleavage to be activated and functions in its native form.
ColG:

The structure of ColG was recently solved revealing an unexpected domain architecture\textsuperscript{35}. The crystal structure shows that the N-terminal collagenase module has a saddle shape. This collagenase module has been divided in an activator domain and a peptidase domain (containing the active site), separated by a glycine-rich linker positioned at the twist of the saddle seat (Figure 9).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{image.png}
\caption{Structure of ColG: a) domain organization, b) Ribbon representation of the collagenase module, and c) Full-length model of ColG in complex with a collagen micofibril\textsuperscript{26}.}
\end{figure}

A construct without the activator domain still showed 100% activity against small peptidic substrates, confirming that the peptidase domain contains the active site. However, this construct was completely inactive towards collagen, showing that both, activator and peptidase domains are necessary for collagen breakdown.

These results made them propose a chewing mechanism for collagen hydrolysis. Collagenase can adopt two conformations: an opened state with a distance of -40Å between domains, and a closed state which allows the collagen triple helix to contact both the activator and the peptidase domain. Collagen is the source of the attractive interaction for the collagenase closing, and once degraded it relaxes to the open state. A variant where the glycine-ringe was short cut, showed one third of the wild type activity towards collagen substrates, proving this chewing mechanism (Figure 10).
Clinical use:

Collagenases from *Clostridium Histolyticum* are already being used to treat diseases in humans. BioSpecifics Technologies developed a preparation with a constant mixture of two collagenases, one of each class\(^37\). The collagenase from class I is 114 kDa and the collagenase from class II is 113 kDa. They are isolated from the anaerobic fermentation of the bacterium and purified. The Food and Drug Administration (FDA) approved this preparation for the treatment of two conditions: Dupuytren's contracture (in 2010) and Peyronie's disease (in 2013). They are commercialized with the name: XIAFLEX, by Auxilium Pharmaceuticals and Pfizer.

- In Dupuytren's contracture collagen accumulates in the palmar fascia of the hands, with the result that the fingers bend toward the palm and cannot be straightened. The injected collagenases enzymatically disrupt the cord.
- A similar situation occurs in Peyronie's disease, with an accumulation of a plaque of types I and III collagen resulting in the curvature of the penis. Patients are treated when the curvature is at least 30 degrees. Injection of collagenases may result in enzymatic digestion of the plaque.

The two collagenases work synergistically by cleaving the collagen molecule at different points. However, there is no clinical data regarding the relative contributions of the individual collagenases to the efficacy of Xiaflex.

Xiaflex is supplied in glass vials containing 0.9 mg of collagenase clostridium histolyticum as a sterile, lyophilized powder. The enzyme is reconstituted with the supplied sterile diluent (0.3 mg/mL calcium chloride dihydrate in 0.9% sodium chloride) prior to intraleosomal injection. The delivery of the enzymes should be carefully located where the collagen accumulation is, avoiding contact with tendons, nerves, blood vessels, or other collagen-containing structures.
The enzymes do not reach the bloodstream in significant amounts and are presumed to largely stay at the point of injection until they are broken down by proteases.

The most common adverse reactions reported in patients with Dupuytren's contracture are swelling of the injected hand, contusion, injection site hemorrhage, and pain in the injected extremity. In the case of Peyronie's disease patients reported penile hematoma, penile swelling and penile pain.

During clinical studies in Dupuytren’s contracture and Peyronie’s disease, patients were tested at multiple time points for antibodies to both collagenases. In both diseases patients had antibodies against both collagenases at 30 days post the first injection. After several injections, every Xiaflex-treated patient developed high titers of antibodies to both collagenases.

Since the clostridial collagenases have some sequence homology with human matrix metalloproteinases (MMPs), anti-product antibodies could theoretically interfere with human MMPs. In vitro studies showed no evidence of cross-reactivity between anti-drug-antibody positive patient sera and a series of relevant MMPs. In addition, no clinical safety concerns related to the inhibition of endogenous MMPs have been observed.
4. Bioactive Sutures

Sutures are still the most common means of wound closure because they are easy to use, efficient, and provide the mechanical support necessary to sustain closure. Recently, scientists have been studying how to use sutures, not only for its mechanical function of wound closing, but also for incorporating bioactivity on them\textsuperscript{38}.

To date, there is only one product of bioactive sutures already in the market, Vicryl Plus from Ethicon (Cornelia, GA)\textsuperscript{39}. Vicryl Plus consists of polyglactin 910 suture coated with triclosan. Triclosan is an antimicrobial agent that has been proved to be effective against the most common organisms that cause surgical site infections (SSI). Percutaneous sutures create a conduit for bacteria to enter the wound, and, like all foreign bodies, when implanted are rapidly coated with tissue protein, creating sites for bacterial colonization. Closing the wound with a suture that expresses antimicrobial activity showed a significant reduction in bacterial adhesion\textsuperscript{40}.

In 2003, Shibuya et al. examined the possibility of covalently linking proteins and cytokines to sutures to enhance the immune response of head and neck cancer patients. The immune system of advanced stage head and neck cancer patients is frequently suppressed. Many groups were studying the possibility of treating cancer by delivering cytokines, such as interleukin-2, that are capable of activating the T lymphocytes of cancer patients. However, local administration requires repeated (daily or weekly) injections, hence the idea of binding to suture material. Polyester suture was acid hydrolyzed followed by reaction with EDC to create a suture-EDC intermediate, that reacted with cytokines to form a covalent bond. The covalently linked cytokines retained their immune-stimulating properties\textsuperscript{41}.

In 2006, Hamada et al. coated monofilament nylon with basic fibroblast growth factor (bFGF) and tested its activity in rabbit tendon repair. This bFGF-coated nylon suture gave excellent results in delivering the growth factor, it accelerated cellular proliferation, and induced an increase of biomechanical strength\textsuperscript{42}.

In 2008, Yao et al. seeded pluripotential embryonic cells onto sutures to help in tendon repair. Delivery of cells to soft tissues remains a challenge and this group used absorbable sutures as a scaffold for stem cell transplantation. They coated sutures with poly-L-lysine and fibronectin and grew stem cells on them \textit{in vitro}. These sutures were implanted in Achilles tendon in rats, where they delivered cells that
remained metabolically active and help to repopulate the acellular zone surrounding the sutures within the tendon. Five weeks later, the transplanted cells survived and the sutures were partly absorbed\textsuperscript{43}.

In 2012, Fuchs et al. also studied the possibility to locally deliver growth factors to the place of injury using coated sutures. They incorporated insulin-like growth factor-1 in a bioabsorbable coating (PDLLA), which they used to coat the suture. They tested the sutures in an animal model, and observed that local deliver of the growth factor took place, enhancing the functional outcome\textsuperscript{44}.

So far, small drugs, peptides and whole cells have been successfully attached to sutures, but to the best of our knowledge, this has not been done yet with enzymes. In this project, we propose to immobilize an enzyme on the surface of the suture that could help in wound healing.
Part II. Objective

In order to improve skeletal muscle healing, our approach in this project is to join the inhibition of fibrosis with suturing the muscle. We propose to prepare bioactive sutures to prevent fibrosis (Figure 11). This will be achieved by immobilization of collagenase enzymes on polyethylene terephthalate (PET) sutures. We hypothesize that closing the injured muscle with collagenase-modified sutures will inhibit the deposition of fibrotic tissue by digesting interfibrillar collagen as it is formed, thus improving muscle healing.

Figure 11. The approach in this project is to develop bioactive sutures to help in muscle healing.

The results of this work should shed further light on the effects of scar tissue on muscle healing after injury, and it will present a new approach to eliminate scar tissue and enhance the regeneration of damage muscle. More broadly, the management of scar formation may also have utility in the treatment of fibrotic diseases, and other areas adversely affected by scarring, such as tendon, spine, and nerve repair.

Specific Aims of the project:

1. Produce and characterize recombinant collagenase that is active in degrading native collagen.
2. Covalently attach collagenase to PET sutures and control the immobilization.
3. Characterize the stability of the collagenase-modified PET sutures.
4. Characterize the degradation of type I collagen gels by collagenase-modified PET sutures.
Part III. Materials and Methods

1. Materials and Chemical Agents

Human ProMMP-1 cDNA was purchased from Origene (SC118645), and Collagenase G gene (construct: Catalytic domain + PKD + CBD + CBD, without prepro signal) was kindly provided by Hans Brandstetter et al. Restriction enzymes and T4 Ligase were kindly provided kindly provided by Sean Yu McLoughlin. TAQ DNA Polymerase in buffer, 100U, was obtained from Fisher Scientific (Denver, CO). Custom-made primers and sequence analyses were performed at Eurofins MWG Operon (Huntsville, AL). E. coli strain DH10B was used for cloning and strain BL21 DE3 for protein expression. The plasmids used were pET-21b and pET-25b, all provided by Sean Yu McLoughlin. For expression, Isopropyl-β-D-thiogalactopyranoside [IPTG] from Research Products International Corp (Mount Prospect, IL) was used.

Chromatographically purified Clostridium Histolyticum Collagenases (>10 ku) were purchased from Worthington Biochemical Corp (Lakewood, NJ).

Polyethylene terephthalate (PET) sutures were obtained from Covidien (Mansfield, MA). We used Surgicat™ sutures 4-0 (0.15 mm in diameter). The sutures are braided, uncoated, non-absorbable and undyed (White). They are prepared from fibers of high molecular weight, long-chain, linear polyesters having recurrent aromatic rings as an integral component. Surgicat Polyester sutures are inert and elicit only minimal local tissue reaction (Figure 12).

![Figure 12. PET sutures.](image)

For the functionalization of PET sutures we used formaldehyde from Fisher Scientific Company LLC (Denver, CO), Acetic Acid Glacial from VWR International (Radnor, PA), Bromoacetic acid reagent grade from Sigma-Aldrich Inc (St Louis, MO) and sodium hydroxide reagent grade from Fischer Scientific. For the coupling of the enzyme with the sutures we used N,N'-Diisopropylcarbodiimide (EDC) and N-
Hydroxysulfosuccinimide sodium salt, ≥ 99.5%, from Chem-Impex International Inc (Chicago, IL). Tween 20 was purchased from Fisher Scientific Company LLC.

As a substrates to measure the peptidolytic and collagenolytic activities we used: n-(3-(2-furyl)acryloyl)-leu-gly-pro-ala, n-(3-(2-furyl)acryloyl)-leu-gly-pro-ala (FALGPA) from Fluka, Sigma-Aldrich Inc (St Louis, MO), DQ gelatin fluorescein conjugate from pig skin from Life Technologies (Carlsbad, CA), and Collagen type I from rat tail from VWR International LLC (Brisbane, CA). We used the cuvette Quartz Fluorometer Cell Sub.Micro, 160ul 10mm Z015 from Starna Cells Inc (Atascadero, CA) for the fluorescence measurements. Tissue culture plates 12-well were from VWR International LLC.
2. Protocols and Methods

2.1. Cloning, Expression and Purification of recombinant proMMP-1 and ColG

Human ProMMP-1 cDNA was purchased from Origene (SC118645). The gene was cloned into two different vectors: one for expression in the cytosol, pET-21b, and one for expression in the periplasm pET-25b. To facilitate later purification of the protein, a C-terminal hexahistidine tag was included.

Collagenase G gene (construct: Catalytic domain + PKD + CBD + CBD, without prepro signal) was kindly provided by Hans Brandstetter et al. The gene was cloned into the vector pET15b-TEV, which also contains an N-terminal hexahistidine tag to facilitate purification, but this time separated from ColG by a TEV cleavage site.

**Table II.** Constructs of MMP-1 and ColG, and expression parameters.

<table>
<thead>
<tr>
<th>Construct</th>
<th>MW (kDa)</th>
<th>Vector</th>
<th>Fusion Tag</th>
<th>E. coli strain</th>
<th>Point of Induction (OD&lt;sub&gt;600&lt;/sub&gt;)</th>
<th>[IPTG] (mM)</th>
<th>Temp (°C)</th>
<th>Period of Expr.</th>
</tr>
</thead>
<tbody>
<tr>
<td>proMMP-1</td>
<td>55.45</td>
<td>pET-21b</td>
<td>C-His&lt;sub&gt;6&lt;/sub&gt;</td>
<td>BL21 de3</td>
<td>0.6-0.7</td>
<td>0.4</td>
<td>25</td>
<td>O.N.</td>
</tr>
<tr>
<td>proMMP-1</td>
<td>55.45</td>
<td>pET-25b</td>
<td>C-His&lt;sub&gt;6&lt;/sub&gt;</td>
<td>BL21 de3</td>
<td>0.6-0.7</td>
<td>0.4</td>
<td>25</td>
<td>O.N.</td>
</tr>
<tr>
<td>ColG</td>
<td>115.6</td>
<td>pET-25b</td>
<td>N-TEV-His&lt;sub&gt;6&lt;/sub&gt;</td>
<td>BL21 de3</td>
<td>1</td>
<td>0.1</td>
<td>25</td>
<td>O.N.</td>
</tr>
</tbody>
</table>

O.N.: Over Night

The recombinant plasmids are transformed into *Escherichia Coli* BL21 (DE3) cells by electroporation. The expression parameters have been optimized to obtain the maximum amount of soluble protein for each construct. In general, low temperatures for longer expression times helps in the expression of soluble protein.

Expression was performed in 2 L baffled flasks containing 700 mL Luria–Bertani medium containing 100 μg/mL ampicillin and with agitation at 200 rpm. When cells reached the desired OD<sub>600</sub>,
expression was induced by adding isopropyl β-D-thiogalactoside (IPTG) and cells were grown over night at 25°C.

In order to recover the recombinant protein, cells were harvested by centrifugation at 4000g for 10 min at 4°C and subsequently resuspended in 25 ml ice-cold lysis buffer containing 50 mM HEPES, 300 mM NaCl, 5 mM CaCl₂ and 5 mM imidazole at pH 8.0. The cells were lysed using an homogenizer and the soluble protein was separated from the other components of the cell by centrifugation at 15000g for 30 min at 4°C.

Purification of the recombinant proteins was done in a Ni–NTA resin (Qiagen). The column was initially pre-equilibrated with the same buffer where the proteins were resuspended in. Once all unbound proteins had been washed from the column, the target proteins were eluted from the column using 250 mM imidazole along with 50 mM HEPES, 300 mM NaCl, and 5 mM CaCl₂ at pH 8.0.

Proteins were dialyzed over night at T=4°C in the desired final buffer:

- proMMP-1: 50 mM HEPES, 10 mM, CaCl₂, pH 7.5
- ColG: 50 mM Tricine, 10 mM CaCl₂, 400 mM CaCl₂, pH 7.5

The purity of the protein was judged using 15-20% SDS–PAGE followed by Coomassie Brilliant Blue G-250 staining and the concentration of the protein was measured with OD₂₈₀ and Bradford Assay.

### 2.2. Functionalization of polyethylene terephthalate (PET) sutures

Poly(ethylene terephthalate), PET, is an inert polymer that lacks active functional groups on the surface to attach biologically active molecules. Thus, we must first functionalize PET and introduce reactive groups on its surface. We chose to introduce carboxylic acid groups because they are convenient for conjugation to a wide variety of biomolecules. We followed a two-step procedure, used by authors such as: Vignesh Muthuvijayan (2009), Kilwon Cho (2005), Peter Kingshott (2003), Zhongping Yang (2000), Stephen Massia (1991), and Stefan Lofas (1990). The procedure consists of initially generating hydroxyl groups on the PET surface, which are later converted into carboxyl groups (Figure 13)⁴⁵–⁴⁹.
Figure 13. Schematic of the two-step reaction to introduce carboxylic acid groups onto PET.

The first step is an electrophilic aromatic substitution. PET sutures are incubated with 18.5% (v/v) formaldehyde solution in 1 M acetic acid for 4h at room temperature. A hydroxymethylation of the aromatic ring takes place that introduces a benzyl hydroxyl group. We term this hydroxylated PET surface: PET-OH. The hydroxyl groups are then carboxymethylated by overnight exposure to 1 M bromoacetic acid in 2 M NaOH at room temperature. We will refer to the carboxylated PET as PET-COOH. Finally, the sutures are washed thoroughly with dH₂O.

2.3. Immobilization of the enzyme onto polyethylene terephthalate (PET) sutures

Although there are 20 different amino acids providing a high complexity of protein structure, only four functional groups account for the vast majority of bioconjugation methods. These are: primary amines (–NH₂), carboxyls (–COOH), sulphhydryls (–SH), and carbonyls (–CHO). The first of these ones, primary amines, exist at the N-terminus of each polypeptide chain and in the side chain of lysine (Lys, K) residues. Because of its positive charge at physiologic conditions, primary amines are usually on the outer surface of proteins, thus, they are usually accessible for conjugation without denaturing protein structure.

The chemistry that we will use to immobilize the enzyme on the sutures will take place between primary amines in the enzyme and carboxylic groups on the suture. The most popular carboxyl-to-amine crosslinker is the reactive group carbodiimide, and the most commonly used carbodiimide is the watersoluble 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) for aqueous crosslinking. Carbodiimides work by activating carboxyl groups for direct reaction with primary amines forming an
amide bond. No portion of their chemical structure becomes part of the final bond, and thus they are considered zero-length carboxyl-to-amine crosslinkers.

EDC first reacts with a carboxyl group. The carboxyl group does a nucleophilic attack on the C=N of the carbodiimide and forms an O-acylisourea intermediate (Figure 14). This is an ‘activated carboxyl group’ which a primary amine in the enzyme can do a nucleophilic attack on, forming an amide bond\(^{50}\). An EDC by-product is released as a soluble urea derivative\(^{51}\).

![Figure 14. EDC (carbodiimide) crosslinking reaction scheme](http://www.piercenet.com/method/carbodiimide-crosslinker-chemistry).

One inconvenience of this reaction is that the O-acylisourea intermediate is unstable in aqueous solutions, and failure to react with an amine results in hydrolysis of the intermediate\(^ {52}\). The hydrolysis reaction is favored because the product of the reaction is a highly stable urea. For this reason, often a water soluble sulfo-hydroxysuccinimide (Sulfo-NHS) is included in EDC coupling to improve efficiency. EDC couples NHS to carboxyls, forming an NHS ester that is considerably more stable than the O-acylisourea intermediate (Figure 15).
**Figure 15.** Carboxyl-to-amine crosslinking using EDC and sulfo-NHS. Three possibilities can take place, O-acylisourea intermediate reacting with a primary amine on the enzyme, being hydrolyzed or reacting with sulfo-NHS to form a more stable intermediate [http://www.piercenet.com/method/carbodiimide-crosslinker-chemistry].

In addition, EDC crosslinking is most efficient in acidic conditions, while the enzyme is more stable at physiologic pH. This leads to a more efficient two-step conjugation procedure as shown in Figure 16.

![Diagram](image)

**Figure 16.** Bioconjugation of the collagenase enzyme onto the PET sutures using a two-step procedure with EDC and Sulfo-NHS.

**Procedure for Two-step Coupling of Proteins Using EDC and Sulfo-NHS:**

(Step I)

Activation of the carboxyl groups on the suture. For best results, this reaction will be taken at pH 5-6 and in a buffer exempt of carboxyls and amines.

**Activation Buffer:** 0.1M MES, 0.5M NaCl, pH 5.0.

1. Equilibrate EDC and NHS for 15 minutes to room temperature before opening bottles.
2. Prepare a 20mM EDC and 50mM sulfo-NHS solution in the Activation Buffer. Add the PET-COOH sutures and react for 15 minutes at room temperature.
(Step 2)
Raise the pH to 7.0-7.5 with another non-amine buffer. This avoids exposing the enzyme's carboxyls to EDC.

**Coupling Buffer:** 100mM sodium phosphate, 150mM NaCl, pH 7.6.

3. Remove the sutures from the first reaction and place them into a solution containing the collagenase enzyme. Different concentrations of collagenase in the Coupling Reaction have been used.
4. Allow the reaction to proceed for 2 hours at room temperature.

Finally, the sutures were extensively washed with buffer to assure that all the remaining protein in the sutures is covalently attached. The sutures were washed in three solutions, at 4°C for one hour each. The buffer used for the washing solutions is the buffer that will be used next in the activity assay.

### 2.4. Activity Assay with FALGPA - peptidolytic activity

FALGPA is a synthetic substrate of *Clostridium histolyticum* collagenases. The name stands for: N-([2- Furfuryl]acryloyl)-Leu-Gly-Pro-Ala (FALGPA). Figure 17 shows the substrate and the catalyzed reaction.

![FALGPA substrate and Collagenase catalyzed reaction](image)

**Figure 17.** FALGPA substrate and Collagenase catalyzed reaction.

Collagenase cleaves the X-Gly bond of collagen and synthetic peptides at places with the sequence: -Pro-X-Gly-Pro-, where X can be almost any amino acid, provided that the imino terminus is blocked. Apparently, there is sufficient structural similarity between the 2-furanacryoyl group and Proline for FALGPA to be an excellent substrate for collagenase. *C. histolyticum* collagenases catalyze the hydrolysis of the substrate in the form FAL + Gly-Pro–Ala. When the bond between Leucine and Glycine is cleaved, a furanacryloyl peptide is released that blue shifts its absorption in the near-ultraviolet. The
peptidolytic activity is monitored by following the decrease in absorbance at any wavelength in the range 324-345 nm. In this case we monitored the absorbance at \( \lambda = 345 \text{nm} \).

The protocol is:

1. Prepare Reaction solution with a final substrate concentration of 1.0 mM FALGPA. Reaction buffer: 50 mM Tricine, 400 mM NaCl, and 10 mM CaCl\(_2\), pH 7.5. In our case the reaction volume was 100 \( \mu \text{l} \): 95\( \mu \text{l} \) 1x Reaction Buffer + 5\( \mu \text{l} \) FALGPA stock 20 mM (dissolved DMSO to prevent hydrolysis). Pipet up and down to mix.
2. Add the free collagenase or the collagenase-modified sutures to the substrate containing solution. Mix to begin the reaction.
3. Monitor the absorbance at \( \lambda = 345 \text{nm} \) for 5 minutes with UV-Visible Spectrophotometer Evolution 260 BIO from Thermo Scientific.

**2.5. Activity Assay with Gelatin (denatured collagen)**

Gelatinase activity (ability to digest denatured collagen) has been measured using the Molecular Probes' EnzChek Gelatinase/Collagenase Assay Kit. This kit provides a fast, sensitive and convenient way to detect gelatin-degrading activity. The substrate of the assay is gelatin heavily labeled with fluorescein, so that the fluorescence is quenched. When gelatinases and collagenases cleave the gelatin, highly fluorescent peptides are released, and this increase in fluorescence can be monitored with a fluorimeter. The fluorescence is proportional to the proteolytic activity.

The protocol is:

1. Prepare Reaction solution with a final substrate concentration of 100 \( \mu \text{g/mL} \). Reaction buffer: 50mM Tris-HCl, 150 mM NaCl, and 5 mM CaCl\(_2\) at pH 7.6. In our case the reaction volume was 200 \( \mu \text{l} \): 180 \( \mu \text{l} \) 1x Reaction Buffer + 20 \( \mu \text{l} \) gelatin stock 1.0 mg/mL (dissolved in dH\(_2\)O). Pipet up and down to mix.
2. Add the free collagenase or the collagenase-modified sutures to the substrate containing solution. Mix to begin the reaction. Incubate the samples at room temperature, protected from light.
3. Measure the fluorescence intensity in a fluorimeter every minute for 5-10 minutes. Digestion products have an absorption maxima at 495 nm and fluorescence emission maxima at 523 nm. For each time point, the signal was measured for 10 seconds and the average value was used.

The fluorescence was monitored with the fluorimeter QuantaMaster™ 40 steady-state, for continuous excitation, property of the BiochemCore of the University of Colorado Boulder. Settings in the fluorimeter:

- Excitation wavelength: 495 nm; Emission wavelength: 523 nm
- Slit = 0.5 nm; PTM = 1050

2.6. Collagen gel - collagenolytic activity

The activity of the enzyme was also tested with native collagen to check the ability to digest triple-helix collagen. This product is prepared and extracted from rat-tail tendon.

Although collagen is normally insoluble because of extensive cross-linking, it can be brought into solution, without denaturation, by cold neutral salt or low ionic strength acidic solutions from some vertebrate tissues. Collagen can be solubilized if it is newly synthesized and it is not yet extensively cross-linked, and/or if a lathyrinic agent is used which either inhibits aldehyde formation or combines with cross-link precursors and inhibits cross-linking. Rat-tail tendon is a good source of collagen for this last reason. This product is supplied as an aqueous solution in 20 mM acetic acid (pH ~3.0) with a concentration of 5 mg/ml of collagen. Once the collagen is brought to neutral pH and room temperature, gelation occurs fast.

We prepared type I collagen gels at 3 mg/mL. The volume of the gel was 1 mL in a 12 wells plate. Procedure:

1. In a sterile tube mix 370 µL dH₂O and 30 µL 7.5% NaHCO₃. Slowly pipet 600 µL of collagen into the tube, and gently pipet solution up and down to mix well.
2. Dispense the collagen into the desired plates, in our case 12 well plates. Gelling occurs rapidly at room temperature.
3. Incubate at 37°C in incubator for 30–40 minutes or until a firm gel is formed.
4. Place the free collagenase or the collagenase-modified sutures on top of the gel and let it react until the degradation of the gel is visible.

5. (If sutures are on top remove the sutures). Wash the collagen gel extensively with dH₂O and stain with Coomassie Brilliant Blue G-250.
Part IV. Results and Discussion

1. Produce and characterize recombinant collagenase that is active in degrading native collagen.

As a first attempt, we tried to express recombinant human proMMP-1 in *E. coli*. It is very common under conditions of high level expression, that recombinant proteins accumulate as insoluble aggregates named *inclusion bodies*. The protein in this state is mostly inactive and denatured. When full-length MMP-1 is expressed it also precipitates in the form of inclusion bodies, and there is almost no soluble proMMP-1 present. In order to obtain a higher amount of active protein two strategies were taken:

1. Improve the solubility of the expressed proMMP-1:

   a) Reducing the rate at which the protein is made, resulting in slower protein production and also in slower proteolytic degradation. The overall result is often an increased expression level of the target protein. This approach was tried by lowering the growth temperature at 25 °C and using a low concentration of inducer, [IPTG] = 0.4mM.

   b) Expressing the protein in the periplasm. Secretion of the protein to the periplasm has some advantages, such as: i) the oxidizing environment of the periplasm allows for the formation of disulfide bonds, which does not occur in the reducing environment of the cytoplasm; ii) the presence of two foldases, disulfide oxidoreductase (DsxA) and disulfide isomerase (DsxC), that catalyze the formation and isomerization of disulfide bonds; iii) less proteolysis because fewer proteins are present; and iv) allows for the accumulation of proteins that are toxic in the cytoplasm. However, expression levels are usually lower and not all expressed protein is secreted into the periplasm, it is also found in the cytoplasm and in the medium. This approach was achieved by the addition of a signal peptide to the N-terminus of the target protein, in this case we used *pelB*.

   Figure 18 shows the comparison between the amount of soluble proMMP-1 obtained in the cytoplasm and the periplasm.
**Figure 18.** Comparison between expression of proMMP-1 (55 kDa) in the periplasm (1-7) and the cytoplasm (8-14). M: protein ladder from bio-rad (precision plus protein dual color standards); Lanes: (1): BL21(de3) cells pre-induction; (2,9) post-induction; (3,10) soluble fraction; (4,11) flow-through affinity column; (5,12) elution; (6,13) soluble post-dialysis; (7,14) insoluble post-dialysis.

Expression in the cytoplasm did not yield any soluble proMMP-1 (55 kDa), even after lowering the protein synthesis rate. All the protein was found in the insoluble fraction. On the other hand, expression in the periplasm did help in improving the solubility of proMMP-1, but the yield was not very high. We obtained 2 mg of proMMP-1 per liter of culture.

2. **Refolding of MMP-1 expressed as inclusion bodies:**

In order to obtain more protein we tried to refold in vitro the proMMP-1 expressed as inclusion bodies. First, the insoluble protein was solubilized and denatured by the addition of urea as a denaturing agent and cysteamine as a reducing agent. After that, refolding of the protein was reached by removal of the denaturant agent via dialysis. MMP-1 contains one disulfide bond so these steps were performed in the presence of the redox Shutting system β-mercaptoethanol/2-hydroxyethyl disulfide to allow the correct disulfide bond formation.53

The results of these attempts were still not satisfactory, as almost no soluble active protein was recovered at the end of the procedure. Almost all proMMP-1 was crushed out of solution during the intermediate dialysis steps and underwent auto-proteolysis. MMP-1 undergoes auto-proteolysis in the
absence of a potent inhibitor, although this phenomenon can be reduced if the protein is stored at a concentration below 10 μM, but then it must be concentrated before use.

Due to the inconvenience of working with MMP-1, we began looking for another collagenase. We were pleased to find that bacterial collagenases, such as ColG, had a broader substrate specificity than MMP-1 and that they were already being used clinically for the treatment of diseases. For this reason, we decided to express Collagenase G (115 kDa) in the lab.

ColG was expressed in the cytoplasm of BL21(de3) cells (Figure 19). When a cell density corresponding to OD600=1 was reached, the expression of ColG was induced by adding 0.1 mM of IPTG and the incubation was done at 25 °C over night. The yield of soluble protein was at least 15 mg per liter of culture.

![Figure 19. Expression of ColG (115 kDa). M: protein ladder; Lanes: (1) pre-induction; (2) post-induction; (3) soluble fraction; (4) flow-through; (5) elution; (6) post-dialysis; (7) concentrated protein.](image)

In these first proofs of concept experiments, only one purification step with an affinity column was performed. The eluted soluble protein is not very pure, indicating that additional purification steps will be necessary. Nevertheless, this preparation allowed us to determine if the enzyme was active against collagenolytic substrates.

The activity of the ColG enzyme was assayed first using the synthetic substrate N-(3-[2-Furyl]acryloyl)-Leu-Gly-Pro-Ala (FALGPA). The activity assay with FALGPA consists in a continuous
spectrophotometric rate determination. FALGPA absorbs at $\lambda=345$nm and ColG catalyzes the hydrolysis of the substrate in the form FAL + Gly-Pro–Ala. Using a constant concentration of 1.0mM FALGPA and varying the concentration of ColG (1-4 mg/ml), the disappearance of absorbance was monitored for 5 minutes. The activity of ColG was plotted against the concentration of enzyme to showed a linear fit (Figure 20).

![Figure 20. Activity of ColG using the synthetic substrate FALGPA; a) continuos spectrophotometric rate determination varying amounts of enzyme and b) activity observed for each concentration of enzyme.]

The activity of ColG was also tested against native collagen. Collagen gels of 3mg/ml were formed from soluble collagen type I from rat tails. Soluble collagen is supplied in acidic conditions and gelation takes places rapidly at room temperature when the pH is adjusted to neutral. Figure 21 shows the digestion of native collagen by ColG, a) before digestion we placed two drops of 10μl each, the top corresponding to ColG and the bottom a control with just buffer. The digestion was allowed to undergo for 30 minutes at 37 °C. After that, gels were washed with dH$_2$O and stained with Coomassie Brilliant Blue G-250. Figure 4.4b shows the result. This assay illustrates that ColG is able to degrade helical regions in native collagen.

![Figure 21. Activity of ColG against native collagen: a) before digestion and b) after 30min of digestion and staining.]

2. Covalently attach collagenase to PET sutures and control the immobilization

Polyethylene terephthalate (PET) is a linear, aromatic polyester that consists of polymerized units of the monomer ethylene terephthalate. It has been shown to have the desirable mechanical strength and durability for biomedical applications, thus it is widely used in medicine for applications including vascular prostheses, heart valve sewing cuffs, implantable sutures and surgical mesh.

2.1. Characterization of PET surface treatment

First, the introduction of carboxylic groups onto the PET suture surface was analyzed using attenuated total reflectance - Fourier transform infrared spectrometry (FTIR-ATR). Both samples, untreated PET sutures and carboxylated PET sutures, contain the same functional groups with the exception of the O-H bands\textsuperscript{54}. Figure 22 shows the magnification of the spectra between the wavelengths 2400-4000 cm\textsuperscript{-1}, where the O-H band appears. While PET does not absorb at these wavelengths, PET-COOH presents a broad absorption peak with maximum at 3421 cm\textsuperscript{-1}, due to OH stretch. The spectrum indicates that the surface of the PET fiber was functionalized successfully with carboxylic acid groups for biomolecule immobilization.

![FTIR-ATR spectra of PET and carboxylated PET.](image)

\textbf{Figure 22.} FTIR-ATR spectra of PET and carboxylated PET.

Scanning electron microscopy (SEM) studies were performed to analyze the possibility of surface deterioration due to chemical modifications. Figure 23 shows the surface morphology of untreated PET, hydroxylated PET and carboxylated PET.
Figure 23. SEM images of a) PET, b) PET-OH, and c) PET-COOH.

The SEM micrographs of the untreated PET and hydroxylated PET have a smooth surface, proving that the first step of the functionalization to introduce benzyl hydroxyl groups does not alter the structural integrity of the suture. However, Figure 23c shows the formation of pits on carboxylated PET, suggesting that degradation of the polymer has taken place.

Authors have used two surface analysis techniques: time-of-flight secondary ion mass spectrometry (TOF-SIMS), and X-ray photoelectron spectroscopy (XPS) to give insight into the chemistry taking place at each step. Zhongping Yang in 2000 found that the carboxylic acid concentration in PET-COOH was about 21% of the theoretical maximum, and therefore they concluded that on average, one COOH group was introduced every five repeat units of PET. However, TOF-SIMS results showed a high concentration of low molecular weight PET oligomers on the surface of the modified polymers compared with the unmodified, that they believed were created by chain scission of PET due to hydrolysis. The action of the base leads to cleavage of ester linkages, resulting in the formation of terminal hydroxyl and carboxyl groups at the new chain ends. Peter Kingshott in 2003 also found that a carboxyl group was introduced in every three PET monomer units. But, AFM measurements showed that significant surface erosion occurred after the treatment, suggesting again that a significant number of carboxyl groups are most likely generated from hydrolysis of PET.

Therefore, the scheme shown in Figure 13 is only approximate, and it is likely that a substantial fraction of reactive groups arise from hydrolytic cleavage of the PET backbone. The presence of pits in PET-COOH (Figure 23c) suggests that a significant number of carboxyl groups are generated from hydrolysis of PET during the second step of the functionalization.
PET is an aromatic polyester, and one of its disadvantages is its hydrophobic character. Conventionally, the hydrophobicity of PET fibers is improved by sodium hydroxide treatment. It has been shown that hydrolysis with aqueous NaOH only affects the outer surface of the fibers and that reaction does not occur in the interior. As the reaction proceeds, surface layers are successively removed by hydrolysis. Figure 24 consists on SEM images showing the morphology of a PET fiber exposed to a 10% aqueous NaOH solution at T=60°C. The untreated PET fiber (a) has a relatively smooth surface, while NaOH treatment causes pitting of the fiber surface, correlating with our results.

![Figure 24. SEM images of: a) starting PET fiber, b) treated 15 min with aqueous NaOH, c) treated for 1 h, and d) treated for 2 h.](image)

The pits on the suture probably serve as weak point or defects at which rupture is initiated. Loss of mechanical strength is a common problem associated with surface modification due to degradation of the polymer.

There are other techniques available to introduce reactive functional groups on PET surfaces, such as: chemical treatments (hydrolysis, reduction, glycolysis, aminolysis and amination), enzymatic modifications, physical treatments (UV irradiation, plasma functionalization, corona discharge, ozone-gas), etc. However, most of them also alter the mechanical strength of PET after modification.

Even with these limitations, many studies have demonstrated immobilization of active biomolecules onto PET sutures. After the treatment, there are a significant number of reactive groups present on PET-COOH for further functionalization, and try to immobilize collagenase on its surface.
2.2. Immobilization of collagenase onto PET sutures

In order to initially characterize the immobilization reaction we did not use the recombinant ColG made in the lab, but instead, we used a commercial preparation. The commercial preparation was used in order to quickly assess the potential of the bioactive sutures. If the results looked promising, we would try to use protein engineering on the recombinant Collagenase G to rationally immobilize it onto the sutures. Worthington Biochemical Corporation sells a chromatographically purified collagenase preparation from *Clostridium histolyticum*, with high collagenase activity (at least 10 ku, where one unit is defined as the amount of enzyme required to liberate 1 μmole of L-leucine equivalents from collagen in 5 hours at 37°C, pH 7.5), and free of other proteolytic activities (≤ 50 caseinase units per milligram), called CLSPA. The batch of collagenases that we used had 970 U/mg dry weight.

Figure 25 shows the SDS-PAGE gel of the proteins present in this preparation. About 95% of the enzymes present have a molecular weight between 90 and 125 kDa. This is in agreement with the molecular weights of *Clostridium histolyticum* collagenases, where crude collagenase mixtures can contain six to eight different molecular weight species ranging from 68 to 130 kDa. There are two less abundant enzymes with molecular weights of 75 and 80 kDa, which probably correspond to the gelatinases, C-terminal truncated forms of the respective full-length collagenase.

We used a conventional immobilization method for the coupling of the enzyme onto the sutures. With a two-step coupling procedure using EDC and sulfo-NHS, the carboxylic groups in the surface of the suture were reacted with primary amines in the enzyme to form an amide bond. The first step, activation, takes place at acidic conditions in the presence of EDC and sulfo-NHS. The second step, coupling, is done in the presence of the collagenase enzymes at neutral pH. All the lysine residues in the protein are susceptible to react and form covalent bonds with the sutures, and because of that, we don't have control over the orientation of the enzyme upon immobilization. Such control is critical in preserving the activity of the enzyme, which can be lost by disruption of the enzyme structure or by blocking the accessibility of the active site of the enzyme (this can happen if residues in or near the
active site participate in the immobilization reaction). Despite the possibility of losing activity upon immobilization, we still tried to prepare bioactive sutures to determine if they retain collagenolytic activity.

After coupling the enzyme onto the sutures, the sutures were extensively washed with buffer. Leaching of the enzyme from the sutures was determined as residual protein concentration in the wash solution, measured with absorbance at 280 nm. Table III shows that after three washes the sutures no longer shed enzyme.

**Table III.** Measurements of OD$_{280}$ of the washing solutions in order to determine leaching of the enzyme.

<table>
<thead>
<tr>
<th></th>
<th>(1)</th>
<th>(2)</th>
<th>(3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer</td>
<td>0.004</td>
<td>0.006</td>
<td>0.006</td>
</tr>
<tr>
<td>1st wash</td>
<td>0.078</td>
<td>0.085</td>
<td>0.072</td>
</tr>
<tr>
<td>2nd wash</td>
<td>0.008</td>
<td>0.005</td>
<td>0.010</td>
</tr>
<tr>
<td>3rd wash</td>
<td>0.002</td>
<td>0.003</td>
<td>0.006</td>
</tr>
</tbody>
</table>

Activity of the commercial collagenase-modified PET sutures was first tested using the FALGPA substrate. Figure 26 confirms the attachment of the enzyme onto the sutures, and demonstrates that the attached collagenases still retain peptidolytic activity.

![Figure 26](image-url)
Activity of the collagenase-modified PET sutures was also studied with native collagen. Controls of non-treated sutures and collagenase-modified sutures were placed on top of the collagen gels and they were allowed to react for four days at room temperature. After that, the sutures were removed, and the gel was washed with dH₂O and stained with Comassie Blue. Figure 27 shows the degradation of the gel by the collagenase on the suture.

![Figure 27. Activity of commercial collagenase modified PET sutures against native collagen (b), a) shows a control with non-treated sutures.](image)

### 2.3. Control the activity of collagenase-modified sutures

The objectives of this section are: (1) to be able to control the immobilization reaction of the enzyme onto the sutures and (2) to obtain reproducible collagenolytic activities. The substrate used to characterize the immobilization reaction was gelatin heavily labeled with fluorescein (EnzChek Gelatinase/Collagenase Assay Kit). A concentration of 100 μg/mL DQ gelatin and at least half an hour incubation period were used in all the experiments to determine the rate of gelatin cleavage by the immobilized enzyme.
First, the activity was measured varying the length of the suture as shown in Figure 28. A concentration of 0.1 mg/mL (97 U/mL) of enzyme was used in all the coupling reactions.

![Figure 28](image)

**Figure 28.** Activity retained on the suture with varying lengths of suture

Figure 4.10a shows that the fluorescence of the substrate did not vary with time. The amount of enzyme in the wash solution was also measured by assaying the activity of the third wash against gelatin. The fluorescence almost did not increase with time, showing that very little, if any, enzyme came out in the third wash. Also, in Figure 4.10a we can see that as the length of the suture increases, so does the enzyme activity. Figure 4.10b shows that the relationship between length of suture and enzyme activity is linear.

The next attempt to control the immobilization reaction consisted of varying the concentration of enzyme 0–0.1 mg/mL (0–97 U/mL) in the coupling reaction. Five centimeters of suture were used in all the measurements. Figure 29 shows the relationship between the concentration of collagenase in the coupling reaction and activity of the sutures.
**Figure 29.** Activity retained on the suture with varying concentration of collagenase in the coupling reaction.

Figure 29 is a saturation-binding curve. As the concentration of enzyme in the coupling reaction increases, the enzymatic activity on the sutures does too. However, at high concentrations of enzyme, all the possible binding sites on the suture are occupied, and there is no increase in activity. The suture has reached its maximum activity.

We then tried to obtain the saturation binding curves at different times of the carboxylation reaction. By varying the reaction time of the second step of the functionalization of PET, different amounts of carboxylic groups will be present on the surface of the suture. Figure 30 shows the binding curve of 1h, 5h, and 10h of the carboxylation reaction.

![Figure 30. Activity varying times of the carboxylation reaction.](image)

To our surprise, all the binding curves looked the same, as if the reaction time of the carboxylation step did not matter. If different amounts of carboxyl groups were present on the suture, we would expect the binding curves to saturate at different maximum activities. This result revealed that there was non-specific binding between the enzyme and the suture.
In order to determine if non-specific binding was taking place, we ran an experiment comparing enzyme activity of treated (carboxylated) sutures to untreated sutures (Figure 31). Both the treated and untreated sutures were five centimeters in length and exposed to a concentration of 0.05 mg/mL (50 U/mL) of enzyme.

![Activity of the control untreated sutures in contact with the enzyme and collagenase-modified sutures.](image)

**Figure 31.** Activity of the control untreated sutures in contact with the enzyme and collagenase-modified sutures.

Figure 31 shows that the untreated sutures retain collagenolytic activity 2156 (Δ fluorescence units)/min, demonstrating that non-specific binding occurs between the collagenases and the sutures. The activity of the sutures with immobilized enzyme is still higher 3038 (Δ fluorescence units)/min, but this result reveals that most of the activity that we were seeing was due to enzyme non-covalently attached to the suture.

PET is an aromatic hydrophobic polyester, and the hydrophobic interaction between the enzyme and the suture is what causes the non-specific binding. Several attempts were taken in order to minimize the non-specific hydrophobic binding: (1) include tween 20 in the coupling and washing solutions, (2) Include NaCl in the washing solutions, (3) lower the pH of the Coupling Reaction, and (4) react the sutures a second time.
(1) Include Tween 20 in the coupling and washing solutions:

Tween 20 is a non-ionic detergent. It is considered to be 'mild' and not likely to denature proteins. Tween 20 prevents the hydrophobic absorption of proteins in solid phase, either by saturating the binding sites on the surface or by hydrating proteins and hindering hydrophobic binding. It is frequently used in immunoassays, such as Western blots and ELISAs, as a washing agent to prevent non-specific antibody binding.

We used tween 20 at 0.05% v/v (above the cmc) in the coupling reaction of the collagenase with the suture and in the washing solutions. Figure 32 shows the result of adding tween 20 in untreated and treated sutures.

![Figure 32. Effect of tween 20 on minimizing non-specific binding: a) untreated sutures, and b) treated sutures.](image)

Tween 20 proved effective in reducing the non-specific hydrophobic binding of the enzyme with the untreated sutures, as no activity was observed in these sutures (Figure 32a). Tween 20 also reduced the activity of the treated sutures with immobilized enzyme, but still some enzyme remained immobilized on the sutures (Figure 32b). The remaining activity of the treated sutures in the presence of tween 20 is 945 (Δ fluorescence units)/min.

Now we know that without the presence of tween 20 in the coupling and washing solutions, 70% of the activity was due to non-specific binding and only 30% was covalent binding of the enzyme to the suture.
We ran another control where the sutures were modified with carboxylic groups, but no coupling agent was added to the reaction with the enzyme (no EDC step). Covalent binding of the enzyme on the suture could not take place. This control was run to look at the possibility that the enzyme was attached by electrostatic interaction to the carboxylic groups on the suture (Figure 33).

![Graph showing fluorescence over time](image)

**Figure 33.** Control where sutures have been treated and contain carboxylic groups on the surface, but no coupling agent is added to the reaction with the enzyme. Formation of covalent bonds could not take place.

Figure 33 shows that the activity of the treated sutures in the presence of tween 20 is mainly due to enzyme covalently attached to the suture. If the coupling agent is not added in the reaction, little to no collagenase remains attached to the suture.

We continued optimizing the immobilization reaction by attempting to covalently attach more enzymes to the suture and by more thoroughly washing non-covalently attached enzymes from the suture.

(2) Include NaCl in the washing solutions:

NaCl is commonly added during protein purification to wash non-specifically bound proteins from an affinity column or from an SPR surface. We expected that adding NaCl would help to wash the non-specifically attached enzyme from the suture. We were not sure about the ability of the collagenases to survive high salt concentrations, so we tried two combinations of salt: 1 M NaCl in each of the washing solutions, and 0.5 M NaCl for only 15 minutes in the first wash step (Figure 34).
(3) Lower the pH of the Coupling Reaction:

This attempt was made in order to get more covalently attached enzyme on the suture. Until this point, coupling of the enzymes with the carboxylic groups on the sutures was done at pH 7.6. At this pH the enzymes are overall negatively charged. In general, it is recommended to do the coupling step at the highest pH possible, because it is important that the lysines are deprotonated to be more prone to nucleophilically attack the carbonyl groups. However, at pH 7.6 lysines are already below its pKₐ, and so, are positively charged. Taking into account that lysines are already positively charge at physiological pH, we thought that overcoming the surface repulsion between the negatively charged groups on the suture and the overall charge of the enzyme would help the enzyme get closer to the surface of the suture. With this, we decided to lower the pH of the coupling solution to 5, where the overall charge of the enzyme is positive. MES buffer at pH 5.0 was used for both the activation and the coupling reactions and tween 20 was also included in the coupling reaction and in the washing solutions.
The activity retained in the sutures after coupling the enzyme at pH 5 is 840 (Δ fluorescence units)/min, similar to the activity obtained previously at pH 7.6 (Figure 35). The enzyme was stable at pH 5, but no more enzymes attached to the sutures.

Figure 35. Effect of lowering the pH in the coupling solution to overcome the charge repulsion between the sutures and the enzymes.

(4) React the sutures a second time:

After the previous result, where we observed that the enzymes were still stable after exposure to pH 5, we tried to react the collagenase-modified sutures a second time. In the case that all the carboxylic groups did not react with enzyme during the first coupling, they will be given another chance to react with enzyme (Figure 36).

The activity of the sutures after being reacted a second time was 595 (Δ fluorescence units)/min, lower than just after one coupling step. Reacting the sutures a second time did not help in getting more enzymes on the sutures either. We observed the appearance of aggregates in the first washing solution of these two-times reacted sutures, meaning that aggregation took place when the collagenases on the sutures were exposed to collagenases in solution.

Figure 36. Coupling of the sutures a second time.

In conclusion, we were able to obtain sutures with an activity against denatured collagen of approximately (Δ fluorescence units)/min per centimeter of suture. Comparing this activity with a calibration of free enzyme in solution, this activity corresponds to 8 ng of covalently attached Clostridium Histolyticum collagenases per centimeter of suture.
3. Characterize the stability of the collagenase-modified PET sutures

Enzyme stability is one of the properties that have generally been considered to improve via immobilization. Thermal stability is thought to increase because collision between molecules is reduced when they are attached to a material. By this same reason, auto-proteolysis is also reduced. In the body, inactivation due to digestion of proteolytic enzymes should also be reduced when the enzymes are immobilized on the suture.

However, this is not always the case. Stability of the enzyme may also decrease upon immobilization. Possible explanations are: i) the microenvironment that the carrier provides, that can promote denaturation of the protein by establishing undesired interactions; and ii) random immobilization of the enzyme on the material may not improve enzyme rigidity.

The impact of immobilization on stability of the collagenses on PET sutures was measured via monitoring inactivation of the immobilized enzyme relative to the native enzyme at 37 °C. Free collagenases in solution and collagenase-modified sutures were incubated in the gelatin assay buffer and samples were periodically removed to test its activity.

Figure 37. Stability of the enzyme at 37 °C in solution and attached to the sutures.

Figure 37 shows that the enzyme on the suture loses activity faster than in solution. While the enzyme in solution still retained some activity after 24h, there was no enzyme activity on the suture after the second hour. As mentioned above, this can be due to undesired interactions between the enzyme and the suture that are favored when the temperature is increased, and to a loss of enzyme rigidity upon immobilization.
4. Characterize the degradation of type I collagen gels by collagenase-modified PET sutures

Finally, we tested the ability of the covalently attached collagenases to degrade native triple helical collagen. Two controls were also run, one with non-treated sutures (Figure 38 left column) and the other with non-treated sutures in contact with collagenase enzymes in the presence of tween 20 (Figure 38 center column). No collagenolytic activity was expected for either of these two controls.

![Figure 38](image)

**Figure 38.** Activity of covalently attached enzyme on the sutures against native collagen (right column). Two controls were also run: non-treated sutures (left column) and non-treated in contact with collagenase tween 20 (center column).

Figure 38 demonstrates that neither of the two controls degraded the collagen. Unfortunately, the sutures with collagenase covalently attached on them did not degrade the type I collagen gel either (Figure 38 right column). Collagenases lost the collagenolytic activity upon covalent immobilization. Previously, when non-specific binding was allowed to occur, the enzyme non-specifically bound to the sutures still retained the collagenolytic activity; however, the enzyme covalently attached to the PET sutures lost this ability.
Part V. Conclusions and Future Work

Formation of scar tissue, which is the result of the body’s natural wound-healing process, is a critical barrier to the regeneration of injured skeletal muscle tissue. Incomplete muscle regeneration due to scarring ultimately compromises the structural integrity of the tissue, prevents full recovery of function, and increases the likelihood of re-injury.

Our approach in this project has been to develop bioactive sutures to prevent fibrosis in injured skeletal muscle. The bioactive sutures consist of collagenase enzymes immobilized on polyethylene terephthalate (PET) sutures. We hypothesize that closing the injured muscle with collagenase-modified sutures will inhibit the deposition of fibrotic tissue by digesting interfibrillar collagen as it is formed, thus improving muscle healing.

Initially, we looked for a recombinant collagenase that is active in degrading native collagen. We began by producing MMP-1, the collagenase found in humans, but it is very unstable and undergoes auto-proteolysis. When looking for another collagenase, we found that bacterial collagenases have broader substrate specificity, being able to digest native triple helical types I and III collagens into a mixture of small peptides, and they are well expressed in E. coli.

We started by characterizing the immobilization reaction of the enzymes onto the sutures with a commercial preparation of Clostridium Histolyticum collagenases. This preparation contained a mixture of the two Clostridium Histolyticum collagenases, ColG and ColH, and its C-terminal truncated gelatinases. While trying to control the immobilization reaction, we observed that there was non-specific binding between the enzyme and the suture. Specifically, around 70% of the activity on the sutures was due to enzyme non-covalently attached to the suture, and only 30% was due to enzyme covalently attached to the suture. We reduced the hydrophobic adsorption of the enzyme to the suture by adding 0.05% tween 20 (v/v) to the coupling and washing solutions. Further attempts were made to covalently attach more enzymes to the suture and to wash more thoroughly the non-covalently attached enzyme. These attempts were: include NaCl in the washing solutions, lower the pH of the coupling reaction, and react the sutures a second time. However, these attempts were ineffective and we were not able to covalently attach more enzymes to the sutures.
We were able to obtain sutures with an activity against denatured collagen of approximately 190 (Δ fluorescence units)/min per centimeter of suture. Comparing this activity with a calibration of free enzyme in solution, this corresponds to 8 ng of covalently attached *Clostridium Histolyticum* collagenases per centimeter of suture.

Although enzyme stability is generally considered to improve upon immobilization of the enzyme on the surface, this is not always the case. The enzymes that we immobilized onto the sutures lost activity much faster than the enzyme in solution. Possible explanations are: i) undesired interactions between the enzyme and the suture, and ii) loss of enzyme rigidity upon immobilization.

Finally, we tested the ability of the covalently attached collagenases to degrade native triple helical collagen. Unfortunately, the sutures with collagenase covalently attached on them did not degrade the type I collagen gel. Collagenases lost the collagenolytic activity upon immobilization. Interestingly, when non-specific binding was allowed to occur, these collagenase-modified sutures were able to degrade type I collagen gel.

Due to the instability of the collagenases immobilized on PET sutures at 37°C, two improvements should be made: 1) use of a more suitable material, such as a more hydrophilic suture and 2) use an immobilization technique that allows control over the orientation of the enzyme upon immobilization.

The orientation of the enzyme is important because reaction of random residues can result in disruption of the enzyme structure, and therefore, loss of activity of the enzyme. In order to minimize the impact on the folding state of the enzyme upon immobilization, a site-specific immobilization should take place. This is possible by the incorporation of a reactive non-natural amino acid on the enzyme\(^{55,56}\). In principle, reactive non-natural amino acids can be incorporated at any position within an enzyme. Thus, one can engineer and control the position within the enzyme that the reaction with the surface may occur. The non-natural amino acid should be incorporated at a site that: is highly exposed to solvent, does not participate in catalysis, and is not involved in contacts or packing interactions with neighboring residues.

Some examples of reactive non-natural amino acids are the azide-containing non-natural amino acids: p-azido-L-phenylalanine, 3-azido-L-tyrosine, azidoalanine, and azidohomoalanine, which have been successfully introduced into proteins. This azide group could be reacted by means of bioorthogonal
azide-alkyne ‘click’ chemistry reactions, if alkyne groups were incorporated onto the surface of the suture. Thus, using this approach, the enzyme could be site-specifically immobilized with the non-natural amino acid on the modified suture.

By engineering the enzyme to incorporate reactive non-natural amino acids, it is possible to control the position within the enzyme that the reaction with the suture will take place, thereby preserving the structure and function of the enzyme. By rationally immobilizing collagenases onto sutures, the collagenolytic activity could be maintained, therefore inhibiting fibrosis and helping in the healing process of muscle injuries.
Bibliography


