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Tissue transglutaminase: A new method of facilitating hydrogel formation for cartilage regeneration and cartilage surface modification

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ABSTRACT

Tissue transglutaminase: A new method of facilitating hydrogel formation for cartilage regeneration and cartilage surface modification

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Articular cartilage is a type of connective tissue that protects the underlying bone from the compressive forces of daily activities. When cartilage is damaged via a traumatic event, chondral defects are formed and require medical intervention. However, current treatments are of inconsistent efficacy and only benefit a subset of patients. Chondrocyte-containing hydrogels have been suggested as potential treatments of chondral defects but often lack the ability to adhere to and integrate with the native cartilage tissue. Tissue transglutaminase provides a potential mechanism of crosslinking hydrogels to native cartilage tissue. Tissue transglutaminase (tTG) is an ubiquitous enzyme that crosslinks lysine and glutamine residues in peptides and proteins. tTG can potentially be used in a multifaceted manner, crosslinking molecules to tissue and crosslinking polymers modified with peptide substrates to form hydrogels. Two tTG crosslinked hydrogel systems were evaluated for *in vitro* culture of chondrocytes, which proliferated and produced a limited quantity of GAG. To examine the ability of tTG to couple peptide-polymer conjugates to cartilage, and hence the hydrogel that contains them, small synthetic peptide substrates of tTG and their PEG conjugates were coupled to the cut surface of cartilage. Serendipidiously, it was determined that tTG was able to couple the peptides to the cut surface and also the articular surface, thus demonstrating that tTG could be utilized to modify the articular surface with drugs and biologically important molecules. This is of potential utility for

the treatment of osteoarthritis, a degenerative joint disease, since some drugs are delivered intra-articularly to treat the disease but drug retention is problematic. Surface modifications to cartilage would make treatment more effective and minimize systemic side effects. To investigate the use of tTG to modify the articular surface of cartilage, a lysine peptide was used to make a peptide prodrug of hydrocortisone for localization to the articular surface of cartilage as a technique of local drug delivery to the joint. These results suggest that tTG may be most useful for coupling biologically important molecules to tissue surfaces. Further modifications and optimization are necessary to improve the utility of tTG in the formation of hydrogels.

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Thesis Overview and Specific Aims

1.1 Motivation and Objective

Cartilage is the connective tissue that protects the underlying bone from compressive forces applied during daily activities. However, sudden impact on the joint, via a fall or various sports activities, can damage the cartilage causing chondral defects. Unfortunately, damage to cartilage usually can not be repaired naturally since chondrocytes have little proliferation potential. Current medical treatments for chondral defects, such as osteochondral plugs, debridement and lavage, and autologous chondrocyte transplants, do not provide consistent results; only a small subset of the affected population benefits from any of these approaches. The use of hydrogels to fill chondral defects has been proposed as an alternative to current treatments. It is hypothesized that hydrogels could serve as temporary matrices for chondrocytes as they regenerate the native cartilage matrix. However, there are three areas in which hydrogels have not been uniformly successful in repairing chondral defects. First, most hydrogels do not have the appropriate modulus to withstand normal joint activity. Second, most hydrogels can not adhere nor integrate with the native cartilage matrix, making them readily dislodged from the site of repair. Finally, most hydrogels lack cell directed degradation.

Since cartilage is aneural and avascular, damage may go undetected by the victim and lead to osteoarthritis. Osteoarthritis is a degenerative disease of cartilage resulting from excessive loading which leads to the inflammation and production of matrix metalloproteinases

that destroy the cartilage. Some osteoarthritis treatments are delivered via intra-articular injection, but only provide temporary relief from symptoms and inflammation. A major problem encountered with intra-articluar delivery is drug loss that occurs via the lymphatics. This reduces the efficacy of treatments, specifically hyaluronic acid and steroids, which have been shown to not only decrease inflammation but also inhibit matrix catabolism, essentially stopping the osteoarthritic process. In order to increase retention of these two drugs, insoluble forms that precipitate in the joint are used. While benefits of this technique have been documented, inflammation can result from these products and exacerbate the problem[1]. Hence other methods of retaining therapeutic agents in the joint space are needed.

Tissue transglutaminase (tTG) may be a beneficial tool in facilitating both the adhesion of hydrogels to native cartilage matrices and the retention of therapeutic agents in the joint. Transglutaminases are a family of calcium-dependent enzymes that crosslink lysine and glutamine residues in proteins and peptides[2]. tTG is the most ubiquitous member of this group, located in all tissues. It is used extracellularly to stabilize the extracellular matrix, forming permanent crosslinks that are chemically and enzymatically resistant[3]. Substrates of tTG had been identified in cartilage, and Jurgensen *et al.* demonstrated that tTG could be used as an adhesive to hold pieces of cartilage together[4], presumably through crosslinking native substrates in the distinct pieces. Previous research in our group demonstrated that synthetic peptide substrates of TG, FKG and GQQQLG, can be used to modify polyethylene glycol (PEG) polymers, thus enabling the formation of a gel through the crosslinking action of tTG[5]. Therefore, it is hypothesized that tTG can also crosslink these gels to the native cartilage tissue during *in situ* gelation, which may lead to better adhesion and integration. Moreover, it is hypothesized that this strategy can be used to couple therapeutic agents to the cartilage surface

and improve retention of therapeutic agents in the joint. Because tTG and its substrates are so commonplace in the body, it is believed that this strategy can be used in tissues other than cartilage.

The goal of this research was to evaluate the use of tTG crosslinked gels for *in vitro* culture of chondrocytes and to explore the use of tTG as a method of immobilizing molecules on biological tissue, specifically cartilage.

1.2 Thesis Outline

This chapter outlines the specific aims of my research. Chapter 2 is a pertinent review on cartilage, chondral defects, osteoarthritis and the potential use of hydrogels to treat chondral defects. Chapter 3 is a review of strategies to immobilize molecules on biological tissue. Chapter 4 is a review of tissue transglutaminase and its current role in biomaterial manipulation. Chapter 5 documents our initial attempts at culturing chondrocytes in an alginate/PEG hydrogel modified with FKG and GQQQLG peptides and crosslinked with tTG. From the evaluation of these hydrogels, it was hypothesized that a hydrogel system without concomitant effects (alginate is also crosslinked with calcium) and with degradable crosslinks may provide superior results. Therefore, Chapter 6 discusses the evaluation of a hydrogel consisting of PEG modified with peptide substrates of tTG and crosslinked with the enzyme. An MMP sensitive sequence was included with the lysine peptide to permit cell directed degradation of the hydrogel. Chapter 7 details the coupling of FKG and GQQQLG peptides and their peptide-PEG conjugates to cartilage tissue with the use of tTG. These studies were performed to verify the ability of tTG to crosslink these peptides and their PEG conjugates to tissue. In Chapter 8, hydrocortisone was

modified with the FKG peptide and tTG was used to couple the drug to the surface. This demonstrated that tTG crosslinking can be used in localized drug delivery to facilitate drug retention.

An overview of the thesis research is given in Figure 1. The multiple uses of tTG and its short synthetic peptide substrates in the delivery of therapeutic agents are illustrated in this figure. Two different hydrogel systems composed of polymers modified with peptide substrates of tTG and crosslinked by the enzyme were evaluated as *in vitro* scaffolds for chondrocytes. Next, the general principle by which peptide substrates of tTG and peptide-polymer conjugates can be coupled to cartilage surfaces was demonstrated. Finally, FKG was modified with hydrocortisone and attached to the articular surface of cartilage. This demonstrated tTG facilitated coupling can be used as a strategy for targeted drug delivery.

1.3 Specific Aims

My overall hypothesis states that small peptide substrates of tTG and any polymeric conjugates of these peptides will be able to be coupled to cartilage using tTG. In addition, I hypothesize that hydrogels made of polymers modified with peptide substrates of tTG and crosslinked by the enzyme will provide an *in vitro* environment conducive to chondrocyte development. These hydrogels, made of polymers modified with peptide substrates of tTG, may potentially improve adhesion to native cartilage. However, since these gels are not degradable, I propose that the inclusion of a MMP sensitive peptide would allow for controlled hydrogel degradation, thus improving cartilage regeneration.

1.3.1 Specific Aim 1(Chapter 5)

Modify PEG with GQQQLG peptide and alginate with FKG peptide, form a hydrogel with these two modified polymers and evaluate the hydrogel as a scaffold for cartilage regeneration.

Hypothesis 1: The formation of an alginate/ PEG hydrogel crosslinked with tTG will support chondrocyte viability and biochemical function.

1.3.2 Specific Aim 2(Chapter 6)

Regenerate cartilage from an *in vitro* culture of chondrocytes in PEG hydrogels made with polymers modified with a more reactive substrates for tTG and MMP-sensitive peptides and crosslinked by tTG.

Hypothesis 2: A hydrogel formed by PEG polymers modified with a new Gln peptide substrate of tTG, which has a higher specificity for the enzyme will form a hydrogel with PEG modified with a Lys substrate that will swell less.

Hypothesis 3: The inclusion of an MMP sensitive peptide by which the hydrogel can be degraded in a cell-directed manner would facilitate cartilage regeneration in the tTG crosslinked PEG hydrogels.

1.3.3 Specific Aim 3 (Chapter 7)

Determine if short synthetic peptide substrates of tTG, FKG and GQQQLG, as well as the respective PEG conjugates of these peptides can be coupled to native cartilage in the presence of tTG.

1.2.4 Specific Aim 4 (Chapter 8)

To synthesize a prodrug that is a combination of a peptide substrate of tTG and hydrocortisone and demonstrate that tTG can be used to immobilize the drug to the cartilage surface.

Hypothesis 5: Because there are native protein substrates of tTG in cartilage, and our synthetic peptides have been successfully utilized as substrates of tTG, these peptides and peptide conjugates will be able to be coupled to native protein substrates of tTG in cartilage via the action of tTG.

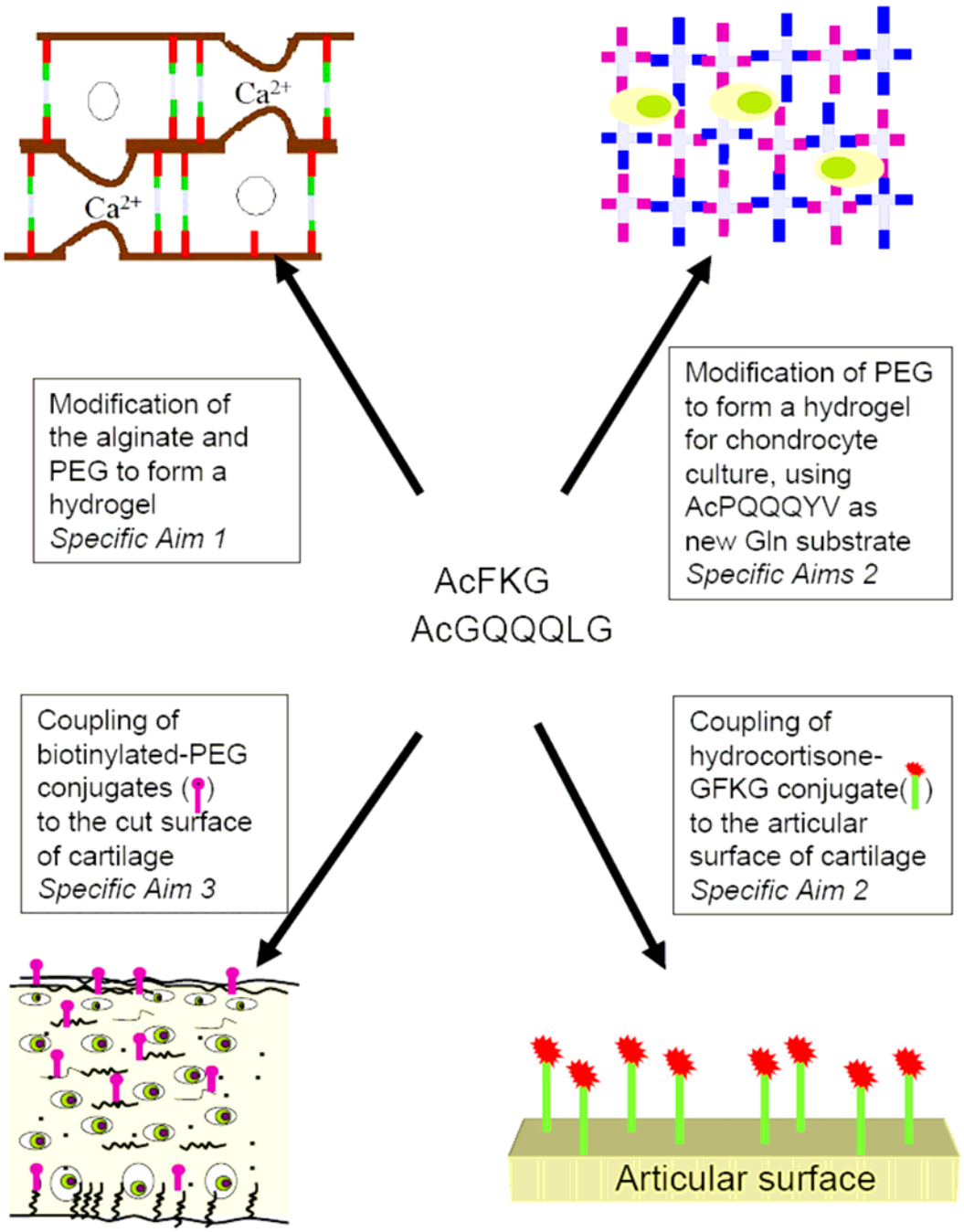


Figure 1-1. Overview of multiple strategies utilizing tTG crosslinking. AcFKG, the lysine substrate, and AcGQQQLG, the glutamine substrate, are used to modify polymers and hydrocortisone in the study of surface modifications and hydrogel formation by tTG.

Chapter 2

Articular Cartilage

2.1 Introduction

Cartilage is a type of connective tissue that provides either structure to the body, as with the ear or nose, or functions as a buffer between two bony structures. Articular cartilage, which is found at weight-bearing joints such as the knee, ankle, or interphalangeal joints, protects the underlying bone from damage due to compressive loads during joint motion by distributing it over the entire joint surface[6-8]. Facilitated by synovial fluid produced by the lining of these joints, it also permits low-friction and rapid movement between the bones[6]. Articular cartilage is avascular, aneural, and alymphatic[8], and is nourished by diffusion of nutrients from blood vessels in the subchondral bone and from the synovial fluid[6]. It is composed of cells called chondrocytes which synthesize and maintain the extracellular matrix (ECM). Both chemical and mechanical changes in the ECM can cause the chondrocytes to modulate ECM metabolism. The two main components of articular cartilage are collagen II which contributes primarily to its tensile strength, and aggrecan which contributes to its compressive resistance[8].

2.2 Organization of articular cartilage

Articular cartilage consists of 4 distinct regions: the superficial zone, middle or transitional zone, deep zone and calcified zone (Figure 1). The cells in the superficial

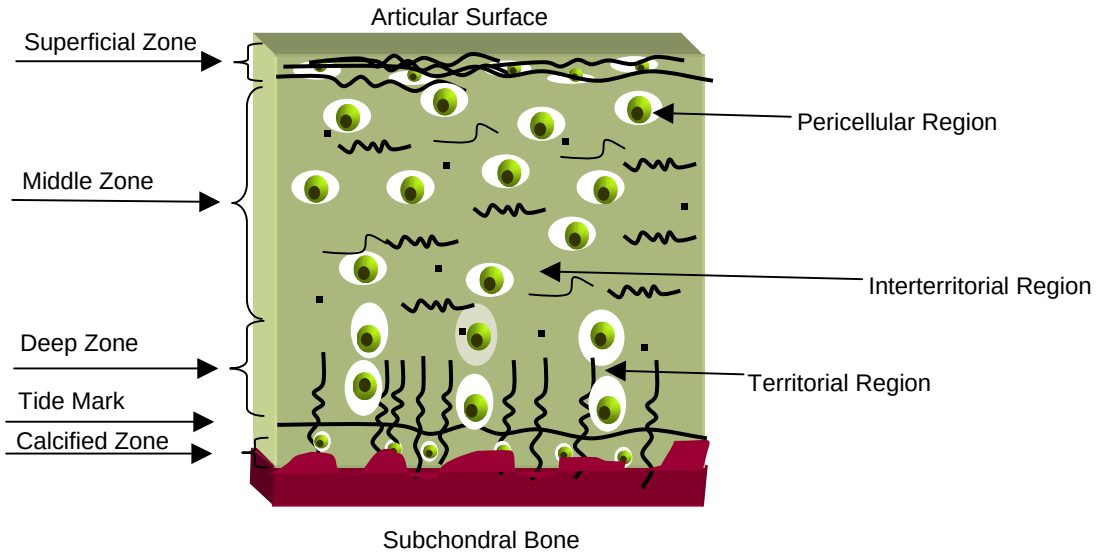


Figure 2-1. Schematic of articular cartilage organization.

zone are similar in shape to fibroblasts, with their long axis parallel to the surface[7]. It is reported that this zone contains a subset of cells that function as chondrocyte progenitor cells that form cartilage appositionally in adults[9]. The ECM in the superficial layer is mostly a dense packing of collagen fibers, which are oriented tangential to the surface. This zone contains very little aggrecan but does contain one large proteoglycan, superficial zone protein, which is used for lubrication[10, 11]. The superficial layer protects the subsequent layers from shear stress[12]. The mechanical properties of cartilage are determined by the organization of the superficial layer; if this layer is disrupted or damaged, cartilage function is compromised and subject to shear stress during joint motion[8, 12].

The middle zone of cartilage is the largest zone[6]. The chondrocytes in this region are rounded and are surrounded by radial fibers of collagen which protects the cells. Further away from the chondrocytes, the collagen fibers are oriented in all directions but parallel to the articular surface[8]. This zone contains a large quantity of proteoglycans and hence heavily contributes to the compressive resistance of cartilage[12].

The deep zone contains cells that are clustered or in columns, which are perpendicular to the articular surface. The cell density is half to a third of that of the superficial zone, but the cell volume is twice that of cells in the superficial zone[6]. Collagen fibers are thickest in this region and are oriented similar to those in the middle zone. In addition, there are fibers that traverse the tide mark into the calcified cartilage, anchoring the cartilage to subchondral bone[7]. The proteoglycan concentration increases to 50% of the ECM in the deep zone[7] and the water content is the lowest[7, 8]. This zone also contributes to compressive resistance.

The calcified zone is formed as a result of endochondral ossification and is a transition zone between non-calcified cartilage and subchondral bone marked by the tide mark. Cells in the calcified zone have less volume than cells in the deep zone[7]. The tide mark is a group of collagen fibrils that is believed to anchor the collagen fibers that travel to the subchondral bone from the uncalcified cartilage as well as provide protection from shear forces[8, 12].

Within each zone of cartilage there exist different areas of matrix organization around the chondrocyte which serves to both protect and transmit mechanical information to the cell. The interterritorial region is the matrix between clusters of cells and makes up the largest portion of cartilage. Collagen fibers in this region are arranged parallel to each other and are organized parallel to the surface in the superficial zone and transition to perpendicular in the deep zone[7]. An abrupt change in collagen fibril orientation marks the transition from interterritorial to territorial region[7]. In the territorial region, collagen fibers form a basket around the cells to protect the cells from mechanical loading and form molecular connections with the ECM in the pericellular region[7]. The territorial region is associated with a cell or cluster of cells and is located directly outside the pericellular region, which includes the ECM within 2 μm of a chondrocyte[7]. It provides hydrodynamic protection to the chondrocyte. The cell and the pericellular matrix together are called a chondron. The pericellular region contains sulfated proteoglycans and many glycoproteins.[8]

2.3 Chondrocytes

Chondrocytes contribute very little to the total volume of cartilage, only about 1-5%, but are responsible for producing and organizing the ECM[8]. Since the ECM varies by zone, cellular

function also differs between zones. Once the cells are positioned in the matrix, they do not move nor do they interact with each other via cell-cell contact. Depending on age, chondrocytes may sometimes be organized in clusters, indicating that they originated from the same progenitors; however, adult chondrocytes rarely divide[7]. Chondrocytes interact with the pericellular ECM via cell surface proteins such as integrins, which are used to interact with fibronectin and collagens I, II and VI[13, 14], CD44 which interacts with hyaluronic acid, a component of aggrecans[14], and annexin V which binds to collagen II[6]. Through these receptors and others, chondrocytes can modulate ECM metabolism in response to mechanical, electrical and chemical changes[8].

Cells in cartilage receive their nourishment from either the synovial fluid or, to a lesser extent, the subchondral bone blood supply[6]. The nutrients in the synovial fluid are transported via diffusion through fenestrated capillaries or by active transport, as is the case of glucose, from blood vessels in the synovium[6]. Because the environment is hypoxic, chondrocytes metabolize glucose via the glycolic pathway to lactic acid[15]. Nutrients from synovial fluid enter the cartilage via diffusion or by mass transport during compressions[6, 16]. The metabolites exits the joint space either through the lymphatics or low molecular weight molecules may diffuse into the plasma[6].

2.4 Noncellular components of cartilage

2.4.1Water

Water constitutes 70 % of the weight of cartilage and is free to diffuse in and out of the porous tissue[6]. It originates from the synovial fluid and is drawn into the tissue by the negatively charged proteoglycans, which attract cations from the synovial fluid, causing water to follow.

The stiffness of collagen prevents too excessive water from being absorbed. The amount of water in the tissue influences the compressive stiffness of cartilage[7].

2.4.2 Collagen

Collagen constitutes the major component (66%) of cartilage's dry weight and gives cartilage its form[17]. Collagen acts to form a lattice to retain the aggrecan (see proteoglycans) molecules in the matrix[12]. There are 8 different types of collagen in cartilage, 90-95% of which being type II[7]. Collagen II fibers are thinner than those of collagen I because of the larger amount of hydroxylylsine, which facilitates crosslinking between fibers, and the presence of other collagens that help organize fibers[6]. The primary role of collagen II is to provide tensile strength to cartilage and it is also believed to play a role in compressive resistance.

Other minor collagens in cartilage include types III, VI, IX, X, XI, XII, and XIV. Collagen VI is located primarily in the pericellular region[17] and plays a role in cell attachment and interactions with other matrix components such as hyaluronan[6, 8]. Collagen IX is found exclusively in cartilage, primarily in the pericellular region[8]. Collagen IX is both a collagen and a proteoglycan, having a chondroitin sulfate binding domain, and this multifunctionality aids in enhancing the stability of the cartilage tissue[6]. Collagen XI, also exclusively found in cartilage, makes up about 5% of total collagen [18] and is buried in the fibrils of collagen II to regulate the fibril diameter[6, 8, 17]. Collagens XII and XIV are similar in structure to IX; they do not form fibers but are associated with collagen II and modulate fibril packing[6]. Collagen X is synthesized exclusively by the cells in the calcified zone[8, 17, 18]. Finally, Collagen III, a minor component is not a major component of cartilage, is found in association with Collagen II[17].

2.4.3 Proteoglycans

Proteoglycans make up about 25%-35% of the dry weight of cartilage[6, 7]. The major proteoglycan in cartilage is aggrecan, which consists of a core protein of 225-250 kDA with covalently attached glycosaminoglycans (GAGs), including chondroitin sulfate and keratan sulfate[6, 8]. Aggrecans have many negatively charged sulfates and carboxylates which attract positively charged ions into the ECM from the synovial fluid, creating a large osmotic pressure This facilitates the flow of water into cartilage[19, 20] and causes the tissue to swell, providing some resistance to compression. In addition, when cartilage is under compression and water and cations are expelled, the negative charges repel each other which also provides compressive resistence[7, 8, 21]. Because aggrecans impact water movement, they also impact nutrient uptake from the synovial fluid[16, 22]. The link protein connects aggrecan molecules to hyaluronan(HA) via noncovalent crosslinks forming the aggregates, which can contain up to 100 aggrecan molecules[6]. Aggrecans not only have a role in general tissue function but also function to facilitate cell-cell and cell-matrix interactions[20]. In addition to aggrecans, there are lots of nonaggregating proteoglycans, that usually function in development and play important roles in collagen fibril formation and stabilization of the ECM[6, 8]. These include decorin, biglycan, and fibromodulin.[8]

While not a proteoglycan, HA plays an important role in retaining aggrecans in the ECM[8]. HA is a polysaccharide of glucuronic acid and N-acetyl glucosamine and is synthesized, secreted and later metabolized by chondrocytes, especially those in the middle zone[8]. HA can transmit mechanical changes in the ECM to chondrocytes via the CD44 receptors on cell surface. [15] Finally, aggrecans bind to HA via the link protein forming large aggregates that help maintain water balance and prevent compression of the cartilage matrix.

2.4.4 Noncollagenous proteins

Noncollagenous proteins make up about 10-25% of the dry weight of cartilage[6, 7]. These molecules play an important role in helping to stabilize the matrix as well as facilitating cell-ECM interactions. Cartilage oligomeric matrix protein (COMP) is one such molecule comprising up to 10% of the dry weight of cartilage, and is located in the interterritorial regions of cartilage where it binds to collagen II and IX[6, 21]. Other noncollagenous proteins include cartilage intermediate layer protein (CILP), which plays a role in calcification[6, 21], anchorin II, which anchors chondrocytes to the collagen fibrils, Glycoprotein-39 which stimulates proliferation of chondrocytes and synoviocytes[6], osteopontin, which participates in calcium mineralization[21] and matrix Gla protein, which is associated with maintenance of the uncalcified matrix[21].

2.5 Chondral Defects

Blunt trauma, like falls, penetrating injuries (i.e. injections), and fractures caused by impact loading during joint motion can cause disruption with the cartilage matrix[7]. When cartilage damage occurs, there is little potential for regeneration because there is no direct blood supply, adult chondrocytes have limited proliferative abilities, and chondrocytes are immobile. In addition, injuries may also kill chondrocytes in the damaged area[7]. Because of the lack of blood vessels, there is no inflammation, hemorrhage, or blood clot formation. In some cases, inflammation occurs because cartilage fragments irritate other joint structure. In fact chondral or osteochondral defects are only identified if other joint structures are affected, as detected by pain, inflammation, and/or effusion. In studies looking at patients presenting with pain and requiring arthroscopy, 25-28% had lesions of some degree, with the median age of this population being

30[23, 24]. Many of those presenting were involved in sports and other physical activities. This indicates that this is a disease of the young and athletic, such patients would likely wish to return to their physical activity after an injury.

The initial response to cartilage injury (chondral defect or partial-thickness defect) is initiated by the chondrocytes surrounding the assault[7]. These cells proliferate, generate new chondrocytes, which attempt to repair the lesion[25]. However, this is not sufficient for reparation of the entire defect and within 48-72 hours[26] of injury, both proliferation and synthesis of new matrix have ceased[7, 12, 16]. If the defect is not repaired, repeat loading can cause propagation of defect and cause the cartilage tissue to become frayed. This would generate free cartilage fragments which in turn, may cause inflammation to other joint tissue, such as the synovium.

When an injury penetrates through the cartilage and into the bone, an osteochondral or full-thickness defect occurs. These injuries cause pain, hemorrhaging from blood vessels in the subchondral bone, fibrin clot formation, and active inflammation. Blood from the underlying bone fills the defect and forms a fibrin clot. Mesenchymal cells migrate into the clot, are transformed in to psuedochondrocytes and form new matrix called fibrocartilage[7, 12]. In a limited number of cases, the defect is repaired and a functional joint surface is formed; however in the majority of cases, the matrix synthesized is either inappropriate or the ECM components are synthesized in the wrong proportion, leading to inferior mechanical properties. The resulting lack of mechanical integrity leads to degradation of the ECM and significant cell loss, resulting in the exposure of the underlying bone[7, 16]. The ability of the fibrin clot to repair the defect may depend on the size of the defect, stability of the injury site and the age of the patient, with smaller defects and children demonstrating superior healing characteristics[7, 12, 16]. Therefore since neither defect can be repaired adequately by innate measures, medical intervention is necessary.

2.6 Treatment of chondral defects

2.6.1 Debridement and lavage

After the initial chondral defect has occurred, there is usually subsequent damage resulting from continual loading and/or degeneration of the cartilage. This damage first generates fibrillations which then may progress to the liberation of cartilage fragments from the surface thereby irritating innervated and vascularized tissue in the joint. This irritation leads to inflammation, effusion, pain and potentially more damage. Fibrillations may also cause changes in the mechanical function of cartilage causing locking or catching[16]. Many surgeons choose to shave the fibrils from the cartilage which is called debridement. Lavage involves the removal of any fragments from the joint arthroscopically by flushing the joint[12]. But the efficacy of debridement and/or lavage has not been confirmed and only 25% of patients show positive results[25]. In a randomized double-blinded (both evaluators and patients) prospective study, researchers found that neither debridement nor lavage of the knee had any advantages over placebo (needle insertion) in terms of pain management or joint function up to two years post procedure[27]. Subsequent studies that have followed have found similar results[28]. Some studies have demonstrated that debridement may actually lead to more fibrillation and cell necrosis[16, 25]. It has been suggested that the benefits of debridement are based on the severity of the patient's illness, with those who receive this intervention early in the disease process yielding a more favorable response[12, 29, 30].

2.6.2 Subchondral abrasion

Subchondral abrasion (drilling or microfracture) is similar to the natural events that occur in osteochondral defects, in which blood from intraosseous vessels is released into the defect, forming a fibrin clot in which mesenchymal cells differentiate into pseudochondrocytes, thus facilitating regeneration of fibrocartilage[7]. While this process relieves pain, it does not generate new cartilage and, depending on a number of factors, may itself degenerate and leave the subchondral bone exposed[16, 31].

2.6.3 Cartilage Grafts

In cartilage grafts, a piece(s) of healthy cartilage is harvested from a donor site and is fitted into the defect. These grafts can be autografts or allografts of cartilage or periosteal or perichondral grafts. Cartilage autografts are isolated from a donor location (usually non-weight bearing so as to minimize further damage)[32] in the same patient and fitted into the defect site. The use of tissue from an autologous source is immunologically preferable, however, there are limited number of locations from which these grafts can isolated[12, 16, 32]. In addition, in order to collect a graft cartilage, a defect is created in another location, thereby making it susceptible to degradation[31, 32]. While cartilage function and joint health is satisfactory as much as 18 years post operation[7], the limited number of sites available for harvesting cartilage do not support this treatment. In addition, it is difficult to procure cartilage grafts with the exact same surface topology[16]. This procedure is recommended for patients under 45 with small lesions and normal appearing adjacent cartilage[31, 33].

Cartilage allografts are healthy cartilage that is removed from a non-patient donor site, usually from a cadaver. This strategy is advantageous because there is no donor site morbidity[7],

and replacement grafts can be isolated from the exact same location, thereby resulting in a better match of the surface curvature[16]. These types of grafts raise concerns about the transmission of infectious agents, such as human immunodeficiency virus, HIV[16]. Cartilage allografts have been used in large joint reconstruction and small pieces have been used in the young and active patients[7]. The tissue can either be fresh, which is preferred, or cryopreserved, which minimizes immunogenicity[16] but compromises chondrocyte viability[12]. Usually allografts are osteochondral plugs, which will have blood that will interact with the recipient's blood[16]. This interaction can cause an immune response leading to inflammation. In smaller transplants, inflammation to the recipient cartilage is not extensive, while larger ones can cause even synovial inflammation[31, 32].

Since the use of autogenous products is preferable to the alternatives, periosteal or perichondral tissue has been used to fill chondral defects. Periosteum is dense connective tissue with osteoblastic progenitor cells that covers bones. Similarly, the perichondrium is a fibrous tissue, which contains chondroblasts, along the bone and cartilage borders. There is evidence that these materials produce cartilage with similar biochemical and viscoelastic properties to native cartilage[16, 34]. However, this benefit may be site specific and may depend on multiple variable factors- the age of the graft, location of the defect to be filled, motion of the joint and the age of the patient[7]. In addition, methods of fixation to the surrounding cartilage are difficult and may cause necrosis[34].

2.6.4 Autologous chondrocyte implant

In autologous chondrocyte transplant, two surgeries are conducted. The first surgery is conducted to harvest the cartilage from which the chondrocytes will be isolated and expanded *in*

vitro. During the second surgery, the chondrocytes are placed in the defect, which is subsequently covered with periosteal tissue. This technique can only be used when the bone is intact[32]. When used in the appropriate type of injury and patient, autologous chondrocyte implant has yielded symptom relief and cartilage regeneration[31]. However, recent studies have suggested that the outcomes of autologous chondrocyte implants are no better than those achieved by osteochondral grafts or abrasion[35-38].

2.7 Osteoarthritis

When the above treatments do not adequately heal the cartilage defects, secondary osteoarthritis (OA) can occur. Secondary OA is defined as cartilage degradation that occurs after another event such as trauma, congenital disorder, or acromegaly[26, 39]. Trauma to the joint may cause OA to develop rapidly or it may develop slowly with symptoms presenting later in life[26]. OA is characterized by cartilage erosion, subchondral sclerosis, and multiple biochemical changes in chondrocytes and synoviocytes, and it usually occurs in the hips, hands, knees, and spines of older individuals[26]. OA is a leading cause of disability and impaired quality of life. It is estimated that 6 million Americans suffer from posttraumatic OA, with a negative economic impact of about $13 billion per year[40].

OA can be described as an imbalance in anabolic and catabolic activities[41]. Early morphological changes in OA include altered distribution of proteoglycans, surface irregularities, formation of cleft which may become ulcerative and expose underlying bone, osteophyte (bony outgrowth) formation, and irregularly shaped fibrocartilage[26, 42]. One of the early changes is an increase in cartilage water content suggesting a weakened collagen II network, however it is unclear as to

whether this is caused by or leads to the loss of proteoglycans[42]. Later in the process, collagen I concentration increases and proteoglycan concentration decreases so much that in late stages of OA, there is virtually no proteoglycan remaining[26]. Metabolically, there is a greater degree of ECM degradation than synthesis, caused by increased production of matrix degrading enzymes. Matrix metalloproteases (MMPs) are responsible for degradation early in the process, with MMP-13 (collagenase III) being the largest contributor to the degradation of collagen II[26, 42]. The production of MMPs 3, 9, and 13 by chondrocytes are up regulated by IL-1 and TNF-α, inflammatory cytokines produced and secreted by synoviocytes and infiltrating leukocytes[26, 43]. Production of these MMP species leads to increased matrix degradation. In addition, IL-1 and TNF-α decrease proteoglycan and collagen synthesis[43], up regulate other pro-inflammatory cytokines, like IL-8, IL-6, and NO and prostaglandin E_2, and stimulate their own production[26]. It is also known that fragments of degraded ECM, such as fibronectin and collagen, can cause increases in the production of some MMPs, such as MMP-3 and -13, as well as pro-inflammatory cytokines[26, 44-46].

As these biochemical changes occur, more cartilage damage is done as the tissue loses the ability to function correctly. The compressive effects of joint motion are greater because of collagen II loss and reduced proteoglycan concentration in the matrix. In OA, synovial inflammation affects the synovial fluid. For example, HA of a lower molecular weight is produced by synoviocytes, which in turn decreases chondrocyte production of lubricating molecules, leading to less lubrication[26]. As cartilage damage progresses, more inflammation occurs, leading to the release of more pro-inflammatory cytokines, which in turn causes more

ECM loss. This cycle continues until, in extreme OA, there is no cartilage remaining and the subchondral bone remodels in order to support loading.

2.8 Treatment of OA

2.8.1 Non-pharmacological Interventions

Nonpharmacological approaches are used in the initial treatment of OA. These include patient education, weight loss, and exercise that strengthens the surrounding muscles. Weight loss and muscle strengthening can help reduce the inflammation and pain associated with OA.[47], Other treatments include temperature application of cold or heat, modified activities of daily life, using orthodics (such as braces and in soles) and alternative treatment strategies such as yoga or magnets.[47]

2.8.2 Pharmacological Interventions

Nonsteroidal anti-inflammatory drugs (NSAIDs) are the most widely used drugs for the pain and inflammation of OA. Ibuprofen, naproxen, aspirin, and cyclooxygenase type 2 (COX -2) inhibitors are all examples of NSAIDs. While these are effective at preventing inflammation, they are associated with systemic side effects. Nonspecific NSAIDs effects are associated with gastric ulcers and renal failure since they inhibit the production of beneficial prostaglandins in those organs..[48] COX-2 inhibitors were designed not to affect prostaglandin producing cells in the GI. However, it was recently discovered that COX -2 inhibitors may be associated with increased cardiovascular problems.[49] The use of COX-2 inhibitors is now determined on a case by case basis, however, the benefits they supplied to a wider population is no longer available.[50]

Corticosteroids are used when effusion and inflammation is not relieved by the use of NSAIDs[47]. This class of anti-inflammatory drugs act to decrease the production of IL-1, a potent pro-inflammatory cytokine, and hence stop all subsequent IL-1 actions, which include inhibition of matrix production and up regulation of matrix metalloproteinases.[51] Corticosteroids are usually administered via intra-articular injection into the diseased joint because of potential deleterious side effects, which include deleterious immunological effects, delayed healing and glucose intolerance[47, 52]. The course of injections and specific corticosteroid drug used is dependent on the physician's training, but no more than 4 injections are recommended because of the systemic side effects. The longevity of drug action is dependent on its solubility, with less soluble formulations acting longer, but typically, relief of symptoms lasts for 2-4 weeks per injection. It is still unclear whether the beneficial action of steroids is solely local or if they have systemic impact[52].

High molecular weight HA is found in the synovial fluid of healthy joints. During the OA process, the total mass of HA in the synovial fluid remains the same, but the molecular weight is reduced, causing effusion and a less viscous synovial fluid[53]. It was first proposed that this low molecular weight HA could be replaced with a higher molecular weight version via intra-articular injections in order to restore the viscosity of the synovial fluid[54-56]. This treatment, called viscosupplementation, has now become a standard procedure in the treatment of OA. When HA of 800 kDa or more is used in a series of 3 weekly injections, symptoms of OA are alleviated over the course of 6 months to 1 year[55, 57]. HA with lower molecular weight can also be used, but 5 injections are required to see a similar relief of symptoms[54]. HA has been shown to revert osteoarthritic chondrocyte and synoviocyte activity to normal levels, to include increased production of HA[44-46, 54, 55, 58]. In addition, it has anti-inflammatory effects in the joint,

decreasing IL-1 production[54, 58]. HA has also been shown to have anti-nocioceptive effects on nerves of the joint, resulting in reduced levels of pain.[55, 59, 60] Intra-articular injections result in rapid clearance of the HA via the lymphatics. In order to minimize this effect, Hylan GF-20 formulation contains small fragments of gel, resulting in a longer residence time in the joint.[53] However, this formulation has been associated with pseudosepsis, which is not fully understood but can be controlled by concomitant use of NSAIDS and corticosteroids[55].

Some other drugs used solely for pain treatment of OA include capsaicin, acetominophen and tramadol. Capsaicin, a compound isolated from jalapeno peppers, works by depleting and preventing the reaccumulation of pain neurotransmitters. It is applied topically but may take up to two weeks before pain relief is achieved[48]. Acetominophen can be used for mild to moderate OA[48] however, since it is metabolized by the liver, caution should be taken when given to individuals with compromised hepatic function. Tramadol is a synthetic analgesic that acts both as a mild opioid receptor agonist and prevents reuptake of serotonin in the CNS[47]. While tramadol does not promote addiction, it is only recommended when the usefulness of other pain relievers have been exhausted [48]. Narcotic analgesics, such as opoids, can be prescribed for severe cases of pain but the potential for addiction may out weigh their benefits[48].

2.8.3 Nutraceutical Intervention

Nutraceuticals are alternative treatments for OA. These include glucosamine, chondroitin sulfate, shark cartilage, avocado and cat claw[47]. These supplements have been demonstrated to relieve osteoarthritic symptoms. In particular, glucosamine and chondroitin sulfate (which contains glucosamine) relieved pain similar to NSAIDs and although pain relief occurred faster with the NSAIDs, the relief provided by glucosamine lasted longer[61]. Glucosamine may prevent

loss of cartilage and potentially regenerate ECM, which was observed during long-term trials where joint space narrowing was not evident following the use of glucosamine[61].

2.8.4 Joint Replacement

When all the above options have been exhausted, joint replacement is considered curative. Joint replacements have a life-time of 10-15 years and may require revision, especially in the young who receive this treatment and are likely to out live their implants[47].

2.9 Future treatment with tissue engineered matrices

Tissue engineering offers potential strategies for the repair of osteochondral or chondral defects. These approaches are not only motivated by the lack of innate cartilage regeneration, but also by the lack of available donor tissue, problems associated with allogenic donations, the inability to match the cartilage surface topology in chondral plugs, as well as differences in defect dimensions[62]. Research in this area focuses on the use of natural or synthetic matrices which can provide a nurturing environment to chondrocytes as they synthesize new ECM. It is anticipated that as the exogenous support matrix degrades, new matrix, similar to native cartilage ECM, will be synthesized and assume the shape of the scaffold[63, 64]. The ideal support matrix should be biodegradable, adhere to cartilage, be nontoxic, facilitate cell attachment, possess adequate mechanical strength, and permit mass transport, i.e. be a interim replicate of the lost cartilage ECM[65, 66]. The benefits of synthetic matrices include minimization of disease transmission and reproducibility of scaffold properties such as degradation rate, mechanical properties, and porosity[67]. Natural polymers provide cytocompatibility and bioactivity[68],

although the ability to modify synthetic polymers with bioactive peptides and molecules has extended these benefits to synthetic matrices. Scaffolds can either be preformed polymer scaffolds or a hydrogels, which can take the shape the defect.

2.9.1 Preformed polymer scaffolds

Solid scaffolds are formed before positioning it in the defect, must be molded to fit the defect and cells must be seeded after the scaffold is formed[64, 68]. These scaffolds include sponges, fibrous scaffolds, and meshes. Fibrous scaffolds include poly(α-hydroxy) esters, particularly polylactide (PLA) polyglycolide (PGA) or copolymers (PLGA) of the two. These polymers are used frequently because they are FDA approved and provide degradability via the ester hydrolysis[64]. Scaffolds of these polymers can be made by salt leaching or gel casting[69]. While these scaffolds have met with success both *in vivo* and *in vitro*, the polymers produce acidic compounds upon degradation which have been linked to toxicity and inflammation[70, 71]. Since these matrices are hydrophobic, they have been modified with bioactive molecules to promote cell growth[64]. Recently, nanofibers of PLA have been synthesized and have been shown to improve chondrocyte activity because their diameter is comparable to the natural ECM[64].

Natural fiber scaffolds have been used to culture chondrocytes. Collagen (both type I and II) scaffolds have had success both *in vivo* and *in vitro*[72-74]. The type of collagen matrix utilized may influence the cell properties and behavior. Collagen II has been demonstrated to provide a suitable environment for chondrocytes, while collagen I promotes dedifferentiation of chondrocytes to fibroblasts[75]. HA scaffolds (HYAFF), modified with benzyl alcohol esters, have

also been used to culture both mesenchymal and primary chondrocytes, both *in vivo* and *in vitro*[76, 77 78].

Recently, silk fibrions have been used to form scaffolds for cartilage regeneration[79]. Silk from silk worms (*Bombyx mori*) or other sources are of interest because the have good mechanical and thermal properties and the fibers can be formed under mild conditions[80]. Wang *et al.* found that chondrocytes were able to maintain their rounded phenotype in the silk scaffolds and produced the appropriate matrix[79]. Silk scaffolds have been used to culture mesenchymal stem cells which differentiated into chondrocytes with the appropriate media supplement[81, 82].

While solid scaffolds can provide mechanical strength, they have drawbacks. First, cells must be seeded on the scaffold, requiring an additional step *ex vivo*. Second, since defects have many different shapes, these matrices would demand that the surgeon physically modify the construct before implantation.

2.9.2 Hydrogels

Because cartilage is over 60% water and has viscoelastic properties, hydrogels have been used as matrices for cartilage regeneration[64]. Hydrogels are hydrophilic polymer networks that contain greater than 30% water by weight and can undergo large deformations[64, 66, 83]. Hydrogels entrap cells rather than promoting their adhesion which is beneficial for chondrocytes because it allows them to maintains their spherical shape[64]. Usually, the precursor gel solution is combined with cells and injected into the defect, which can be accomplished using minimally invasive procedures[66, 68]. The solution takes the shape of the defect and is then allowed to gel by a variety of mechanisms such as changes in heat, light, pH, or ionic concentration[68]. The materials used to

make the hydrogel is dependent on a number of factors, such as mechanical properties, degradability, mass transport and biological properties[66].

Hydrogels used for cartilage regeneration are formed through a number of gelation mechanisms. Alginate is a natural polymer that is crosslinked into a hydrogel via ionic interactions. It is a polysaccharide derived from brown seaweed which forms a hydrogel in the presence of divalent cations (except magnesium), which interacts with the negative carboxylic groups on guluronate, one of the monosaccharides in alginate[84]. Chondrocytes have been cultured in alginate, demonstrating that this matrix can maintain chondrocyte phenotype as the they produce new ECM[85, 86]. The composition of alginate can influence chondrocyte behavior; alginate containing a high level of guluronate can form more crosslinking thus improving the mechanical properties of the gel[87], while alginate with a high concentration of mannuronate promoted cell growth[88]. Alginate hydrogels have been shown to be an appropriate matrix for cartilage regeneration both *in vivo* and *in vitro*[89, 90]. One drawback of alginate hydrogels is its degradation by diffusion of the divalent cations from the polymer network and hence dissolution of the polymers and loss of mechanical integrity[83]. The dissolution of the gel is influenced by the external concentration of the crosslinking cation and can be unpredictable. LeRoux *et al.* demonstrated that this occurs when alginate is gelled and then transferred to a isotonic solution[91]. In order to make the degradation more predictable, alginate was partially oxidized which reduced the MW of the chain, allowing for faster predictable degradation[92].

Self-assembling peptides have also been used to form hydrogel scaffolds for chondrocyte culture. These hydrogels can be formed under physiological conditions and are considered mild forms of gel formation. Kisiday *et al.* demonstrated that these self-assembled peptide hydrogels

provided a favorable environment for chondrocytes[93, 94]. Since these hydrogels are made of peptides, caution must be taken when used *in vivo* due to possible immunogenicity[93].

While not a hydrogel, the elastin-like polypeptide (ELP) coacervate forms an aggregate above its critical temperature of 35 ^{o}C and this aggregate has mechanical properties similar to weak hydrogels. The ELP coacervate has been used to culture chondrocytes[95]. In order to improve the mechanical properties of the coacervate, glutamines and lysines were introduced into the ELP polymers and once the coacervate had formed, it was crosslinked enzymatically with tTG[96]. This led to a three-fold improvement in the mechanical properties of the coacervate.

There are many covalently crosslinked hydrogels that have been investigated for cartilage regeneration. PEG is used in many covalently cross linked hydrogels because it is hydrophilic and biocompatible[80]. Alginate-PEG have been crosslinked to form a hydrogel and has been utilized successfully to cultured chondrocytes[97]. PEG has been modified with acrylate groups and crosslinked with a cysteine-containing peptide[98]. PEG modified with acrylate groups has also been used successfully in a number of photopolymerizable hydrogels. PEG acrylate has been used as a copolymer with PLA in photopolymerized hydrogels in order for the gel to be degradable. In studies of these gels, it was determined that the higher the molecular weight of the PEG polymers yielded better cartilage regeneration[99, 100]. Mesenchymal stem cells (MSCs) were cultured in a photopolymerized PEG diacrylate hydrogel, containing TGF-β and HA, which led to the differentiation of the MSCs into chondrocytes[101]. Hwang *et al.* modified the PEG diacrylate with glucosamine and used it to form a gel, which improved chondrocyte viability[102].

Poly(N-isopropyl acrylamide) (PNIPAm)is a polymer that is photocrosslinked to form a hydrogel. However, its interesting property is that at its lower critical solution temperature

(LCST), the gel undergoes a swell-collapse transition. When the temperature is above the LCST, it is hydrophobic and excludes water (collapse) , while below the LCST, it is hydrophilic and absorbs water (swell)[80]. These gels can be cycled through this transition a number of times, making them thermoreversible. PNIPAm has been combined with other polymers to form matrices conducive to chondrocyte culture[103-106].

Fibrin hydrogels, which are similar to blood clots formed in subchondral abrasion, are enzymatically crosslinked by Factor XIII, the circulating member of the transglutaminase family. In short term experiments, chondrocytes have been successfully cultured in these gels.[107, 108] These hydrogels, however, have not met with great success in cartilage regeneration. Brittberg *et al.* used fibrin adhesive and found that it did not support cartilage regeneration nor integration with adjacent tissue[109]. Another problem associated with fibrin gels is the source of the components; bovine products used to form the gel can cause immunological reactions[80]. In addition, viral infections in isolated fibrin gel products (from human or otherwise) may not be detectable with current screening technologies.

2.9.3 Cell type

Many of the matrices used for cartilage regeneration can be used with a variety of cell types. The most obvious of these is primary chondrocytes which are programmed to produce cartilage ECM. However, sources of primary chondrocytes are limited since chondrocytes do not proliferate. In addition, the procurement of primary chondrocytes, either autologous or allogenic, would require the formation of another defect, making the cartilage at the donor site susceptible to degradation. In addition, when cultured in a monolayer in order to expand cell number, chondrocytes tend to de-differentionation[63, 110].

Adult mesenchymal stem cell is another more attractive cell source. These cells are obtained form the bone marrow and have the potential to become chondrocytes[63, 110]. There are also chondroprogenitor cells in the periosteum and perichondrium[65]. These cells can be expanded in vitro and then with the appropriate biochemical signals, such as bone morphogenetic protein (BMP-2) and fibroblastic growth factor (FGF-18), become chondrocytes[63, 111]. Finally, embryonic stem cells can be differentiated into chondrocytes. These cells have the capacity to differentiate into any cell type. However, there are ethical and social issues associated with their use[110].

Because there are a large number of factors in treating cartilage defects including defect size, cartilage health surrounding the defect, and defect depth, it may be difficult to adopt one therapeutic method for the repair of all defects. Understanding more about the biology of cartilage defects will help in designing appropriate scaffolds with the necessary growth factors and signaling molecules[62]. In addition hydrogels, while they can support cell growth and development, do not have sufficient adhesion to native tissue nor mechanical properties beneficial to cartilage[65]. Finally, although there are many different matrices for cartilage regeneration, no strategy has been able to demonstrate reliability and predictability in cartilage repair[65].

Chapter 3

Tissue surface modification

3.1 Introduction

Delivering therapeutic molecules via tissue surface modification can potentially play a significant role in modulating biological responses in the target environment. Tissue surfaces can be modified with molecules that would promote or inhibit cell adhesion, with drugs that will be delivered to the target tissue at high efficiency, as well as with tissue engineered scaffolds to enable tissue regeneration. However, many current tissue surface modification techniques have limitations including a paucity of chemical groups for reacting with the tissue surface, issues of tissue specificity, and potential toxicity. Developing a tissue surface modification approach that is versatile, non-toxic and works at physiologic conditions could enhance the existing state of the art. Here we examine different strategies that have been used to modify tissue surfaces, to include covalent coupling, ionic interactions, and mucoadhesion.

3.2 Covalent coupling

1-Ethyl-3-carbodiimide (EDC) chemistry has been used to crosslink molecules to tissue surfaces. Carbodiimides form an *O*-acylisourea active intermediate with carboxylic acids, which makes it more susceptible to the formation of an amide with a primary amine. EDC is preferable to other carbodiimides because both it and its urea byproduct are water soluble[112]. Because the active intermediate of EDC is short lived due to hydrolysis, N-hydroxy succinimide (NHS) is

usually used in conjunction with EDC to extend the life of the active ester[112]. This method was used to immobilize hyaluronic acid (HA) to the surface of tendons in order to minimize adhesions. In this method, the carboxylic acids of HA were activated by NHS and crosslinked to the amines in the collagen of the tendon[113]. This technique was also used to simultaneously form a hydrogel of HA and gelatin and to crosslink the hydrogel to the tendon surface to facilitate a reduction in adhesion. However, the reduction in adhesion observed *in vitro* was not replicated *in vivo*[114-116].

Because the EDC/NHS reaction is quite inefficient, requiring 20- 30 fold more EDC, NHS, and amine than the carboxylic acids to be modified[112, 117], some researchers first modify the carboxylic acid group with NHS by other methods, i.e. using a different carbodiimide in organic solvents, followed by purification prior to modifying with the amine. This approach was used to modify islets to render them immunoprotected. First, PEG molecules were made amine reactive with NHS in a separate reaction and then reacted with the amines in proteins on islets' surfaces[118, 119]. Citric acid was also modified in this manner and then used as a crosslinking agent between collagen and porcine soft tissue[120, 121]. A conjugate molecule consisting of NHS-PEG-Biotin was used to modify arteries *in vitro*, where the biotin was used a target for NeutrAvidin microspheres. This was used as a model to demonstrate how drugs, DNA, cells and other important therapeutic agents could be targeted to modified surfaces[122]. While EDC has the advantage of being water soluble, it has been shown to be cytotoxic in some cases. In hydrogels crosslinked with EDC, hepatocytes cultured therein had a significant amount of cell death[123], and similar results were seen with fibroblasts cultured in gelatin hydrogels crosslinked with EDC[124].

Polymers containing cyanate groups- isocyanates, diisocyanates, and cyanuric acid- are used for crosslinking reactions with amines in proteins on tissue surfaces. PEG isocyanate was used to modify de-endothelialyzed arteries in order to reduce platelet adhesion[125, 126] as well as to modify islets for immunoprotection[119, 127]. PEG diisocyanate was used to modify collagen and preclotted Dacron to reduce platelet adhesion, thus reducing thrombosis[128]. In another study, PEG was coupled to the surface of sheep red blood cells with cyanuric acid. These PEG-modified cells were injected into mice and were able to evade the immune system of the animal[129]. One limitation of cyanate reactions is hydrolysis in aqueous buffer solutions; therefore, when used to modify surfaces, cyanates must be delivered rapidly[130].

Another method of covalent coupling to tissue is via cis diols in polysaccharides using phenylboronic acid. A poly-L-lysine (PLL) polymer was grafted with PEG and phenylboronic acid. This modified PLL was able to adhere to both cell and tissue surfaces via ionic and covalent interactions. The cationic PLL polymer facilitated the ionic interactions with the tissue while the phenylboronic acid moiety provided covalent crosslinks to the polysaccharides in the tissue or on the cell surface, while the PEG in this construct served to provide immunoprotection. While this polymer system worked well in immunoprotecting red blood cells, it did not function in preventing peritoneal adhesion[131, 132]; the failure was attributed to polysaccharide variability in different tissues[131].

Compounds containing aldehydes can also be used for crosslinking to amines in tissue, resulting in the formation of imines. This chemical strategy was used with chondroitin sulfate, modified chemically to contain both aldehyde and methacrylate groups, to serve as a tissue adhesive. In one study, this modified chondroitin sulfate was used to form an adhesive with polyvinyl alcohol covinylamine (PVA-A) for corneal repair. In this application, the aldehyde

groups in the modified chondroitin sulfate were used to crosslink to amines in the target tissue as well as to amines in the PVA-A[133]. In other studies, the aldehyde groups were used to crosslink to the target tissue, while the methacrylate groups were used to crosslink with a acrylate photopolymerizable hydrogel[134, 135]. This dual functionality of the modified chondroitin sulfate allowed the hydrogel to be attached to the tissue surface. Finally, a gelatin hydrogel was crosslinked to amines in tissue using glutaraldehyde, demonstrating that this hydrogel system can function as a tissue adhesive[136-138]. Stability of imines is a limitation of the aldehyde coupling strategy. Imines are generally not stable unless they are precipitated or water is removed[139]. Therefore, environmental factors such as pH and temperature *in vivo* can cause a cleavage of the imine bond[140].

Another approach used to crosslink molecules to the targeted tissue is the through oxidation of certain chemical moieties. In tissue initiated photopolymerization, the tyrosines in collagen were oxidized by hydrogen peroxide to form radicals and then a photopolymerizable polymer (2-hydroxy-ethyl methacrylate) in a precursor gel solution was introduced to the area. This solution was then crosslinked to form a gel which was also crosslinked to the tissue[141].

Fibrin sealants are stabilized by forming crosslinks between fibrin molecules using Factor XIII, a circulating transglutaminase which forms crosslinks between lysines and glutamines in substrates of the enzyme. It is through that Factor XIII forms covalent crosslinks between the fibrin in the sealant and proteins in tissue, i.e. fibronectin, when used as an adhesive[142]. Fibrin glues have been used to attach both collagen scaffolds[143] and periosteum[144] to osteochondral defects and also has been used to form covalently attached barriers on the dura mater, preventing bone morphogenetic protein (BMP) from entering the tissue after eluting from a BMP-loaded collagen sponge[145]. Other forms of transglutaminase have also been used as adhesives; gelatin

and microbial transglutaminase were combined to form an adhesive[146, 147], and tissue transglutaminase has been used as an adhesive to bond two pieces of cartilage[4] and to bond a PEG polymer modified with peptide substrates of tTG to guinea pig skin[148].

3.3 Ionic interactions

Because tissues and cells have negatively charged molecules on their surfaces, it is possible to adsorb positively charged molecules to the surface. These cationic molecules can be modified with therapeutic molecules to promote or minimize specific interactions. This was accomplished with PLL polymer modified with PEG molecules which was then adsorbed to tissue. The presence of PEG made the tissue impervious to protein deposition, thus preventing post surgical adhesions in a model of abdominopelvic surgery model in mice[149]. A similar strategy was also used to stabilize red blood cells[150].

The layer–by-layer (LbL) approach involves the layering of polyelectrolytes with opposite charges on a surface in alternating fashion. Some benefits of LbL include rapidity of layer formation and attachment of drugs or bioactive molecules to the polymers[151]. In addition, most of the polymers used in this strategy are water soluble. The LbL approach was used to modify a gelatin adsorbed layer, which is representative of a collagen ECM, with alternating layers of polylysine and alginate, making it bioinert[149]. This same strategy was used to provide immunoprotection to islets[152] and to reduce platelet interaction with the blood vessel wall using a LbL of HA and chitosan[153]. To explore the ability to control platelet behavior, a LbL of cationic poly(dimethyldiallylammonium chloride) and anionic poly(styrene sulfonate) were successfully assembled on platelets. In addition, the outmost layer was modified with IgG and targeted with

an anti-IgG antibody. The authors suggest that this method can be used to control the platelet microenvironment and prevent or promote platelet interaction with each other as well as with the blood vessel wall[154]. In an effort to provide a microenvironment that facilitates stem cell development, stem cells were coated with a LbL of PLL and HA and were successfully cultured[155]. While changes in pH may not completely dissolve electrostatically assembled LbL structures, it can affect their interaction with each other and their internal organization, potentially making the structures more or less permeable depending on the environmental pH[149, 156]. New strategies for LbL assembly, such as forming covalent bonds and hydrogen bonding, may provide the means to improve the stability of these structures[157].

3.4 Mucoadhesions

Mucoadhesion occurs when molecules are adsorbed to a mucus layer, i.e. the mucus membrane, including buccal, stomach, intestinal, and vaginal mucosae. This method is considered a favorable method of drug delivery because it localizes delivery, leading to increased residence time. It may be possible to target specific tissues with appropriate mucoadhesion molecules, i.e. lectins to fucose, with this method. [158, 159] Some examples of mucoadhesive polymers are polycyanoacrylate, carboxymethyl cellulose, and chitosan[158, 160]. Mucoadhesion occurs partially by electrostatic interactions with negatively charged mucin. However, in some cases, hydrophobic interactions, Van der Waal forces, hydrogen bonding, molecule diffusion, and entanglement may play a role in adhesion[159, 160]. In addition, mucoadhesion can be affected by pH and ionic concentration, molecular weight of the polymer, and polymer flexibility[159, 160].

While mucoadhesion does not usually involve the formation of covalent bonds, some thiomers are now being synthesized so that on adhesion, disulfide bonds can be formed[160, 161].

3.5 Conclusion

Tissue surface modifications can be used to inhibit interactions between cells and tissue, to deliver therapeutic agents, and to potentially attract other molecules and cells to the targeted tissue. There are many surface modification strategies that utilize either covalent bonds or ionic interactions as a means of crosslinking the molecule(s) of interest to the tissue. However, many of these methods can potentially be adversely affected by environmental conditions such as pH and ionic concentration. These limitations and others, such as potential toxicity, make it desirable to develop a superior method of tissue surface modification. Ideally, this method would be biological, nontoxic and active under physiologic conditions. Tissue transglutaminase is potentially a superior candidate for tissue surface modification because of its wide distribution and large number of available tissue substrates in a variety of tissue types[162, 163]. In addition, the crosslinks formed by tTG are very stable, being resistant to chemical and enzymatic degradation[3].

Chapter 4

Tissue Transglutaminase

4.1 Introduction

Tissue transglutaminase (tTG) is a member of the transglutaminase (EC 2.3.2.13) family of enzymes that catalyze the crosslinking of the side chain of glutamine residues (acyl donors) to the side chain of lysine residues (amine donors) to form a γ-glutaminyl-ε-lysine isopeptide bond in a calcium-dependent manner (Figure 1)[2]. Transglutaminases are found in a variety of organisms to include mammals[2], worms[164, 165], soybean[166], fish[167], and microbes[168]. While there are over 8 different types of transglutaminase in humans, tTG is the most ubiquitous member occurring both intra- and extracellularly[3, 169] and in almost every tissue. Although tTG has some signal transduction activity as a GTPase[170], and can actually deamidate glutamines when sufficient amines are not present[171], it is most recognized for its ability to form the isopeptide bond.

4.2 Enzyme action

Three events must occur sequentially in order for tTG to form the isopeptide bond: 1) Calcium must bind in its pocket; 2) The glutamine substrate must enter the catalytic site; and the 3) Lysine enters the catalytic site. In order for the enzyme to become receptive to the glutamine substrate, calcium first must bind in its region of the enzyme[3, 172], inducing conformational changes to the enzyme which permits the glutamine substrate binding to the catalytic center[3, 173].

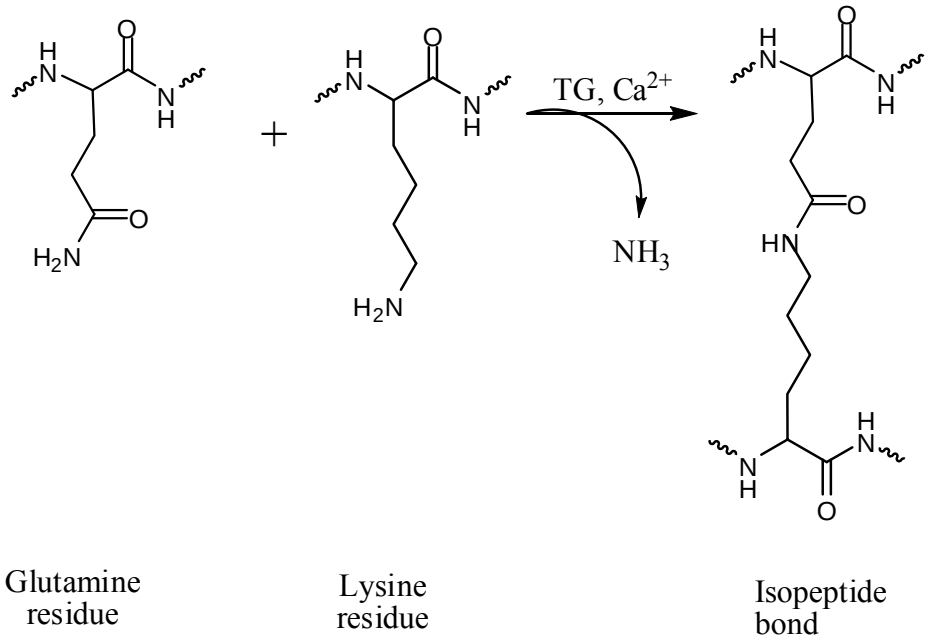


Figure 4-1. Transglutaminases catalyze the crosslinking of proteins and peptides to form isopeptide bonds via lysines and glutamines and require calcium for action.

The binding of Ca^{2+} to tTG is modulated by the binding of GTP in a different location on the enzyme, causing an increase intramolecular interactions in the enzyme which interferes with the conformational changes induced by Ca^{2+3}.

After Ca^{2+} binds to tTG, the glutamine substrate is allowed to bind. The binding of the glutamine substrate forces another conformational change in the enzyme, thus opening the catalytic center[167, 173]. Only glutamines in peptides or proteins are reactive, indicating the importance of residues adjacent to glutamine in interactions with the enzyme. Liver tTG has secondary interaction with the glutamine-bound peptide, extending at least 4-5 residues away from the reactive glutamine of the substrate[173]. Once the substrate is bound, the carboxamide of the reactive glutamine enters the catalytic center and covalently reacts with a cysteine residue (Cys^{277}) to form a thioester[2]. The remainder of the active site is hydrophobic which is believed to provide an environment for the methylene groups alpha and beta to the carboxamide. Because the reactive site is physically constrained, the alpha and beta methylene groups cannot be substituted[173]. When D-glutamine is included in an peptide that otherwise contains only L isomers of the other amino acids, the peptide will still bind, however; there is no reaction because of the incorrect orientation of the carboxamide side chain[173].

The secondary interaction of the glutamine peptide with the enzyme may impact the interaction of the enzyme with the lysine macromolecular substrates because the amine does not bind until the thioester intermediate has formed[173]. The secondary interaction with the lysine substrates may be limited to the amino acids directly adjacent to the active lysine[173]. However, while these secondary interactions provided by adjacent residues may improve substrate binding they are not mandatory as with the glutamine substrates because primary amines, such as cadaverine, spermine, putresine, hydroxyamine and ethanolamine can also function as

substrates[174]. As with the carboxamide, the methylene groups close to the reactive amine cannot be branched because of the structural constraints of the reactive site[173].

4.3 Glutamine substrate specificity

Specific requirements for tTG reactive glutamine and lysine substrates are not fully understood, although it is believed that the enzyme has more stringent requirements for the glutamine (acyl donor) than for the lysine (amine donor)[173]. For the glutamine substrate, it has been suggested that a Leu residue flanking the C-terminus of a glutamine increases reactivity[162]. However, recent combinatorial library studies suggest that the amino acid directly adjacent to the reactive glutamine is not as important as amino acids 2-3 residues away on the C-terminus[163, 175]. In particular, nonpolar amino acids such as Pro and Phe and polar amino acids (Tyr, Met, and Ser) have demonstrated positive impact on substrate reactivity in those positions[162, 176, 177]. Basic amino acids are rarely identified as advantageous to reactivity in any position close to Gln; however, His[177, 178] as well as Arg [162], have been identified adjacent to the reactive glutamines[179]. Studies have also demonstrated that an increase in the number of glutamines increases the tTG reaction with the amine donor and in such substrates, basic amino acids, flanking the reactive Gln, may increase the reactivity by increasing solvation[179, 180]. A number of studies have demonstrated that the amino acids on the N-terminus are not important in determining reactivity[163, 175, 181]. While some general trends about the substrate have been observed, research to understand the acyl donor continues. For example it is known that substrates with the sequence of QXP react well with tTG, while small positional shifts of the proline residue, such as QP or QXXP sequences, negatively impacts substrate reactivity[182]. Finally, the use of peptides

increases the rate of the transamidation reaction; however, there are potentially quaternary interactions between tTG and full length proteins that facilitate (or inhibit) binding. For example, Gorman and Folk demonstrated that a pentadecapeptide of β-casein had a higher reaction rate than the full protein, however, the binding of the protein was superior to that of the peptide[183].

4.4 Lysine substrate specificity

Traditionally, the lysine substrate has been viewed as requiring less specificity when compared to the glutamine substrate because it binds after the glutamine. tTG has been found to react with a wide variety of primary amines as well as peptide/protein bound lysines[3, 173]. The amine donor utilized is a function of the glutamine used since the thioester intermediate must be formed before the lysine is permitted to bind[173]. Nevertheless, certain peptide features do enhance lysine reactivity. One such feature is the presence of a C-terminal adjacent glycine or valine[184] or an N-terminal hydrophobic residue juxtaposed to lysine[5, 173] Groenen, *et al.*, studied short synthetic lysine peptides as substrates of tTG and found that N-terminally adjacent Arg and Ser residues increased lysine reactivity, while Pro and Gly had deleterious effects[184]. Another study discovered that in the N-terminal adjacent position to the reactive Lys, the presence of Val, Arg, Phe, Ser, and Ala produced the optimum reactivity, while Asp, Pro, His, Gly, Trp, Tyr and Asn did not[185].

4.5 Biological Effects of tTG

4.5.1 Cellular

Eighty percent of the tTG in the body is found intracellularly with the remainder being in cell membranes or excreted[2] via an unknown mechanism[170]. Intracellularly, tTG has two opposing roles: it acts to hydrolyze GTP in signal transduction, which inhibits its secondary function of transamidation of proteins and peptides[2, 3, 170]. The function that the enzyme assumes depends on the amount of GTP and Ca^{2+} available. For example in apoptosis, when there is an increase in intracellular calcium, tTG may become active; isopeptide bonds have been demonstrated to be elevated in apoptotic bodies[2].

Transglutaminase on the cell surface acts as a coreceptor of integrins, coordinating binding of ECM proteins. For instance, in the case of fibronectin, tTG binds to the gelatin binding domain of fibronectin to hold it in place for integrin interactions[170]. In addition, it also crosslinks the fibronectin (as well as other ECM proteins), to improve cell adhesion and to organize the matrix around the cell[186]. tTG has been found to affect cell motility by causing more GTP to bind to the integrin receptors intracellularly, which decreases adhesion[170]. Increased transglutaminase activity has also been associated with organogenesis, to include heart, lung, and skeletal tissue[186].

4.5.2 Extracellular

Outside of the cell, tTG functions to stabilize the ECM by forming large polymeric structures that are resistant to proteolytic/chemical degradation and mechanical stresses[170, 187, 188]. It has been suggested that tTG is excreted from the cell in the GTP bound form[189]. Some proteins

involved in forming these large structures include fibronectin, collagens, osteonectin, osteopontin,[190] and vitronectin. Other proteins may also participate but are hard to identify since the crosslinked ECM are usually high molecular weight and insoluble, and as such the structure is so complicated that the proteins can not be identified[3]. In addition to crosslinking the matrix, tTG also plays a role in the immobilization of latent transforming growth factor beta (TGF-β) complex to the ECM from which active TGF-β is released[191]. The enzyme is very active when matrix remodeling is occurring, as during organogenesis, wound healing and maturation[192].

4.5.3 Cartilage

In cartilage, tTG activity and tTG itself have been localized to the pericellular region of chondrocytes[193-195]. In developing mammals, tTG activity can be detected throughout the full thickness of cartilage but seems to be highest extracellularly in the lower hypertrophic zone and seem to parallel osteonectin expression and cartilage mineralization[192, 194, 195]. The extracellular content of tTG was inversely related to the intracellular amounts with intracellular amounts being highest in the proliferative zone[194, 196]. In human adults, Summey *et al.* found that tTG extracellular content was greatest in the superficial zone. tTG was also localized intracellularly but its distribution was the same in all zones of cartilage[193]. In osteoarthritis (OA), tTG expression is up regulated at the surface, with an increase in isopeptide bond formation in the pericellular region[197], and can be induced by IL-1[198]. Transglutaminase activity is increased in aging cartilage, not for remodeling but for permanent matrix organization and may lead to the pathogenesis of calcium pyrophosphate dehydrate athropathy[197, 199].

Tissue transglutaminase is believed to affect many proteins extracellularly. Bowness and Tarr discovered that most tTG isopeoptide bonds occurred in the noncollagenous protein and collagen fractions of cartilage but not in the proteoglycan fractions[200]. Aeschlimann *et al*. identified osteonectin and collagen II as acyl donors in transglutaminase crosslinking[194]. tTG has been demonstrated to crosslink osteopontin, which increases its adhesion to collagen I. The increased adhesion is believed to be due to either the actual crosslinking of the polymerized osteopontin to collagen or perhaps the crosslinking exposes epitopes previously hidden which allows for improved noncovalent binding to collagen better[201]. Since it is found extracellularly in areas of mineralization, it is believed that cross-linking of calcification proteins may provide a nucleation site for mineralization[186]. Jurgensen *et al.* demonstrated that tTG can be used to adhere cartilage surfaces[4]. Fibronectin was found to be the protein acted upon by membrane-bound tTG in chondrocytes cultured in a monolayer[202].

4.5.4 Other Biological Effects

Tissue transglutaminase activity has been suspected in some neurodegenerative diseases that involve CAG expanding repeats such as Huntington's disease[203]. The associated proteins form aggregates in the cytoplasm and nucleus of the neurons, which have increased amounts of tTG produced. It has been suggested that tTG inactivates other proteins by crosslinking them to these insoluble aggregates[180]. tTG has also been implicated in other neurological disorders such as Alzheimer's[203, 204].

Celiac Sprue (celiac disease or gluten enteropathy) is the chronic disease of maladsorption of nutrients in the small intestines which is associated with the gliadin protein of

gluten which contains 32-56% glutamines[205]. In this disease, both IgA and IgG antibodies to tTG[187] are present but it is unclear what exact role these antibodies play because there is an increased production of tTG and their presence does not inhibit tTG activity[205]. tTG is believed to deamidate glutamines in the gliadin proteins, thereby making them more immunogenic[206]. Deamidation of glutamines have similar substrate requirements as the glutamine used in tramsamidation[176]. Fleckenstein demonstrated that at pH of 6, deamidation would occur faster than at pH 8 when in competition with transamidation[177]. In addition, deamidation occurred even faster when a primary amine was not included[177, 207]. Also, the tTG can react with the gliadin protein forming complexes that may be immunoreactive as well as crosslinking of gliadin protein to ECM components may make its clearance difficult[205].

Tissue transglutaminase is associated many other events in the human body. It is responsible for crosslinking phospholipase A_2, an enzyme in inflammation[191, 208]. tTG is implicated in autoimmune disease such as rheumatoid arthritis, lupus erythematosus, Type I diabetes but its role is not fully understood[191]. It is also involved in early fibrosis of liver and kidney that follows inflammation, crosslinking matrix component. This fibrosis could be due to tumor necrosis factor alpha (TNF-α) up regulation of tTG and its later export to ECM[191]. tTG has also been found in atherosclerotic plaques as well as fibro-lipid plaques of aortas[209].

4.6 Uses in Tissue Engineering

The use of tTG and other transglutaminases is becoming more prevalent in tissue engineering. Fibrin sealant is a mixture of components involved in the blood clotting that reproduces the last steps in the process, namely the conversion of fibrinogen to fibrin by

thrombin, the formation of loose fibrin clot, and finally the crosslinking of the fibrin via thrombin- and Ca^{2+}- activated Factor XIII, a transglutaminase[142, 210]. Fibrin sealants are used as post surgical closures, promoting wound stabilization and assisting in healing[210].

Hydrogels crosslinked by the members of this transglutaminase family are gaining greater use. The use of enzymes to assist in the formation of hydrogels is attractive because enzymes are active at physiologic conditions[211]. Fibrin gels formed by Factor XIII have been used for neurite extension[212-214], cardiovascular tissue regeneration[215] and to promote angiogenesis[216, 217]. Fibrin gels have also been used to culture chondrocytes[218] and create matrices for bone repair[219]. Sanborn *et al.* modified PEG with short synthetic peptide substrates of Factor XIII and formed a hydrogel[220].

Hydrogels formed by tTG has not been as widely evaluated as those of Factor XIII. Sperinde *et al.* formed hydrogels through the crosslinking of glutamine modified poly(ethylene glycol) (PEG) polymers and a lysine containing polyaminoacid[221]. Hu *et al.* subsequently employed rationally designed peptide substrates of tTG to modify PEG polymers to form an adhesive hydrogel[5, 148]. Taking advantage of the activity of tTG in forming more stable crosslinks, it was recently used to crosslink an elastin-like polymer (ELP) coacervate that contained lysine and glutamine residues which led to improved mechanical properties over the ELP alone[96].

Microbial transglutaminase crosslinked hydrogels are being explored more because microbial transglutaminase does not require Ca^{2+} and because it can be more economically produced than other forms of transglutaminase sources[188]. It has been used extensively in the food industry for preservation of food and as a thickener in some dairy products[168]. Microbial TG has been used to crosslink gelatin into an adhesive gel with properties similar to fibrin

sealants[147], and it has been evaluated as a sealant for retinal reattachment[146]. Microbial TG was also used with a modified HA and gelatin to form a hydrogel[222].

Finally, there have been additional applications of transglutaminases in bioengineering. Factor XIII has been used to couple biomolecules to fibrin matrices to provide cells with nutrients and/or signal molecules and peptides for infiltration[212, 216, 223]. tTG has been used to modify the surface of an insoluble matrix with RGD in order to promote cell migration[224] and was used to enhance cell adhesion and spreading on collagen matrices and synthetic polymers coated with fibronectin[225, 226]. Orr *et al.* used fibrin sealant to secure periosteal tissue to an osteochondral defect[227]. Ting and Lin introduced a unique way to detect cells expressing a protein of interest. They first genetically modified a cell surface protein with a short glutamine peptide substrate of tTG, then to detect the expression of this glutamine modified protein, they used tTG to crosslink an fluorescent amine donor molecule to cell surfaces expressing it[228].

4.7 Conclusions

Tissue transglutaminase, with its ability to crosslink glutamines and lysines in proteins and small peptides under physiologic conditions, is an enzyme of potential utility in bioengineering applications. Tissue transglutaminase peptide substrates can be used to modify synthetic polymers and these modified polymers can be crosslinked into hydrogels or more complex structures. Because tTG has many natural substrates that are part of the ECM, such as collagen and fibronectin, these biomolecules can be easily included in with modified synthetic polymers to promote cell-matrix interactions. Since effective peptide substrates of tTG can be short, i.e. 3 to 6 residues, as demonstrated by Hu and Messersmith[5], they offer a facile method for modifying

biomolecules as well as other compounds of biological interest. These modified molecules can then be coupled to either modified synthetic polymers or biological tissue directly. This presents a unique opportunity and great potential for introduction and immobilizing therapeutic agents to localized regions of interest in the body.

Chapter 5

Alginate-PEG hydrogel cross-linked with tTG for in vitro culture of chondrocytes

5.1 Introduction

Chondral defects are created by traumatic events to cartilage, such as falls or sporting injuries. However, chondrocytes are generally unable to sufficiently replace the damaged cartilage[7, 26]. In addition, since cartilage is avascular, it lacks the ability to readily receive nutrients from the blood supply that may assist with wound healing. Oftentimes, these defects go undetected, leading to further cartilage damage and degeneration, eventually progressing to OA[26]. When the defect is detected, because of pain due to the irritation on other joint structures, medical intervention is required. However, current clinical strategies, such as debridement and lavage[25], autologous chondrocyte implant[35-38], or osteochondral plug transplant [31, 32], are only successful with a subset of patients. Moreover, some strategies require that further damage be inflicted, as with osteochondral plugs that require puncturing of the subchondral bone in order to release blood and cells which may promote adhesion and integration with the native cartilage tissue[7].

Alginate hydrogels have been demonstrated to be a useful biomaterial for cartilage regeneration, both *in vitro*[85, 86] and *in vivo*[229]. Alginate, a polysaccharide isolated from algae, is a

block copolymer composed of two monosaccharide, mannuronate (M) and guluronate (G), each having carboxyl groups. The quantity and sequence of the monomers in the polymer are dependent on the species of algae and the environmental conditions in which the algae grows[230, 231]. Traditionally, alginate is gelled with Ca^{2+}, which forms crosslinks between proximal contiguous guluronate segments within and between polymer chains[230]. However, since alginate gelation occurs via ionic crosslinking, its physical properties are not predictable or controllable in biological aqueous environments because of ion exchange of Ca^{2+} for Na^{+}[91].

There have been many efforts to improve the stability of alginate hydrogels using covalent crosslinks and other interactions. In some cases, these strategies are used in conjunction with the traditional calcium crosslinking[232]. First, EDC/NHS chemistry has been used to activate the carboxyl groups on alginate which are then reacted with various polyamines to crosslink the alginate[232-234]. Lee *et al.* demonstrated that the properties of alginate hydrogels formed by crosslinking with various polyamines depended on the type and concentration of amine used[234]. Another strategy that has been used is the oxidation of the monosaccharide rings with periodate to generate aldehydes which are then covalently crosslinked to polyamines[232, 235]. In addition, alginate has been modified with methacrylate groups, allowing hydrogel formation through photocrosslinking[236]. Studies by De Boisseson *et al.* of alginate modified with hydrophobic molecules revealed that hydrogels formed by hydrophobic interactions and calcium ions were more stable than those crosslinked by calcium alone[237]. Finally, barium has been used to replace calcium in the ionic hydrogel system to improve hydrogel stability in aqueous solutions[231, 238].

In this study, tissue transglutaminase (tTG) was used to covalently crosslink a PEG-alginate hydrogel in order to produce a hydrogel with improved stability. Tissue transglutaminase is an calcium-dependent enzyme that forms covalent crosslinks between lysine

and glutamine residues in peptides and proteins to form isopeptide bonds[2]. This mechanism has been used to form hydrogels with tTG[221, 239] and Factor XIII[220, 240], another member of the transglutaminase family. Transglutaminases have also been used to modify tissue engineered scaffolds with biologically active molecules[224, 241, 242]. In addition to covalent crosslinking of the hydrogel, tTG has the potential to also crosslink the gel to the native cartilage matrix. Jurgensen *et al.* demonstrated that tTG could be used as an adhesive to form cartilage-cartilage bonds[4]. It was hypothesized that tTG crosslinked the native substrates of the enzyme that are in cartilage[200, 243, 244], such as collagen II[196, 244] and fibronectin[245], creating adhesive bonds. Recently in our lab, we have developed rationally designed short synthetic peptide substrates of tTG, AcGQQQLG and Dopa-FKG, which were used to modify PEG polymers, which were then crosslinked into a hydrogel[5]. For this study, the glutamine peptide substrate was used to modify a linear PEG while the lysine peptide substrate used, Phe-Phe-Lys-Gly-Cys-NH_2, was slightly modified from the original peptide to enable it to be conjugated to alginate. Phenylalanine (Phe) was substituted for the 3,4-dihydroxy-L-phenylalanine (Dopa) and the cysteine was added to provide a motif for crosslinking to alginate. Here we describe the synthesis of these polymers and their enzymatic crosslinking by tTG into hydrogels. Figure 5-1 is a schematic of the anticipated crosslinks that would occur in such a hydrogel. It is hypothesized that there will be both covalent crosslinks formed by tTG and ionic crosslinking between Ca^{2+} and alginate. These hydrogels were then evaluated for their ability to serve as cartilage regeneration matrices for chondrocytes.

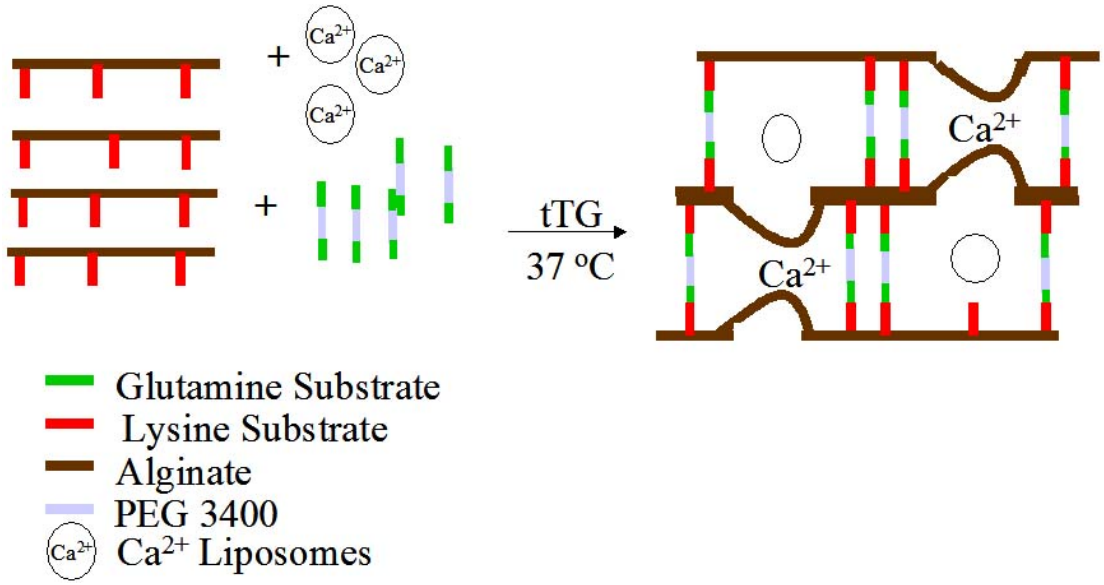


Figure 5-1. Illustration of the PEG-alginate hydrogel formed. The modified polymers, liposome-entrapped Ca^{2+}, and tTG were used to form the hydrogel. The liposomes were designed to release Ca^{2+} at 37 °C. Calcium is required for tTG activation, and can also be utilized to form crosslinks between alginate polymers. Therefore, we expected both the formation of the isopeptide bond of tTG and Ca^{2+} cross-linking of alginate.

5.2 Materials and Methods

5.2.1 Materials

Gly-2-ClTrt-Resin was purchased from Peptides International, Louisville, KY. All Fmoc-Amino acids, Rink-amide resin, and pronase were purchased from EMD Bioscience, San Diego, CA. Alginate was purchased from Pronova, Oslo, Norway. PEG diamine ($\bar{M}_w$ = 3.4 kDa) was purchased from Nektar Inc, Birmingham, AL. Bovine metacarpophalangeal joints were purchased from a local slaughter house. Tissue transglutaminase was purchased from Sigma, St. Louis, MO. DMEM/F-12 media and the Live/Dead cell viability/cytotoxicity stain kit were purchased from Invitrogen, Carlsbad, CA. Collagenase-P was purchased from Worthington Biochemical Co., Lakewood, NJ. Finally, 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC), N-hydroxysulfosuccinimide(Sulfo-NHS) and [N-e-maleimidocaproic acid] hydrazide (EMCH) were purchased from Pierce, Rockford, IL. Fetal bovine serum (FBS) was purchased from HyClone, Logan, UT. 1,2 dipalmitoyl-*SN*-glycero-3-phosphatidylcholine (DPPC) to 1,2 dimystiroyl-*SN*-glycero-3-phophatidylcholine (DMPC) were purchase from Avanti Polar Lipids, Alabaster, AL.

5.2.2 Methods

5.2.2.1 Synthesis of FFKGC-NH_2 and modification of alginate.

FFKGC-NH_2 peptide was synthesized on Rink-Am resin (1 g, 0.46 mmol/g) using standard Fmoc solid phase peptide synthesis. Each coupling reaction was performed for 3 hours with a 10 min pre-activation of four equivalents of Fmoc-amino acid: benzotriazole-1-yl-oxy-tris(dimethylamino)-phosphoniumhexafluorophosphate (BOP): *N*-hydroxybenzotriazole (HOBt):

diisopropylethylamine (DIEA) (1:1:1:1) in N-methylpyrrolidinone (NMP) at room temperature. The Fmoc was removed by 20% piperidine in NMP for 1 hour. Progression of the coupling reaction was monitored using the Kaiser test. After Fmoc removal, the resin was washed with NMP and dichloromethane (DCM), two times each. The resin was dried under vacuum and then treated with 95% trifluoroacetic acid (TFA), 1% water, 2% ethanediol, and 2% triisopropylsilane for 3 hours. Purity was determined using RP-HPLC equipped with a C18 column and gradient of 5 to 15% acetonitrile in water. The mass was confirmed using MALDI-TOF MS analysis on a PE Voyager DE-Pro MALDI-TOF Mass Spectrometer (Perspective Biosystems, MA).

The scheme for the synthesis of the modified alginate is outlined in Figure 5-2. A 2% solution of high-mannuronic acid alginate in 0.1 M MES and 0.3 M NaCl at pH 6.5 was combined with EDC, Sulfo-NHS and EMCH. The COOH:EDC:sulfo-NHS:EMCH ratio was 8:2:1:2. The reaction was performed at room temperature for 2 hours after which the un-reacted solutes and byproducts were removed using centrifugal ultrafiltration for 2.5 hours. The lysine substrate, FFKGC-NH_2, was then added at equimolar quantity to EDC and the reaction allowed to proceed at room temperature for 3.5 hours. The un-reacted peptide was removed by centrifugal ultrafiltration and solvent exchanged to water. The solution was considered pure when the filtrate tested negative for amines utilizing the Kaiser test. The solution was then lyophilized and stored at –20 ºC until use. The amount of peptide coupled to alginate was determined using ninhydrin solution according to the method previously reported by Eiselt *et al.*[97] and lysine to generate a standard curve.

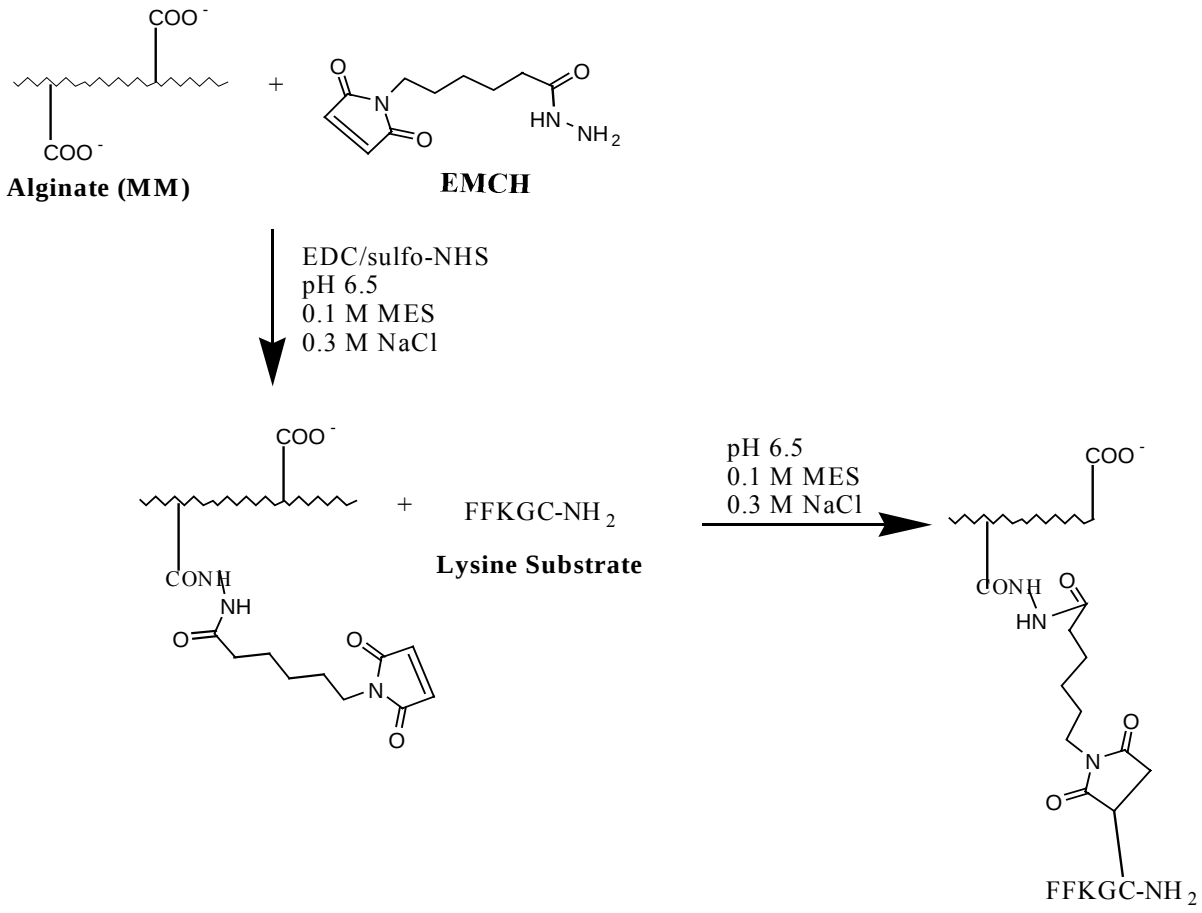


Figure 5-2. Chemical scheme for the modification of alginate with the FFKGC-NH_2.

5.2.2.2 Synthesis of protected AcGQQQLG for PEG-peptide conjugates.

The protected peptide portion, AcGQ(Trt)Q(Trt)Q(Trt)LG, was synthesized as described for the lysine substrate but on Gly-2-ClTrt-resin (1.0 g, 0.77 meq/g). After final Fmoc removal, the peptide was acetylated with 4 equivalents of acetic anhydride combined in a 1:1:1:1 ratio with BOP:HOBt:DIPEA. After completion of the coupling reactions, the resin was washed with NMP and dichloromethane (DCM) two times each. The peptide was cleaved from the resin with the amino acid side chains protected using 1% TFA in DCM for 15 min and the solution was collected in 10% pyridine in methanol (MeOH). This was repeated 2 additional times. The protected product was obtained by concentration of the TFA solution and the addition of 4 ^{o}C water. The product was collected by centrifugation and then dried over vacuum.

5.2.2.3 Conjugation of peptides to PEG.

The coupling of the AcGQ(Trt)Q(Trt)Q(Trt)LG peptide to PEG is illustrated in Figure 5-3. The coupling of the protected AcGQ(Trt)Q(Trt)Q(Trt)LG to the PEG diamine ($\bar{M}_w$ 3.4 kDa) was performed overnight with a 10 min pre-activation of 6 equivalents of the protected peptide: BOP: HOBt: DIEA (1:1:1:1) in DCM at room temperature. The peptide and the PEG were combined in a 3:1 ratio and reacted for 12 hours. The product was precipitated in ether and dried under vacuum. The dried product was treated with 95% trifluoroacetic acid (TFA), 2.5% water, and 2.5% triisoproylsilane for 3 hours. Purification was performed using semi-preparative RP-HPLC. The mass was confirmed using MALDI-TOF MS analysis on a PE Voyager DE-Pro MALDI-TOF Mass Spectrometer (Perspective Biosystems, MA).

5.2.2.4 Formation of hydrogel and rheological studies.

Liposomes, composed of DPPC:DMPC in a ratio of 9:1, were used as the vehicle for calcium delivery. This ratio of DPPC:DMPC yielded liposomes that release their contents at 37 ºC. The liposomes were prepared, using a 0.22 M solution of $CaCl_2$, by the method described by Westhaus and Messersmith[240]. The gel solution consisted of 1.7% (m/v) modified alginate, 1.7% (m/v) modified PEG, tTG at 8.3 units/mL, 4.5 or 9 mM $CaCl_2$, 0.22 M NaCl, 4 mM DTT, 2 mM EDTA, and 42 mM Tris, pH 8.0. Oscillatory rheometry was used to study hydrogel formation and was performed on a Bohlin VOR rheometer. A cone and plate configuration was used, the cone angle being 1º and the cone diameter being 50 mm. The strain was 1% and the amplitude was 0.1 Hz. All solution components were mixed and placed on the thermostatted plate at 25 ºC and equilibrated for 3 minutes. The temperature was increased linearly over 12 minutes to 37 ºC and then held at 37 ºC for 15 minutes. Mineral oil was placed on the edge of the cone to

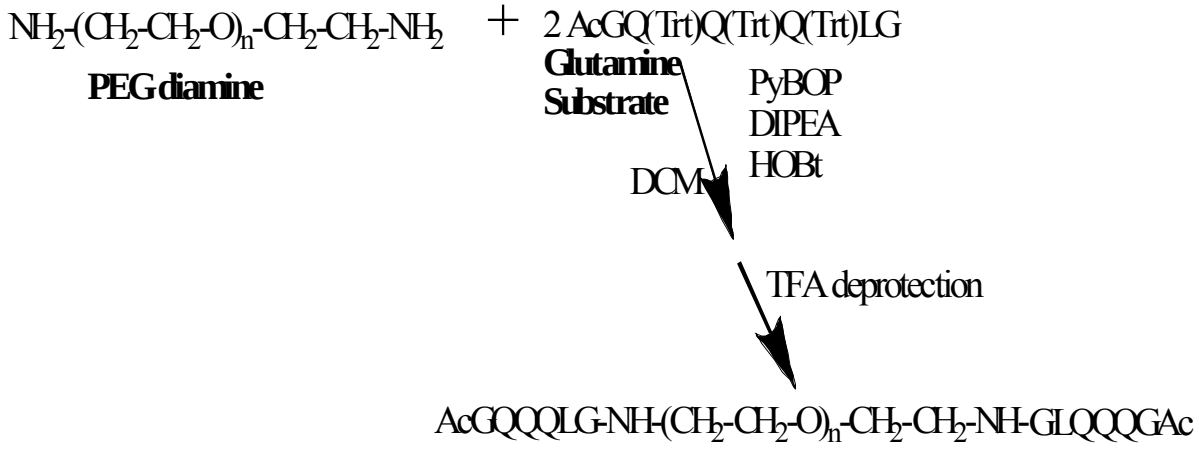


Figure 5-3. Chemical modification of PEG diamine with the glutamine substrate.

minimize dehydration of the hydrogel. At the completion of the oscillatory experiment for each gel examined, a frequency sweep was performed, varying from 0.01 to 10 Hz with an applied strain of 1 %. This was with a strain sweep, varying from 0 to 10% strain with a frequency of 0.1 Hz.

5.2.2.5 *In vitro* culture of chondrocytes in hydrogel.

Chondrocytes were isolated from cartilage of adult bovine metacarpophalangeal joint by sequential digestion, first with pronase in DMEM-F12 media with 0.1 % (v/v) gentamicin for 1.5 hrs, followed by collagenase-P digestion in DMEM-F12 media supplemented with 5% fetal bovine serum (FBS) overnight[86]. Cells were then rinsed 3 times with Hank's balanced salt solution, and counted with a hemocytometer.

Chondrocytes were combined with the precursor gel solution containing 4.5 mM $CaCl_2$ described above at a seeding density of 50 x 10^6 cells/mL. One half millilitre of precursor gel solution was allowed to solidify (having a thickness of 1 mm) in the well of a 24-well plate for 20 minutes at 37 ^oC, followed by five 20 minute incubations in DMEM/F-12 media. After this initial time, the cell/hydrogel constructs were cultured in the well using DMEM-F12 media supplemented with 10% FBS, 0.1% gentamicin, and 50 μg/ mL ascorbic acid for 8 weeks. The media was changed twice daily. Samples were collected at 1, 2, 4, 6, and 8 weeks for biochemical analysis.

5.2.2.6 Biochemical Assays.

The cell-gel constructs were frozen and lyophilized, and then digested with 126 μg/mL papain, 5 mM cysteine, 5 mM EDTA at pH 6.0 at 60 ^oC overnight. Each digest was analyzed for DNA content fluorometrically (λ_{ex} = 352 nm, λ_{em} = 452 nm) using Hoeschst 33258 as described

by Kim *et al.*[246]. The quantity of GAG in the constructs were analyzed spectrophotometrically with dimethylene blue complexation using chondroitin sulfate to generate a standard curve[247]. A portion of the digest was frozen and lyophilized, then acid hydrolyzed with 6 N HCl at 110 ^{o}C for 20 hours. The amount of collagen in the hydrolysate was determined with p-dimethylaminobenzaldehyde and chloramine T[248]. The quantity of collagen was calculated from the hydroxyproline amount based on a previous report that collagen consists of 10% hydroxyproline[169].

5.2.2.7 Histology.

Samples were stained with Safranin-O for GAGs and [^{35}S] sulfate incorporation studies were conducted to further evaluate GAG production. These samples were prepared by Dr. James Williams at Rush University, Chicago, IL.

5.3 Results

5.3.1 Modification of alginate

The mass spectrum of FFKGC-NH_2 had prominent peaks at m/z of 300.8 and 321.4 which represented the divalent peptide and the divalent peptide complexed with sodium, respectively (Figure 5-4). The molecular ion peak was also present at 600.3 m/z (calculated 598.75 Da). The synthesis yield exceeded 90% and purity was greater than 85% as analyzed via HPLC (Figure 5-5). An impurity with a retention time of 20.5

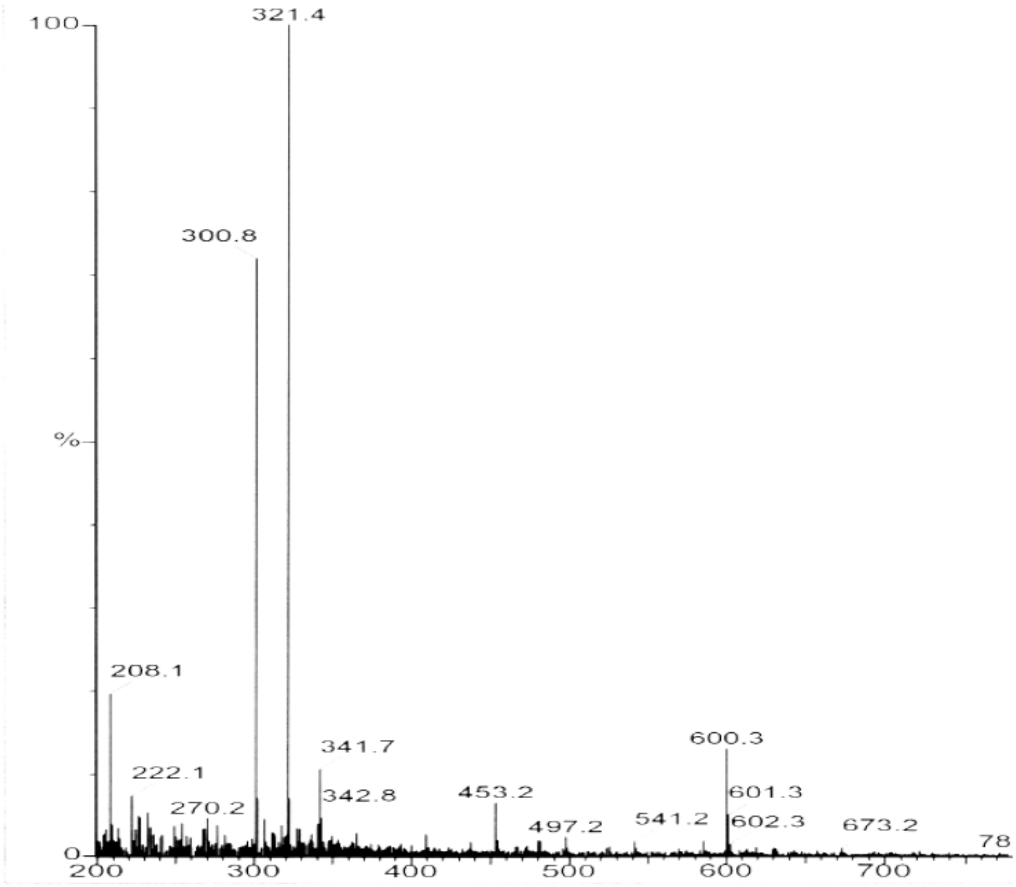


Figure 5-4. MALDI-TOF mass spectrum of FFKGC-NH_2. 600.3 $(M)^+$, 300.8 $(M)^{2+}$, and 321.4 $(M+Na)^{2+}$.

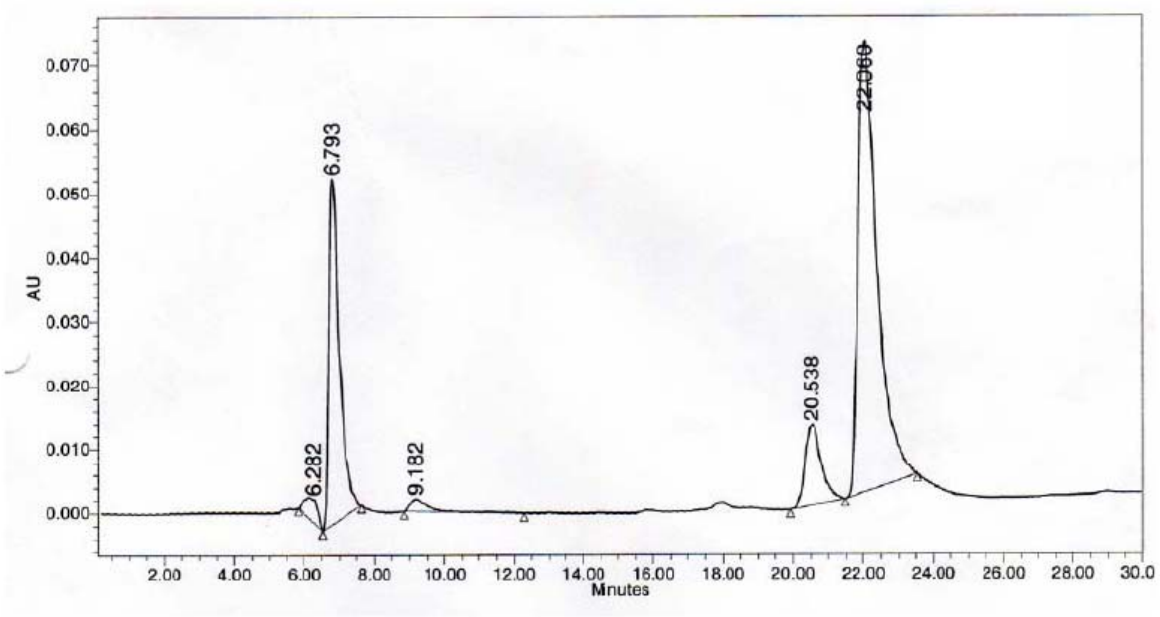


Figure 5-5. Chromatogram of FFKGC-NH_2. FFKGC-NH_2 had a retention time of 22.1 minutes using a 5 to 15% acetonitrile in water gradient. The compound with a retention time of 20.5 minutes represents a 13% impurity and acetic acid had a retention time of 6.8 minutes.

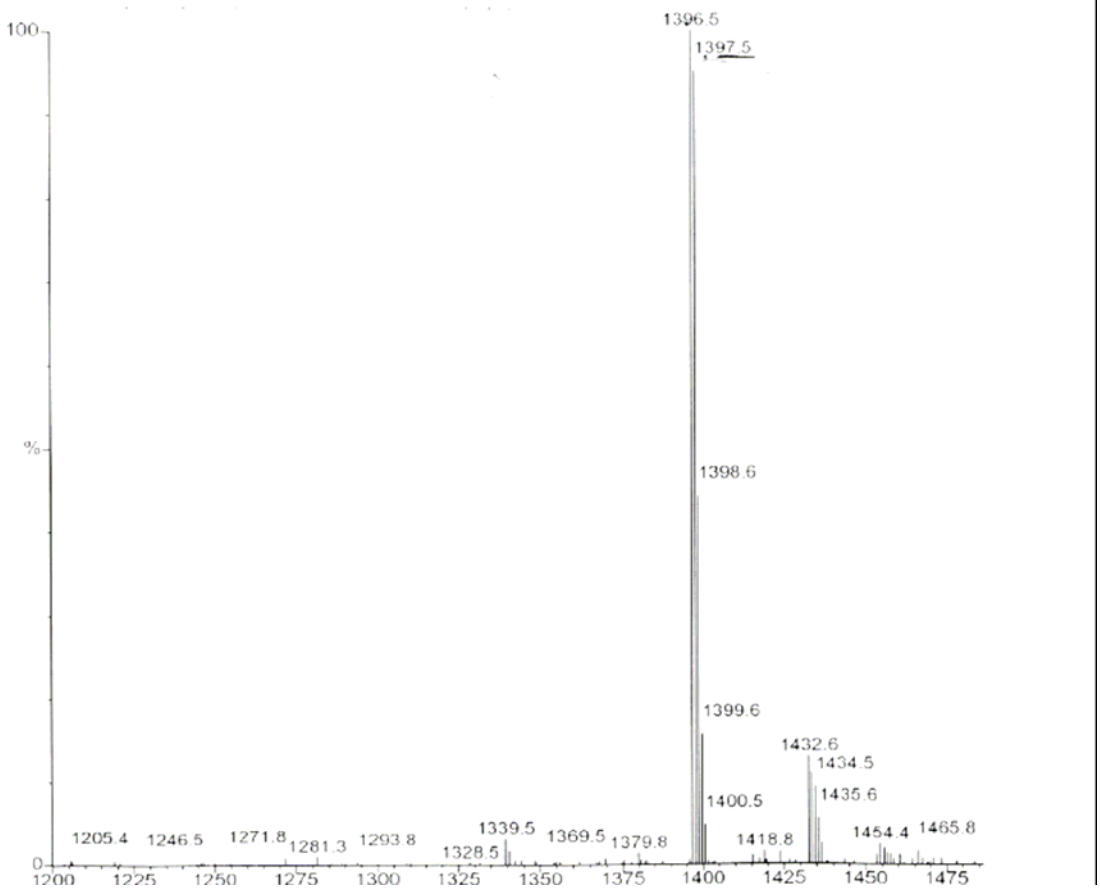


Figure 5-6. MALDI-TOF mass spectrum of the protected glutamine peptide, Ac-G(Q(trtl))$_3$LG. 1396 $(M)^+$; 1432.6 $(M +2Na)^+$.

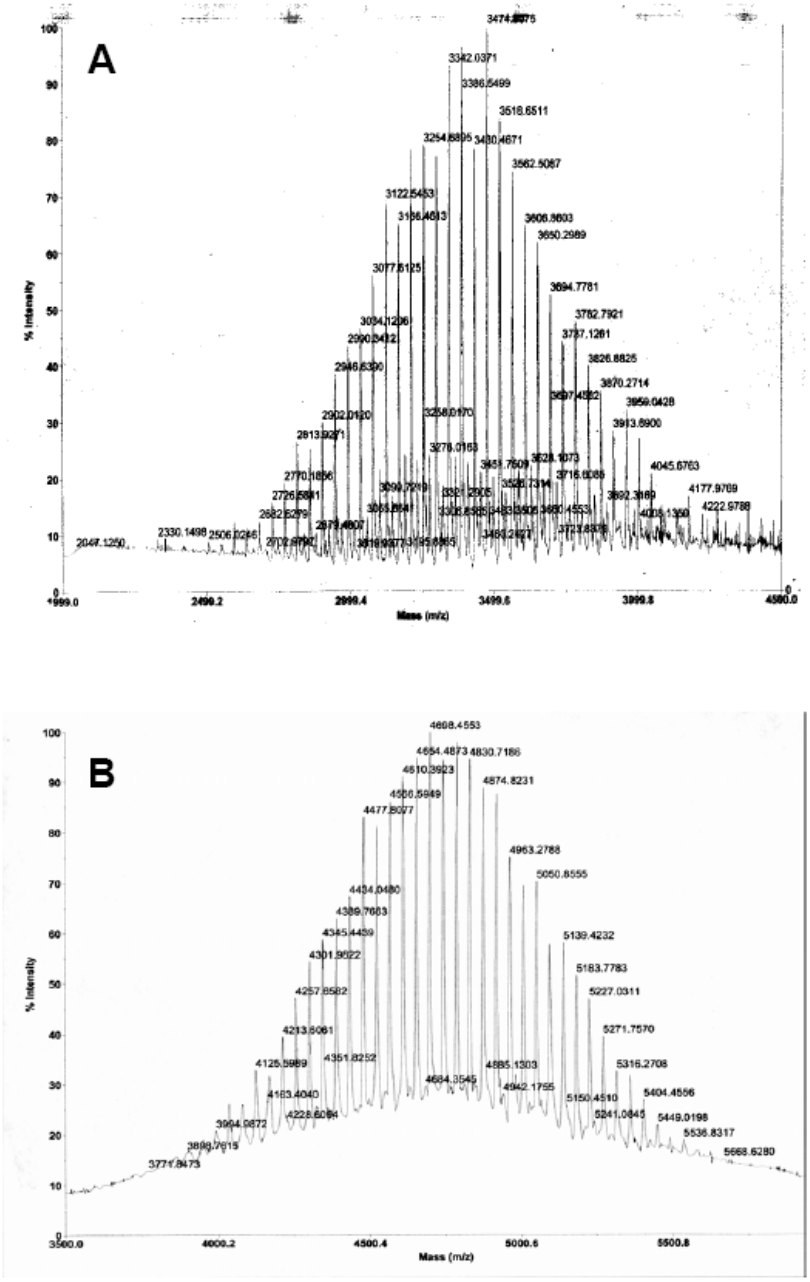


Figure 5-7. MALDI-TOF spectra of unmodified and Ac-GQQQLG modified PEG. A. Mass spectrum of unmodified PEG diamine ($\bar{M}_w$ = 3.4 kDa). B. Mass spectrum of AcGQQQLG modified PEG ($\bar{M}_w$ = 4.7 kDa).

minutes was also detected in the sample. When the peptide was conjugated to alginate, ten percent (± 1%, standard deviation) of the carboxylic acid groups on alginate were modified, which yielded a reaction efficiency of about 40%. The final yield of modified alginate was 60-70%, with the remainder being lost during centrifugal filtration purification.

5.3.2 Modification of PEG

The mass spectrum of protected AcGQ(Trt)Q(Trt)Q(Trt)LG had a prominent peak at m/z = 1396.5, which was the molecular ion peak (calculated 1398.6 Da) and a smaller peak at 1432.6, which was the molecular ion complexed with 2 Na^+ (Figure 5-6). After conjugation of the protected peptide to the PEG amine and the deprotection of the peptide, the observed $\bar{M}_w$ of the PEG-conjugate by MALDI-TOF MS was 4.7 kDa, which was 1.3 kDa more than the unmodified PEG ($\bar{M}_w$ = 3.4 kDa) (Figure 5-7). This is the approximate total mass of two deprotected Gln peptides, $AcGQ_3LG$ (each with a calculated mass of 671.7 Da) used to modify the PEG. Both PEG spectra had the characteristic molecular weight distribution of a polydispersed polymer, with peaks increasing by multiples of 44 Da, the mass of the ethylene oxide unit.

5.3.3. Rheological studies of hydrogels

The hydrogel formation and its viscoelastic properties were evaluated using oscillatory rheology. Samples were placed on the rheometer stage at 25 ^oC and allowed to equilibrate for 3 minuntes. The temperature was then increased linearly to 37 ^oC and again equilibrated there for 15 minutes. Hydrogel formation was initiated when calcium was released from the liposomes,

which occurred at 30 ºC in samples containing tTG (both for the 4.5 and 9 mM), but at 33 ^{o}C for samples without tTG (Figure 5-8) as evidenced by the rapid increase in storage modulus at those temperatures. At 4.5 mM Ca^{2+}, the hydrogel formed with tTG had a plateau storage modulus of 200 Pa but formed a very weak hydrogel, having a storage modulus of 20 Pa, when tTG was not included (Figure 5-8A). Likewise at 9.0 mM Ca^{2+}, the samples with tTG had a larger plateau storage modulus (500 Pa) than without tTG (300 Pa) (Figure 5-8B). In all cases, the maximum loss modulus observed (Table 5-1) was significantly less than the storage modulus, demonstrating that the hydrogels created had properties of an elastic solid. The hydrogels formed with tTG were both robust since variations in both strain and frequency resulted in no fluctuation in the storage modulus at 37 ^{o}C (Figure 5-9). To examine the extent of tTG crosslinking, 200 μL of either tTG-containing hydrogel was incubated with an equivolume amount of 12 M EDTA. In the presence of EDTA, the hydrogel formed with 9 mM Ca^{2+} dissolved while the hydrogel formed with 4.5 mM Ca^{2+} remained intact.

5.3.4 Study of hydrogel-cell constructs

Over the 8 weeks of *in vitro* culture, primary chondrocytes in the tTG crosslinked hydrogels containing 4.5 mM Ca^{2+} did not proliferate as measured by DNA concentration, which remained constant at about 250 ng/mg wet weight throughout the experiment (Figure 5-10A). Figure 5-10B shows that there was a statistical ($p<0.05$) increase in GAG production from week 1 to 2, where it had a maximum value of 4.1 ± 1.1 (S.E.) μg/μg DNA but did not increase over the duration of the experiment. Figure 5-10C demonstrates that collagen production by chondrocytes had a statistical increase from week 1 to 2 but did not increase over

Table 5-1. The range of loss moduli observed for the various PEG-alginate hydrogels formed.

Sample	Minimum loss modulus recorded (Pa)	Maximum loss modulus recorded (Pa)
4.5 mM Ca, no tTG	1	5
4.5 mM Ca, with tTG	1	20
9.0 mM Ca, no tTG	3	40
9.0 mM Ca, with tTG	6	20

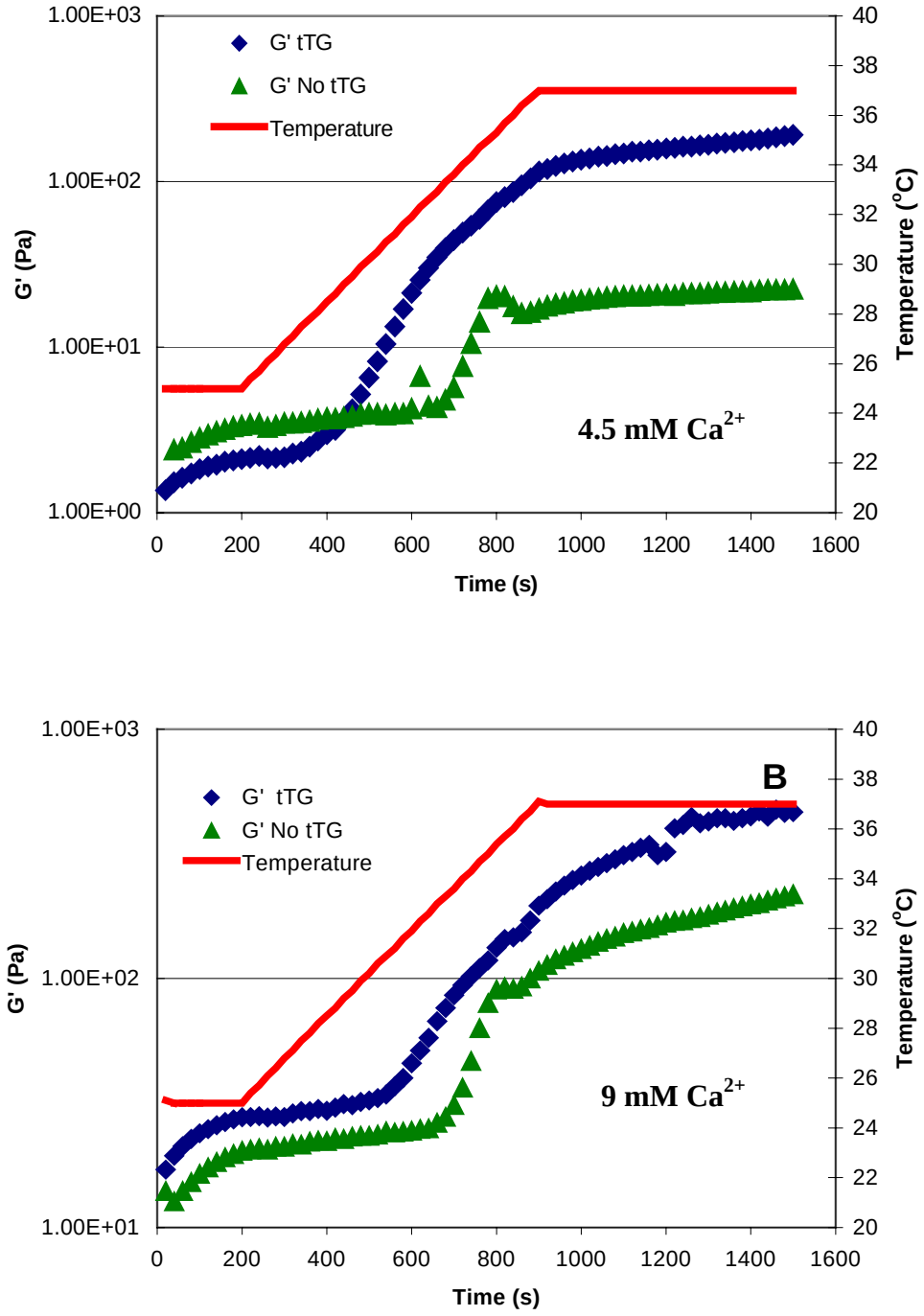


Figure. 5-8. Rheological study of PEG-alginate hydrogels formed with different amounts of calcium and tTG. A. 4.5 mM Ca^{2+}. B. 9.0 mM Ca^{2+}.

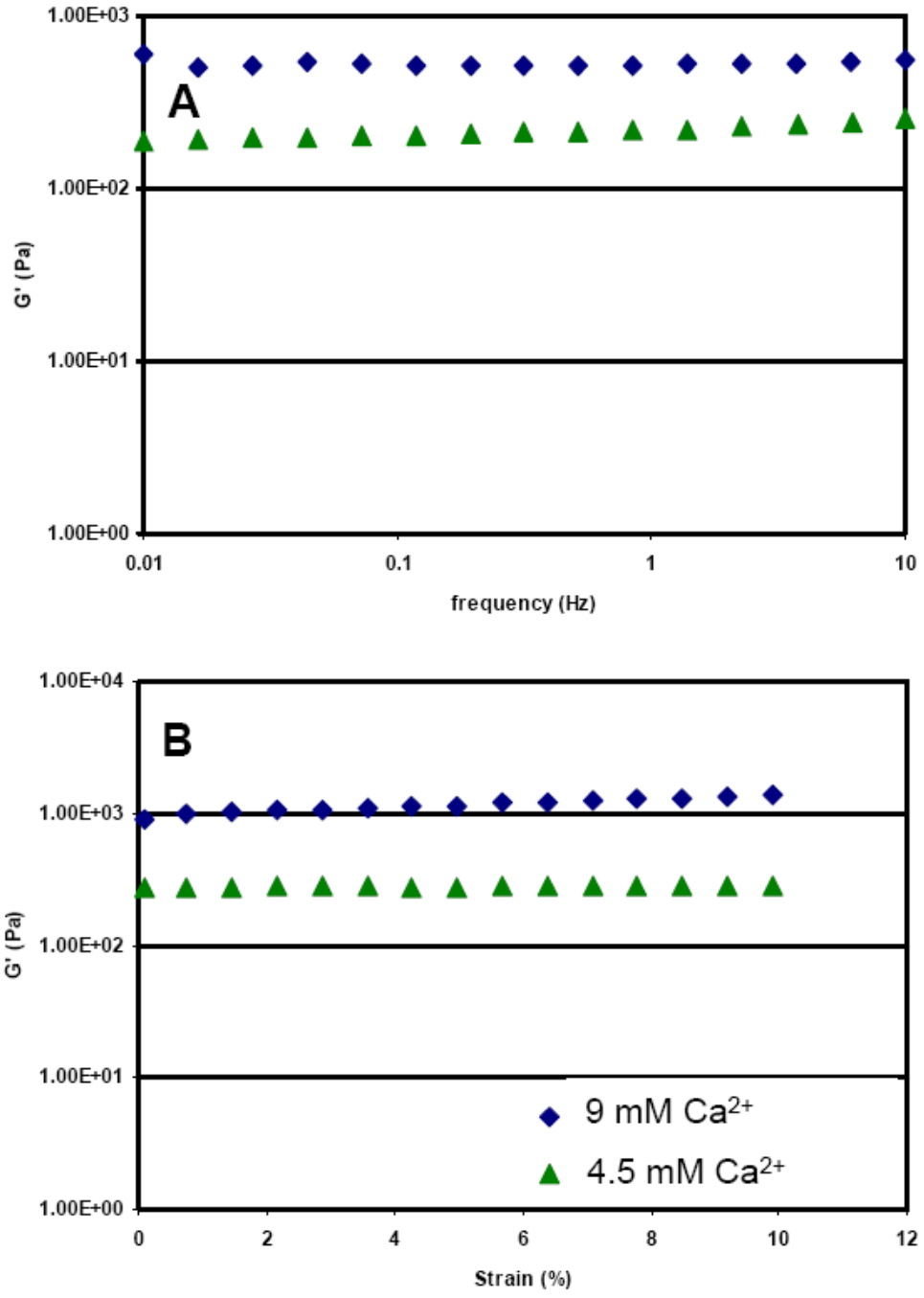


Figure 5-9. Frequency and strain dependence of storage modulus of PEG-Alginate hydrogels crosslinked with tTG. A. Frequency sweep. B. Strain sweep.

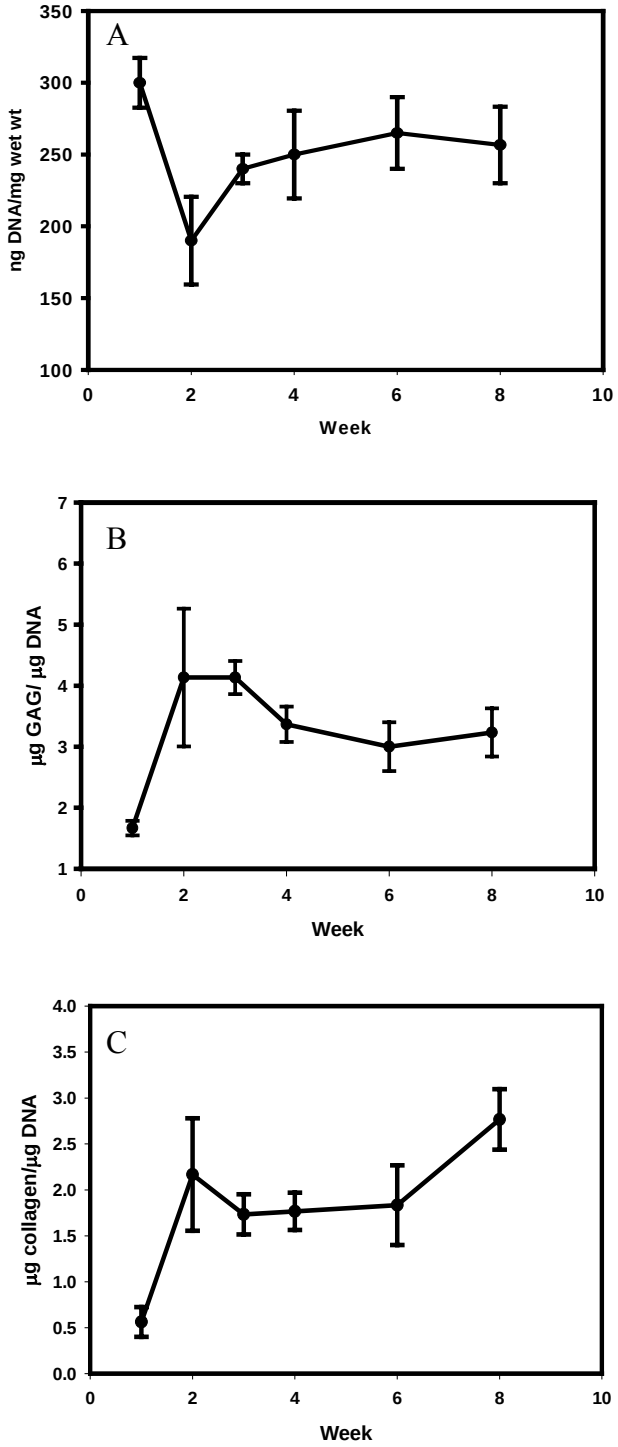


Figure 5-10. Biochemical Analysis of 8 week-study of chondrocytes cultured in PEG-alginate gels. A) DNA analysis; B) GAG analysis: C) Collagen analysis. Error bars represent 1 standard error (S.E.)

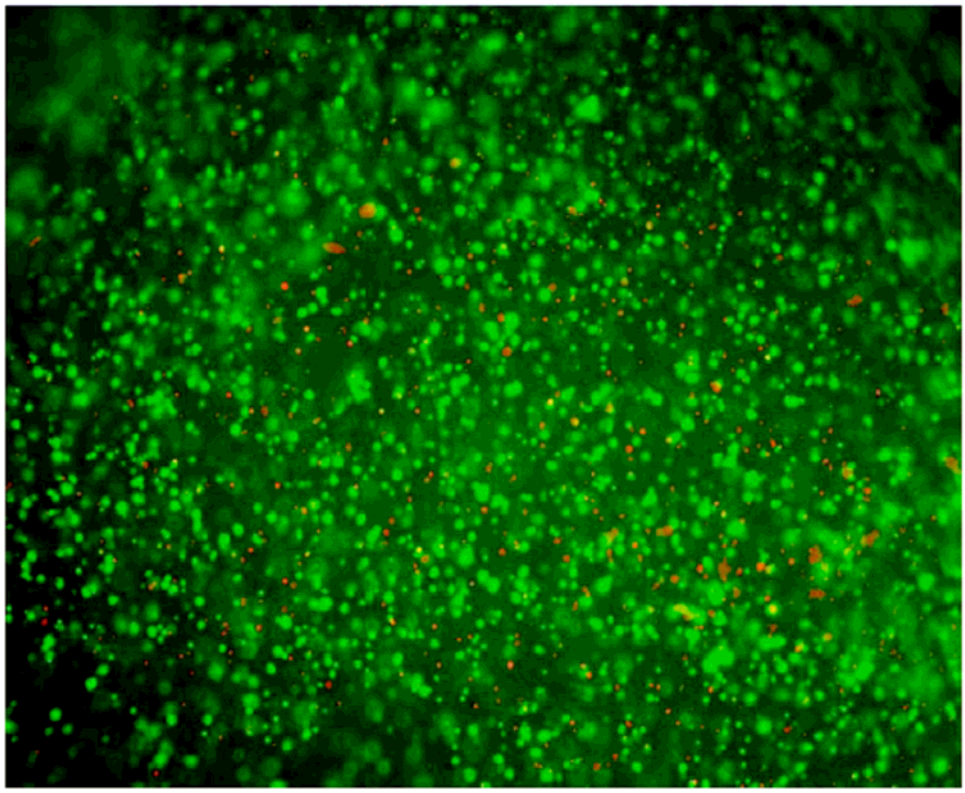


Figure 5-11. Image of live-dead staining of chondrocytes in PEG-alginate hydrogel at 8 weeks. Image is at 5X magnification. Calcein was used to detect live cells and ethidium to detect dead cells.

the remaining 6 weeks. Live/dead assay with calcein/ethidium homodimer demonstrated that, qualitatively, the majority of the cells were alive at 8 weeks (Figure 5-11).

At the completion of the eight week study, the hydrogel/cell constructs were stained with Safranin-O. The chondrocytes (blue) were round and surrounded by lacunae (Figure 5-12A). In addition, GAGs were detected in the pericellular region of chondrocytes as observed by the lighter staining pink tissue around the cell. As expected, the majority of the matrix stained a dark pink because the alginate was not degradeable. This intense staining prohibits any visualization of GAGs that may have diffused throughout the matrix. The [^{35}S]-sulfate uptake confirmed that chondrocytes were still actively synthesizing GAGs at week 8 (Figure 5-12B) and was detected primarily around the cell.

5.4 Discussion

Alginate hydrogels are recognized as suitable biomaterials for *in vitro* maintenance of chondrocytes, providing an environment that facilitates chondrocyte proliferation and production of ECM components[85, 86]. However, because alginate is crosslinked with Ca^{2+} cations, its rheological properties can be adversely affected by ionic profile of the aqueous environment. If the Ca2+ concentration is not sufficiently large, Ca2+ in the crosslinked gel can be exchanged Na+ in solution causing the hydrogel to disassemble. This is particularly a problem for hydrogels composed of high mannuronate alginate, which has been demonstrated to be a better matrices for culturing chondrocytes than the corresponding high guluronate form[87, 88]. Because the matrix is less crosslinked, it is easier for Ca^{2+} ions to exchange with Na^{+} in the aqueous environment

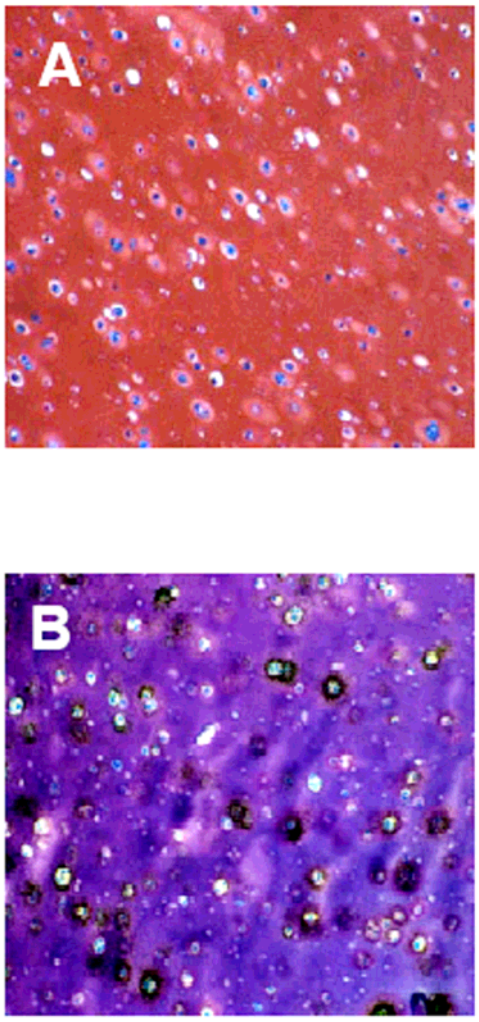


Figure 5-12. Histological evaluation of chondrocytes in the PEG-alginate gels at 8 weeks. A. Safranin-O stainining. Lighter pink staining areas around the cells demonstrate GAG production. B. [^{35}S] sulfate study. Black staining indicates the location of the [^{35}S] sulfate incorporation into newly synthesized GAGs. Magnification is 4X for both images.

than those made with high guluronate alginate[41]. It is possible that covalent crosslinks in alginate can stabilize its rheological properties and make these gels not susceptible to variations in the aqueous environment.

In the current study, a PEG-alginate hydrogel covalently crosslinked by tTG was evaluated as a scaffold for *in vitro* culture of chondrocytes. These polymers were modified with short synthetic peptide substrates of tTG, AcGQQQLG and DopaFKG, which were rationally designed in our laboratory[5]. Similar peptides have been used previously to modify PEG polymers which were crosslinked into a hydrogel[5] and to modify bioactive peptides which were then coupled to a scaffold via tTG[224]. The use of tTG to crosslink the hydrogel has the added benefit of potentially crosslinking the peptide-modified hydrogel to native cartilage tissue during *in situ* gelation in a chondral defect, creating adhesive bonds between the hydrogel in cartilage and native substrates to include collagen II[196, 244], fibronectin[245], and osteonectin[244, 249].

As with other hydrogels[220, 240], liposomes were used in this hydrogel system to deliver Ca^{2+} for gelation and were designed to release its contents at 37 ^{o}C. However, in this study, the liposomes released their contents prematurely. When transglutaminase was included in the PEG-alginate precursor solution, the premature release occurred at an even lower temperature. It is hypothesized that the interaction of the modified polymers, transglutaminase and liposomes caused this premature release. Liposomes stored in alginate have a time dependent change in permeability due to insertion of alginate into the phospholipid bilayer[250]. It has also been demonstrated that protein adsorption on and penetration into the phospholipid membrane can lower the transition temperature of the liposomes in a concentration dependent manner[251]. In addition to concentration dependence, disruption of the lipid membrane is also protein

dependent[251]. This may explain why liposome disruption occurred in these hydrogels and not in hydrogels crosslinked with Factor XIII[220, 240].

The calcium released had two competing pathways for use in the PEG-alginate hydrogel system, both giving rise to gelation: 1) formation of natural ionic crosslinks with alginate and 2) the calcium-dependent tTG crosslinking. In order to minimize the use of calcium for ionic crosslinking of alginate, low guluronate alginate was used. Second, alginate has a greater affinity for sodium ions than for calcium ions[87, 252]. Therefore, a hyperosmotic saline solution was used to promote interactions between sodium and alginate and afford the enzyme an opportunity to utilize the calcium. The hyperosmotic conditions were not anticipated to have long-term effects on chondrocytes since the constructs were washed in DMEM/F-12 a number of times after gelation and cultured in FBS supplemented DMEM/F-12 media[253]. With these conditions, tTG and at a calcium concentration of 4.5 mM, a gel was achieved that was not solubilized by EDTA, indicating that tTG was able to utilize the calcium and form covalent crosslinks between the PEG and the alginate. However, at 9.0 mM Ca^{2+} with tTG, even though the storage modulus of the formed hydrogel was greater than at 4.5 mM Ca^{2+} with the same conditions, the hydrogel was readily dissolved with EDTA, demonstrating that the alginate was crosslinked with calcium ions and very little tTG crosslinks were used to stabilize the gel. This may suggest that a critical amount of Ca^{2+} is needed before an ionically crosslinked gel is formed.

The hydrogel formed with 4.5 mM Ca^{2+} and tTG had a similar storage modulus to the PEG hydrogel modified with similar peptides[5] and to the gelatin hydrogel cross-linked with microbial transglutaminase[254] despite differing amounts of polymer, enzyme and Ca^{2+} utilized. Crescenzi *et al*., in their study of modified-HA/gelatin gels crosslinked with microbial tTG, suggested that the enzyme may become entrapped as it forms crosslinks around it[222]. This may indicate that these

transglutaminase crosslinked gels can only achieve a specific critical modulus after which time the enzyme becomes immobilized.

The PEG-alginate hydrogel was able to maintain chondrocyte viability throughout the 8-week experiment of *in vitro* culture. Matrix production during that time was limited to directly around the cells. These hydrogels were compared to a gel-like coacervate of an elastin-like polypeptide with a similar modulus[95]. Both of these gel/chondrocyte constructs had similar levels of GAGs and collagen production. However, when compared to alginate beads[86], the PEG-alginate hydrogel system did not promote matrix production and proliferation at the same rate; this may be related to the lack of degradability of the PEG-alginate hydrogel. Bryant *et al.* demonstrated that matrix production is directly related to degradeability[255, 256]. In addition, the hydrogel matrix could undergo structural changes as the new matrix is produced and this in turn could lead to changes in extracellullar matrix production by the entrapped cells[53]. Therefore, the small amounts of matrix produced and localized around the cell, combined with the lack of degradability of the hydrogel, may have inhibited further matrix production. Finally, lack of cell proliferation and matrix produced per cell had been previously reported with high cell densities[256, 257]. However, when this cell density was used to culture chondrocytes in an alginate hydrogel, it generated the best storage modulus and total matrix production[257].

5.5 Conclusion

In summary, alginate and PEG were modified with short peptide substrates of tTG and were crosslinked by tTG to form a hydrogel. This hydrogel matrix was used to culture chondrocytes *in vitro,* and the cells were shown to produce collagen and GAG, two major

components of cartilage. However, the non-degradable nature of this matrix may not support the production of these components at the level required to regenerate cartilage.

Chapter 6

Evaluation of a PEG hydrogel crosslinked by tissue transglutaminase for in vitro culture of chondrocytes

6.1 Introduction

Articular cartilage is important for the proper joint function and protection of the underlying bone. It permits frictionless joint motion and dissipates compressive forces across the surface, thereby minimizing transmission of forces to the bone[41, 42, 258]. When cartilage is damaged during a traumatic event, the chondrocytes generally cannot mount a response sufficient to replace the lost cartilage[7, 26]. Unless there is medical intervention, degradation of cartilage will continue, eventually leading to osteoarthritis[26]. Depending on many factors, including defect size, patient age, and the health of the remaining cartilage, the defect can be repaired with debridement and lavage. However, the efficacy of debridement and lavage has not been confirmed, and only 25% of patients show positive results[25]. Subchondral abrasion is another possible treatment. In this approach, the subchondral bone is punctured and blood and cells from the subchondral bone are allowed to fill the defect[7]. Autologous or allogenic osteochondral plugs can also be used to fill the defect, but this treatment compromises the cartilage at the donor site[31, 32]. Autologous chondrocyte implantation has recently been commercialized, however the results from this treatment have been mixed[35-38]. Therefore, other methods of treating cartilage defects and of facilitating repair are needed.

Hydrogels have been suggested as a potential method of repairing cartilage defects because cells can be entrapped in the precursor gel solution, which can then be gelled *in situ* to

fit the defect[64, 66]. While many different hydrogels have successfully been used to culture chondrocytes *in vitro*[80, 83], there are only a few strategies that permit the crosslinking of the hydrogel to the native cartilage matrix *in vivo*. Tyrosines in proteins on the cartilage surface have been oxidized with hydrogen peroxide and then used to initiate crosslinking of a hydrogel and attachment of that hydrogel to cartilage[141]. Wang *et al.* achieved crosslinking of a PEG diacrylate hydrogel to the native matrix with a two step approach using chondroitin sulfate polymer containing both aldehyde and methacrylate groups[134]. First, the aldehyde groups were used to form imine bonds with amines in proteins on the cartilage surface and then the methacrylates were used to photocrosslink the hydrogel to the chondroitin sulfate polymer, thereby indirectly crosslinking the hydrogel to the cartilage surface. Englert *et al.* demonstrated that glutaraldehyde and genipin can be used to form cartilage-cartilage bonds[259]. Genipin has also been used to crosslink a PEG hydrogel while simultaneously crosslinking the hydrogel to the native cartilage in the treatment of osteochondral defects[260]. Fibrin gels containing cells were demonstrated to adhere to and integrate with native cartilage[261, 262]. This crosslinking of the fibrin gel was accomplished with Factor XIII, a transglutaminase that forms γ-glutaminyl-ε-lysine isopeptide bonds between lysine and glutaimine residues in a calcium-dependent manner[2]. There are substrates of Factor XIII in cartilage[263] to which fibrin gel components can be crosslinked. Tissue transglutaminase (tTG), another member of the transglutaminase family, also has substrates in cartilage[186] that may be used to adhere hydrogels to cartilage. Jurgensen *et al.* demonstrated that tTG can be used to form crosslinks between pieces of cartilage[4] and it has been used to crosslink an elastin-like polypeptide (ELP) coacervate used for chondrocyte

culture[96]. Therefore, it is hypothesized that hydrogels containing substrates of tTG can be crosslinked to native cartilage

In our lab, we have rationally developed peptides that are substrates of tissue transglutaminase. These peptide substrates, FKG and GQQQLG, have been used to form a hydrogel that is adhesive to guinea pig skin[5, 148] and have been crosslinked to native cartilage, demonstrating that polymers modified with these peptides can be coupled to cartilage[264]. However, since these peptides were rationally designed based on the literature of tTG substrates, Hu *et al.* screened both lysine and glutamine peptides in combinatorial libraries to identify additional substrates with superior enzymatic properties[265]. Because tTG is not very discriminating with respect to the lysine peptide, the lysine library yielded many substrates with good specificity for tTG. However, in the glutamine peptide library, PQQQYV was found to be a superior substrate with not only a higher specificity but also higher reactivity than GQQQLG.

In this study, we examine the ability of a PEG hydrogel crosslinked by tTG to provide a matrix for chondrocyte culture *in vitro*. The PEG polymers were modified with AcFKG and AcPQQQYV. In order to facilitate cell-activated degradation, a MMP sensitive sequence[266] was combined with the lysine peptide to create AcFKGGPLSLRSG. MMP-2 is expressed by healthy chondrocytes throughout the cartilage matrix[267-269]. The enzymatic activity of this peptide towards both tTG and MMP-2 was studied and MMP-2 induced *in vitro* degradation of PEG-based hydrogels was examined. Both glutamine and lysine substrates were evaluated with cells to determine whether these peptides could be coupled to membrane proteins of chondrocytes via transglutaminase. Finally, chondrocyte-containing hydrogels were formed from these polymers and chondrocytes cultured *in vitro* for 6 weeks after which biochemical and histological analyses to assess matrix production were conducted.

6.2 Materials and Methods

6.2.1Materials

Gly-2-ClTrt-Resin was purchased from Peptides International, Louisville, KY. All Fmoc-Amino acids, active MMP-2, 7- amino- actinomycin D, and pronase were purchased from EMD Bioscience, San Diego, CA, and 4-arm PEG tetraamine ($\bar{M}_w$ = 10 kDa) was purchased from SunBio, Orinda, CA. Methoxy-PEG amine (mPEG-NH_2) ($\bar{M}_w$ = 2.0 kDa) was purchased from Nektar Therapeutics, Inc, Huntsville, AL. Adult bovine metacarpophalangeal joints were purchased from a local slaughter house. Guinea pig liver tissue transglutaminase (tTG), dansyl-glutamine and Masson trichrome staining kit were purchased from Sigma, St. Louis, MO. Dansyl-e-aminocaproyl-QQIV (Dansyl-e-aca-QQIV) was a gift from Dr. Laszlo Lorand, Northwestern University. DMEM/F-12 media and the Live/Dead cell viability/cytotoxicity stain kit were purchased from Invitrogen, Carlsbad, CA. Collagenase-P was purchased from Worthington Biochemical Co., Lakewood, NJ. Phycoerythrin-streptavidin was purchased from Vector Labs, Burlingame, CA.

6.2.2 Methods

6.2.2.1 Synthesis of AcFKGGPLSLRSG and its mPEG-NH_2 conjugate for enzyme studies.

AcFKGGPLSLRSG peptide was synthesized on a Gly-2-Cltrt resin (1 g, 0.77 mmol/g) using standard Fmoc solid phase peptide synthesis. Each coupling reaction was performed for 3 hours with a 10 min pre-activation of four equivalents of Fmoc-amino acid: benzotriazole-1-yl-oxy-tris(dimethylamino)-phosphoniumhexafluorophosphate (BOP): *N*-hydroxybenzotriazole (HOBt): diisopropylethylamine (DIEA) (1:1:1:1) in N-methylpyrrolidinone (NMP) at room

temperature. The Fmoc was removed by 20% piperidine in NMP for 1 hour. After Fmoc removal, the peptide was acetylated with the use of 4 equivalents of acetic anhydride. At the completion of the coupling reactions, the resin was washed with NMP and dichloromethane (DCM), two times each. The resin was dried under vacuum and then treated with 95% trifluoroacetic acid (TFA), 2.5% water, and 2.5% triisopropylsilane for 3 hours. Purification was performed using semi-preparative RP-HPLC. The mass was confirmed using MALDI-TOF MS analysis (Figure 6-1) on a PE Voyager DE-Pro MALDI-TOF Mass Spectrometer (Perspective Biosystems, MA). The determined mass is reported in Table 6-1.

For the mPEG-NH_2 conjugate, AcFKGGPLSLRSG-PEG_{2000}, the peptide portion was synthesized as above. After the acetylation reactions, the resin was washed with NMP and dichloromethane (DCM), two times each. The resin was treated with 30% hexafluoroisopropanol (HFIP) in DCM 3 times for 10 minutes each. The product was obtained by concentration of the HFIP solution and addition of 1:1 diethyl ether:hexane. The coupling of the protected peptide to the $mPEG_{2000}$ amine was performed overnight with a 10 min pre-activation of 1.5 equivalents of the AcFK(boc)GGPLS(tbu)LR(Pbf)S(tbu)G: BOP: HOBt: DIEA (1:1:1:1) in NMP at room temperature. The reaction completion was determined via the Kaiser test. The product was collected by precipitation in diethyl ether and dried overnight under vacuum and then treated with 95% trifluoroacetic acid (TFA), 2.5% water, and 2.5% triisopropylsilane for

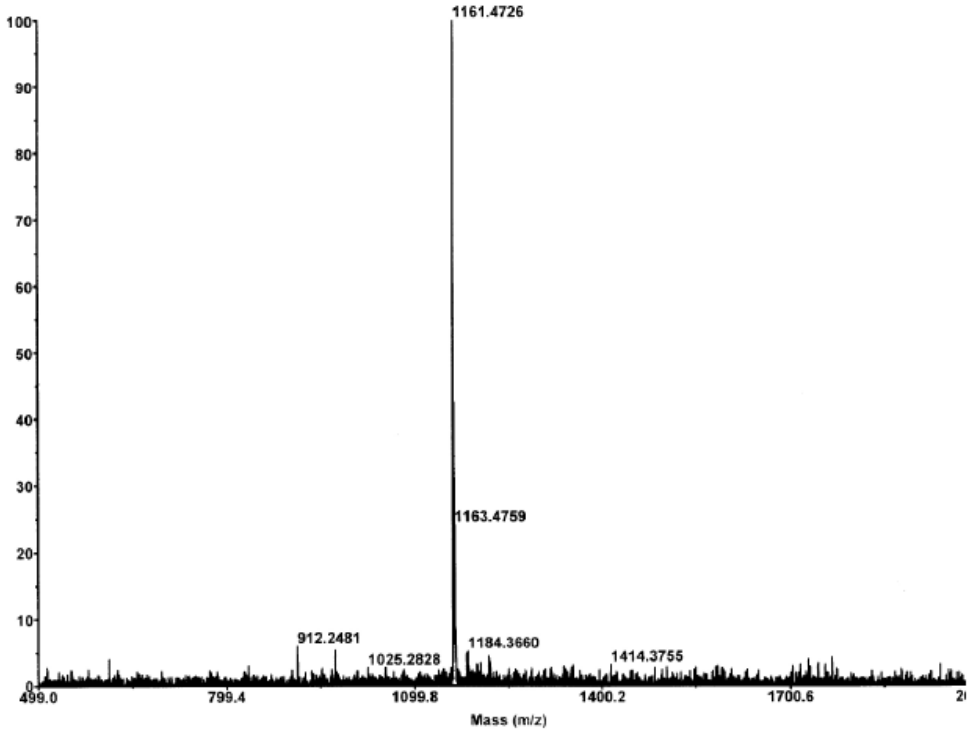


Figure 6-1. MALDI-TOF mass spectrum of AcFKGGPLSLSRG. 1161.5 $(M)^{+}$.

Table 6-1. Characterization and enzyme activities AcFKGGPLSLRSG.

Enzymes and substrates tested	**Determined Mass (Da)**	**Expected Mass (Da)**	**Specificity k_{cat}/K_m ($min^{-1}mM^{-1}$)**	**k_{cat} (min^{-1})**
tTG				
AcFKGGPLSLRSG	1161.5	1161.5	312	21.8
AcFKG			560[5]	26.3[5]
MMP-2				
AcFKGGPLSLRSG	1161.5	1161.5	5100 ± 390	
AcFKGGPLSLRSG-PEG	3200*	3200*	6100 ±180	
IPVSLRSG			4900 ± 360[270]	
VPLSLYSG			3700 ± 240[270]	

*value shown for AcFKGGPLSLRSG-PEG is average mass value for a polydispersed sample as determined by MALDI-TOF MS. Underlined residues represents those combined in the peptide used in this study.

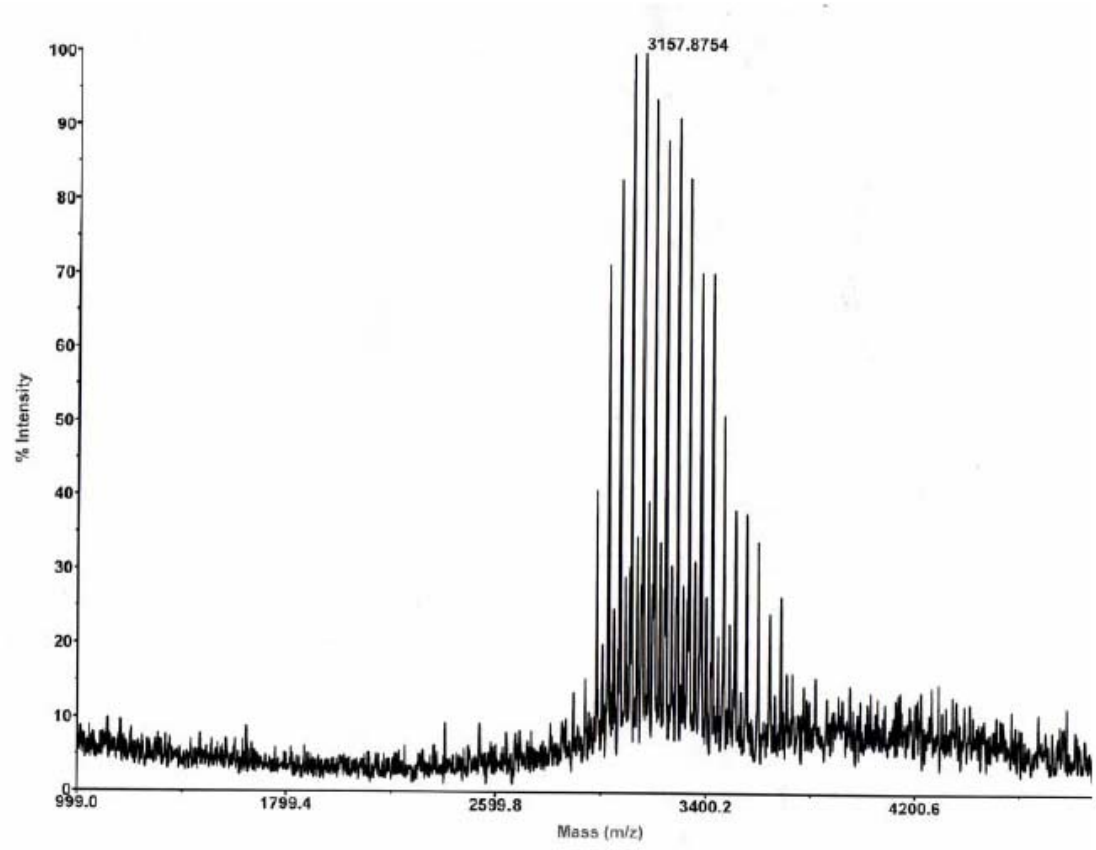


Figure 6-2. MALDI-TOF spectrum of AcFKGGPLSLSRG-PEG. $\bar{M}_w$ = 3.2 kDa.

3 hours. The crude product was obtained by concentration of the TFA solution and addition of diethyl ether. Purification was performed using semi-preparative RP-HPLC. The mass was confirmed using MALDI-TOF MS analysis (Figure 6-2). The determined mass is reported in Table 6-1.

6.2.2.2 Synthesis of protected peptides for PEG-peptide conjugates for hydrogel formation.

The peptide portions, AcFKG, AcFKGGPLSLRSG, and AcPQQQYV, were synthesized as previously described. After Fmoc removal, the peptide was acetylated with 4 equivalents of acetic anhydride. After completion of the coupling reactions, the resin was washed with NMP and dichloromethane (DCM) two times each. For AcFK(Boc)G and AcFK(Boc)GGPLS(tbu)LR(pbf)S(tbu)G, the resin was treated with 30% hexafluoroisopropanol (HFIP) in DCM 3 times for 15 minutes each. The protected peptide products were obtained by concentration of the HFIP solution and addition of 1:1 diethyl ether:hexane, then dried overnight. For AcPQ(trtl)Q(trtl)Q(trtl)Y(tbu)V, the resin was treated with 1% TFA in DCM for 15 min and the solution was collected in 10% pyridine in methanol (MeOH). This was repeated 2 additional times. The protected product was obtained by concentration of the TFA solution and the addition of 4 ^{o}C water. The product was collected by centrifugation and then dried over vacuum.

6.2.2.3 Conjugation of peptides to 4-arm PEG tetraamine.

The coupling of the protected AcFK(Boc)G to the 4 arm PEG tetraamine to form **K-PEG** was performed overnight with a 10 min pre-activation of 6 equivalents of the protected peptide: : BOP: HOBt: DIEA (1:1:1:1) in 50:50 DCM:dimethylformamide(DMF) at room temperature. The peptide and the PEG tetraamine were combined in a 6:1 ratio. The product was precipitated in methanol at -20 ^{o}C and dried under vacuum. The dried product was treated with 95% trifluoroacetic acid (TFA), 2.5% water, and 2.5% triisoproylsilane for 3 hour. The crude product

was obtained by concentration of the TFA solution and addition of diethyl ether. Purification was performed via dialysis in 2500 kDA membranes. The mass was confirmed using MALDI-TOF MS analysis (Figure 6-3). Protected AcFK(boc)GGPLS(tbu)LR(pbf)S(tbu)G to form **K-MMP-PEG** (MALDI-TOF MS given in Figure 6-4) and AcPQ(trtl)Q(trtl)Q(trtl)Y(tbu)V to form **Q-PEG** (MALDI-TOF MS given in Figure 6-5) were treated the same as the AcFK(boc)G except that the coupling reactions were completed in DMF and DCM, respectively.

6.2.2.4 tTG enzyme reaction with AcFKGGPLSLRSG.

Enzymatic reactions were conducted on this peptide at various concentrations using 0.27 mM dansyl-ε- aca- QQIV (QQIV) and 0.08 units/mL of tTG in 50 mM Tris, 2.5 mM DTT, 7.5 mM $CaCl_2$, and 1 mM EDTA at pH 8 and 25 ^{o}C in a total reaction volume of 200 μL. The reactions were conducted for 15 minutes and a fraction of the samples were taken at 3, 6, 9, and 15 minutes. The reaction was quenched at each time point with an equivolume amount of 2% TFA and 200 mM dansyl-glutamine as the internal standard. Samples were then analyzed on RP HPLC at 280 nm and the amount of product formed was quantified using dansyl-glutamine as an internal standard. Kinetic constants were calculated using SigmaPlot 9.0 using the initial velocities versus substrate concentration fit to the Michaelis-Menten equation. A molecular weight of 77 kDa was used for tTG.

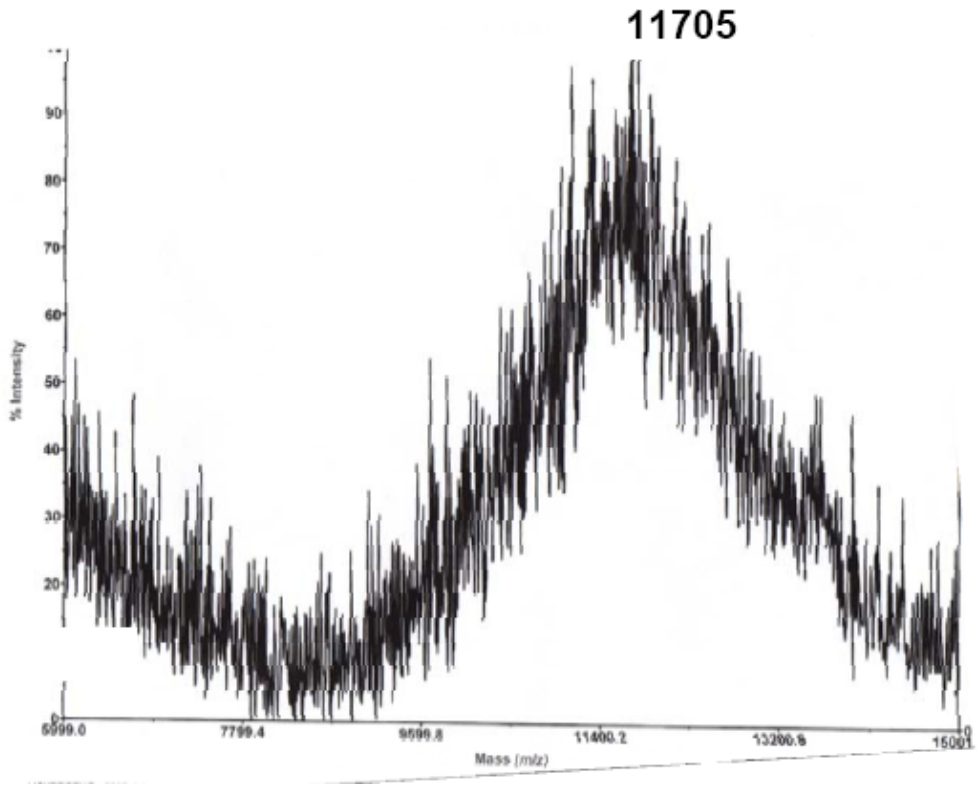


Figure 6-3. MALDI-TOF spectrum **K-PEG**. $\bar{M}_w$ = 11.7 kDa.

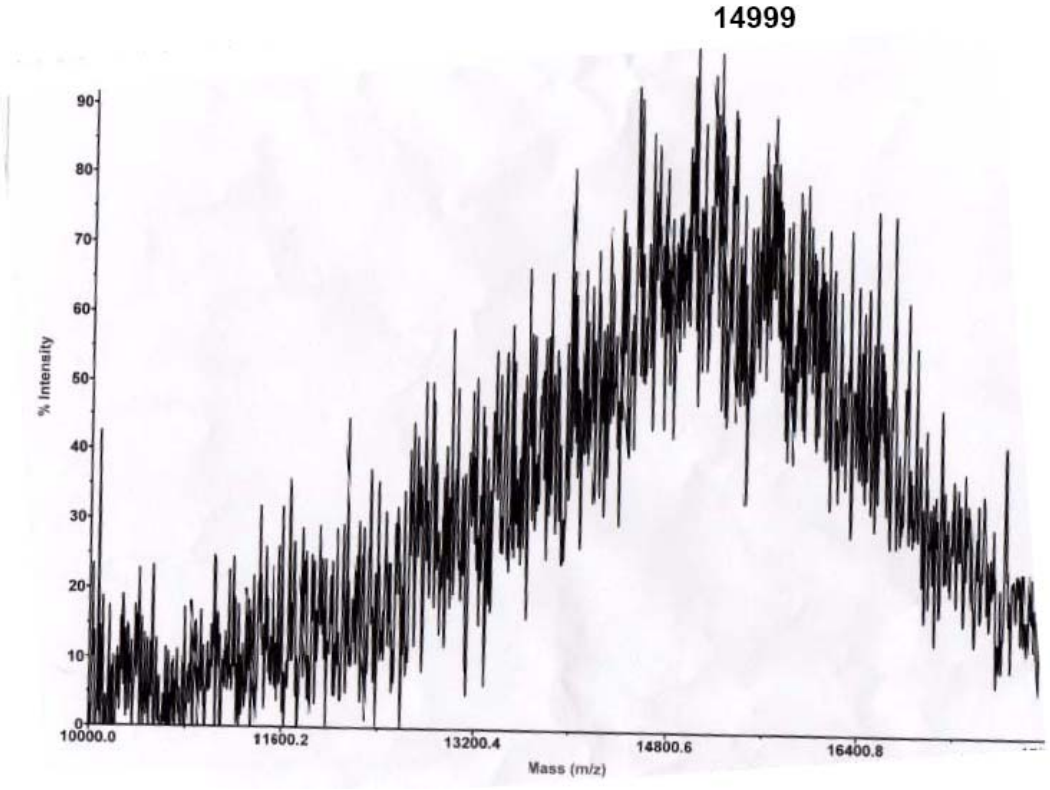


Figure 6-4. MALDI-TOF spectrum of **K-MMP-PEG**. $\bar{M}_w$ = 15.0 kDa (calculated $\bar{M}_w$ = 14.9 kDa.)

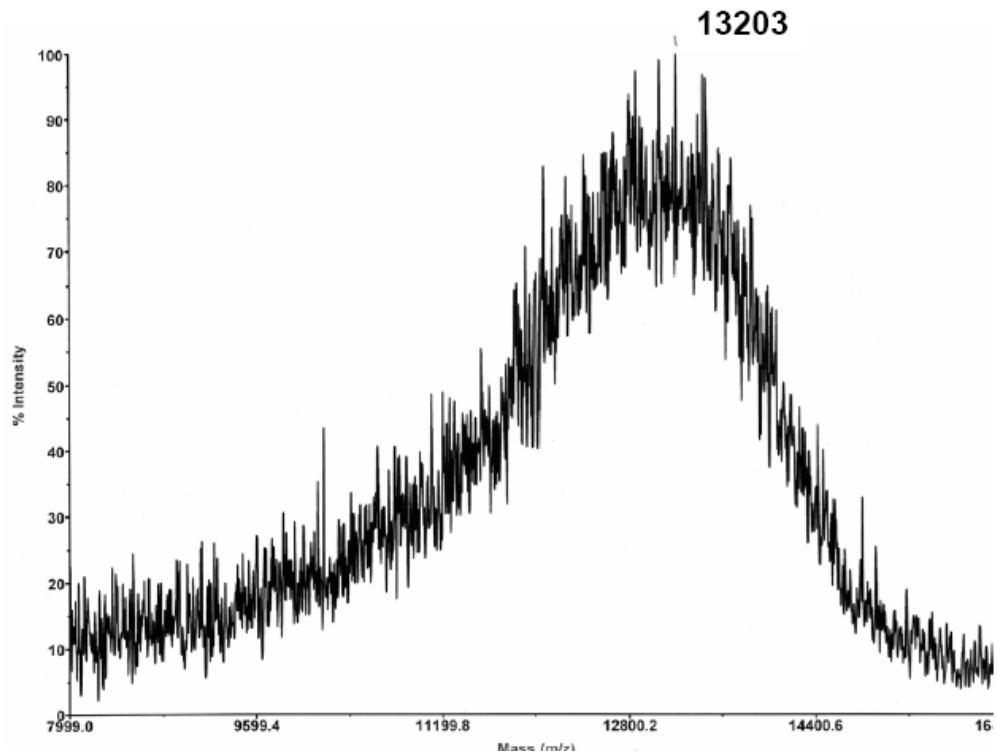


Figure 6-5. MALDI-TOF spectrum of **Q-PEG**. $\bar{M}_w$ = 13.2 kDa.

6.2.2.5 MMP-2 enzymatic reaction with AcFKGGPLSLRSG and AcFKGGPLSLRSG-PEG_{2000}.

Enzymatic reactions were conducted with these compounds at 100-1000 μM with 20 nM MMP-2 in 25 mM HEPES, 200 mM NaCl, 10 mM $CaCl_2$, pH 7.4 at 37°C. The reactions were conducted for 10 minutes and a 20 μL fraction of the samples were taken at 2, 4, 6, 8, and 10 minutes. The reaction was terminated with 30 mM EDTA. For analysis, 100 μL of 3.6mM fluorescamine in acetonitrile was added to the 20 μL sample and fluorescence was measured using the Tecan Safire$^{2\ TM}$ microplate reader, with excitation at 390 nm and emission at 490 nm. The amount of cleaved product was determined from L-leucine standards in reaction buffer. For reactions linear over the substrate concentration range, k_{cat}/K_m values were determined from the 100 μM reaction velocity, assuming [S] << K_m. For non-linear reactions, k_{cat}/K_m values were determined from a Lineweaver-Burke plot.

6.2.2.6 Hydrogel formation.

To optimize the hydrogel formulation, nondegradable hydrogels were formed with two different concentrations of the polymers, **K-PEG** and **Q-PEG**, and tTG in 200 mM MOPS, 10 mM $CaCl_2$, at pH 7.4 (gelling buffer), 372 mOsm at 37 ^oC. The hydrogels were swollen overnight in the buffer, after which the swollen gel mass was measured. The gels were then frozen and lyophilized. The masses of the dried samples were measured. The mass swelling ratio was calculated by dividing the mass of the dried sample by that of the completely swollen gel.

6.2.2.7 MMP-2 degradation of hydrogels

Hydrogels for *in vitro* degradation studies were made by combined so that the final solution was 10% (w/v) **Q-PEG** in the gelling buffer with an equivolume of solution containing 10 units/mL

tTG and 10% (w/v) of the appropriate mixture of **K-PEG** and/or **K-MMP-PEG**. Table 6-2 contains the composition of the various gels tested. Hydrogels were allowed to completely form for 1 hour at 37 ^{o}C. Once the hydrogels were formed they were swollen overnight in 25 mM HEPES, 200 mM NaCl, 10 mM $CaCl_2$, pH 7.4, after which the swollen gel mass was measured. Gels were exposed to 1.4 nM MMP-2 in swelling buffer and the residual hydrogel swollen mass was monitored for 53.5 hours.

6.2.2.8 Cell-Hydrogel constructs

Chondrocytes were isolated from cartilage of adult bovine metacarpophalangeal joint by sequential digestion, first with pronase in DMEM/F12 media with 0.1 % (v/v)gentamicin for 1.5 hrs, followed by collagenase-P digestion in DMEM-F12 media supplemented with 5% fetal bovine serum (FBS) overnight.[86] Cells were then rinsed 3 times with Hank's balanced salt solution, and counted with a hemocytometer.

Cells were suspended in 20% (w/v) **Q-PEG** in the gelling buffer to give a concentration of 50 million cells per mL. An equivalent amount of solution containing 10 units/mL tTG and 20% (w/v) **K-PEG** or **K-MMP-PEG** in the gelling buffer was added to the **Q-PEG**/cell suspension and mixed well to yield **Gel 1** and **Gel 3**. For the samples cultured in transwells, 40 μL of precursor solution were transferred to the transwell and allowed to gel at 37 ^{o}C for 30 minutes. For both culturing strategies, DMEM-F12 media was added to rinse the constructs and replaced one half hour later with media supplemented with 10% FBS, 0.1% gentamicin, and 50 μg/ mL ascorbic acid. The media was changed every two days. Constructs cultured in the transwell were collected at 1, 3,

Table 6-2. Composition of tested tTG crosslinked hydrogels.

	K-PEG (m/v)	**K-MMP-PEG (m/v)**	**Q-PEG (m/v)**
Gel 1	10	0	10
Gel 2	5	5	10
Gel 5	0	10	10

Values are concentrations in final gel solution.

and 6 weeks for biochemical analysis at 6 weeks. Live/dead analysis was conducted on 2 gels using the viability/cytotoxicity kit per the manufacturer's instructions. The gels were imaged using a Leica epifluorescent microscope (λ_{ex} = 495 nm, λ_{em} = 520 for calcein and λ_{ex} = 530 nm, λ_{em} = 620 nm for ethidium) and a SPOT RT digital camera (Diagnostic Instruments, Sterling Heights, MI).

6.2.2.9 Biochemical Assays

The cell-gel constructs were frozen and lyophilized, and then digested with 126 μg/mL papain, 5 mM cysteine, 5 mM EDTA at pH 6.0 at 60 ^{o}C overnight. Each digest was analyzed for DNA content fluorometrically (λ_{ex} = 352 nm, λ_{em} = 452 nm) using a Hoeschst 33258 as described by Kim *et al*.[246] The quantity of GAG in the constructs were analyzed spectrophotometrically with dimethylene blue complexation using chondroitin sulfate to generate a standard curve.[247] A portion of the digest was frozen and lyophilized, then acid hydrolyzed with 6 N HCl at 110 ^{o}C for 20 hours. The amount of collagen in the hydrolysate was determined with p-dimethylaminobenzaldehyde and chloramine T.[248] The quantity of collagen was calculated from the hydroxyproline amount based on a previous report that collagen consists of 10% hydroxyproline.[169]

6.2.2.10 Histology

After 6 weeks of culture, constructs of the **Gel 1** and **Gel 3** were fixed in 4% paraformaldehyde overnight. They were then transferred to 20% sucrose solution overnight followed by incubation in 30% sucrose solution for 3 days. Thereafter, they were equilibrated in a 1:1 solution of 30% sucrose: freezing media and then frozen in the solution. The samples were cut in 25 micron sections using a cryostat (Microm HM505N, Carl Zeiss). Samples were stained

with Safranin-O, Masson trichrome. Samples were imaged using a Leica microscope (brightfield) and a SPOT RT digital camera.

6.2.2.11 Coupling of peptides to cell surfaces.

Cells were isolated as described **Section 6.2.2.8** section and the **B2K** (Biotin-$(EO)_2$-FKG) and **B2Q** (Biotin-$(EO)_2$-GQQQLG) were synthesized and prepared as described in Chapter 7. One million cells were transferred to an microcentrifuge tube and pelleted by centrifugation at 1000 g for 10 min followed by removal of supernatant. The cells were suspended in 100 μL of the gelling buffer containing 1 mM of either **B2K** or **B2Q** and 0.5 units/mL tTG and incubated at 37 ^{o}C for 30 min. Control samples without tTG were also prepared. The cells were pelleted and 1% bovine serum albumin in PBS (blocking buffer) was added and incubated for 30 minutes at room temperature. The cells were pelleted and 500 μL of phycoerythrin-StreptAvidin (1:2000 dilution) in the blocking buffer was added and incubated for 30 minutes. Finally, the cells were washed and pelleted 3 times, then suspended in the analysis buffer to which 10 mL of 1nM 7-amino-actinomycin D was added. The samples were analyzed on a flow cytometer and data analyzed using Magellan software to determine the mean fluorescence intensity for each sample.

6.3 Results

6.3.1 Enzymatic evaluation of AcFKGGPLSLRSG and its mPEG amine conjugate

AcFKGGPLSLRSG is a combination of the AcFKG peptide, which is a substrate for tTG,[5] and PLSLRSG, which is a MMP sensitive peptide modified from the one reported by Turk *et al*[270]. The peptide was initially designed to be a substrate of both MMP- 2 and 9 by combing portions of the two peptides; upon examination of the specificity of the two peptides, MMP-2 is

able to cleave both. to be a substrate of To determine if the activities of either tTG and MMP-2 were altered in the hybrid peptide, the activity of AcFKGGPLSLRSG with these enzymes were examined. Table 6-1 lists the enzymatic kinetic parameters for the peptide and mPEG conjugate. The tTG specificity and the k_{cat} of AcFKGGPLSLRSG were 312 $mM^{-1}min^{-1}$ and 21.8 min^{-1}; these values were similar in magnitude but slightly lower than the corresponding values reported for AcFKG. Evaluation of the peptide and the PEG conjugate with MMP-2 resulted in specificities of 5500 $mM^{-1}min^{-1}$ and 6100 $mM^{-1}min^{-1}$, respectively. These values were similar to those reported by Turk *et al.* for a similar peptide[270].

6.3.2 Hydrogel formation

A schematic of the hydrogel formation of **Gel 1** is shown in Figure 6-6a. **Q-PEG** always comprised 50% of the polymer in the precursor solution while the concentration of **K-PEG** and **K-MMP-PEG** were varied. An image of a hydrogel represented in the schematic is shown in Figure 6-6b. Hydrogel mass swelling ratios for different formulations of **Q-PEG** and **K-PEG** were evaluated and are reported in Table 6-3. At 8% (w/v) polymer (**QPEG:KPEG**, 1:1) the hydrogels had a higher mass swelling ratio than at 20% (w/v) polymer. Increasing the amount of tTG by a factor of two decreased the swelling ratio by less than 5%. However, a 2.5 fold increase in polymer concentration at either concentration of tTG led to a decrease in the swelling ratio by at least 20%. All subsequent studies were performed using 20% (w/v) polymer and 5 units/mL tTG.

6.3.3 MMP-2 degradation of hydrogels

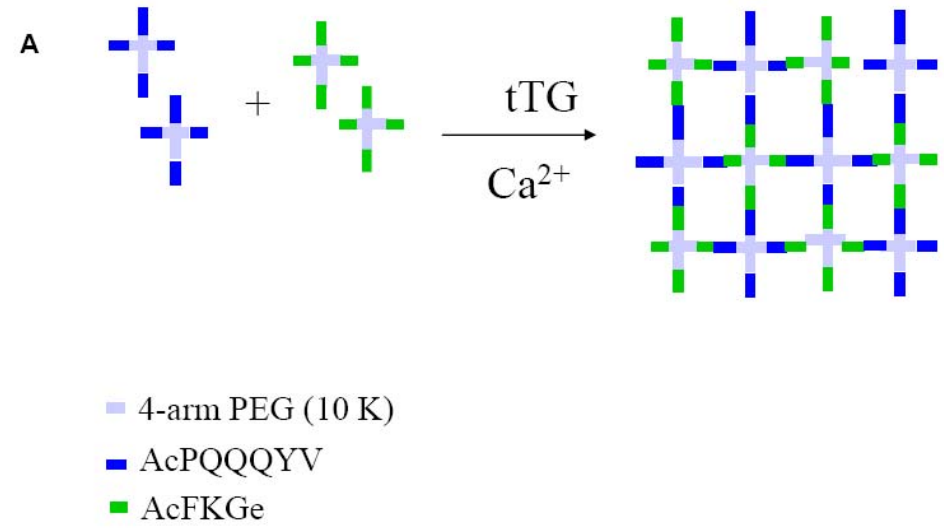
Hydrogels were formed with various concentrations **K-PEG** or/and **K-MMP-PEG** but totaling 10 % (m/v), while **Q-PEG** was always used at 10% (m/v) of the precursor gel solution. When incubated with exogenous active MMP-2, **Gel 3** increased in mass during the first 2 hours and then began to degrade in a linear fashion over the remaining 48 hours after which time no gel could be detected in the solution (Figure 6-7). The **Gel 1** hydrogel initially swelled but then maintained its mass over the course of the experiment. The **Gel 3** continued to swell over the course of the experiment, increasing its mass by 20% at the termination of the experiment, which was statistically ($p < 0.05$) greater than the mass at the start of the experiment.

6.3.4 Cell-Hydrogel constructs

Primary chondrocytes were cultured up to 6 weeks in hydrogels of various compositions (Table 6-2). Live/dead analysis indicated that cell viability was almost 100% in both constructs as little ethidium could be detected in the cells Cells in both hydrogel types had similar rounded morphology at one and three weeks. At week six, there were fewer cells visualized with calcien staining than in the previous weeks, however few ethidium-stained cells were detected. In addition, at this time point, clusters of cells could be visualized with calcien staining unlike in the previous weeks when clusters were not present. An enlarged view of one of the clusters demonstrated that there are multiple cells in each cluster.

6.3.5 Biochemical Assays

Over the course of the 6 week experiment, the dry mass of the **Gel 1** cell-hydrogel constructs significantly increased, while the **Gel 3** constructs did not have a significant mass change (Figure 6-9a). The amount of DNA in the **Gel 1** increased significantly from



B

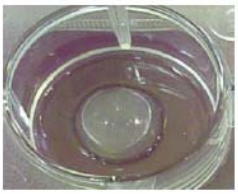


Figure 6-6. Formation of PEG hydrogel crosslinked with tTG. A. Schematic of the formation of the tTG crosslinked hydrogel with Q-PEG and K-PEG (1:1). Some hydrogels had a portion or all of the K-PEG replaced by K-MMP-PEG. B. Image of an KPEG:QPEG (1:1) hydrogel.

Table 6-3. Effects of polymer concentration and tTG concentration on mass swelling of the hydrogel formed.

Wt % polymer KPEG:QPEG (1:1)	**tTG (units/mL)**	**Mass swelling ratio (S.D.)**
8	5	13.5 (0.8)
8	10	12.9 (0.0)
20	5	9.8 (1.1)
20	10	9.7

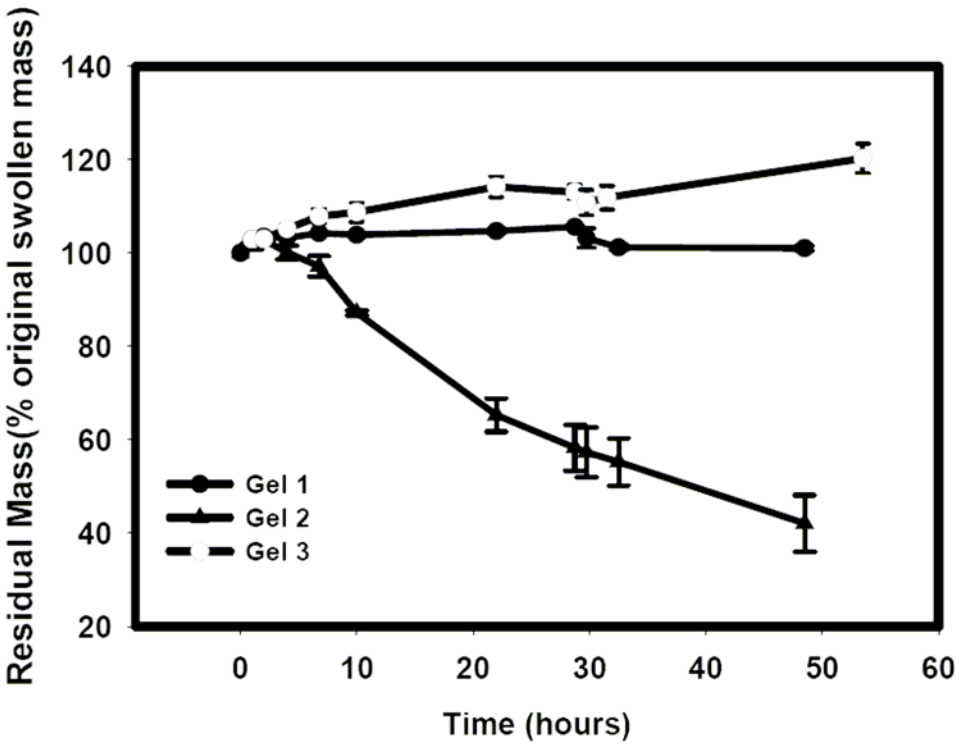


Figure 6-7. In vitro degradation of the hydrogels by exogenous active MMP-2. All hydrogels contained 50% Q-PEG. The remaining 50% was composed of K-MMP-PEG or/and K-PEG.

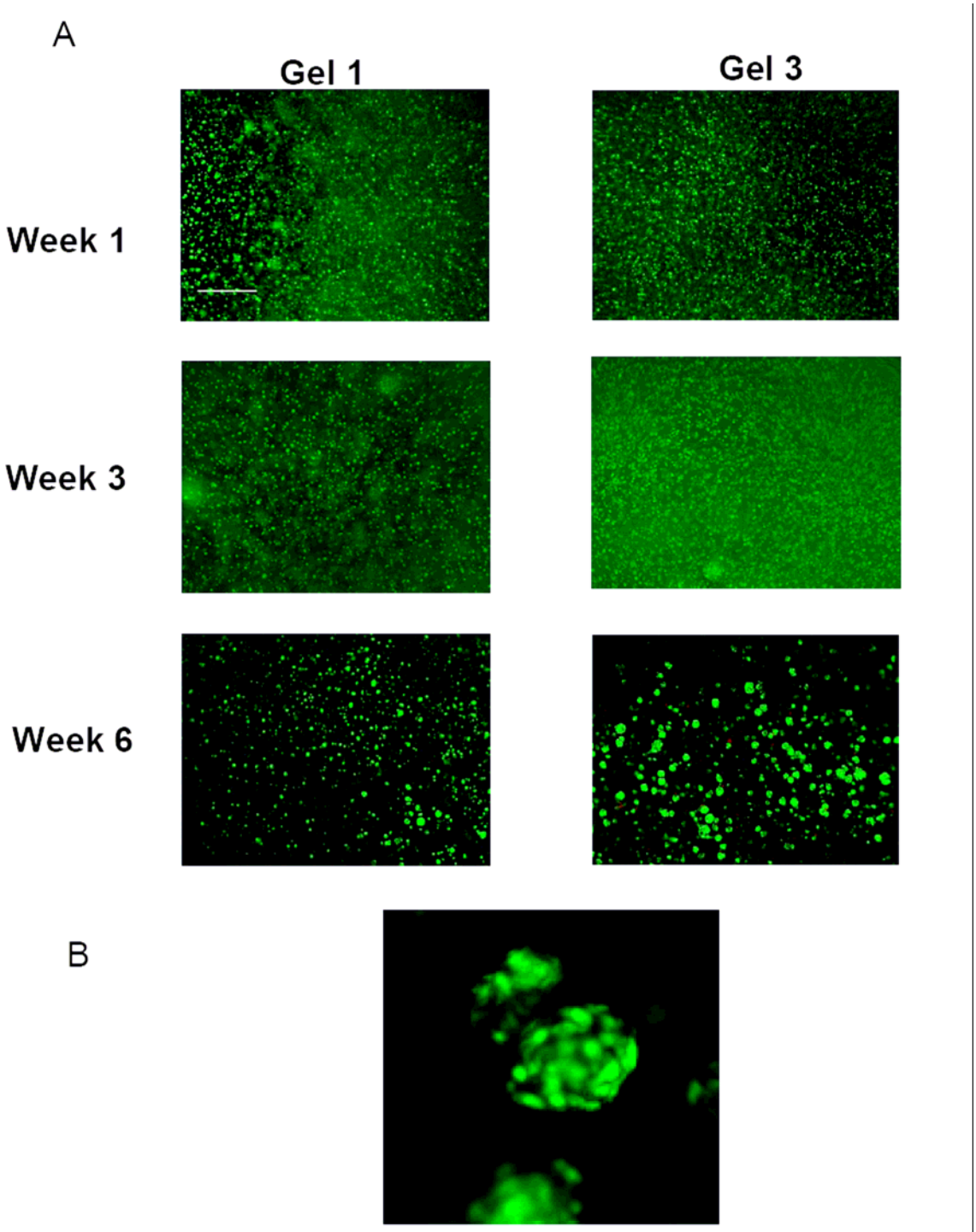


Figure 6-8. Live/dead staining of chondrocytes in hydrogel constructs. A. Live/dead staining of cells in constructs at week 1, 3, and 6. 5X magnification. B. Enlarged image of cell cluster in the **Gel 3** constructs at week 6 demonstrating that each cluster has multiple cells.

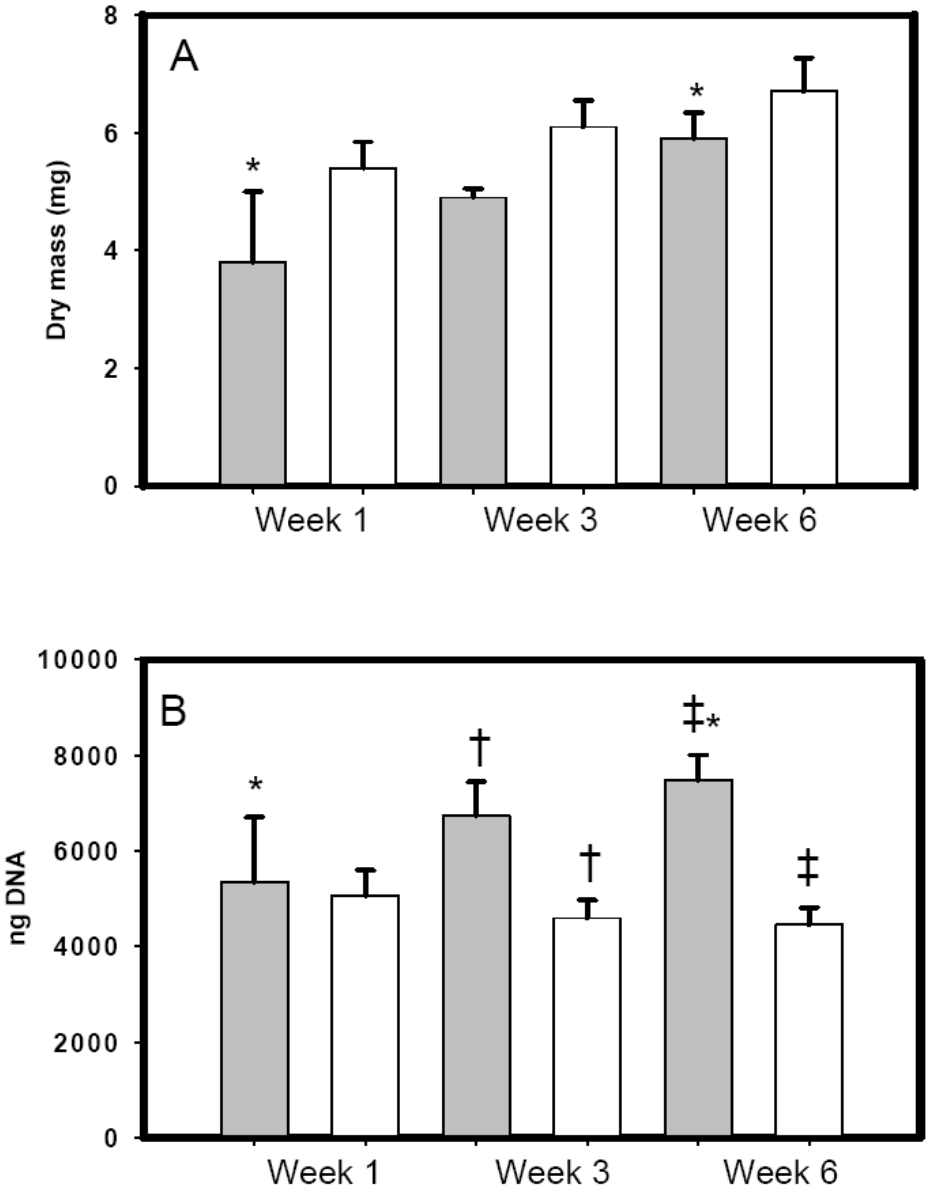


Figure 6-9. Hydrogel mass and cell proliferation during the 6-week experiment. A. Graph of dry mass during 6 week culture of chondrocytes. *, $p<0.05$ when 10% (m/v) **K-PEG** sample is compared between week 1 and week 6. b. Graph quantifying DNA in samples, *, $p<0.05$ comparing week 1 to week 6 of **Gel 1**; †, $p<0.05$ comparing **Gel 3** to **Gel 1** hydrogels at week 3; ‡, $p<0.05$, when comparing **Gel 3** to **Gel 1** hydrogels at week 6. Gray bars represent **Gel 1** samples; white bars represent **Gel 3** samples.

week 1 to week 6, however this was not observed for the **Gel 3** constructs (Figure 6-9b). At weeks 3 and 6, there was significantly more DNA in the **Gel 1** than in the

The amount of GAG produced per cell increased during the 6 weeks of the experiment in both treatments; however, there was no significant difference in the amount produced per cell between the 2 types of constructs (Figure 6-10a). Finally, collagen production was statistically the similar throughout 6 weeks for both types of constructs (Figure 6-10b).

6.3.6 Histology

At 6 weeks, the constructs were examined histologically with Safranin-O for negatively charged GAG and Masson trichrome for collagen. (Figure 6-11) Upon examination, both constructs were positive for GAG, located in the cell clusters. While the **Gel 1** constructs had cell clusters throughout, the cell clusters were localized to the surface of the **Gel 3** constructions. The Masson trichrome stain detected collagen in the same region as GAG, localized around cell clusters.

6.3.7 Coupling of peptide to cell surfaces

Chondrocytes' cell membranes may contain proteins that are substrates of tTG which potentially could be crosslinked to the gel components. To determine if proteins in the cell membranes of chondrocytes would be crosslinked to the peptide substrates in the presence of tTG, cells were separately incubated with biotinylated peptides, **B2K** and **B2Q** and analyzed via flow cytometry. The frequency histograms demonstrated that **B2K** and **B2Q** were coupled to the cell membrane of chondrocytes in the presence of tTG as evidenced by the

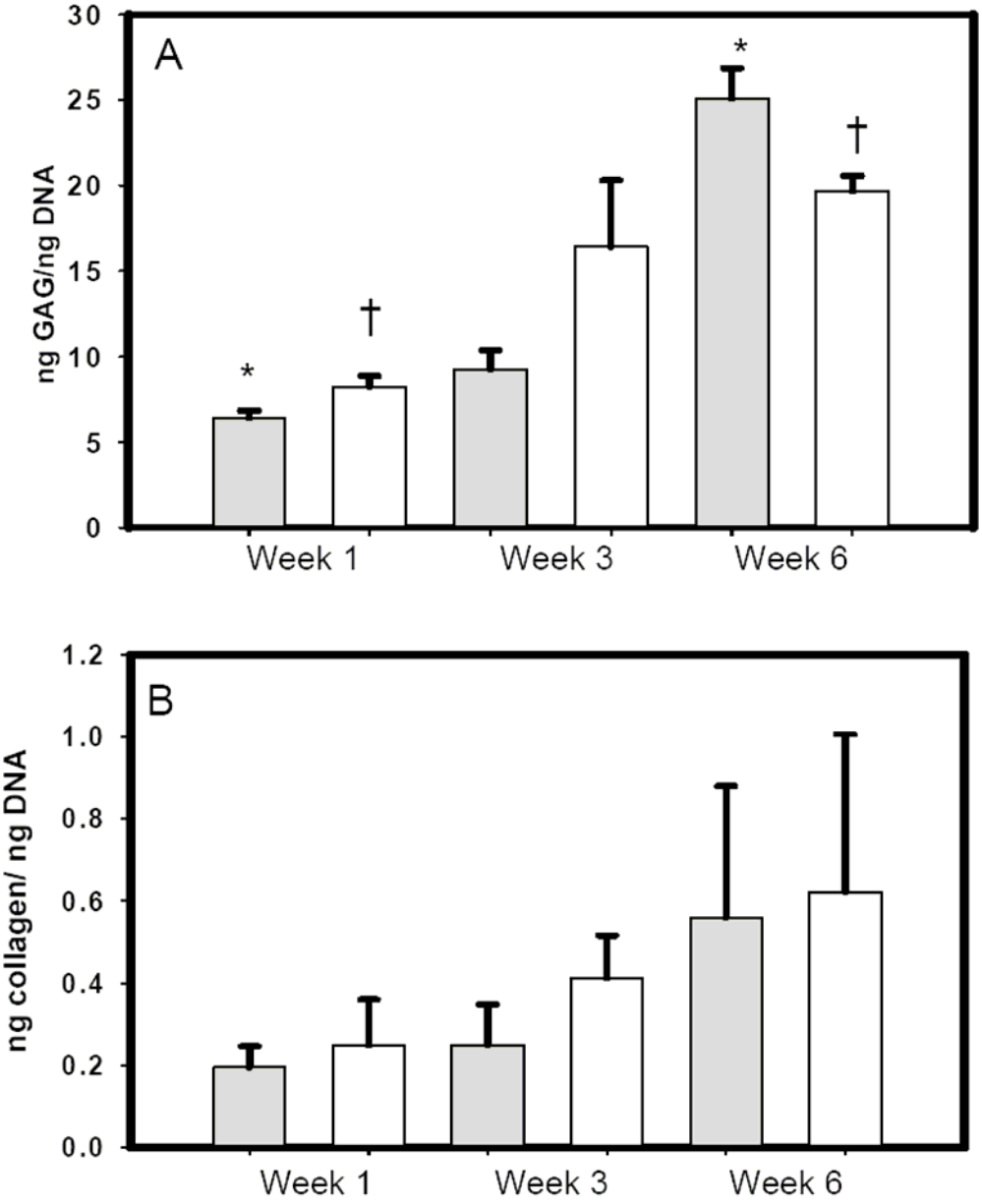


Figure 6-10. Matrix production in cell-hydrogel constructs. A. Graph quantifying the amount of GAGs in hydrogel samples for the 6 week experiment. *, $p<0.05$ comparing **Gel 1** week 1 to week 6; †, $p<0.05$ comparing week 1 and week 6 of **Gel 3**. B. Graph quantifying collagen production in hydrogels. Gray bars represents **Gel 1** samples; white bars represents **Gel 3** hydrogel samples.

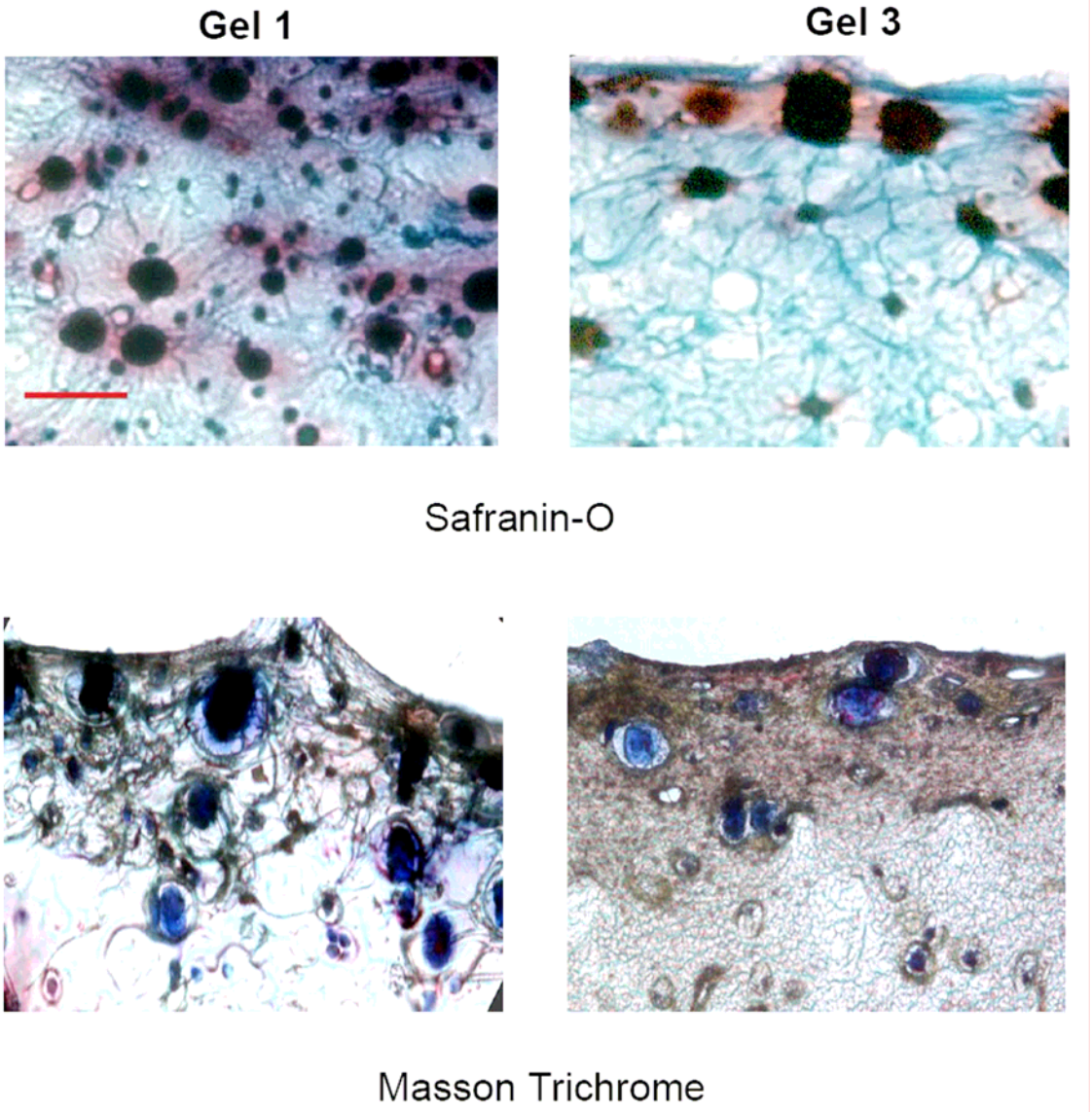


Figure 6-11. Histology of **Gel 1** and **Gel 2** hydrogels. Hydrogels stained with Safranin-O for GAG (top images) and with Masson trichome for collagen (bottom images). Bar represents 400 μm.

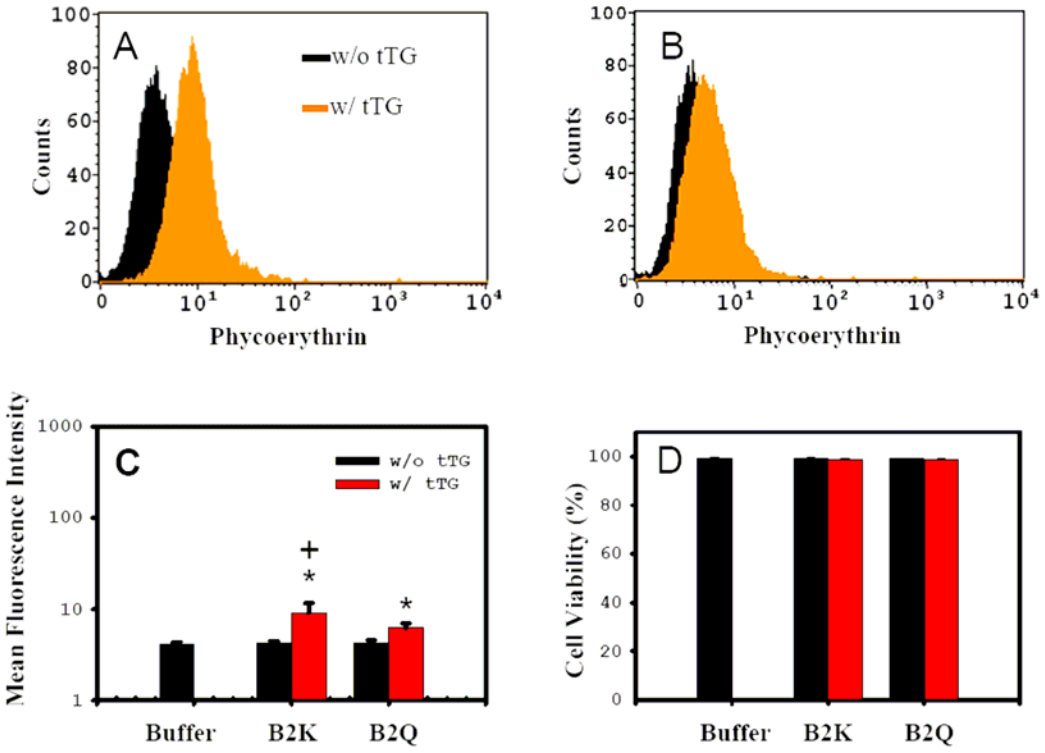


Figure 6-12. Flow cytometry analysis of peptide coupling to cell surfaces. A. Frequency histogram of cells incubated with **B2K**. B. Frequency histogram of cells incubated with **B2Q**. C. Graph of mean fluorescence intensity for each treatment. $p < 0.05$; *, when compared to treatments without tTG or to buffer only $p<0.05$, +, when compared to **B2Q** with tTG.

increase in detected fluorescence intensity as compared to without tTG (Figure 6-12a and b). The mean intensity of the fluorescence from the cells was statistically greater after incubation with **B2K** with tTG than for **B2Q** with tTG ($p < 0.05$) (Figure 6-12c). The mean fluorescence intensity of the cells when incubated with either peptide treatments in the presence of tTG was statistically greater than incubation with buffer alone or **B2K** or **B2Q** without tTG. Finally, the cells remained viable in the presence of the peptides and tTG as determined by the exclusion of 7 amino-actinomycin D, a compound that binds to DNA when the cell membrane is compromised (Figure 6-12d).

6.4 Discussion

When cartilage is damaged by a traumatic event, the cellular response is insufficient to repair the damaged tissue and medical interventions are necessary. However, current medical treatments for chondral defects do not reliably regenerate cartilage because their success is dependent on a number of factors, most of which are not fully understood. Therefore, some defects do not heal properly and patients go on to develop osteoarthritis, a degenerative disease of cartilage. It has been suggested that hydrogels could be an alternative treatment for chondral defects because the precursor gel solution can fill and take the shape of the defect and then the solution can be gelled *in situ*.[80] More importantly, these hydrogels could be used as scaffolds that support chondrocytes as they regenerate the native matrix. However, hydrogels have fallen short in 2 ways: adhesion to native cartilage and cell directed degradation of the hydrogel.

Tissue transglutaminase may offer a method of facilitating hydrogel formation and crosslinking of hydrogels to cartilage. tTG is an widely distributed enzyme that is known to have substrates in cartilage such as collagen II[196, 244], fibronectin[245] and osteonectin[244, 249]. Jurgensen *et al.* demonstrated that tTG could be used to bond two pieces of cartilage, achieving adhesion by forming covalent crosslinks between its native substrates in cartilage[4]. Recently, in our lab, we developed synthetic peptide substrates of tTG, which were used to modify PEG polymers and then these polymers were crosslinked to form a hydrogel[5]. These peptides and their PEG conjugates were also coupled to the cartilage[264]. Taken together, these results suggest that tTG can be used to form covalent crosslinks between cartilage and any hydrogel that contains polymers modified with peptide substrates of tTG. Therefore, we investigated the ability of tTG crosslinked hydrogels to sustain and promote *in vitro* culture of chondrocytes. To provide potential cell-directed degradability to these hydrogels, an MMP sensitive peptide sequence was combined with the lysine peptide.

The mass swelling ratios of the hydrogels formed by **K-PEG** and **Q-PEG** in the presence of tTG were influenced more by changes in the polymer concentration than by changes in the enzyme concentration. Increasing the polymer content by a factor of 2.5 decreased the swelling ratio 20-25%, while a doubling of the enzyme concentration decreased the swelling ratio by only 7-13%. It has been suggested that altering enzyme concentration can not effectively reduce hydrogel swelling, because an increase in the enzyme concentration results in more rapid gelation, which in turn localizes the enzyme and prohibits it from performing further crosslinking.[222, 271] Sun *et al*. demonstrated that there is an optimal amount of transglutaminase that would give favorable gel properties; however, at larger concentrations, the elastic modulus of the gel will be lower[271]. Other than increasing transglutaminase concnetration, hydrogels

crosslinked by this mechanism can be improved by changing the concentration of the polymer substrates[221] or by increasing the number of arms or crosslinking points on the polymer. Park *et al.* demonstrated that swelling decreased in a gel formed with 8-arm PEG as compared to a 4-arm PEG[98].

The hydrogels formed with K-PEG and Q-PEG were not degradable and therefore the peptide AcFKGGPLSLRSG, containing an MMP sensitive sequence along with the lysine substrate of tTG, was evaluated as a possible substitute for the AcFKG peptide on PEG in order to incorporate cell-directed degradability. The MMP-sensitive sequence, PLS↓LRSG[270], was selected because chondrocytes constitutively produce MMP-2.[267-269] Enzymatic reactions demonstrated that this conjoined peptide remained active towards tTG and MMP-2, since enzymatic values were very similar to the individual parent peptides. It is known that tTG is not as discriminatory with respect to the lysine substrate as with the glutamine substrate, and that any secondary interactions of the enzyme with residues would occur with ones that are adjacent to the lysine[173]. It was anticipated that the tTG kinetic values would not be greatly influenced, since the additional residues were several residues away from the lysine. From previous coupling studies of these peptides to cartilage[264], it was expected that the addition of PEG would also not adversely impact the kinetic values of tTG. For MMP-2, the 3 amino acid residues adjacent to the cleavage site on the C- terminus are of most importance[272] and since the modification with the lysine substrate of tTG was distant from the cleavage site on the N-terminus, this was likely the reason there was no impact on MMP-2 kinetic values. When a PEG polymer was added to AcFKGGPLSLRSG, the MMP-2 specificity for the peptide increased; this was previously observed with a 10 kDa PEG[273].

The *in vitro* degradation with exogenous MMP-2 yielded expected results with the **Gel 3** and **Gel 2** hydrogels, exhibiting complete degradation or the lack thereof, respectively. However, since the matrix of the 5% **K-MMP-PEG** gel still contained 5% **KPEG** to form its crosslinking junctions, this gel swelled significantly to 20% of its original swollen mass. Preliminary experiments demonstrated that this swelling led to cell death, and hence **Gel 2** were not tested with cells.

To prevent excessive hydrogel swelling, the constructs were cultured in transwells with a 3 μm pore size that only permitted nutrients to pass through. The cells were initially viable in this configuration; however decreased cell viability at 6 weeks in both types of constructs was likely due to swelling of the hydrogel during later time points. As observed in the coupling of **B2K** and **B2Q** peptides to cell surfaces with tTG, it is possible that the cells were crosslinked into the hydrogel networks and when the hydrogels swelled, cell lysis occurred. Lin *et al.*, during studies of tTG specifically labeling cells expressing a glutamine substrate, also observed that excess reagents or prolonged reaction time caused tTG to label other proteins on the cell surface[228]. This may explain the lack of ethidium staining of dead cells; if the cell membrane was compromised and/or ruptured, the DNA could potentially be removed from the gel. Although there were fewer viable cells at 6 weeks, the clusters of cells that were viable were larger than in previous weeks, indicating that the cells that did survive also proliferated. While the **Gel 1** hydrogels increased in mass and cell number (as indicated by the increase in DNA), the **Gel 3** hydrogels experienced a decrease in cell number. This could be due to cell migration out of the gel and onto the membrane of the transwell. These chondrocytes were not included with those in the gel for DNA analysis. In preliminary experiments where the gels were cultured

unconfined, we observed that chondrocytes in **Gel 3** would change morphology, become spindle-shaped and migrate out of the hydrogel. It is also hypothesized that this layer of cells could either have prevented nutrient flow through the membrane, or perhaps depleted the media of nutrients as it flowed through the membrane. Both of these 2 possibilities could have contributed to the reduction in cell viability in **Gel 3**.

Glycosaminoglycans are a marker of chondrocyte phenotype since they must produce these molecules to protect cartilage[85]. The cultured chondrocytes produced GAG during the 6 week experiments. This indicates that the cells were likely chondrocytic in phenotype which was also verified by the histology. However, during the same period, collagen production was not statistically increased over the 6 weeks. It is known that proliferation and matrix production are adversely affected by static conditions[274]. Therefore our culture conditions could have inhibited matrix production.

6.5 Conclusion

Tissue transglutaminase has been demonstrated to create cartilage-cartilage bonds as well as being able to crosslink PEG modified with peptide substrates of tTG to cartilage. This demonstrates the possibility of using tTG for generating adhesive bonds between cartilage and tissue engineered scaffolds that contain substrates of tTG for cartilage regeneration. This study demonstrates the first use of a PEG hydrogel crosslinked with tTG for *in vitro* chondrocyte culture. These gels were able to culture chondrocytes in a limited fashion. It was determined that, under these conditions, the constructs with the MMP sensitive site did not work as well as the constructs without it, in part because the hydrogel matrix was degraded too rapidly, allowing

chondrocytes to migrate out. A less reactive MMP sensitive sequence may be beneficial. Culturing the chondrocytes in transwells was done to minimize gel swelling and the detrimental effects of swelling on the entrapped chondrocytes. However, even with these conditions the gels still had some swelling, which caused cell lysis. tTG crosslinking of hydrogels may be more feasible if used with a polymer that does not swell as much as PEG.

Chapter 7

Facile Coupling of Synthetic Peptides and Peptide-polymer Conjugates to Cartilage via TransglutaminaseEnzyme

7.1 Introduction

Strategies for chemically coupling natural or synthetic molecules to biological surfaces are important tools for drug delivery, tissue repair, and fixation of tissue engineered scaffolds for tissue regeneration. Several methods are capable of attenuating, inhibiting or promoting interactions between tissue surfaces as well as between the cells and extracellular matrix (ECM) proteins that comprise them. Electrostatic interactions have been employed by Elbert *et al.* in the form of poly-L-lysine-graft-(poly(ethylene glycol) polymers that chemisorb to proteins on tissue surfaces, and this approach was explored as a strategy to minimize postsurgical adhesions[275]. Winblade *et al*. employed phenylboronic acid modified polymers[131, 132] to provide reversible covalent crosslinks to *cis*-diols in sugar residues of glycoproteins and polysaccharides. Layer by layer (LbL) assembly of polyelectrolytes has been used to apply polymer coatings onto model biological surfaces[149], the surface of blood vessels,[153] and pancreatic islets[152].

Specific functional groups found in ECM proteins have been exploited for covalent surface modification strategies. For example, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS) have been used extensively to couple macromolecules containing carboxylic acids to protein amines[113, 276, 277]. Amine-reactive PEG diisocyanates have been used to modify pancreatic islets in order to provide immunoprotection[127, 276], to create a barrier to platelet adhesion on damaged arteries[125, 126], and on preclotted Dacron and other model

biological surfaces[128]. Aldehyde modified chondroitin sulfate, which also reacts with tissue amines, has been used as a tissue adhesive in both the cornea[133, 278] and cartilage[134, 135]. Photochemical oxidation of native tyrosine residues in collagen II has been used to improve the integration of photopolymerized hydrogels with cartilage[141].

In contrast to chemical or photochemical approaches, we seek to develop a general strategy for tissue surface modification that relies instead on biological enzyme mediated crosslinking reactions. Transglutaminases (TG) are calcium-dependent enzymes that catalyze crosslinking between lysine and glutamine residues to form ε-(γ-glutaminyl) lysine isopeptide bonds[2]. There is growing interest in the use of transglutaminase enzymes for tissue repair and reconstruction. Calcium-independent microbial TG[168] has been used to develop gelatin hydrogels for biomedical adhesives[146] as well as for *in vitro* expansion of cells[279]. Factor XIII, the circulatory form of TG, has been used to form fibrin matrices for *in vitro* and *in vivo* studies of neurite growth[213], angiogenesis[215, 217], and cartilage regeneration[107, 262, 280]. The incorporation of bioactive peptides and proteins into these matrices was achieved by including a Factor XIII reactive peptide domain within the molecule[219, 223, 241]. Synthetic polymers have also been modified with Factor XIII substrate peptides, which were then crosslinked by the enzyme into a hydrogel[220]. A transglutaminase enzyme found in many connective tissues and often referred to as tissue transglutaminase (tTG) was used by Sperinde and Griffith to form hydrogels through crosslinking of glutamine modified poly(ethylene glycol) (PEG) polymers and a lysine containing polyaminoacid[221]. Hu *et al.* subsequently employed rationally designed peptide substrates of tTG to modify PEG polymers to form an adhesive hydrogel[5, 148], tTG was recently used to crosslink an elastin-like polymer (ELP) coacervate that contained lysine and glutamine

residues[96]. Finally, tTG has also been used to couple biomolecules to insoluble peptide assemblies[224], and was used to enhance cell adhesion and spreading on collagen matrices and synthetic polymers coated with fibronectin[225, 226].

tTG has attractive features for enzymatic coupling of molecules to tissue surfaces because it operates under mild physiologic conditions and has a number of known protein substrates found in the ECM[3, 186] and on cell surfaces[228]. In this paper, we explore the use of (tTG) enzyme as a simple and biocompatible method for coupling synthetic molecules to tissue surfaces, using cartilage as a model tissue. We chose cartilage for this initial study because it is known to contain tTG[193, 196] as well as several ECM substrates of tTG[200, 244, 249, 263]. Furthermore, Jurgensen *et al.* previously demonstrated the development of adhesive bonds between cartilage surfaces brought into contact in the presence of tTG, suggesting that protein substrates of tTG are exposed at tissue surfaces and available for reaction with soluble enzyme[4]. Short synthetic peptides containing either lysine or glutamine residues[5] and conjugates of these peptides with polymers were bound to cartilage surfaces through the action of tTG enzyme. Several cartilage ECM components were identified as possible substrates for the reaction, including collagen II, fibronectin, osteopontin and osteonectin. Given the existence of macromolecular tTG substrates in many tissues, this facile approach to tissue surface modification should be broadly applicable to a variety of tissues for localization of therapeutic agents and for enhancing (or inhibiting) adhesion at tissue-tissue and tissue-device interfaces.

7.2 Materials and Methods

7.2.1 Materials

Rink amide resin was purchased from Anaspec, San Jose, CA. Fmoc-amino acids were purchased from Peptides International, Louisville, KY. O-(N-Fmoc-3-aminopropyl)-O'-(N-diglycolyl-3-aminopropyl)-diethyleneglycol (MW 558.6) (Fmoc-NH-$(EO)_2$-COOH) was purchased from Novabiochem, San Diego, CA and Fmoc-NH-PEG-NHS ($\bar{M}_w$ = 3.4 kDa) was purchased from Nektar Therapeutics, Inc, Huntsville, AL. Biotin, guinea pig liver tissue transglutaminase (tTG), chondroitinase ABC, fibronectin, and peroxidase conjugated ExtrAvidin were purchased from Sigma, St. Louis, MO. Adult bovine metacarpophalangeal joints were purchased from a local slaughter house. Fluorescein and peroxidase conjugated anti-biotin antibodies and the DAB substrate kit were purchased from Vector Labs, Burlingame, CA. Anti-Collagen II and Texas Red conjugated donkey anti-rabbit antibodies were purchased from Abcam, Cambridge, MA. Collagen II was purchased from Chondrex, Redmond, WA, osteonectin from Biodesign, Saco, ME, and osteopontin from R&D Systems, Minneapolis, MN. ECL Plus western blotting detection reagents were purchased from GE Healthcare Biosciences, Piscataway, NJ.

7.2.2 Materials

7.2.2.1 Synthesis of peptide conjugates.

The molecules synthesized for use in this study and their abbreviations are listed in Table 7-1. The peptide portions of the molecules were synthesized on a Rink amide resin (1 g, 0.45 mmol/g) using standard Fmoc solid phase peptide synthesis. Each

Table 7-1. Structures, abbreviations and mass analyses for synthetic peptide conjugates used in this study.

Structure of peptide-PEG	**Abbreviation**	**Expected Mass* (Da)**	**Observed Mass* (Da)**
Biotin-$(EO)_2$-FKG-NH_2	B2K	894.08	893.49
Biotin-$(EO)_2$-GQQQLG-NH_2	B2Q	1173.34	1172.57
Biotin-$(EO)_2$-FOG-NH_2	B2O	880.05	879.50
Biotin-$(EO)_2$-GNNNLG-NH_2	B2N	1131.26	1131.95
Biotin-PEG-G-NH_2	B72	3600	3600
Biotin-PEG-FKG-NH_2	B72K	3800	3800
Biotin-PEG-GQQQLG-NH_2	B72Q	4100	4100

*values shown for B72, B72K and B72Q are average mass values for polydisperse molecules.

coupling reaction was performed for 3 hours with a 10 min pre-activation of four equivalents of Fmoc-amino acid: benzotriazole-1-yl-oxy- tris(dimethylamino)-phosphoniumhexafluorophosphate (BOP): *N*-hydroxybenzotriazole (HOBt): diisopropylethylamine (DIEA) (1:1:1:1) at room temperature. The Fmoc was removed by 20% piperidine in N-methylpyrrolidinone (NMP) for 1 hour. After Fmoc removal from the final amino acid, either Fmoc-NH-$(EO)_2$-COOH or Fmoc-NH-PEG-NHS was added. Fmoc-NH-$(EO)_2$-COOH was treated as Fmoc-amino acid and used at 2 equivalents. The Fmoc-NH-PEG-NHS was used at 2 equivalents and combined with DIEA (1:2) in dichloromethane (DCM), added to the resin and reacted for 2 days. The unreacted peptide on the resin was acetylated with the use of 4 equivalents of acetic anhydride. The Fmoc was then removed and biotin was coupled using 4 equivalents by the previously described procedure for coupling Fmoc-amino acids. At the completion of the reaction, the resin was washed with NMP, DCM, and methanol (MeOH), two times each. The resin was dried under vacuum and then treated with 95% trifluoroacetic acid (TFA), 2.5% water, and 2.5% triisopropylsilane for 3 hours. The crude product was obtained by concentration of the TFA solution and addition of diethyl ether. Purification was performed using semi-preparative RP-HPLC and the masses were confirmed using MALDI-TOF MS analysis on a PE Voyager DE-Pro MALDI-TOF Mass Spectrometer (Perspective Biosystems, MA). The peptide-PEG conjugates were then frozen and lyophilized (Figure 7-1 to 7-7).

7.2.2.2 Cartilage isolation and preparation.

For the coupling of peptide conjugates to cartilage sections, osteochondral plugs were extracted from adult bovine metacarpophalangeal joints using a hole punch with a

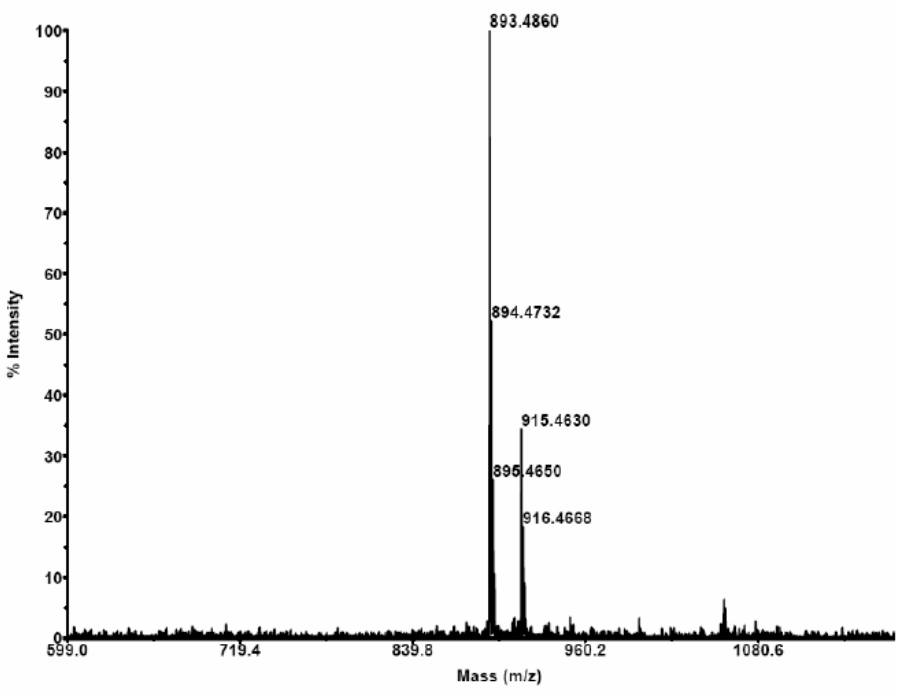


Figure 7-1. MALDI-TOF mass spectrum B2K.893.49 $(M)^+$; 915.46 $(M+Na)^+$.

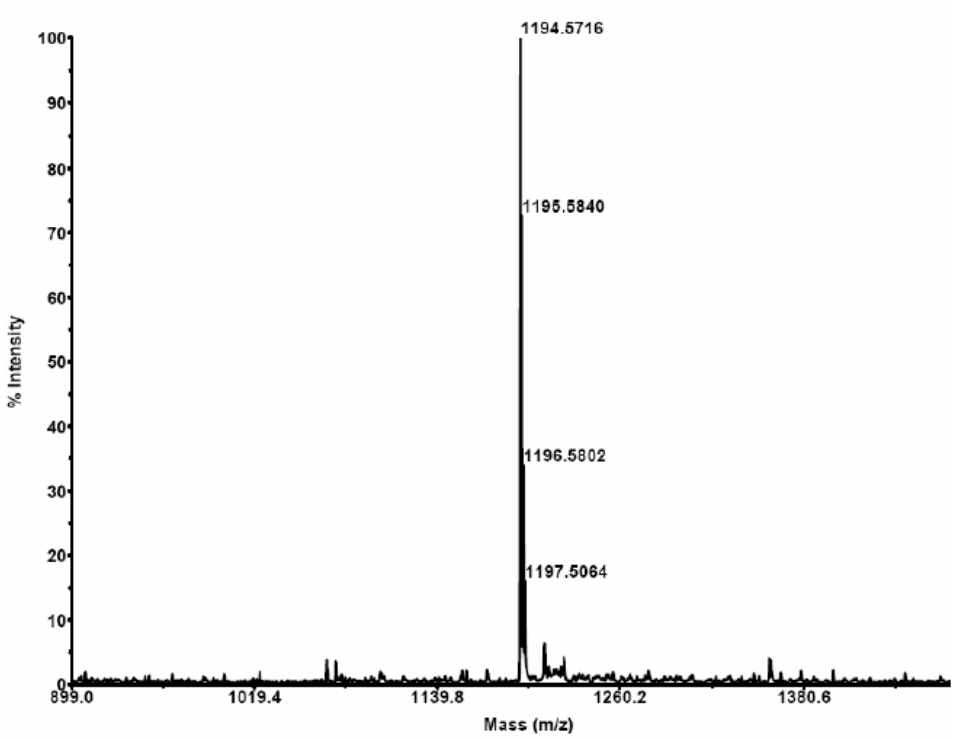


Figure 7-2. MALDI-TOF mass spectrum B2Q.1194.57 $(M+Na)^+$.

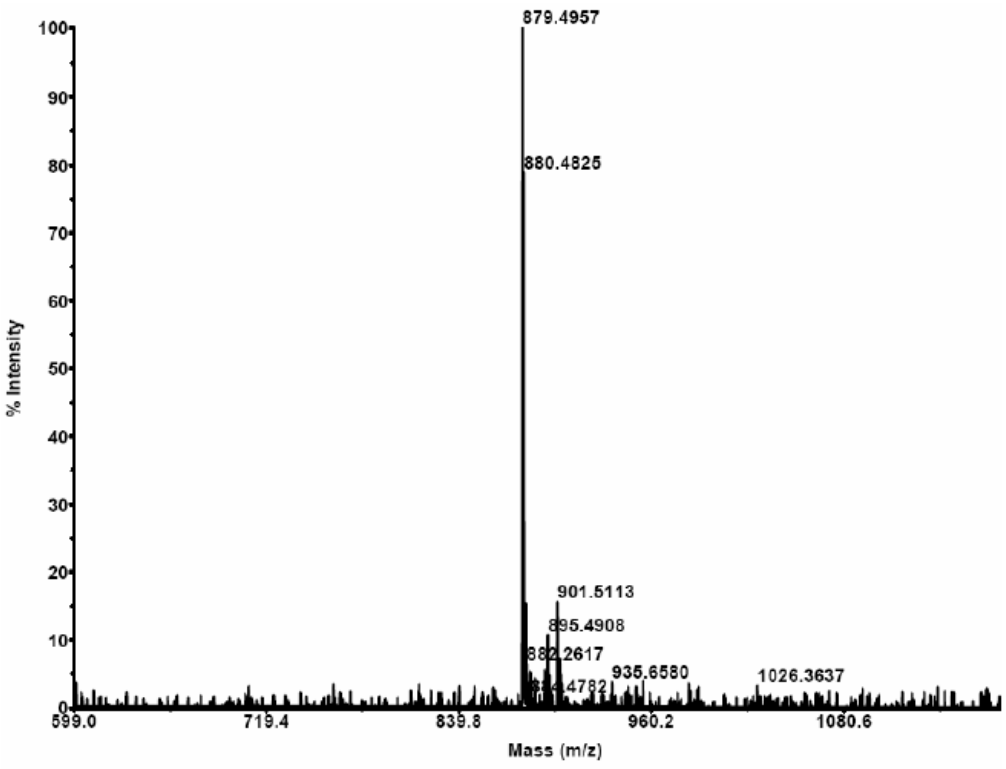


Figure 7-3. MALDI-TOF mass spectrum B2O.879.50 $(M)^+$; 901.51 $(M+Na)^+$.

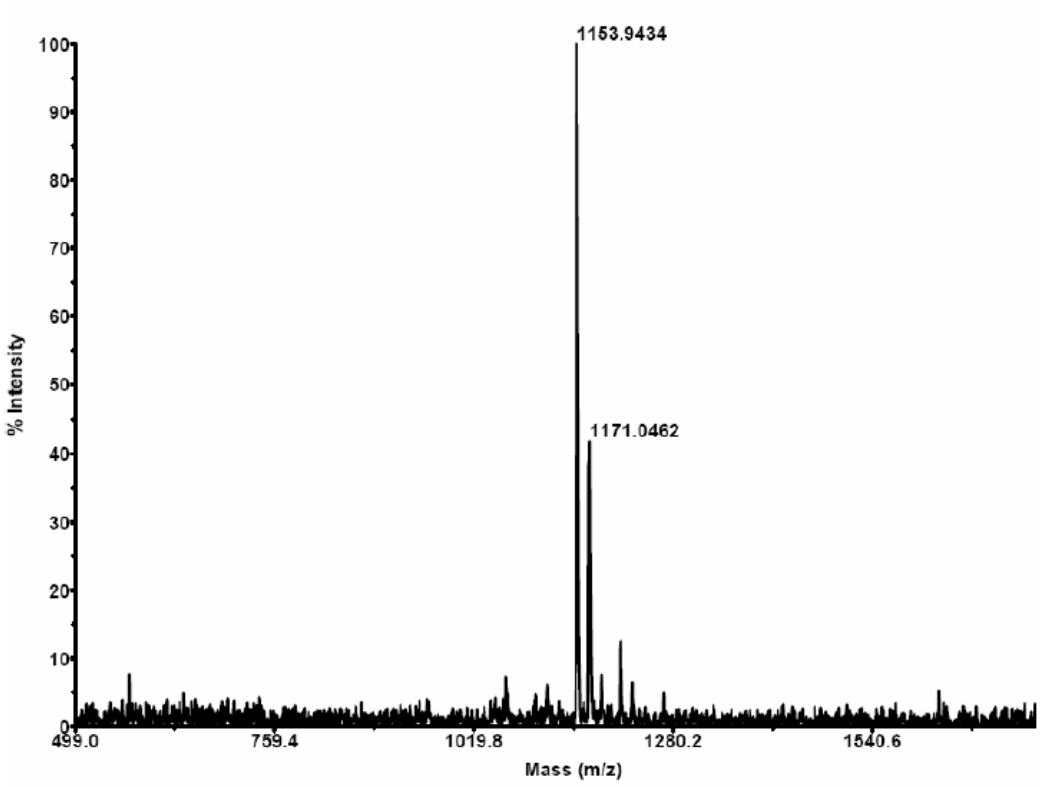


Figure 7-4. MALDI-TOF mass spectrum B2N.1153.95 $(M+Na)^+$; 1171.05 $(M+K)^+$.

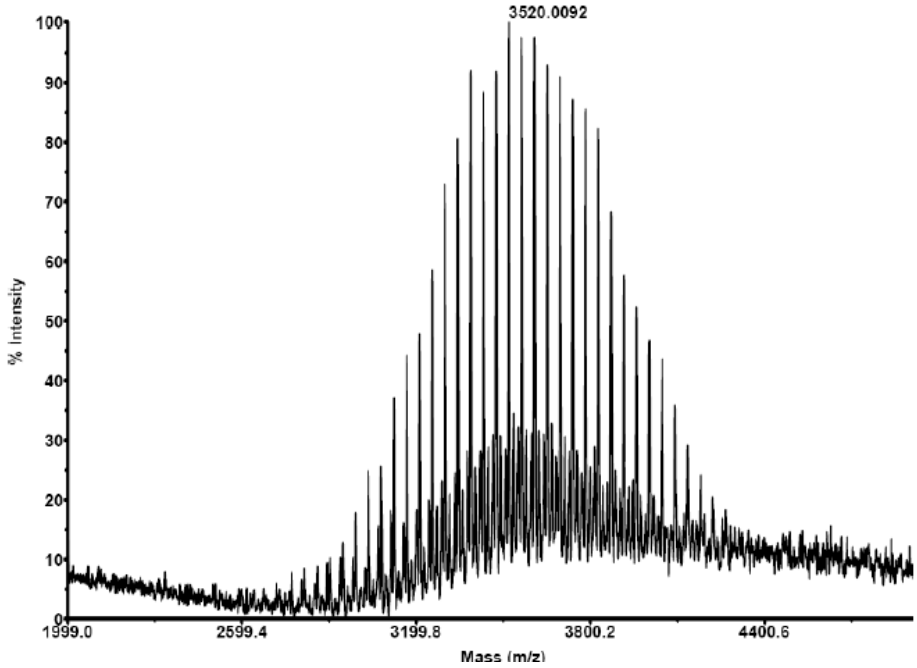


Figure 7-5. MALDI-TOF mass spectrum B72.

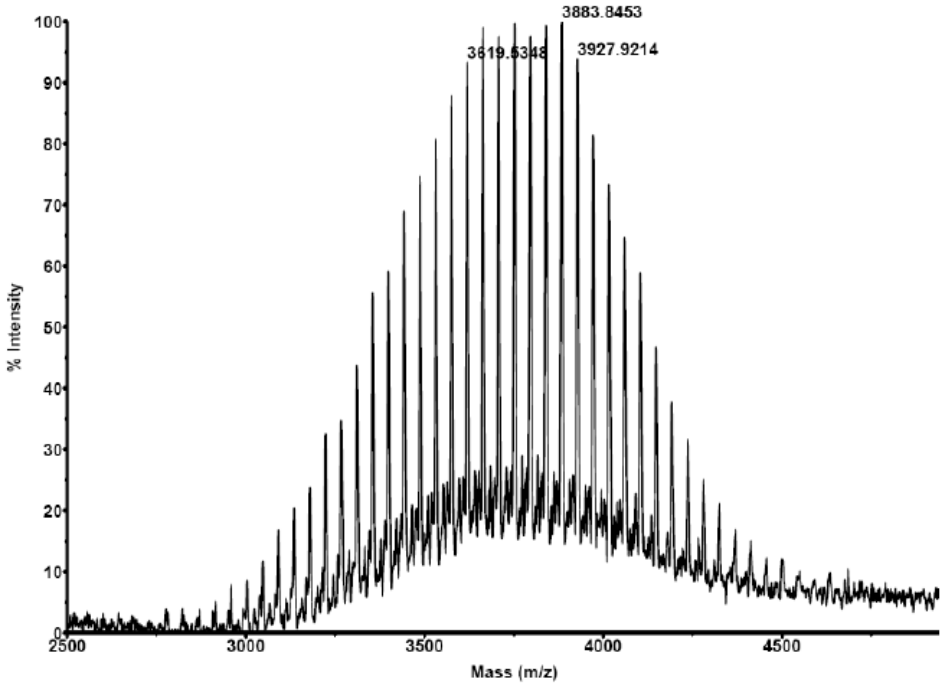


Figure 7-6. MALDI-TOF mass spectrum B72K.

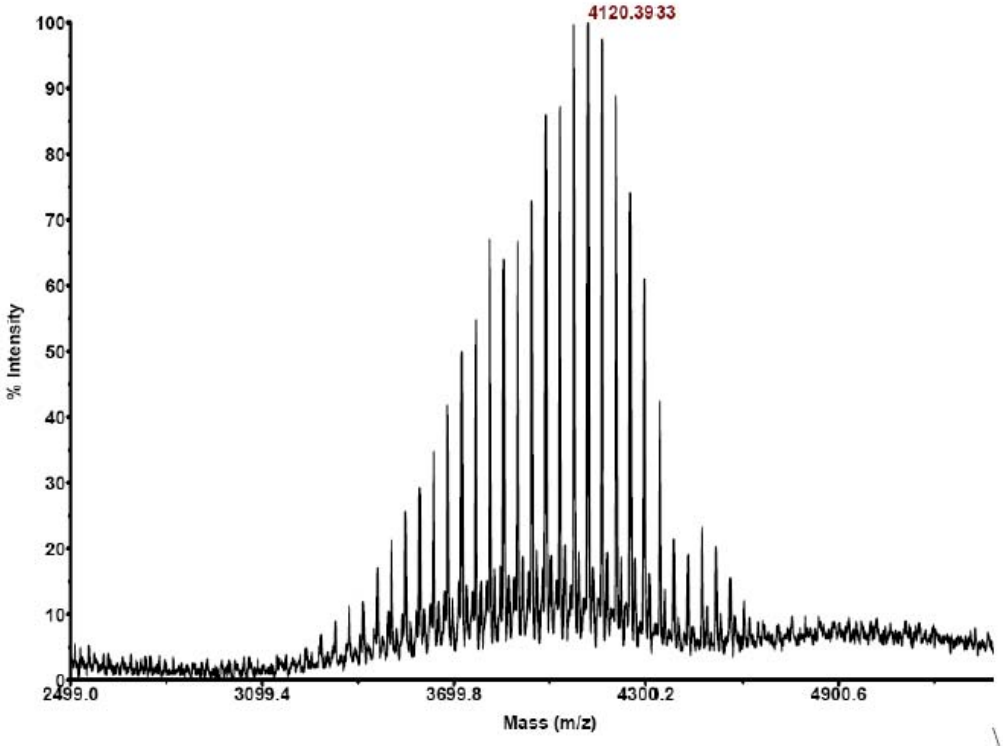


Figure 7-7. MALDI-TOF mass spectrum B72Q.

diameter of 0.793 cm. Samples were then frozen in tissue freezing media and stored at -80 ^{o}C until use. Vertical sections (25 μm), which included the articular surface as well as a portion of the underlying bone, were cut using a cryostat (Microm HM505N, Carl Zeiss) and the freezing media was removed with 3 washes of Tris buffered saline (TBS) (50 mM Tris, 100 mM NaCl) at pH 7.4. For the penetration studies, four millimeter by two millimeter rectangular samples of cartilage were excised from adult bovine metacarpophalangeal joint to a depth of 200-250 microns from the articular surface. Samples were frozen as described earlier for later use, at which time they were thawed and rinsed in TBS.

7.2.2.3 Coupling of peptide conjugates to cartilage sections

A general scheme of the covalent coupling of the peptide-PEG conjugates to cartilage is shown in Figure 7-8. Partial depolymerization of glycosaminoglycans[281] in the tissue was accomplished by digesting with chondroitinase ABC at 40 mU/mL in 50 mM Tris, 70 mM sodium acetate and 0.01% bovine serum albumin (BSA) at pH 8 for 30 minutes at 37 ^{o}C. Samples were then rinsed 3 times with TBS and blocked with 1% BSA in TBS for one hour at room temperature. The tTG reaction conditions used were similar to a previously described method[196]. The cartilage sections were incubated in 500 μL of 50 μM peptide or peptide-PEG conjugate, 0.5 U/mL tTG, 5 mM $CaCl_2$, 2.5 mM dithiothreitol and 100mM Tris, at pH 8.3 for 30 min at 37 ^{o}C. Control experiments were performed in the absence of tTG. After completion of the tTG reaction, the solution was removed and samples were rinsed 3 times with 1% BSA in TBS for 5 minutes each. The cartilage sections were then incubated with fluorescein conjugated anti-biotin antibody at 10 μg/mL in 1% BSA in TBS for 2 hours at room temperature. Samples were rinsed 4

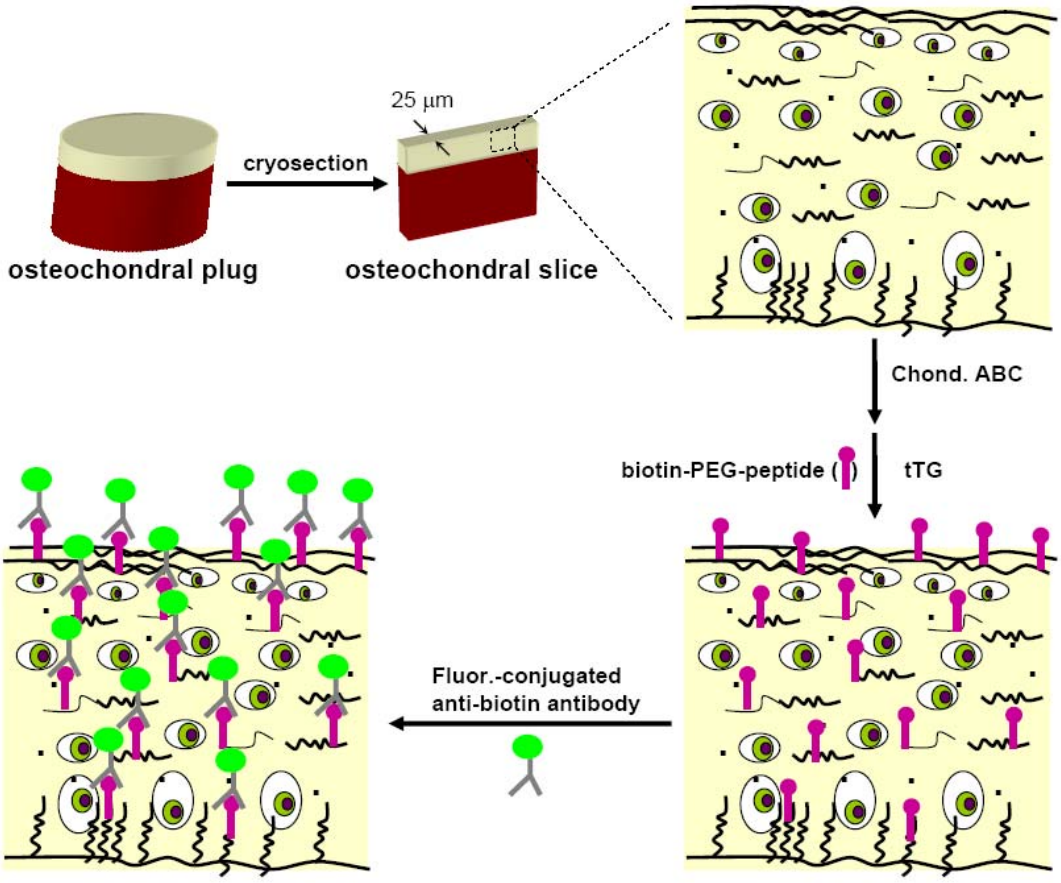


Figure 7-8. Schematic overview of the experimental methods used for reaction of synthetic peptides with cartilage tissue surfaces and for detection of tissue-bound peptide-PEG conjugates.

times with TBS for 5 minutes each, followed by blocking with 10% donkey serum for 1 hour and incubation with rabbit anti-collagen II antibody in TBS containing 10% donkey serum for 1 hour. The samples were rinsed 3 times with 10% donkey serum in TBS and then incubated with Texas Red conjugated donkey anti-rabbit antibody. Samples were rinsed again with TBS and then imaged using a Leica epifluorescent microscope (λ_{ex} = 495 nm, λ_{em} = 520 for fluorescein and λ_{ex} = 596 nm, λ_{em} = 620 for Texas Red) and a SPOT RT digital camera (Diagnostic Instruments, Sterling Heights, MI).

7.2.2.4 Image analysis

Images were analyzed using Metamorph image analysis software (Molecular Devices, Sunnyvale, CA). Fluorescein fluorescence was measured at three randomly selected areas of the middle zone on each cartilage section image and then averaged. A similar procedure was utilized to determine Texas Red fluorescence. The fluorescein fluorescence intensity was then normalized to the fluorescence of the Texas Red for each cartilage section image in order to account for potential ECM differences between sections. Finally, the average green fluorescence for each peptide-PEG conjugate was then normalized by dividing by the red fluorescence.

7.2.2.5 Depth of peptide coupling in cartilage

Four millimeter by two millimeter rectangular samples of cartilage were thawed and rinsed, after which the chondroitinase ABC digestion and tTG reactions with the peptide and peptide-PEG conjugates were performed as stated above (Figure 7-9). After the tTG reaction, the cartilage was removed and rinsed 3 times with TBS for 5 minutes each. The samples were then frozen in tissue freezing media followed by cutting using a cryostat. Twenty micron thick vertical sections were collected from each cartilage piece,

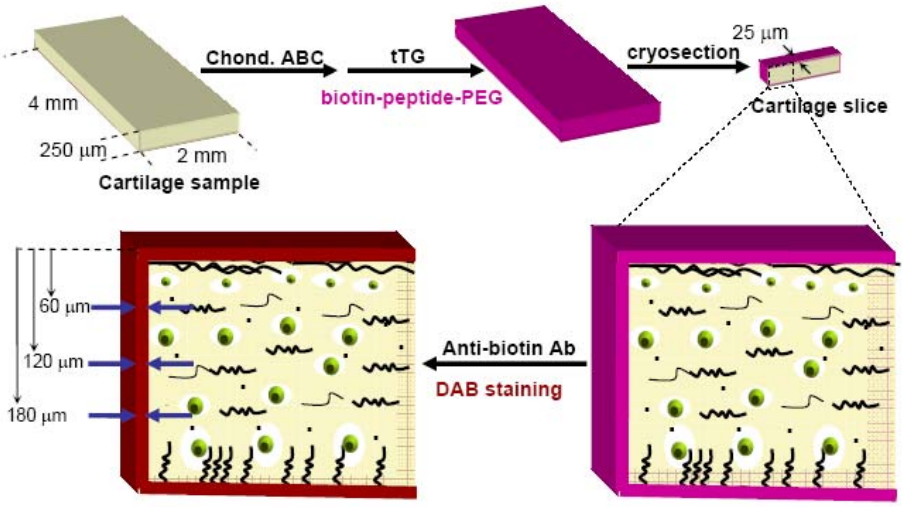


Figure 7-9. Schematic illustration of the method used for determination of depth of peptide coupling to cartilage. A 4mm by 2mm by .25mm piece of cartilage was treated with Chondroitinase ABC and then reacted with tTG and biotinylated peptide-PEG. After cryosectioning the slice was treated with anti-biotin antibody followed by DAB staining through a secondary antibody. The thickness of DAB staining was measured at 60, 120 and 180 μm from the articular surface as indicated by the blue arrows.

rinsed in TBS, blocked with 1% BSA TBS containing 0.1% Tween (TBS-T), and incubated with peroxidase conjugated anti-biotin antibody at 5 μg/mL in 1% BSA in TBS-T for 2 hours at room temperature. Samples were rinsed 4 times with TBS and 4 times with distilled water. Finally, the anti-biotin antibody was detected using a DAB kit which contains the peroxidase substrate 3,3'-diaminobenzidine yielding a brown color. Samples were imaged using a Leica microscope (brightfield) and a SPOT RT digital camera.

Adobe Photoshop (Adobe, Inc, San Jose, CA) was used to measure the depth to which the peptide and peptide-PEG conjugates were detected. The depth of staining into the cartilage along the cut surface perpendicular to the articular surface was measured at distances of 60, 120 and 180 microns from the articular surface for each sample. The average depth of staining for each sample was calculated from these three measurements. For each peptide and peptide-PEG conjugate, an overall mean depth of staining was determined from the measurements of three samples.

7.2.2.6 Reaction of peptides with cartilage ECM proteins

Fibronectin, collagen II, osteonectin, and osteopontin were separately combined at 0.1% (w/v) in 100 mM Tris and 5 mM $CaCl_2$ with 500 μM peptide-PEG conjugates and 0.05 U/mL tTG. The reaction was allowed to proceed for 30 min at 37 ^{o}C and then stopped with the addition of iodoacetamide (1 mM final concentration). Control experiments without transglutaminse were also performed. The proteins were then separated using SDS-PAGE with either 7.5% (fibronectin and collagen II) or 10% (osteonectin and osteopontin) gels, transferred to PVDF membranes which were then blocked with 2% BSA in TBS with 0.1% Tween (TBS-T). The modified proteins were probed with peroxidase conjugated ExtrAvidin. Detection of bound

ExtrAvidin was accomplished using ECL Plus Western Blotting Detection Reagents and imaged on a Storm 860 imager (GE Healthcare Biosciences, Piscataway, NJ). Band intensities were measured using ImageQuant (GE Healthcare Biosciences). Statistical analysis was performed using one-way ANOVA and Tukey's post-hoc test with 95% confidence intervals with SPSS (SPSS, Chicago, IL). A portion of the tTG reaction mixtures of the osteonectin and osteopontin with both B72Q and B72K were dialyzed against 10 mM acetic acid and the PEG-modified proteins were analyzed by MALDI-TOF MS.

7.3 Results

7.3.1 Fluorescence evaluation of the peptide conjugates coupled to cartilage

In the presence of tTG enzyme, B2K, B2Q, B72K, and B72Q (Table 7-1) were enzymatically crosslinked to the cartilage sections as evidenced by the images of the tissue sections stained with fluorescein anti-biotin antibody (Figure 7-10a, d, g, and h). Fluorescence was detected in all regions of the cartilage, from the articular surface to the calcified zone, although fluorescence intensity was greatest near the lacunae. The B2K and B72K treated samples qualitatively appeared to be more fluorescent than the B2Q and B72Q modified cartilage sections.

The catalytic role of tTG in coupling the peptide-PEG conjugates to cartilage was verified with several controls. First, incubation of B2K and B2Q with the cartilage in the absence of tTG resulted in no observed fluorescence (Figure 7-10b and e), indicating that tTG was required for the coupling of the peptide-PEG conjugates to cartilage. Secondly,

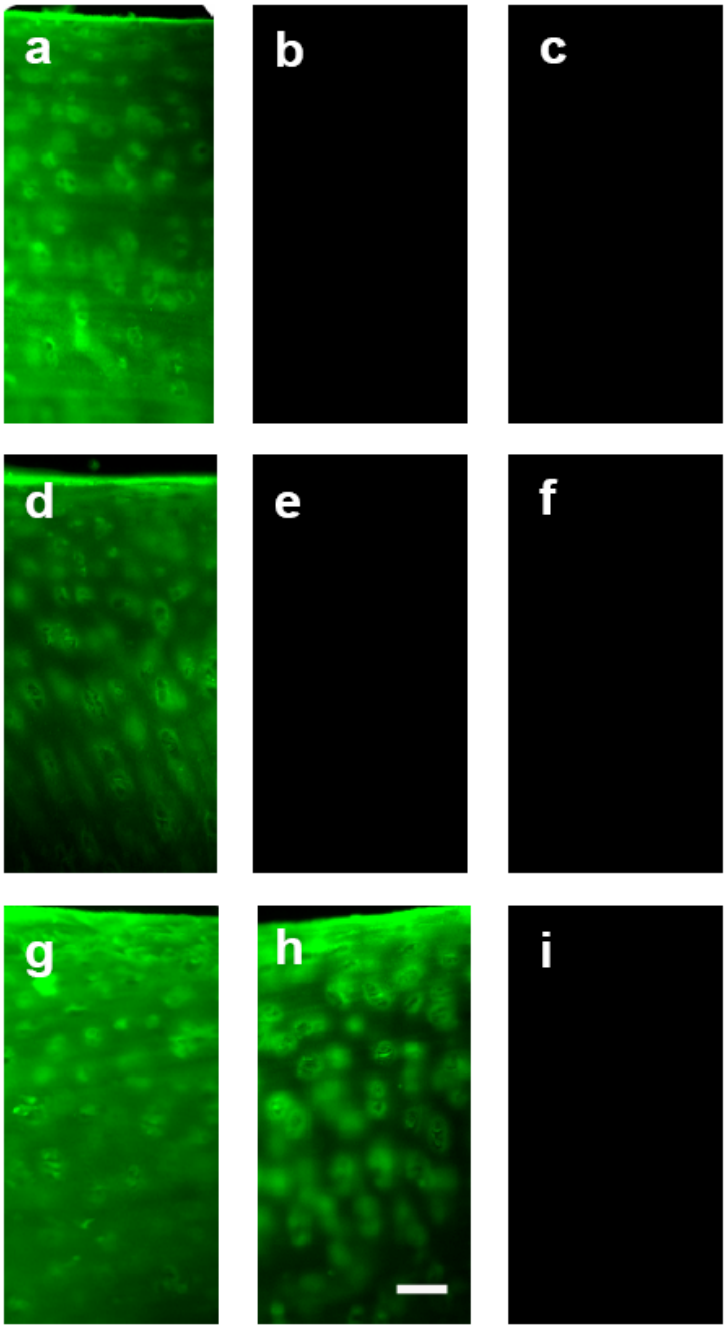


Figure 7-10. Representative images of fluorescent anti-biotin antibody stained cartilage sections after reaction with peptide-PEG conjugates and tTG. a) B2K; b) B2K without tTG; c) B2O; d) B2Q; e) B2Q without tTG; f) B2N; g) B72K; h) B72Q; i) B72. Scale bar represents 25 μm for all images. The articular surface is located at the top of each image.

when cartilage sections were incubated with B2N or B2O in the presence of tTG, no coupling was detected as evidenced by the lack of fluorescence in panels 7-10c and f. While asparagine and ornithine are structurally similar to glutamine and lysine, respectively, both are known to be poor substrates for tTG due to their shorter side chains.[162, 173, 282] Finally, nonspecific binding of the biotin-PEG component of B72K and B72Q to the cartilage can be ruled out as shown by the lack of fluorescence when the cartilage sections were incubated with B72 and tTG (Figure 7-10i).

Texas Red staining of type II collagen proved to be a useful internal reference for quantifying bound peptide, as red fluorescence was detected in all regions of the cartilage (Figure 7-11a) and was unaffected by the reaction with peptides and tTG enzyme as can be seen by the level of red fluorescence from the treated samples as compared to that of the untreated samples (Figure 7-11b). This allowed the amount of peptide bound to the cartilage to be quantified by ratioing green fluorescence from the fluorescein conjugated anti-biotin antibody to that of the red fluorescence resulting from type II collagen staining. Normalization in this manner demonstrated that the average fluorescence intensity from bound peptide was nearly 2 times more intense for B2K and B72K samples as compared to B2Q and B72Q samples (Figure 7-11c), however, not statistically significant. The presence of a longer PEG linker did not significantly affect peptide coupling as the fluorescence of B2K and B72K and B2Q and B72Q were similar.

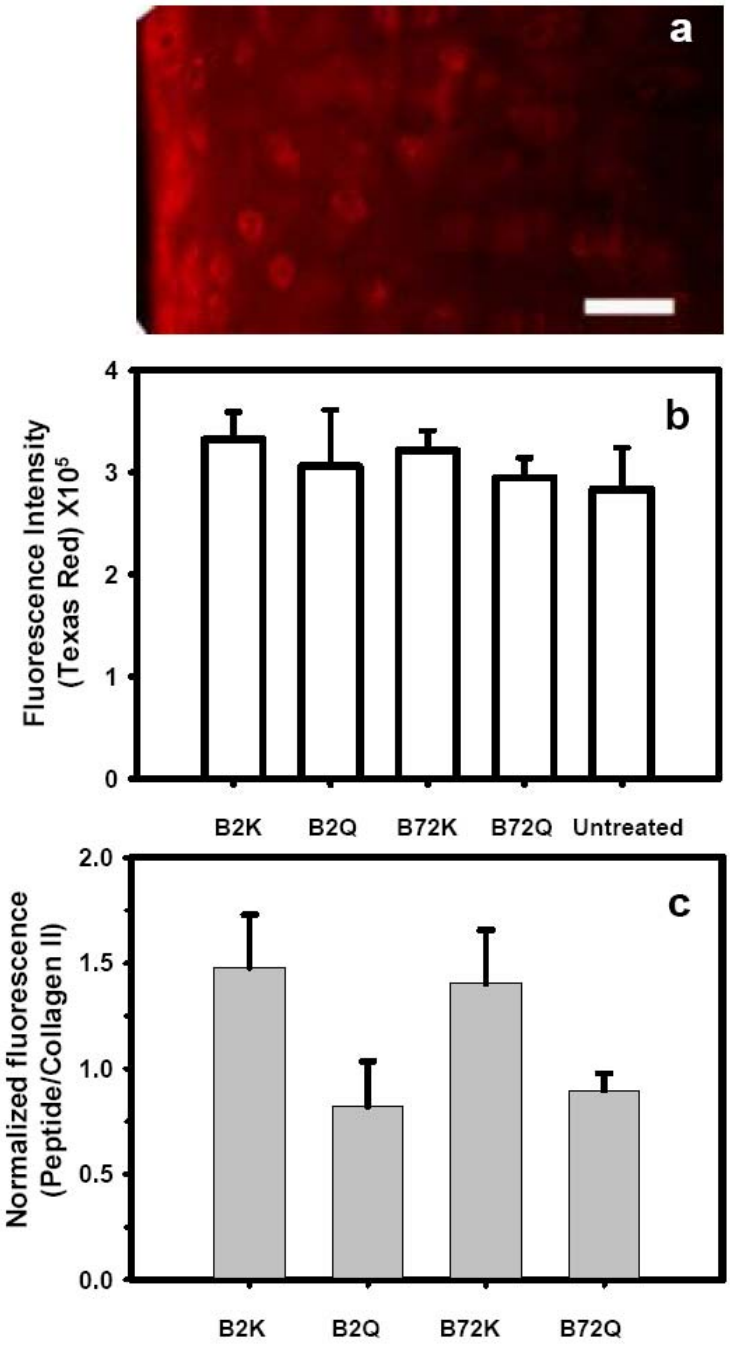


Figure 7-11. Quantification of peptide-PEG coupled to cartilage. a) Typical Texas Red fluorescence image of untreated cartilage (the articular surface is shown at the left). Scale bar represents 25 μm. b) Texas Red fluorescence intensity of cartilage sections treated with the peptide-PEG conjugates and tTG. (c) Normalized fluorescein fluorescence intensity of the peptide–PEG treated cartilage. Error bars in b and c represent standard deviation.

7.3.2 Depth of peptide coupling

In a separate series of experiments, 2 mm by 4 mm by 200-250 μm pieces of cartilage were incubated with each peptide-PEG conjugate and tTG. At the completion of the reaction, the cartilage pieces were frozen and sectioned and the coupled peptide conjugates were detected with a perioxidase anti-biotin antibody. The bound antibody was detected with DAB reagent which forms a brown precipitate in the presence of perioxidase.[283] A representative stained section is shown in Figure 7-12b. The brown DAB staining was detected on the cut and articular surfaces as indicated by the arrows in Figure 7-12b. The depth of peptide penetration and coupling was measured along the cut edge at 60, 120, and 180 μm from the articular surface as shown in Figure 7-8. No relationship was found between the thickness of DAB staining and distance from the articular surface. The average depth of peptide coupling for B2K was 13 μm. BQ2, B72Q, and B72K were detected at an average depth of 9.8 μm, 8.6 μm, and 9.8 μm, respectively (Figure 7-12c), but these differences were not found to be significant. In the absence of tTG, no staining was observed (Figure 7-12a). Finally, a control experiment in which undigested cartilage tissue was treated with tTG and B2K revealed staining to a depth of 5.8 microns (data not shown), indicating that tissue digestion with chondroitinase was not required for the coupling reaction to occur but was enhanced (as compared to 13 microns, $p < 0.05$) with its usage.

7.3.3 Reaction of peptide conjugates with cartilage ECM proteins

To identify components of the cartilage ECM that were reactive toward the peptide conjugates, enzyme reactions were conducted in solution with fibronectin, collagen II, osteonectin, and osteopontin. Coupling of peptide conjugates to all proteins was observed as

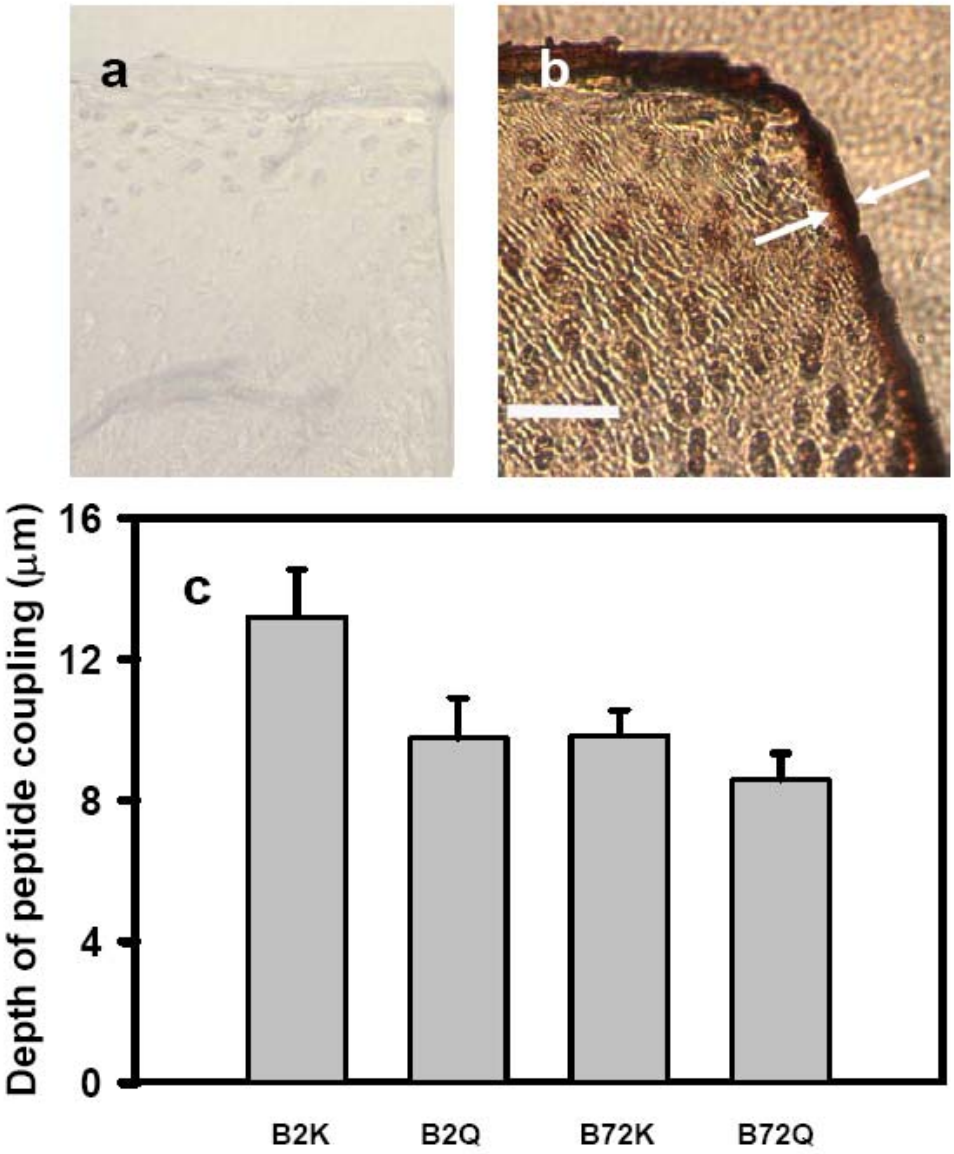


Figure 7-12. Depth of peptide-PEG conjugation to cartilage as determined by DAB staining and image analysis. a) Representative image of cartilage treated with B72K peptide but no tTG. b) Representative image of cartilage treated with B2K peptide and tTG. Arrows indicate the region of the cartilage tissue near the exposed surface that has been stained with DAB. c) Graph comparing the thickness of tissue modified by the 4 peptide conjugates. Error bars represent standard deviation. The scale bar in (b) indicates 50 μm for both images.

evidenced by western blot analysis (Figure 7-13a). With the exception of osteopontin, the cartilage proteins reacted more with lysine peptides (B2K and B72K) than with glutamine peptides (B2Q and B72Q) as evidenced by the amount of loaded protein required to detect the biotinylated conjugates. Preferential reactivity of the lysine peptides became evident when the band intensity was normalized by the amount of protein loaded (Figure 7-13b-e). For fibronectin, type II collagen, and osteonectin the intensity for B2K and B72K was statistically greater than for B2Q or B72Q ($p<0.05$). As observed with the tissue staining experiments, no differences were found between B2K and B72K and between B2Q and B72Q. For osteopontin, B2Q demonstrated a significantly greater normalized band intensity than the B2K and B72K ($p<0.05$). Control experiments without transglutaminase demonstrated that there were not non-specific interactions between the peptides and proteins. In addition, reactions of the proteins with B2N, B2O and B72 in the presence of tTG did not result in detectable crosslinking on western blot.

The MALDI-TOF spectrum of osteonectin modified with B72K demonstrated that the majority of the protein was modified with one PEG molecule but there were additional peaks indicating that the protein had been modified with up to 4 PEGs (Figure 7-14a). By contrast, when the spectrum of B72Q coupled osteonectin was evaluated, the peak corresponding to unmodified osteonectin was the most intense, indicating that a large portion of the protein had not been modified (Figure 7-14b). There were also 3 additional peaks visualized, suggesting that osteonectin had been modified with up to 3 molecules of B72Q. The MALDI-TOF mass spectra of osteopontin revealed that majority of this protein was unmodified and that it had reacted with up to two B72Q while only reacting with one B72K (Figures 7-15).

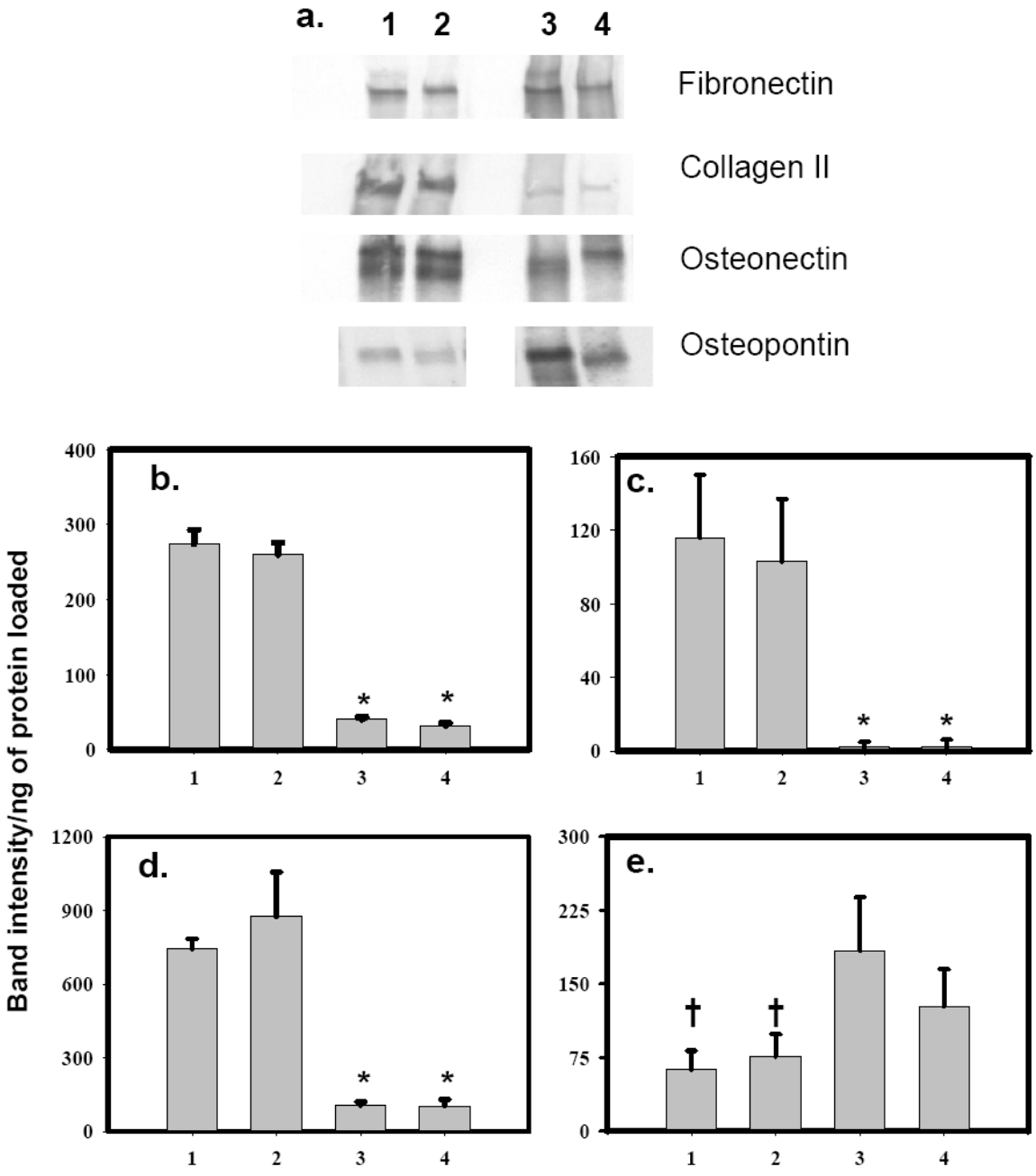


Figure 7-13. Reactivity of peptide-PEG conjugates toward cartilage ECM proteins. a) Western blot analysis of individual cartilage proteins, showing their reactivity with each peptide-PEG conjugate. Lane 1 = B2K, Lane 2 = B72K, Lane 3 = B2Q, and Lane 4 = B72Q. b-e. Comparison of the band intensity normalized by the amount of protein loaded. b) Fibronectin. c) Type II collagen. d) Osteonectin. e) Osteopontin. Bar labels are for each peptide-PEG conjugate as defined in panel a. *, p <0.05 as compared to B2K or B72K. †, p <0.05 as compared to B2Q. Error bars represent standard deviation.

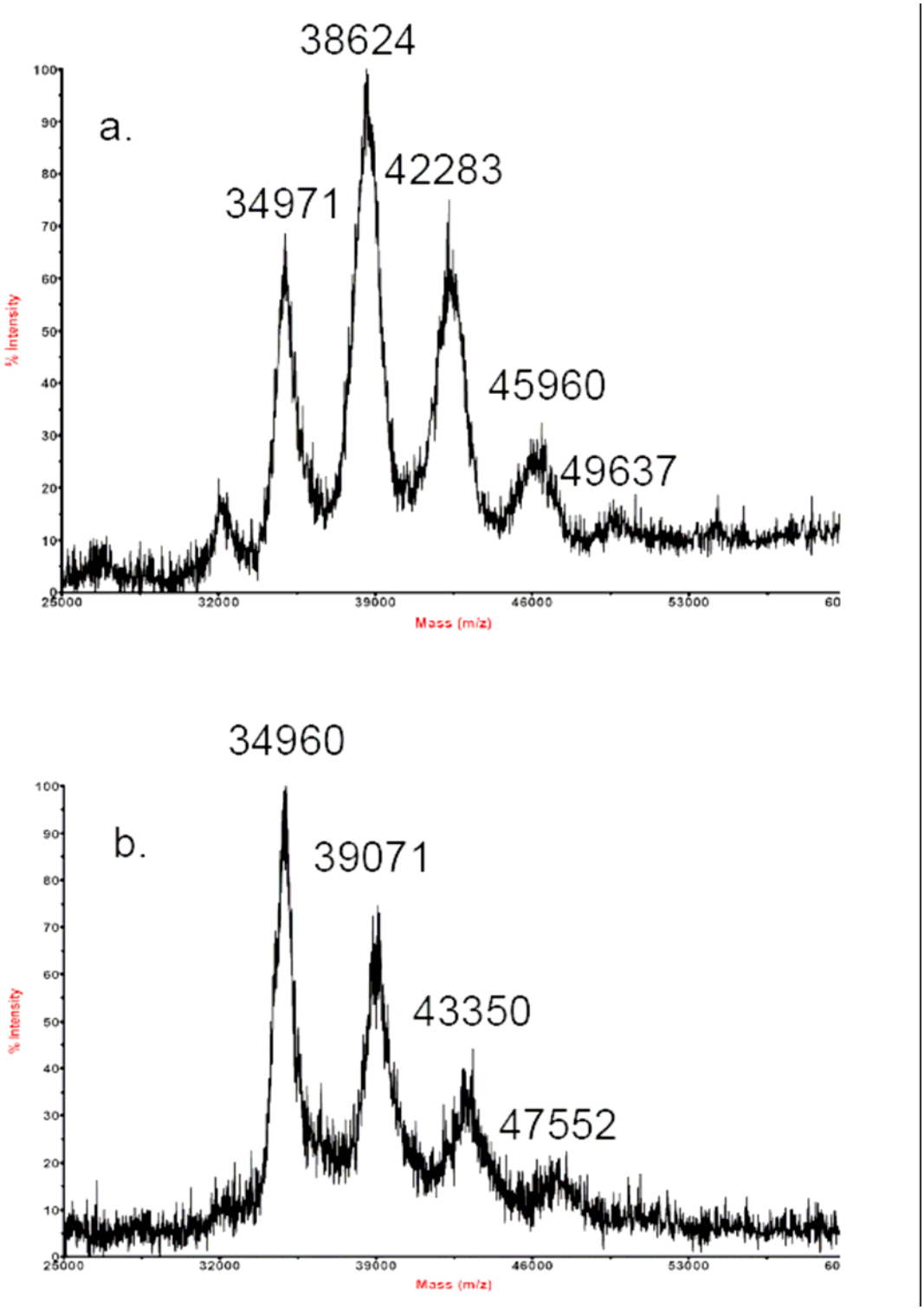


Figure 7-14. MALDI-TOF MS spectra of osteonectin modified with PEG-peptides using tTG. a) Reaction with B72K. b) Reaction with B72Q. The peak at 34900 is the molecular ion peak of

osteonectin. The difference in mass between peaks is approximately 4000 Da, the molecular weight of the peptide-PEG conjugates.

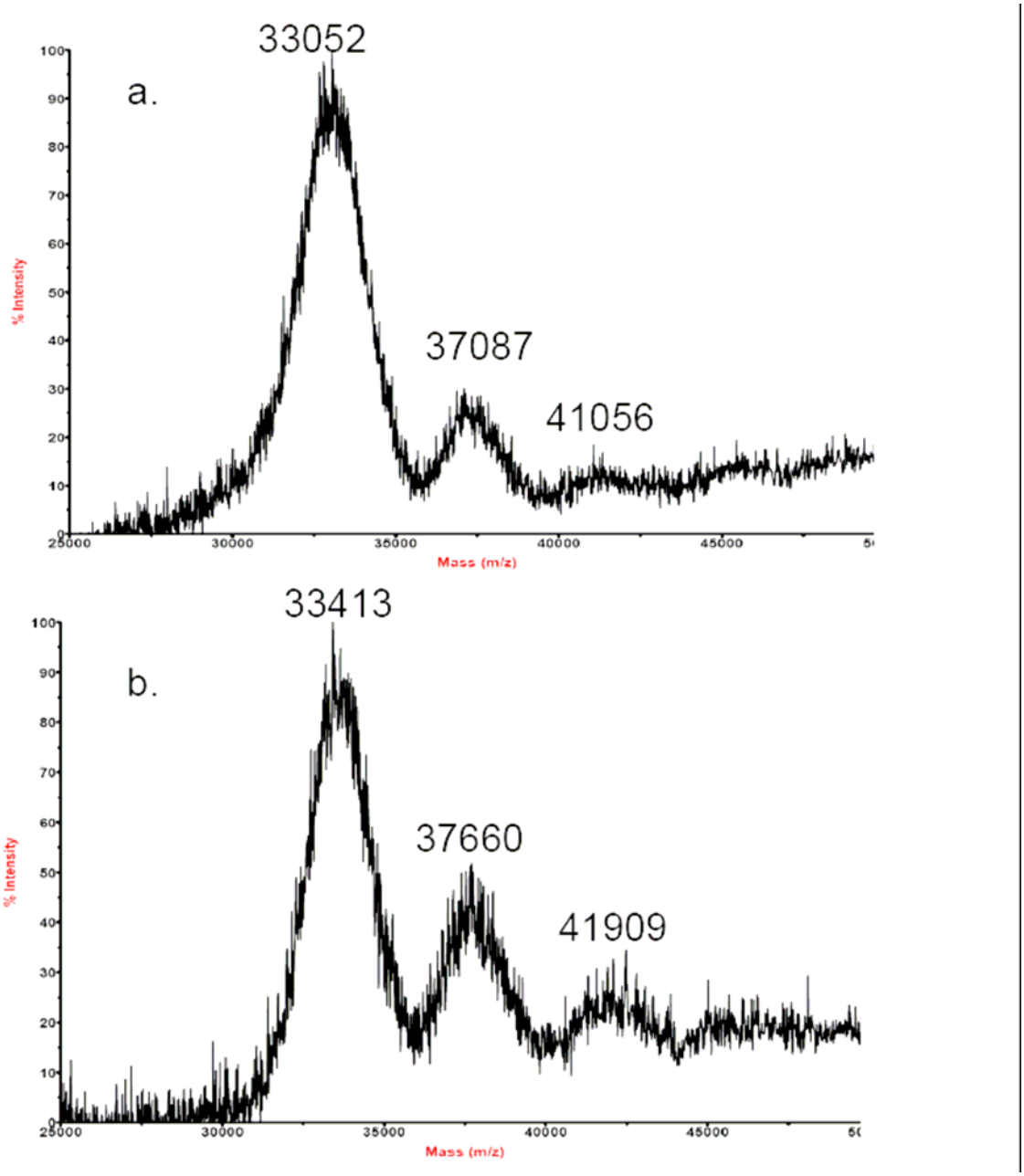


Figure 7-15. MALDI-TOF MS spectra of osteopontin modified with PEG-peptides using tTG. a) Reaction with B72K. b) Reaction with B72Q. The peak at 33000 is the molecular ion peak of osteopontin. The difference in mass between peaks is approximately 4000 Da, the molecular weight of the peptide-PEG conjugates.

7.4 Discussion

tTG is widely distributed within many connective tissues and has been implicated in organogenesis, tissue repair and in tissue stabilization.[3, 186] Cartilage was chosen for our studies in part because a prior study by Jurgensen *et al.* showed that cartilage tissue surfaces adhered strongly to each other when incubated with tTG enzyme, suggesting a high level of reactivity of cartilage ECM components as substrates for tTG.[4] Several components of the cartilage ECM have been identified as substrates for tTG, including osteopontin,[284, 285] collagen II,[196, 244] collagen XI,[286] fibrillin,[287] fibronectin,[245] and osteonectin.[244, 249] Some other known ECM substrates of tTG found in other tissues include collagen I,[225, 288] collagen V,[286] vitronectin,[289, 290] and laminin.[291]

To demonstrate the general approach of tissue surface modification using tTG enzymes, we sought to design a simple and versatile molecular model consisting of a short synthetic peptide substrate that could ultimately be conjugated to a therapeutic molecule such as a drug, growth factor or synthetic polymer. Specific requirements for tTG reactive glutamine and lysine residues are not fully understood, although it is believed that the enzyme has more stringent requirements for the glutamine (acyl donor) than for the lysine (amine donor).[173] It has been suggested that a leucine residue flanking the C-terminus of a glutamine increases reactivity.[162] However, recent combinatorial library studies suggest that the amino acid directly adjacent to the reactive glutamine is not as important as the amino acids 2-3 residues away on the C-terminus side.[163, 175] Proline and phenylalanine as well as other nonpolar amino acids have been suggested to have favorable roles in those locations.[176, 177] In contrast, tTG is considered more tolerant of lysine substrates, as it has been found to react with a wide variety of primary amines as well as

peptide bound lysines.[3, 173] Nevertheless, certain peptide features do influence lysine reactivity, such as the presence of a C-terminal adjacent glycine[184] or an N-terminal hydrophobic residue juxtaposed to lysine.[5, 173]The specific lysine and glutamine peptides used here were rationally designed and identified as good tTG substrates in our prior study.[5]

Our findings demonstrate that both FKG-NH_2 and GQQQLG-NH_2 are bound to cartilage surfaces via tTG as demonstrated by tissue staining experiments. Conjugates of these peptides with 3.4 kDa PEG were similarly active, suggesting that the presence of the polymer had little effect on the ability of tTG to catalyze the coupling of the peptide to the tissue surface. Diffuse staining over the cartilage matrix as well as more intense focal staining in the pericellular region were observed (Figure 7-10). The intense staining in the pericellular regions could be explained by tTG reactive noncollagenous proteins which are believed to mediate the interaction between cells and the ECM and can be found predominantly in the areas surrounding the chondrocytes.[21] It is known that some noncollagenous cartilage ECM proteins, such as fibronectin, react well with tTG[186, 188, 200] and therefore it was anticipated that our peptides would react with the proteins in this region. Intense staining at the articular surface could be due to the high concentration of collagen there,[6] although sample geometry and imaging considerations prevent us from making that conclusion without further detailed experiments.

The peptide conjugates were bound to ECM to a depth of approximately 8-13 microns from the cut tissue surface. The depth of tissue modification can be affected by many factors, including the ability of the enzyme and peptide reactants to diffuse into the tissue before reacting with ECM components. No difference was found for the depth of modification when lysine (B2K) or glutamine (B2Q) peptides were coupled to a 3.4 kDa PEG (B72K and B72Q, respectively), suggesting that the diffusion of the peptide components through the tissue is

unlikely to be a limiting factor. The masses of all peptide conjugates are well below that of tTG (77 kDa),[292] making it more likely that diffusion of tTG to be a limiting factor. The surface coupling reaction did not require pretreatment of the tissue with chondroitinase, although the depth of tissue modification was greater when the tissue was partially digested. Deeper tissue modification, if desired, may be achieved with a higher concentration of chondroitinase, longer digestion times,[293] or through the use of a different enzyme such as collagenase or hyaluronidase.[294] To correlate the observed tissue modification to specific matrix components of the tissue, we also conducted tTG reactions in solution using selected cartilage ECM proteins and the peptides. One of the major ECM components of cartilage is collagen II, which can be found throughout the cartilage matrix.[6] Collagen II used in these studies was isolated by pepsin digestion, which yields primarily the triple helical region of collagen II.[295] Both lysine peptide conjugates, B2K and B72K, were reactive toward collagen II, suggesting that glutamine residues in the triple helical region act as substrates for tTG. The B2Q and B72Q containing peptides were less reactive toward collagen II in solution, which could be due to loss of lysine residues as a result of hydroxylation during collagen II interchain crosslinking and glycosylation.[296]

B2K and B72K were significantly ($p<0.05$) more reactive than B2Q and B72Q with all proteins except osteopontin. There are a few possible explanations for these results. First, FKG has a significantly higher specificity (560 mM^{-1} min^{-1}) than GQQQLG (34.1 mM^{-1} min^{-1}),[5] indicating that the amine donor (FKG) is more reactive with the enzyme than the acyl donor (GQQQLG). However, the reactivities of the glutamine and lysine residues in the ECM proteins may also play important roles and could explain the higher reactivity of osteopontin toward the glutamine peptide conjugates (B2Q and B72Q). In addition, unlike our short peptide constructs that are expected to have no secondary or tertiary structure, the existence of alpha helices, beta

sheets and higher order protein structures of proteins may shield some lysine and glutamine residues from the enzyme. For example, it has been demonstrated that when fibronectin is exposed to tTG and [^{14}C]putrescine in solution 4 glutamines are reactive.[245] However, after partial digestion, as many as 8-9 glutamines were reactive toward tTG. Thus, the total number of lysine and glutamine residues in an ECM protein may not always be a reliable indicator of potential reactivity toward tTG.

7.5 Conclusion

In summary, our results demonstrate that synthetic peptide and peptide-polymer conjugates can be enzymatically coupled to cartilage under mild conditions through the formation of isopeptide bonds between the peptide and ECM proteins. Cartilage is only one example of a tissue whose free surface is readily accessible through minimally invasive administration of solutions containing tTG and synthetic molecules; others include the tissue surfaces of the oral cavity, gastrointestinal and reproductive tracts, the surface of the eye, and tissue surfaces exposed during invasive surgical procedures. The reactive cartilage ECM components identified in this study can also be found in many other connective tissues, suggesting that this strategy can be broadly applicable. We anticipate that this facile and versatile method for modifying tissue surfaces can be employed with a wide range of therapeutic biomolecules, growth factors and functional polymers.

Chapter 8

Enzymatic coupling of a hydrocortisone prodrug to the articular surface of cartilage: A novel method of localized drug delivery

8.1 Introduction

Osteoarthritis (OA) [41, 297] and rheumatoid arthritis (RA)[298] affect over 70 million people in the US with debilitating pain and inflammation, in large part due to the action of the potent catabolic cytokine interleukin 1 (IL-1). The effects of IL-1 include increased production of matrix metalloproteases[43] and other inflammatory cytokines[299, 300], inhibition of extracellular matrix (ECM) synthesis[300], and cell death[301, 302]. Glucocorticoids down regulate the transcription and translation of IL-1, and hence are a major treatment strategy for reducing the pain and inflammation of OA and RA[303, 304]. These drugs are administered as insoluble suspensions[305] via intra-articular injections in order to maximize their effects at the inflamed joint and to minimize their adverse systemic effects[306-308]. However, when these compounds are deposited in the joint space, the crystals formed may induce inflammation as well[1]. In addition, a significant portion of the drug dose is still cleared from the joint, entering the circulatory system and exerting deleterious systemic effects including a drop in endogenous cortisol levels[309, 310], facial flushing, temporary disruption in glucose metabolism, and immunosuppression[311].

Several approaches have been proposed to increase the retention of glucocorticoids in the inflamed joint; however, they all depend on physical deposition of the drug-laden vehicle in the intra-articular space. Liposomes of glucocorticoid palmitate esters have been shown to be an effective treatment of inflammation by reducing knee swelling more than the free drug alone[312-

[314]. Another method proposed by Payan *et al*.[315] to improve glucocorticoid retention in the joint was via modification of another intra-articularly delivered drug, hyaluronan (HA), with methyl prednisolone. Cortivo *et al.* demonstrated that this modified HA had better antioxidant properties on chondrocytes than either drug individually[316]. Microspheres and nanospheres have also been utilized as local drug delivery vehicles to joints[317-322]. However, their performance is dependent on a number of factors to include the molecular weight and type of polymer used to form the spheres[320], the extent of crosslinking[318, 319] and sphere diameter[321]. It has been shown that while more microspheres are retained in the joint space, they elicit a more vigorous immune response than nanopheres[322]. Conversely, nanospheres can be readily phagocytosed by macrophages and are transported through the synovial lining, providing anti-inflammatory effects deep into the tissue[322]. Schulze *et al.* demonstrated that polyvinyl alcohol covered supraparamagnetic ion oxide particles (SPION) could be used to localize therapy to the joint with the use of a magnet.[323] The efficacy of these particles depended on both the size of the particles and of the joint being infused[324]. Finally, radioactive elastin-like polymer (ELP), which aggregates in the articular joint space, is another potential method of delivering therapeutic peptides and proteins to the joint, either by engineering these molecules as part of the ELP, or by utilizing ELP as a trap to store drugs in the joint.[325]

Unlike the aforementioned techniques of localized drug delivery which depend on deposition of the drug delivery vehicle, we propose to enzymatically couple the drug delivery vehicle to the cartilage surface. Transglutaminases are calcium-dependent enzymes that catalyze the crosslinking of lysine residues to glutamine residues in proteins and peptides to form ε-(γ-glutaminyl) lysine isopeptide bonds (Figure 8-1)[2]. Two members of this family, tissue

transglutaminase, the ubiquitous form of the enzyme, and Factor XIII, the circulatory form, have been used to couple growth factors containing enzyme substrates to tissue-engineered scaffolds[224, 326, 327]. Both have also been used with cartilage in various approaches. Factor XIII, as a component of fibrin sealant, was used to secure periosteal tissue in the repair of a osteochondral defect[144]. Jurgensen *et al.* used tTG as an adhesive link to bond two pieces of cartilage together, presumably crosslinking the native substrates in the ECM[4]. Recently, we have developed short synthetic peptide substrates of tTG that were used to modify polymers. These modified polymers were then used to form hydrogels[5, 265] and were coupled to the cut surfaces of cartilage via the enzymatic activity of tTG[264]. This coupling is possible because of the availability of tTG substrates in cartilage such as osteopontin[284, 285], collagen I[225, 288], collagen II[196, 244], collagen XI[286], fibrillin[287], fibronectin[245], and osteonectin[244, 249].

In the current study we present a novel strategy for immobilizing drugs to tissue surfaces for localized therapeutic delivery. Hydrocortisone hemisuccinate was conjugated with a peptide substrate of tTG and then coupled to the articular surface of cartilage (Figure 8-2). The presence of bound hydrocortisone on the articular surface was detected using immunohistochemistry and quantified with a Cortisol Parameter Assay kit. The dependence of coupling on both hydrocortisone prodrug concentration and time was evaluated. The prodrug molecule was also evaluated after conjugation with PEG, because PEG has been used previously to camouflage proteins from circulating enzymes and

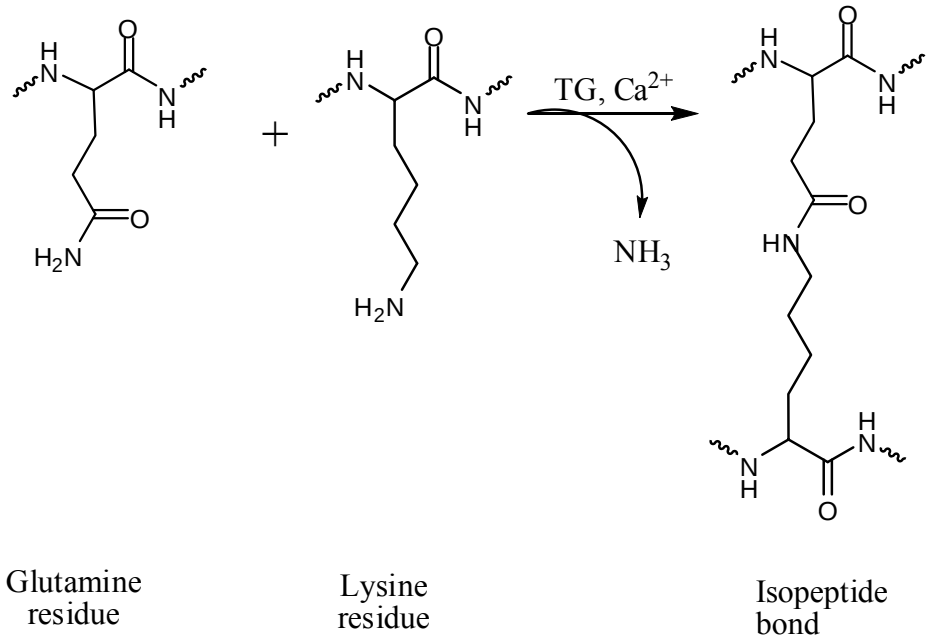


Figure 8-1. Transglutaminase reaction. The calcium-dependent transglutaminases catalyze the crosslinking of proteins and peptides by forming isopeptide bonds between lysine and glutamine residues. TG = transglutaminase.

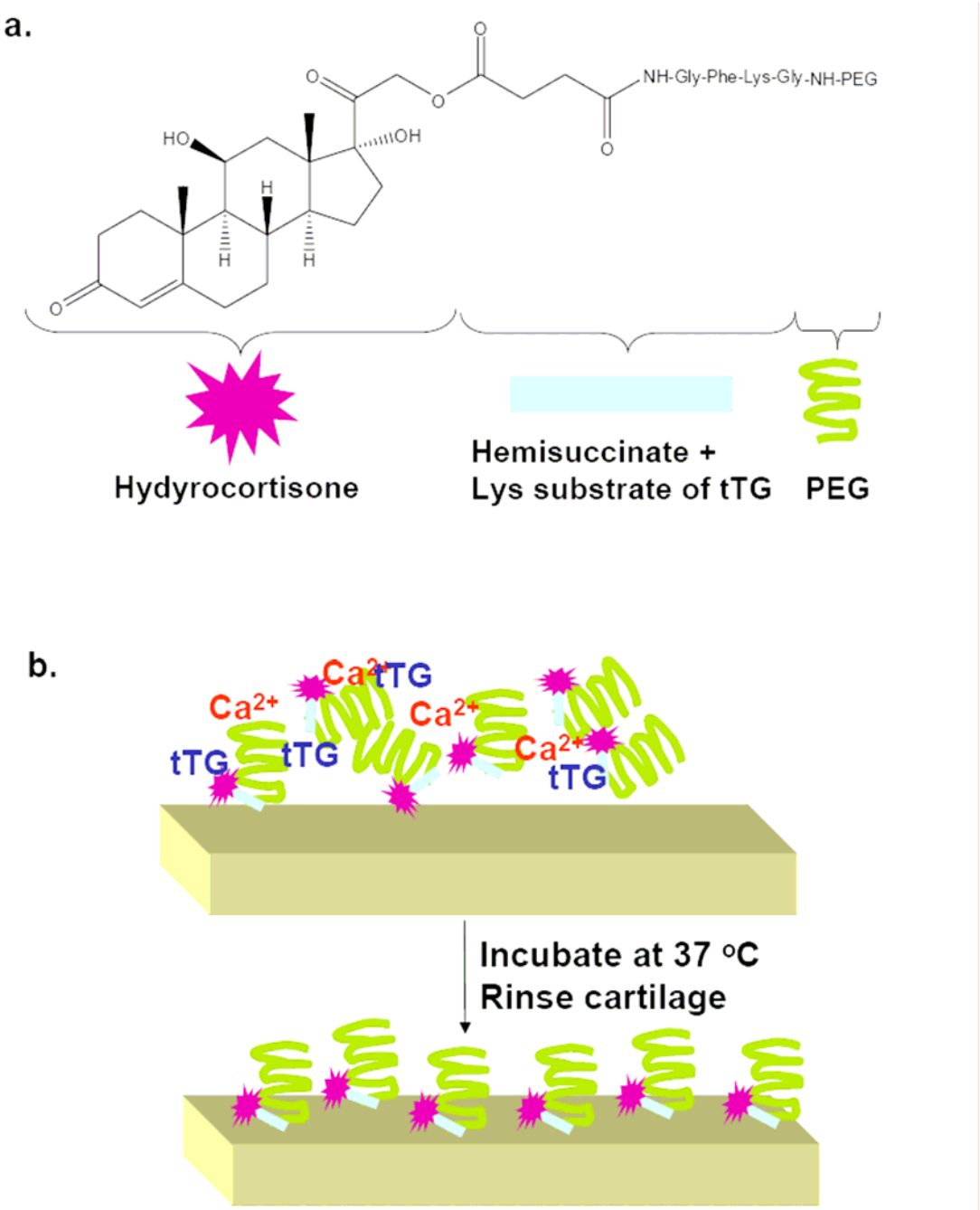


Figure 8-2. Diagram of the coupling of the prodrug to the articular surface of cartilage. a.) Molecular structure of HyC-GFKG-PEG prodrug synthesized and pictographic representation of each component b.) Illustration of cartilage articular surface reacting with HyC-GFKG-PEG in

the presence of Ca^{2+} and tTG. After the reaction is complete and the unused reactants are removed, the HyC-GFKG-PEG coupled to the surface remains.
immune cells, and has also been used to make biological surfaces inert to protein deposition and cell adhesion[328, 329]. Therefore, the prodrug-PEG conjugate may be protected from hydrolytic enzymes on the articular surface. The stability of the hydrocortisone prodrugs on the articular surface was examined in phosphate buffered saline (PBS), normal synovial fluid, and osteoarthritic synovial fluid. Finally, to demonstrate the ability to modify other joint structures, the hydrocortisone prodrugs were coupled via tTG to the meniscus.

8.2 Materials and Methods

8.2.1 Materials

Gly-2-ClTrt-Resin was purchased from Peptides International, Louisville, KY. All Fmoc-Amino acids, anti-hydrocortisone antibody, and fluorescein conjugated goat anti-mouse secondary antibody were purchased from EMD Biosciences, San Diego, CA. mPEG amine ($\bar{M}_w$ = 5.0 kDa) was purchase from Nektar Therapeutics, Inc, Huntsville, AL. Hydrocortisone 21-hemisuccinate, 6α-methyl-prednisolone, DEAE cellulose, and papain were purchased from Sigma, St. Louis, MO. Twenty-two week-old bovine knees were purchased from a local slaughter house. Phosphate buffered saline was purchased from Invitrogen, Carlsbad, CA. Goat serum was purchased from Abcam, Cambridge, MA. Complete Mini protease inhibitor was purchased from Roche Applied Science, Indianapolis, IN. Cortisol Parameter Assay kit was purchased from R&D Systems, Minneapolis, MN. Guinea pig livers were purchased from Charles River, Wilmington, MA. Bovine synovial fluid was purchased from Animal Technology,

Tyler, TX. The human osteoarthritic synovial fluid was a gift from Dr. James Williams, Rush University, Chicago, IL.

8.2.2 Methods

8.2.2.1 Tissue transglutaminase isolation.

Guinea pig liver tissue transglutaminase was isolated according to a procedure adapted from Folk.[330] DEAE cellulose was prepared according to the manufacturer's specifications and a column (27 cm x 3 cm) was packed with resin dispersed in 5 mM Tris, 2 mM EDTA, pH 7.5 (1X buffer). The column was then rinsed with 200 mL of 1X buffer. At this stage, all reagents were stored and all procedures performed at 4 ^{o}C. Five guinea pig livers (200-250 g) were homogenized in 200 mL of 250 mM sucrose. The homogenate was centrifuged at 26000 RPM for 1 hour. The supernatant from the centrifuged homogenate was collected and loaded on the prepared DEAE column followed by a rinse with 150 mL of 1X buffer. tTG was then eluted with a 1 L linear gradient of 0.1 to 0.7 M NaCl in 1X buffer solution and collected in 8 mL fractions.

To determine which fractions contained tTG activity, 100 μL of each fraction was combined with 100 μL of 1M Tris-acetic acid pH 6.0, 150 μL water, 75 μL of 0.2 M Cbz-Gln-Gly pH 6.0, and 25 μL each of 0.1 M $CaCl_2$, 0.02 M EDTA, and 2 M hydroxylamine pH 6.0. The components were mixed thoroughly and placed in a 37 ^{o}C bath for 10 min. To terminate the reaction, 500 μL of a solution containing 5% $FeCl_3$ (in 0.1 N HCl): 2.5 N HCl: 15% trichloroacetic acid (1:1:1) was added to each reaction. The presence of tTG activity in a fraction was confirmed by the generation of a purple to brown color (Figure 8-3). The corresponding fractions, generally 0.3 M to 0.6 M NaCl in 1X buffer, were then pooled.

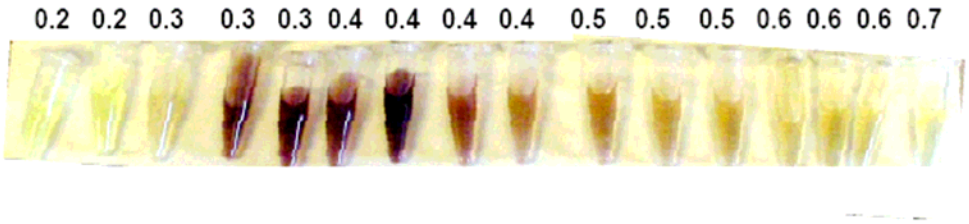
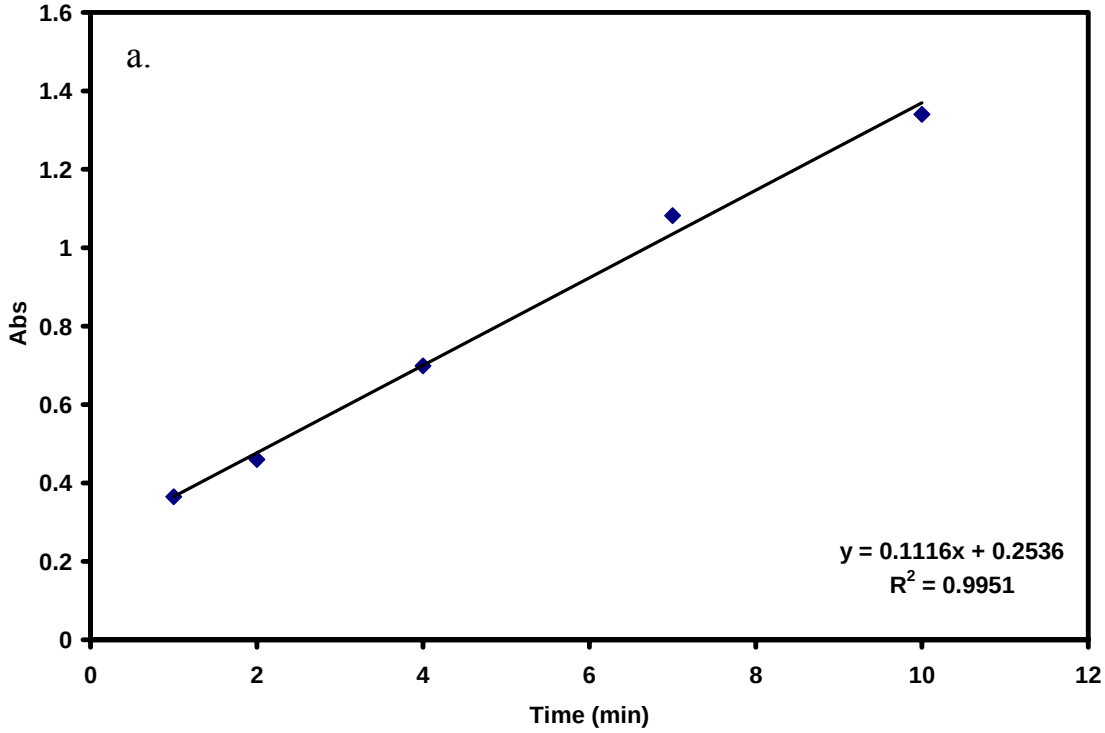


Figure 8-3. Detection of guinea pig tTG in column fractions. Fractions (designated above each tube by the concentration of NaCl) were examined for tTG activity. Brown to purple color is indicative of increasing amounts of tTG. Fractions of 0.3 M to 0.6 M NaCl in 1X buffer were pooled.

The tTG was precipitated from the pooled fractions with 20 mL of freshly prepared 1% (w/v) protamine sulfate in water. The precipitate was collected by centrifugation at 15,000 RPM for 15 min. The tTG was extracted from the protamine precipitate with 20 mL of 0.05 M ammonium sulfate in 1X buffer. The suspended precipitate was placed in a glass tube and homogenized with a Teflon pestle to extract tTG from the precipitate, then the suspension was centrifuged at 15,000 RPM for 5 min. The ammonium sulfate solution containing the dissolved tTG was collected and the extraction procedure was repeated with the remaining precipitate 2 more times using 15 mL and then 10 mL of the ammonium sulfate solution. The 45 mL of ammonium sulfate solution containing tTG was dialyzed overnight against 0.5 M NaCl in 1X buffer with 5 mM DTT. The tTG solution was then frozen and lyophilized, and the product was stored at -20 ^{o}C until use.

The activity of tTG was determined by dissolving a 3.7 mg portion of the lyophilized enzyme in 550 μL of cold water. One hundred microliters of the enzyme solution was combined with 100 μL of 1M Tris-acetic acid pH 6.0, 150 μL water, 75 μL of 0.2 M Cbz-Gln-Gly pH 6.0, and 25 μL each of 0.1 M $CaCl_2$, 0.02 M EDTA, and 2 M hydroxylamine pH 6.0. A different solution was made for each time point - 1, 2, 4, 7, and 10 min. All solutions were incubated at 37 ^{o}C and removed at the appropriate time. To terminate the reaction, 500 μL of a solution containing 5% $FeCl_3$ (in 0.1 N HCl): 2.5 N HCl: 15% trichloroacetic acid (1:1:1) was added to each reaction. The absorbance of each solution was measured at 525 nm. A plot of time vs. absorbance was generated and the slope was determined by linear regression (Figure 8-4a). The tTG activity was calculated at 0.49 units per mg from the slope (0.1116 absorption units per minute), the molar



b. Example calculation for the determination of tTG

$$\frac{Slope}{340} = \frac{0.1116 \text{Abs} / \text{min}}{340 \text{Abs} * \text{L} / \text{moles}} \text{ x } \frac{1L}{1000mL} \text{ x } \frac{1000000\ \mu\text{moles}}{1\text{moles}} = \frac{0.328\text{units}}{\text{mL}}$$

100 μL of tTG was added to the 1 mL.

$$\frac{0.328\text{units}}{100\mu\text{L}} = \frac{0.00328\text{units}}{\mu\text{L}} \text{ x 550 μL (total tTG solution made)} = 1.805 \text{ units}$$

3.7 mg of lyophilized product was added to the 550 μL.

$$\frac{1.805\text{units}}{3.7\text{mg}} = \frac{0.49\text{units}}{\text{mg}}$$ This is the activity of the lyophilized product.

Figure 8-4. Determination of guinea pig tTG activity. a. Graph of transglutaminase activity dependence on time as measured by absorbance at 525 nm. b. Example calculation to determine tTG activity.

absorptivity, 340 Abs/M^{-1}, and 1 unit of tTG = micromoles/minute. An example of the calculation is given in Figure 8.4b.

8.2.2.2 Synthesis of hydrocortisone prodrugs.

Hydrocortisone-GFKG (HyC-GFKG) prodrug was synthesized on a Gly-2-Cltrt resin (1 g, 0.77 mmol/g) using standard Fmoc solid phase peptide synthesis. Each coupling reaction was performed for 3 hours with a 10 min pre-activation of four equivalents of Fmoc-amino acid: benzotriazole-1-yl-oxy-tris(dimethylamino)-phosphoniumhexafluorophosphate (BOP): *N*-hydroxybenzotriazole (HOBt): diisopropylethylamine (DIEA) (1:1:1:1) in N-methylpyrrolidinone (NMP) at room temperature. The Fmoc was removed by 20% piperidine in NMP for 1 hour. After Fmoc removal from the final amino acid, hydrocortisone 21-hemisuccinate was added to the resin. It was treated as a Fmoc-amino acid and used at 2 equivalents. At the completion of the reaction, the resin was washed with NMP and dichloromethane (DCM) two times each and then treated with 50% trifluoroacetic acid (TFA) in DCM for 1 hour. The crude product was obtained by concentration of the TFA solution and addition of diethyl ether. Purification was performed using semi-preparative RP-HPLC using a C-18 column (250 x 22 mm, 10 μm, Vydac) with an acetonitrile gradient of 15-46% in water with 0.1% TFA for 120 min. The mass was verified by ESI-MS analysis on a LCQ LC-MS system (Finnigan, Thermoquest, CA) and the presence of hydrocortisone was verified by RP-HPLC on a C18 column (250 x 4.6 mm, 10 mm, Vydac) with an acetonitrile gradient of 20-55% in water with 0.1 % TFA for 30 min with detection at 215 and 242 nm. The purified HyC-GFKG was then frozen, lyophilized and stored at -20 ^{o}C until use.

For synthesis of HyC-GFKG-PEG, the HyC-GFKG portion was synthesized as described above. After the coupling reactions were completed, the resin was washed with NMP and dichloromethane (DCM) two times each and then treated with 30% hexafluoroisopropanol (HFIP) in DCM 3 times for 10 minutes each. HyC-GFK(boc)G product was obtained by concentration of the HFIP solution and addition of 1:1 diethyl ether:hexane. The coupling of the protected peptide to the $mPEG_{5000}$ amine was performed overnight with a 10 min pre-activation of 1.5 equivalents of the HyC-GFK(boc)G: BOP: HOBt: DIEA (1:1:1:1) in NMP at room temperature. The product was collected by precipitation in diethyl ether and dried overnight under vacuum. Boc was removed with 50 % TFA in DCM for 1 hour. The crude product was obtained by concentration of the TFA solution and addition of diethyl ether. Purification was performed using semi-preparative RP-HPLC on a C-18 column with an acetonitrile gradient of 25-55% for 120 min. HyC-GFKG-PEG was then frozen and lyophilized. The mass was confirmed using MALDI-TOF MS analysis on a PE Voyager DE-Pro MALDI-TOF Mass Spectrometer (Perspective Biosystems, MA) and the presence of hydrocortisone verified on RP-HPLC with a C-18 column with an acetonitrile gradient of 24-55% and detected at 215 and 242 nm. The final product was stored at -20 ^{o}C until use.

8.2.2.3 Hydrolysis of the hydrocortisone prodrugs in solution.

The hydrolysis of 1.0 mM HyC-GFKG in PBS at pH 7.4 was studied at 4, 23, and 37 ^{o}C. The prodrug hydrolysis was also examined in PBS at pH 4.0 at 4 ^{o}C, and in a solution of 10 mM $CaCl_2$, 40 mM HEPES, 100 mM NaCl, pH 7.4 at 37 ^{o}C. The hydrolysis of 1.0 mM HyC-GFKG-PEG was evaluated in PBS at pH 7.4 at 37 ^{o}C. A 30 μL portion of each sample was collected at 0.5, 1, 2, 4, 7, 24, and 48 hours and further hydrolysis inhibited by the addition of 60 μL of 0.5

mM 6α-methyl-prednisolone in 1% TFA and 20% acetonitrile in water and stored at -20 ^{o}C until analysis. Samples were analyzed with RP-HPLC and eluted with a linear gradient of 24%-55% acetonitrile in water with 0.1% TFA. The amount of free hydrocortisone as well as the amount of the prodrug remaining was calculated by measuring the peak area in relation to the internal standard. The $T_{50\%}$, half-life, of the prodrug was calculated from 0.693/S, where S is the mean slope giving the best correlation coefficient in the linear part of the logarithmic curve of unhydrolyzed prodrug versus time. The hydrolysis of 1.0 mM HyC-GFKG was also studied in 75% normal synovial fluid in PBS at 37 ^{o}C. Thirty microliter samples were collected at 10, 20, 30, and 60 minutes and analyzed as previously described.

8.2.2.4 Cartilage and meniscus isolation and preparation.

Cartilage pieces (15 mm x 17 mm x 5 mm) from the femoral trochlea and meniscus pieces of the same dimensions were collected from 22-week old calves and stored at -80 ^{o}C until use. Upon use, pieces were thawed in phosphate buffered saline (PBS) then rinsed with a solution of 10 mM $CaCl_2$, 40 mM HEPES, and 100 mM NaCl, pH 7.4.

8.2.2.5 Coupling of hydrocortisone prodrugs to the articular surface of cartilage sections.

A general scheme of the covalent coupling of hydrocortisone prodrugs to cartilage is shown in Figure 8-5. Cartilage sections were incubated in 3 mL of 1.0 mM HyC-GFKG, 1 U/mL tTG, 10 mM $CaCl_2$, 40 mM HEPES, and 100 mM NaCl at pH 7.4 for 60 min at 37 ^{o}C. Control experiments were performed in the absence of tTG. At the end of the coupling reaction, the cartilage was rinsed 3 times in 3 mL PBS for 5 min each at

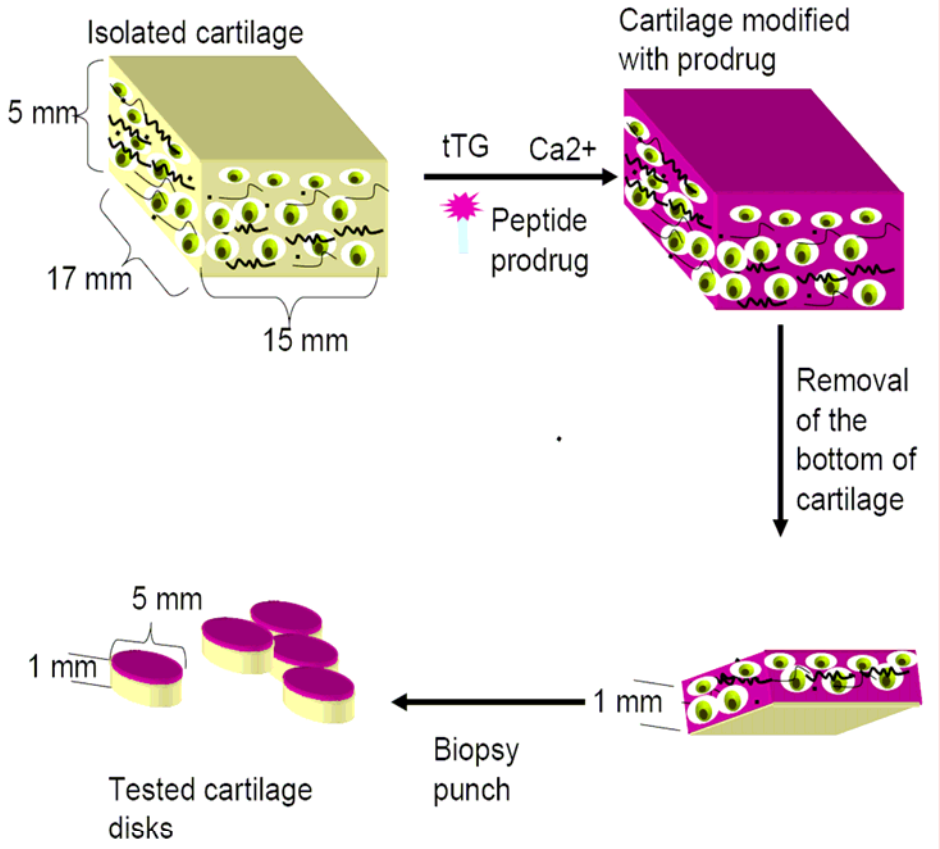


Figure 8-5. Schematic of articular surface modification with prodrugs. Both the HyC-GFKG and HyC-GFKG-PEG were treated in the same manner for the tTG reaction. After the coupling reaction, 4 mm were cut from the bottom of the cartilage piece. A biopsy punch was used then to isolate disks with 5 mm diameter, having only the articular surface modified with the prodrug.

room temperature. After rinsing, approximately 4 mm of tissue was removed from the bottom of the piece. Five 5 mm disks were cut from each cartilage piece using a biopsy punch, with the thickness of each disk being between 0.5 and 1.5 mm. The disks were rinsed overnight in 3 mL PBS containing protease inhibitors at pH 4 and 4 ^{o}C. Finally, the disks were rinsed for 1 hour at 4 ^{o}C in PBS to remove any residual protease solution.

The disks were blotted dry and the mass determined, after which they were digested in 750 μL of PBS with 125 μg/mL papain, 5 mM EDTA and 5 mM cysteine at pH 6 overnight. A portion of the solution containing the digested disk was subjected to analysis by the Cortisol Parameter Assay kit (used per the manufacturer's protocol) to determine the amount of hydrocortisone in the sample. The assay plate was analyzed using the Tecan plate reader system. The quantity of hydrocortisone detected in each sample were corrected for the mass of the disk.

8.2.2.6 The effect of time and concentration on prodrug coupling.

The relationship between concentration and coupling was evaluated for HyC-GFKG at 0.1, 0.5, 1.0, 1.5, and 2.0 mM. The effect of time on the amount of HyC-GFKG coupled was evaluated at 0.25, 0.5, 1.0, and 2.0 hours using 1.0 mM of the prodrug. These experiments were conducted and analyzed in the same fashion as the coupling reactions previously described in .2.5.

8.2.2.7 Effect of PEG on prodrug coupling.

In order to study the effect of PEG on coupling, 1.0 mM HyC-GFKG-PEG was coupled to the articular surface of cartilage in the same manner as HyC-GFKG. All experimental parameters were the same as described on 8.2.2.5.

8.2.2.8 Release of the coupled hydrocortisone prodrugs on the articular surface of cartilage.

HyC-GFKG and HyC-GFKG-PEG prodrugs were coupled to the articular surface as described previously in section 8.2.2.5 except that all solutions were filter sterilized and manipulation of the cartilage was performed in a sterile laminar flow hood. Control experiments in which hydrocortisone and hydrocortisone-GFKG without tTG were incubated with the cartilage (as described in section 8.2.2.5) were conducted and evaluated similar to the test samples. After the overnight rinse in PBS with proteases and the subsequent PBS wash, 3 disks were incubated with sterile PBS, 3 with normal synovial fluid, and 3 with osteoarthritic synovial fluid. Each disk was incubated with 100 μL of the respective fluid at 37 ^{o}C. Ten microliters of the fluid were removed from each treatment at 4, 12, 24, and 48 hours. To maintain a 100 μL incubation volume, 10 μL the respective fluids were added at each time point. At the end of the experiment, the digested as previously described. All samples were analyzed using the Cortisol Parameter Assay kit.

8.2.2.9 Coupling of the HyC-GFKG to the meniscus.

HyC-GFKG at a concentration of 1 mM was coupled as described and analyzed as described in 8.2.2.5.

8.2.2.10 Immunohistochemistry.

After tissue modification, select pieces of cartilage and meniscus were frozen in tissue freezing media and stored at -80 ^{o}C until use. The frozen discs of cartilage and meniscus were cut in vertical sections (25 μm thick), which included the articular surface, using a cryostat (Microm HM505N, Carl Zeiss). The freezing media was removed with 3 washes of PBS.

Samples were blocked with 4% goat serum in PBS (blocking buffer) for 1 hour. The cartilage sections were incubated with 10 μg/mL mouse anti-hydrocortisone antibody for 1.5 hr in the blocking buffer at room temperature. Samples were rinsed 3 times with PBS for 5 minutes each and then incubated with fluorescein conjugated goat anti-mouse antibody (1/200) in blocking buffer for 1 hour. Samples were rinsed again with PBS and then imaged using a Leica epifluorescent microscope (λ_{ex} = 495 nm, λ_{em} = 520 nm for fluorescein) and a SPOT RT digital camera (Diagnostic Instruments, Sterling Heights, MI).

8.2.2.11 Statistical analysis.

Statistical analysis was performed using one-way ANOVA and Tukey's post-hoc test with 95% confidence intervals with SPSS (SPSS, Chicago, IL).

8.3 Results

8.3.1 Synthesis of prodrugs

After synthesis and purification of HyC-GFKG, the collected product had a determined molecular mass of 852.78 Da by ESI-MS (calculated 851.99) (Figure 8-6). On RP-HPLC, the retention time was 21.2 min with a 20-55% acetonitrile:water gradient and peaks were detected at both at 215 and 242 nm (Figure 8-7). For HyC-GFKG-PEG, the detected $\bar{M}_w$ was 5.8 kDa, which was 800 Da more than that for unmodified PEG, indicating that the HyC-GFKG had been conjugated to the PEG (Figure 8-8). On RP-HPLC, the retention time of HyC-GFKG-PEG was 24.0 min with an acetonitrile gradient of 24-55% and peaks were detected at both 215 and 242 nm (Figure 8-9). There was an additional peak at 23.0 min, accounting for a 7% impurity in the

sample. The peaks with a retention time of 3.4 min at 215 nm in both chromatograms (Figure 8-7a and Figure 8-9a) represent trifluoroacetic acid.

8.3.2 Effect of temperature and pH on hydrolysis of the hydrocortisone prodrugs.

In all solutions tested, the prodrugs were hydrolyzed, releasing hydrocortisone which had a retention time of 10.1 min on HPLC with a 24-55% gradient of acetonitrile in water (Figure 8-10). The 6 α-methyl prednisolone, used as an internal standard, had a retention time of 14.0 min while the unhydrolyzed HyC-GFKG had a retention time of 16.0 min. Table 8-1 lists the $T_{50\%}$ of the prodrugs at different hydrolysis conditions. As

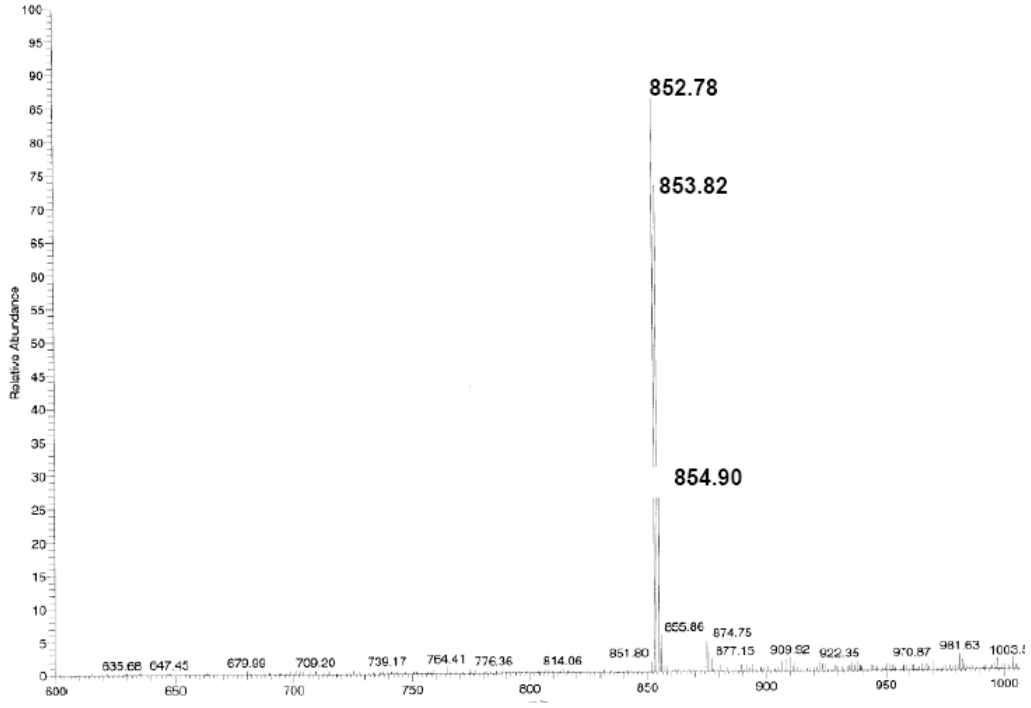


Figure 8-6. ESI-MS of HyC-GFKG. Peak at 852.78 represents the $(M+H)^+$.

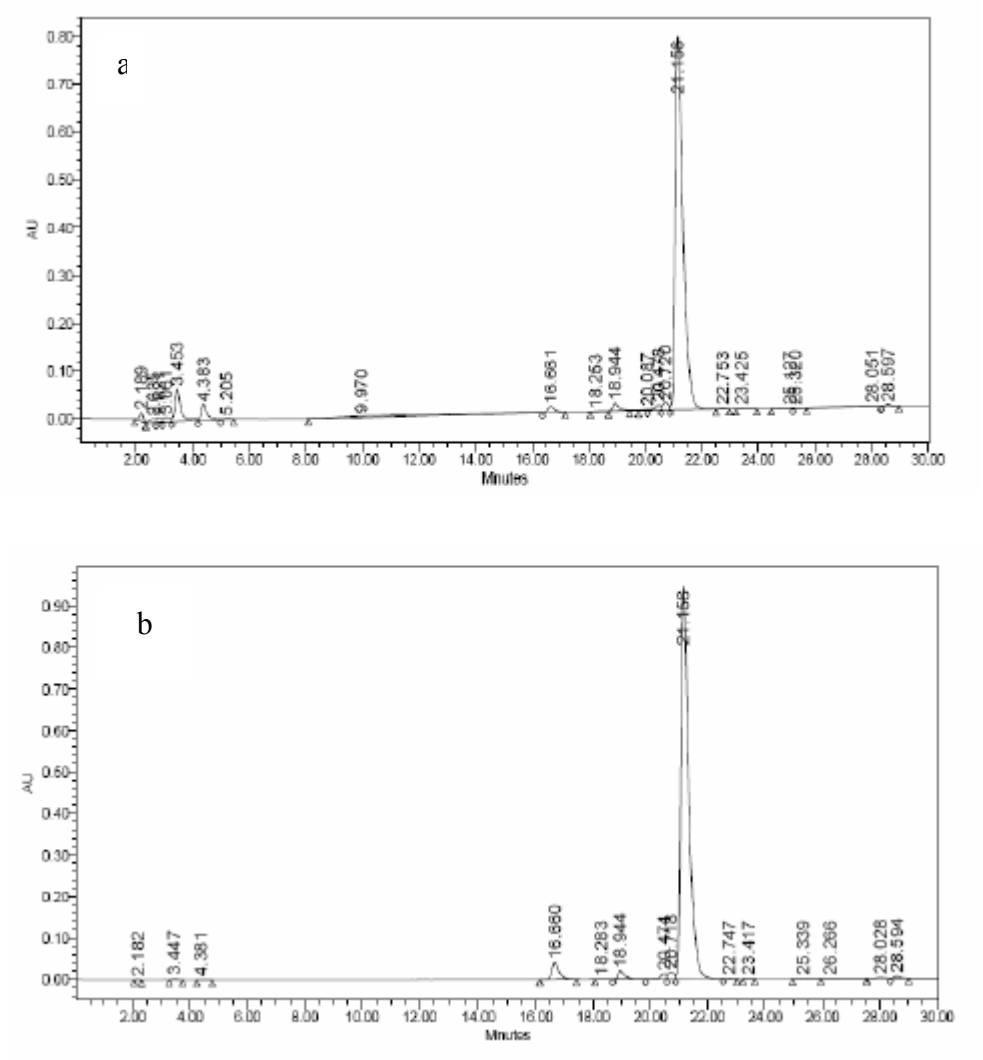


Figure 8-7. Chromatograms of HyC-GFKG. Peak at 21.2 min retention time is HyC-GFKG. a.) Detection at 215 nm. The peak with a retention time of 3.4 min is TFA. b.) Detection at 242 nm, the maximum absorption of hydrocortisone.

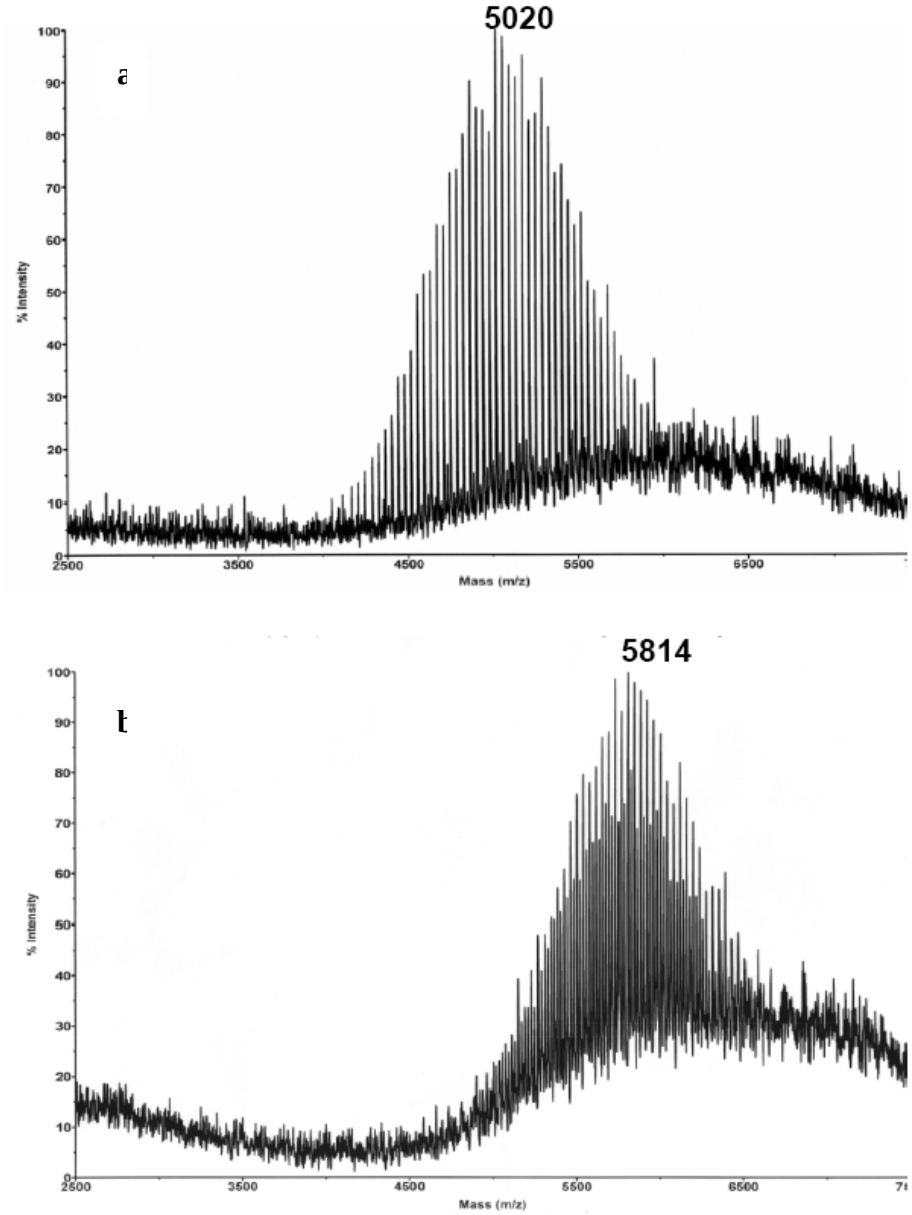


Figure 8-8. MALDI-TOF mass spectra of PEG and HyC-GFKG-PEG. a.) Mass spectrum of unconjugated PEG with a $\bar{M}_w$ of 5 kDa. b.) After conjugation of HyC-GFKG, the molecular weight of the PEG increased to a $\bar{M}_w$ of 5.8 kDa.

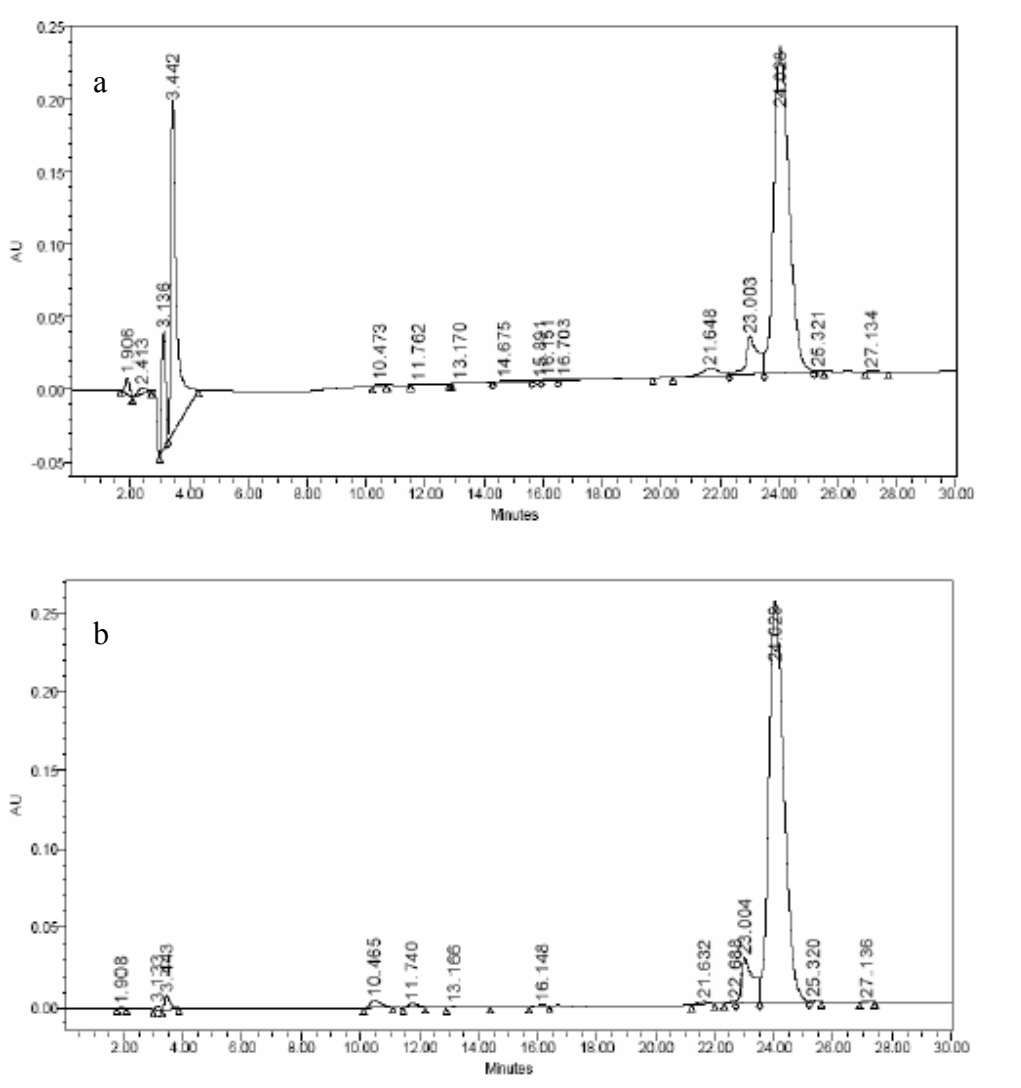


Figure 8-9. Chromatograms of HyC-GFKG-PEG. Peak with a retention time of 24.0 min is HyC-GFKG-PEG. The additional peak with a retention time of 23 min is an impurity (7%) in the sample. a.) Detection at 215 nm. The peak with a retention time of 3.4 min is TFA. b.) Detection at 242 nm.

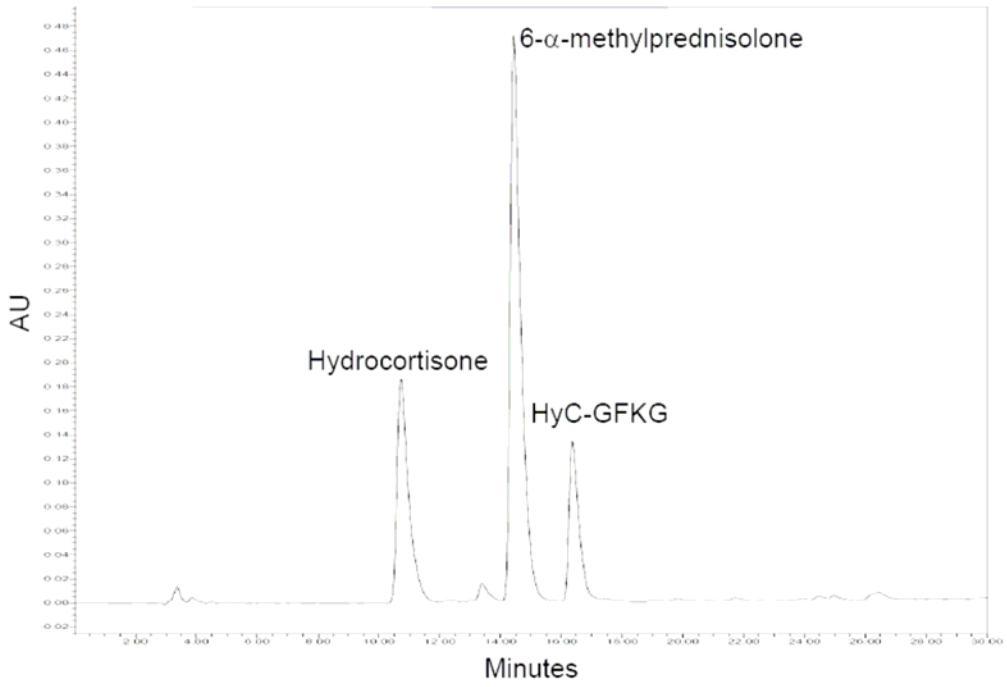


Figure 8-10. Representative chromatogram of the aqueous hydrolysis of HyC-GFKG at 242 nm. 6-α-methylprednisolone was used as the internal standard.

Table 8-1. Solution hydrolysis of prodrugs at various conditions.

Hydrolysis Conditions	$T_{50\%}$ (minutes)
HyC-GFKG	
PBS, pH 7.4, 37 ^{o}C	50.2
PBS, pH 7.4, 23 ^{o}C	198
PBS, pH 7.4, 4 ^{o}C	3465
PBS, pH 4.0, 4 ^{o}C	115500
HEPES, pH 7.4, 37 ^{o}C	27.6
Synovial Fluid, 37 ^{o}C	11.1
HyC-GFKG-PEG	
PBS, pH 7.4, 37 ^{o}C	54.1

temperature decreased, the $T_{50\%}$ of hydrolysis increased in PBS buffer (pH 7.4), from 50.2 min at 37 ^{o}C to 57.8 hours at 4 ^{o}C. $T_{50\%}$ of HyC-GFKG and of HyC-GFKG-PEG were comparable in PBS at 37 ^{o}C. A decrease of 3 pH units, from 7.4 to 4.0 at 4 ^{o}C, also led to an increase in $T_{50\%}$ of hydrolysis by a factor of 33. In HEPES buffer at 37 ^{o}C, the $T_{50\%}$ was lower than in PBS. Finally, the prodrug hydrolysis was 4 times more rapid in synovial fluid than in PBS at 37 ^{o}C.

8.3.3 Coupling of HyC-GFKG to articular surface

In the presence of Ca^{2+} and tTG, HyC-GFKG was coupled to the articular surface as evidenced by the florescence of the fluorescien antibody, which detected the presence of hydrocortisone in the prodrug (Figure 8-11a). The most intense fluorescence was on the articular surface; however, fluorescence was also detected in the tissue. The majority of detected hydrocortisone was coupled by tTG to the articular surface, since in the absence of tTG, the fluorescence intensity was greatly reduced both with the prodrug and with hydrocortisone (Figure 8-11b and c). When the disks were digested and the amount of bound hydrocortisone was quantified via the Cortisol Parameter Assay kit, it was determined that the amount of hydrocortisone bound to the disks incubated with hydrocortisone alone had an average of 0.00085 nmoles hydrocortisone/mg of cartilage. Disks incubated in the presence of HyC-GFKG without tTG had an average of 0.0036 nmoles of bound hydrocortisone/mg of cartilage, and disks incubated in HyC-GFKG with tTG had an average of 0.0194 nmoles of bound hydrocortisone/mg of cartilage (Figure 8-11d). The amount of bound hydrocortisone detected in the sample incubated with tTG was statistically ($p<0.05$) greater than that

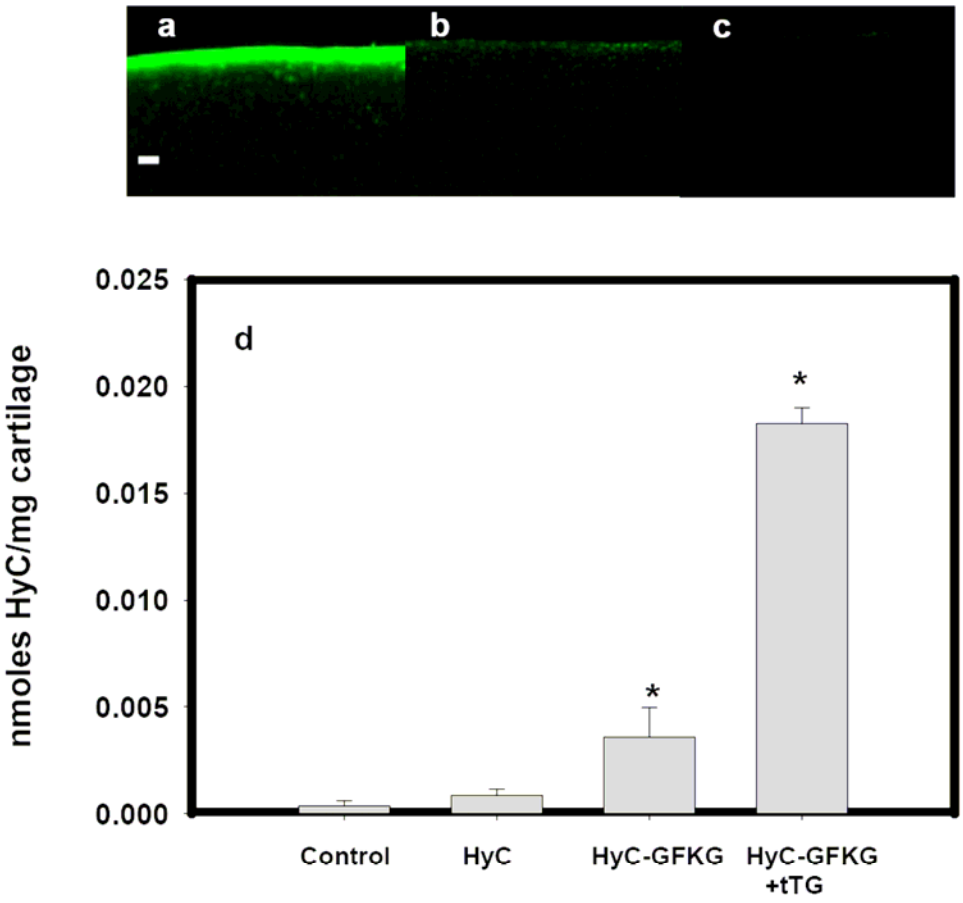


Figure 8-11. Detection of HyC-GFKG on articular surface of cartilage. a-c) Immunodetection of the bound HyC-GFKG with an anti-hydrocortisone antibody and fluorescein conjugated secondary antibody. Bar in (a) represents 30 μm. Incubation of cartilage with a) HyC-GFKG with tTG; b) HyC-GFKG without tTG; c) hydrocortisone. d) Graph quantifying the amount of hydrocortisone (HyC) detected using the cortisol assay kit. Control are samples incubated with only buffer. The error bars represent standard deviation. *, $p<0.05$.

incubated without tTG. However, there were no statistical differences between the control samples.

8.3.4 The dependence of HyC-GFKG coupling on initial solution concentration

To determine the effect of concentration on coupling of the prodrug, the cartilage pieces were incubated with different concentrations of HyC-GFKG. At each concentration examined, the samples that were incubated with tTG had significantly ($p<0.05$) larger quantities of HyC-GFKG detected than the respective control samples without tTG, except at 0.1 mM (Figure 8-12). As the concentration of HyC-GFKG increased from 0.1 to 1 mM, there was a linear increase in the amount coupled by tTG to the articular surface. The amounts coupled at 0.1 mM and 0.5 mM were statistically lower ($p<0.05$) than the amount of HyC-GFKG coupled at 1.0 mM. Increasing the concentration of HyC-GFKG to 1.5 mM or 2.0 mM did not increase the amount of prodrug coupling.

8.3.5 Time dependence of coupling

Over the two hours that the coupling reaction was observed, there was a continual increase in the amount of the hydrocortisone prodrug coupled to the surface, but the rate of coupling decreased as time increased (Figure 8-13). The amount coupled at 15 and 30 minutes was statistically less than at 1 hour ($p < 0.05$). However, the amount detected at 1 and 2 hours were statistically the same. During that same 2 hour period, the control samples did not have a statistical increase in the amount of hydrocortisone detected ($p > 0.05$).

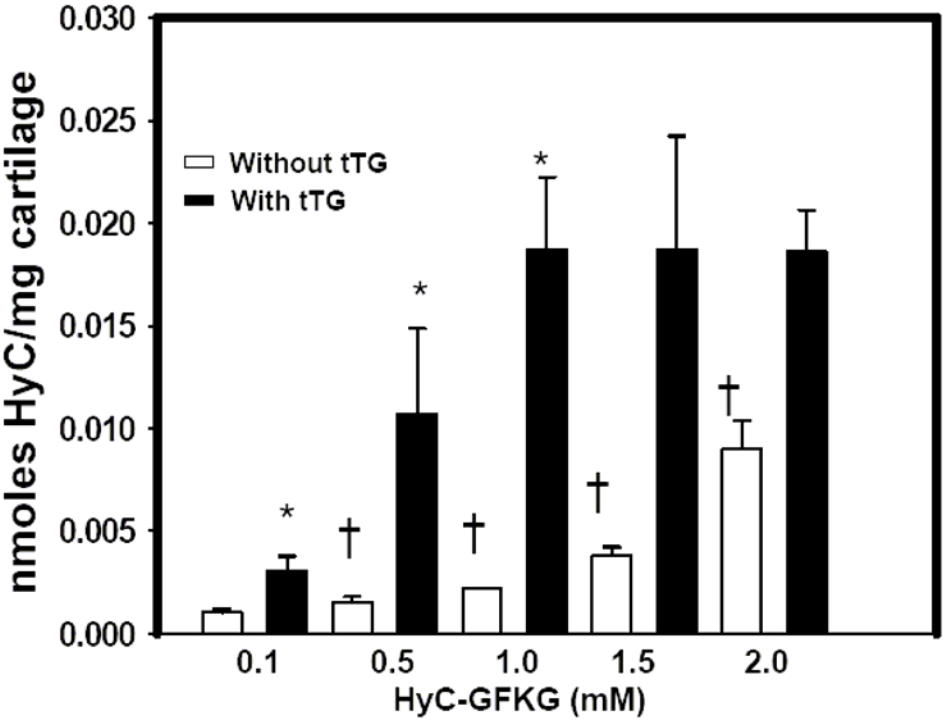


Figure 8-12. Effect of concentration on quantity of HyC-GFKG coupled to the articular surface. Graph quantifying the amount of hydrocortisone (HyC) detected on cartilage after incubation with 0.1 mM to 2 mM HyC-GFKG. Error bars represent standard deviations. *, $p<0.05$ as compared to 1.0 mM with tTG. †, $p<0.05$ as each is compared to the sample at the same concentration but coupled with tTG.

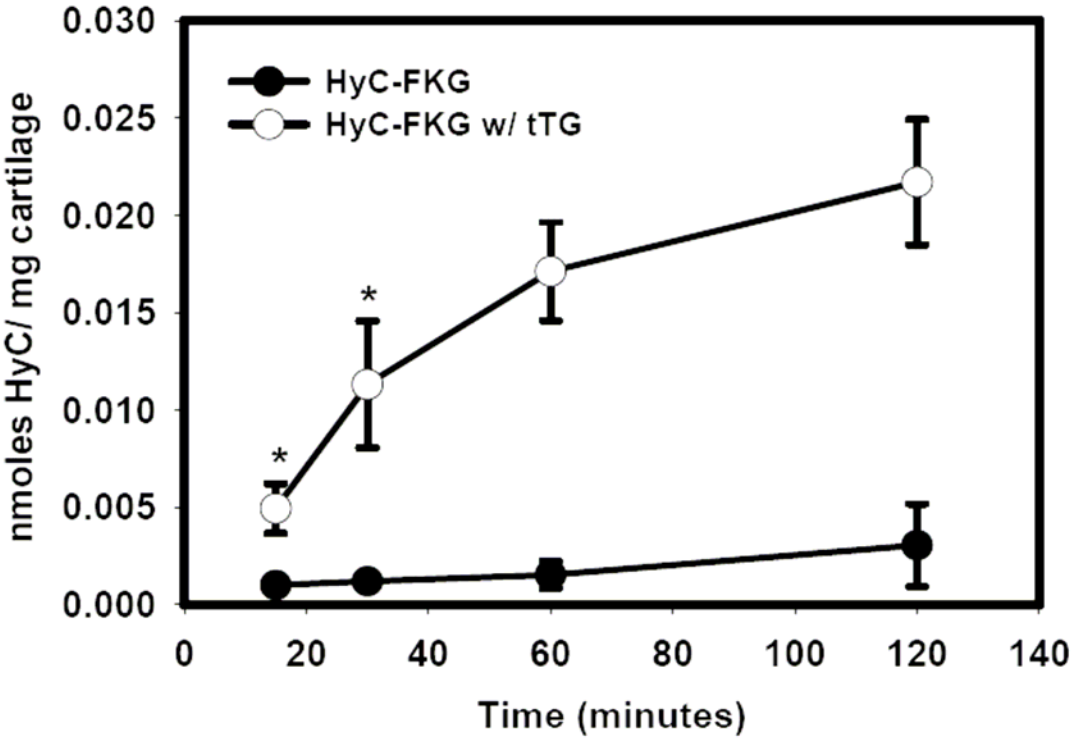


Figure 8-13. Time dependence of coupling of HyC-GFKG. The amount of hydrocortisone (HyC) detected increased as time increased for the samples incubated with tTG. There was no statistical increase in the samples that did not contain tTG. *, $p<0.05$ comparing the amount of hydrocortisone detected after 15 and 30 minutes of coupling to the amount after 60 minutes of coupling.

8.3.6 Effect of PEG on produg coupling

HyC-GFKG-PEG was coupled to the articular surface of cartilage in an amount equivalent to that seen for HyC-GFKG, as indicated by the amount of hydrocortisone detected ($p > 0.05$) (Figure 8-14a). In addition, the levels of nonspecific binding were the same for the two prodrug controls without tTG ($p > 0.05$). The immunohistochemistry confirmed the results obtained by the hydrocortisone ELISA. The HyC-GFKG and the HyC-GFKG-PEG prodrugs qualitatively appear to be coupled to the cartilage surface in equal quantities (Figure 14b and d) and the control sections exhibited very little fluorescence, indicating that the amount of nonspecific prodrug adsorption was insignificant for both prodrugs (Figure 14c and e).

8.3.7 Release of the prodrugs from articular surface in PBS buffer and synovial fluids

From 4 to 48 hours, there was a statistical increase in the amount of hydrocortisone released from the cartilage for samples treated with tTG and HyC-GFKG, regardless of the fluid the cartilage was in ($p < 0.05$) (Figure 8-15a). At 4 hours, there was statistically more released hydrocortisone detected in the osteoarthritic synovial fluid than in PBS. When the time increased to 12 and 24 hours, there was statistically more released hydrocortisone detected in both synovial fluids than in PBS. However at 48 hours, all 3 solutions had statistically the same amount of released hydrocortisone. Similar results were obtained for the HyC-GFKG-PEG samples (Figure 8-15 b).

Two control experiments were conducted to examine the release of HyC-GFKG and hydrocortisone when incubated in PBS and both synovial fluids (Figure 8-15c and d). Both of these negative controls had similar results in each fluid. At each time point, the amount of

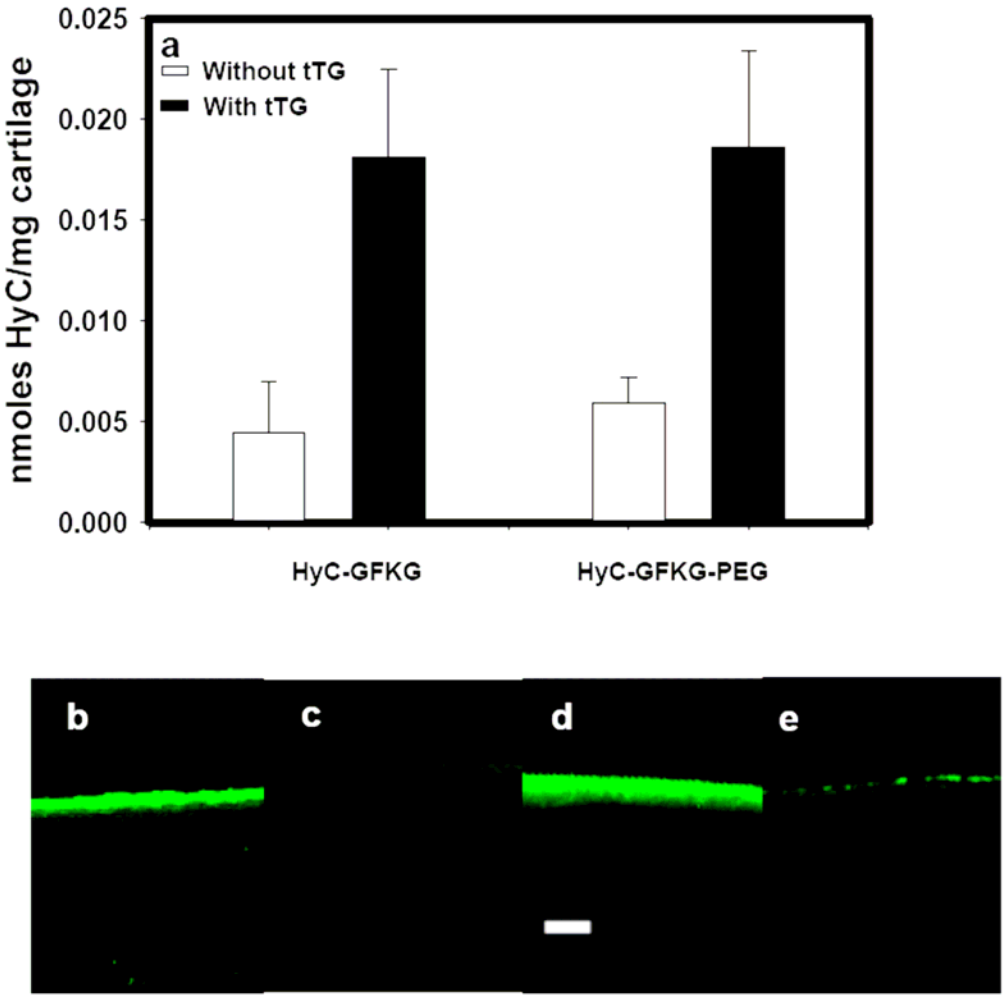


Figure 8-14. Comparison of the amount of HyC-GFKG and HyC-GFKG-PEG coupled to the articular surface. a) Graph quantifying the amount of hydrocortisone detected/mg of cartilage when incubated in either HyC-GFKG or HyC-GFKG-PEG. Error bars represent standard deviation. b-e)Immunohistochemistry. Detection of hydrocortisone with anti-hydrocortisone antibody and a fluorescein conjugated secondary antibody under different coupling conditions. b) HyC-GFKG with tTG; c) HyC-GFKG without tTG; d) HyC-GFKG-PEG with tTG; HyC-GFKG-PEG without tTG. Bar represents 30 μm.

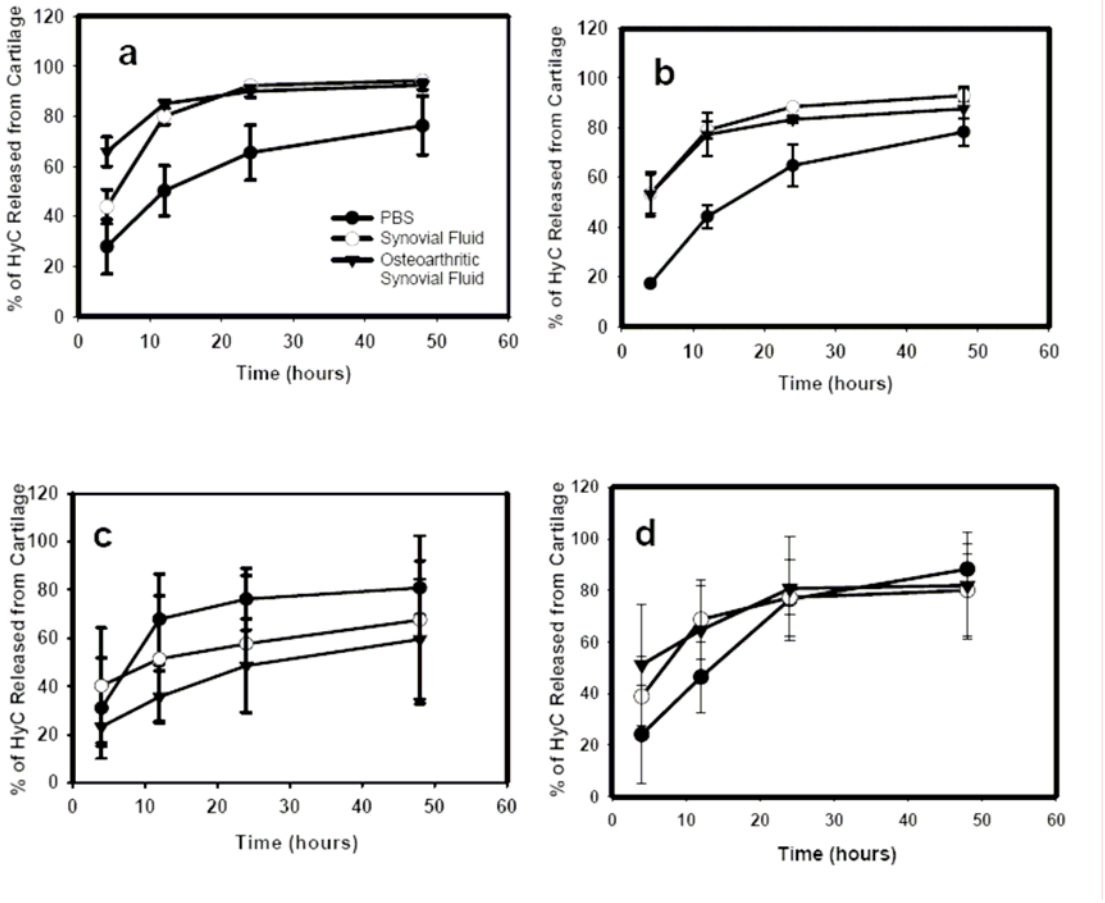


Figure 8-15 Release of hydrocortisone from the articular surface during 24 hours of incubation with PBS, normal synovial fluid, and osteoarthritic fluid. Prior to the 48 hour experiment cartilage was treated as specified. a.) Cartilage treated with HyC-GFKG and tTG; b.) Cartilage treated with HyC-GFKG-PEG and tTG; c.) Control cartilage treated with HyC-GFKG; and d.) Control cartilage treated with hydrocortisone. Samples incubated with tTG had a statistical increase in the amount of hydrocortisone detected in all 3 fluids from 4 to 48 hours ($p < 0.05$). In addition, there was significantly more hydrocortisone detected in both synovial fluids than in PBS ($p<0.05$). However, no statistical significance was detected for either of the control samples without tTG.

hydrocortisone detected in each of the 3 fluids were statistically the same. In addition, there was no statistical change in the amount of released hydrocortisone detected from 4 to 48 hours.

8.3.8 Modification of meniscus

Modification of the meniscus with HyC-GFKG was detected on the surface of the tissue with the anti-hydrocortisone antibody and the corresponding fluorescein conjugated secondary antibody (Figure 8-16a). As with cartilage, when tTG was not included in the incubation only a small amount of hydrocortisone could be detected on the tissue (Figure 8-16b). When the amount coupled was compared to that coupled on cartilage, there was statistically more prodrug coupled to the meniscus than on the caritilage ($p < 0.05$) (Figure 8-16c). The amount of non tTG-mediated prodrug binding was statistically equivalent for both tissue types.

8.4 Discussion

Glucocorticoids provide relief for both the pain and inflammation of OA and RA by inhibiting IL-1, a potent inflammatory cytokine[51]. Because glucocorticoids can cause many adverse biological effects, these drugs must be administered via intra-articular injection in order to target the treatment to the affected joint[306-308]. However, current methods of maintaining the drug in the joint, via deposition from a suspension[305], can cause synovitis and usually still results in drug loss to the circulatory system via the joint lymphatics[52]. New strategies being investigated to retain glucocorticoids in the joint space also rely on deposition and insolubility, which can lead to similar problems as with current treatments:

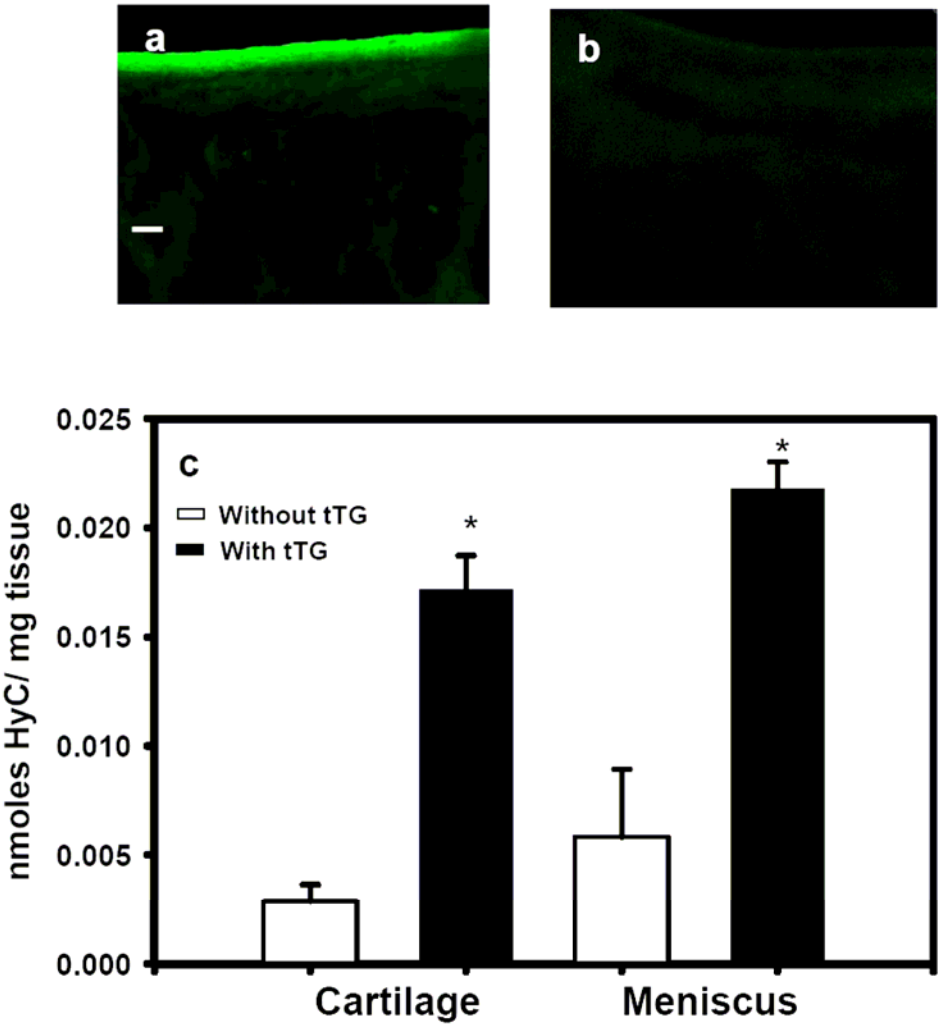


Figure 8-16. Coupling of HyC-GFKG to the meniscus. a and b) Immunohistochemistry of the menisucus with anti-hydrocortisone antibody and the corresponding fluorescein conjugated secondary. a) Incubation of the meniscus with HyC-GFKG and tTG. b) Incubation of the meniscus with HyC-GFKG. Bar represents 60 μm. c) Graph comparing amount of hydrocortisone detected in cartilage with that in the meniscus. *, $p<0.05$, comparing the amount of hydrocortisone detected in cartilage to that in the meniscus.

elimination via the lymphatics and potential inflammation. Therefore, there exists a need for an improved method of retaining therapeutic agents in the joint.

In this study, we demonstrate a novel and straightforward approach of localized drugdelivery to the joint by covalently coupling a hydrocortisone prodrug to the articular surface of cartilage via an enzyme, tissue transglutaminase. This ubiquitous enzyme forms chemically and enzymatically resistant isopeptide bonds between glutamine and lysine residues in proteins and peptides at physiological conditions[3]. In our lab, we have developed short synthetic peptide substrates of tTG that have been used to modify polymers, which were then covalently coupled to cartilage[5, 264]. One of these peptide substrates, FKG (lysine substrate), was used to modify hydrocortisone hemisuccinate which was subsequently covalently attached to the articular surface. While our lab has investigated both glutamine and lysine substrates of tTG[5, 265], the lysine substrate was selected for this study because the articular surface of cartilage is composed primarily of collagen II, which crosslinks readily with lysine substrates in the presence of tTG.[195, 201, 263] It has also been demonstrated that lysine substrates crosslink to collagen II in larger quantities than glutamine substrates[264]. In addition, the lysine peptide has a higher solubility than the glutamine peptide in aqueous solutions.

The conjugation of hydrocortisone hemisuccinate to GFKG formed a prodrug that released hydrocortisone by the hydrolysis of the ester bond in both buffer solutions and synovial fluid. Hydrolysis in buffer solutions was likely facilitated by the amine group on lysine, which acted as an intramolecular catalysis of ester cleavage[331, 332]. In synovial fluid, the presence of esterases was likely responsible for the increased degradation of the prodrug over that observed in the buffer solutions[333, 334]. The hydrolysis of the ester bond, mediated by the amine of the lysine substrate, while undesirable, can be modulated by introducing moieties that sterically

block interactions between the amine and the ester[331, 332, 335]. One method of achieving this is through the manipulation of peptide sequence and length[336, 337]. Patel *et al.* demonstrated that the sequence of a dipeptide (Gly and Val) ester of gancyclovir could affect the kinetics of aqueous hydrolysis by a factor of 10^{336}. Both the peptide's sequence and length may influence the ability of esterases to hydrolyze the ester bond by affecting the ability of the enzyme to bind the ester substrate. Recently, Hu *et al.* screened a library of tetramer lysine peptide substrates of tTG and found that several were good substrates of tTG[265]. This is because the enzyme has a broader substrate specificity for the lysine peptide than for the glutamine substrate[3, 173]. It is therefore possible that a lysine peptide other than FKG may provide more steric inhibition to the intramolecular hydrolysis by the amine, while still retaining acceptable levels of reactivity with tTG.

Despite its finite stability, the prodrug HyC-GFKG was coupled to the articular surface of cartilage via tTG at about 0.019 nmoles/ mg of cartilage tissue. Most of the bound prodrug was attached to the articular surface but a minute amount was detected in the tissue. The amount of prodrug coupled to the articular surface increased as the incubation time was increased. Prodrug coupling was likely limited by the stability of the drug and by the competitive inhibition of the hydrolyzed peptide, which remained a substrate of the enzyme. Nevertheless, it could be determined that the coupling occurred similar to standard enzyme kinetics. For example, at HyC-GFKG concentrations between 0.1 and 1.0 mM, there was a linear relationship to the amount of prodrug bound to the surface. At these concentrations, the amount of prodrug coupled was limited by the concentration of the substrate. However, at concentrations of 1.0 mM and above, the amount of prodrug coupled was limited by k_{cat} because an increase in concentration did not yield an increase in the amount of prodrug bound. It may be possible for more prodrug to be

coupled with an increase in enzyme concentration, which would increase the rate of coupling of the prodrug early in the reaction, thus minimizing the effects of drug hydrolysis.

The release of hydrocortisone from the coupled HyC-GFKG on the articular surface was examined in PBS, normal bovine synovial fluid, and human osteoarthritic synovial fluid. In both PBS and normal synovial fluid, the rate of release of hydrocortisone from the coupled prodrug into the fluid was less than the rate of hydrolysis of the prodrug in solution, but the release in synovial fluid was still more rapid than in PBS. The coupling of the prodrug by tTG resulted in the chemical production of an amide, which was bonded to proteins in the articular surface, thus eliminating the intramolecular catalytic cleavage of the ester and decreasing the release of the hydrocortisone from the prodrug. In addition to aqueous hydrolysis, the hydrocortisone can be liberated from the surface by esterases[333, 334] in the synovial fluid or by matrix metalloproteinase degradation[338] of the cartilage matrix. Esterases in the synovial fluid were likely responsible for the increased rate of hydrolysis above that occurring in PBS. These enzymes are excreted into the synovial fluid by a subset of synoviocytes that are phagocytic in origin[339]. During OA, these synoviocytes increase production of degradative enzymes therefore, it was unexpected that the rate of hydrolysis of the prodrug was the same in the normal and OA synovial fluid. This could be related to the amount of prodrug on the surface which was to small to determine differences in rates of hydrolysis. It is possible that esterases from these bovine and human are can sinilar. It has been shown that esterases from different species and different organs within any animal may or may not have the same rate of hydrolysis. In order to increase the lifetime of the cartilage on the surface, Payan *et al.* added HA to the mixture which increased the viscosity of the solution and reduced hydrolysis, likely because of decreased mobility of the enzyme[315]. Another method

of decreasing hydrolysis of the ester is to modify the structure of the ester, although the structural features that promote stability of the ester are not well understood.

Both healthy and osteoarthritic chondrocytes synthesize and excrete MMPs that can degrade the cartilaginous matrix. In addition, MMPs are excreted into the synovial fluid both by chondrocytes and synoviocytes. Therefore, *in vivo* degradation of the cartilaginous matrix by MMPs may prematurely release the bound prodrug. However, the prodrug is hydrolyzed and becomes active because it must be in its active form to enter cells by passive diffusion through the membrane. In the current experiments, MMP activity was not likely a mechanism of liberation of the prodrug on the articular surface because chondrocyte viability was adversely affected by storage at -80 ^{o}C[340], the acidic[341] rinse to remove excess prodrug, and the long-term glucose deprivation[341]. In addition, any MMPs produced were likely washed out with the multiple washing steps.

PEG has been used to modify biological surfaces, making them inert to cells and proteins as well as used to camoflauge proteins from cells and action of enzymes in the circulatory system[119, 329]. For these reasons, PEG was added to the hydrocortisone- peptide to form a new prodrug. Modification of with PEG did not adversely affect prodrug coupling nor did it have an effect on the rate of aqueous hydrolysis. It was previously demonstrated that the addition of PEG does not adversely affect enzyme action[264, 273, 342]. However, in another study of PEGs of varying molecular weight, 200 to 2000, used to modify hydrocortisone hemisuccinate, aqueous hydrolysis was not significantly influenced by the molecular weight of the PEG; however hydrolysis by esterases was reduced with increasing molecular weight[342]. In the current study, there was no difference between the PEGylated and non-PEGylated prodrugs coupled to the articular surface in any of the fluids utilized. This could be due to the length of the PEG, which

may not properly cover the articular surface. In addition, the locations on the articular surface to which the the prodrug is bound may not be close together and hence coverage of the cartilage is insufficient. This may be accomplished by using a longer PEG, such as 10 kDa to 40 kDa, may provide better coverage [343].

The articular surface of cartilage is only one joint structure to which the prodrug would be coupled via tTG if injected intra-articularly. The meniscus was also modified with the prodrug, similar to the articular surface of cartilage. The outer portion of the meniscus is primarily composed of collagen I, which is also a substrate of tTG, while the composition of the inner portion of the meniscus is composed primarily of collagen II[6], similar to the articular surface. It is also expected that the synovial lining which covers many joint structures such as ligaments will also be modified with the prodrug *in vivo* since it is also composed of collagen I. Collagen I may have more sites for attachement because of

Collagen I is a component of many biological tissues. Therefore, tTG can be used to immobilize therapeutic agents modified with substrates of tTG to tissues other than cartilage. In addition, there are other ECM components such as fibronectin[344], vitronectin[289, 290], and laminin[291] that are substrates of tTG and can be used for coupling molecules to tissue surfaces. Variables such as coupling concentration and number of potential sites available on the tissue will need to be investigated since ECM organization will determine exposed epitopes.

8.5 Conclusions

This study demonstrates the first use of tTG couplingof molecules to the articular surface as a method of localized drug delivery, in this case, hydrocortisone. This approach to retaining drugs at a target location is simple and can be conducted at physiologic conditions. Moreover, this strategy is not limited to the intra-articular space, but can be used to immobilize therapeutic agents on other biological tissues. Methods of conjugating the therapeutic agent to the appropriate peptide substrate of tTG, as well as tissue specific characteristics, will need to be evaluated in order to improve the efficacy of this method as it applies to different tissues.

Chapter 9

Recommendations for Future Experimentation

9.1 Introduction

In vivo, tissue transglutaminase (tTG) is used to crosslink proteins that contain reactive lysines and glutamines. These crosslinkings assist in stabilizing the ECM and facilitate the coupling of growth factors to the ECM[3]. In the current study, tTG was used in a similar manner: to form (and stabilize) hydrogels consisting of polymers modified with synthetic peptide substrates of tTG and to couple modified molecules to cartilage tissue. Two hydrogel formulations were used to evaluate the tTG crosslinking strategy as a means of entrapping chondrocytes for the treatment of osteochondral defects. It was determined that while tTG could be used to form a hydrogel, the swelling of the gel could affect cell viability. In addition, peptide-PEG conjugates were coupled to the native proteins in cartilage via tTG in order to demonstrate the potential for improved hydrogel integration with the native cartilage tissue during *in situ* gelation. It was determined that not only were peptide-PEG conjugates coupled to the cut surface of the cartilage tissue but also to the articular surface, which was hypothesized to have greater potential and applicability. Since drugs delivered locally via intra-articular injection are usually lost to the circulatory system via the lymphatics, coupling the drug to the articular surface could be a useful strategy for retaining the drug in the intra-articular space. This strategy of localized therapeutic delivery and long-term retention has potential in tissues other than cartilage because the ECM of many tissues is composed of substrates of tTG. This was demonstrated by coupling a hydrocortisone-peptide prodrug to the articular surface of cartilage

and released in the presence of biological fluid. However, further study of the current system of surface modification to cartilage is needed to better understand the impact the biological environment has on coupling and of the tTG on the local tissue.

9.2 Future utilization of tTG crosslinking for hydrogel formation

It is evident from the experiments discussed in Chapters 5 and 6 that the use of tTG crosslinked gels may not be a feasible approach for entrapping chondrocytes. Both hydrogel constructs had limited matrix production and in the PEG hydrogel, cells were fatally damaged during swelling, which is hypothesized to be the result of cell surface proteins crosslinking with hydrogel components. It is possible that hydrogels consisting of polymers that result in limited swelling may be compatible with tTG crosslinking, as indicated by the PEG-alginate gel. In addition, because of the culture conditions used for the PEG hydrogels, it is still unclear whether degradability can improve matrix production, although studies with free swollen hydrogels indicate that degradability would be beneficial. Therefore, hydrogel degradation with an appropriate matrix that does not compromise the cell still needs to be investigated.

One potential use of these hydrogels is as an adhesive between osteochondral plugs and the native cartilage tissue. In Chapter 7, peptide-PEG polymers were shown to penetrate the native cartilage matrix by about 10 microns, which may be of sufficient depth to perform an adhesive role. Jurgensen *et al.* demonstrated that tTG could be used as an adhesive for cartilage-on- cartilage bonds[4] and Hu *et al.* demonstrated that a PEG hydrogel crosslinked with tTG was adhesive with guinea pig skin[148]. It is therefore possible for tTG crosslinked hydrogels to be used

as an adhesive between two segments of cartilage tissue, allowing osteochondral plugs to remain in the defect and connected to the native tissue.

9.3 Therapeutic modification to the intra-articular space and other tissue surfaces

9.3.1 Future experiments

Tissue transglutaminase, as illustrated in the previous two chapters, can be utilized as a means of coupling polymers and molecules to the cartilage surface. Therefore, tTG coupling has the potential to be utilized as a means of retaining therapeutic agents in the joint to which the drug has been administered. While the studies performed in Chapter 7 and 8 are promising, more studies are required to better understand the intricacies of tTG coupling to the articular surface of cartilage. This section will discuss the necessity for evaluation of 1) a prodrug containing a glutamine peptide substrate, 2) potential effects of matrix catabolism on both prodrug coupling and premature release, and 3) short-term *in vivo* experiments that will elucidate benefits and limitations not detectable by *in vitro* study. These areas of study would assist in the optimization of this strategy of drug retention.

The prodrug evaluated in Chapter 8 contained only a lysine peptide substrate of tTG for reasons previously mentioned. A glutamine substrate was not evaluated, since upon coupling of the peptide to collagen II in solution, western blot analysis showed that the lysine was more reactive than the glutamine (see Chapter 7). Although the articular surface is composed primarily of collagen II, there may be other collagens (such as Type I)[6] and noncollagenous proteins to which a prodrug containing the glutamine substrate may be efficiently coupled. In addition, the collagen II tested in Chapter 7 was only the triple helical region, in which many of the lysines are

hydroxylated for use in crosslinking and glycosylation[296]. However, *in vivo*, the telomeric regions of collagen II would also be available to which the glutamine substrate may be also be coupled. Therefore, the examination of a prodrug with the glutamine peptide is essential. Moreover, coupling a prodrug containing the glutamine substrate and one containing the lysine substrate sequentially should increase the total amount of prodrug immobilized on the articular surface.

The isopeptide bonds formed by tTG during coupling are resistant to chemical or enzymatic cleavage[3]. Therefore, tTG couplings would retain the therapeutic agent at the site of application until it is released from its peptide anchor or the anchor is released due to ECM degradation. In osteoarthritic cartilage, there is an increase in MMP-3 and MMP-13 which would degrade the collagenous matrix[345]. This degradation could affect the number of tTG coupling sites available and/or the rate of premature drug release. To study this *in vitro,* either of the aforementioned MMPs or iodoacetate[346, 347] could be used as a model of the cartilage degradation present in osteoarthritis. The compounds can be applied before coupling to examine the effect of degradation on available sites, or after coupling to evaluate premature removal of the peptide. The relationship between the amount of prodrug coupled or removed under degradative conditions and the severity of degradation may elucidate limitations or benefits of this technique at different stages in the disease. Potentially, the degradation may even unveil epitopes previously hidden and increase the coupling of lysine and/or glutamine substrates.

After these *in vitro* experiments have been used to optimize retention of the prodrug, initial *in vivo* experiments can be performed. These experiments would elucidate the behavior of the drug delivery system under normal physiological conditions and assess the feasibility of using this strategy for treatment. All components of the prodrug delivery and retention system

must be assessed for their potential to cause inflammation. While the peptide and drug itself are not anticipated to be problematic, transglutaminase is of concern. In OA, tTG is known to cause pathological calcification of cartilage[197, 348] and in RA, tTG production is increased, with a corresponding increase in tTG antibodies[349]. Transglutaminase has been demonstrated to increase inflammation by its action on phospholipase A[191] as well as cause tissue adhesions[350]. Therefore, this *in vivo* model will assess the tTG effects independent of disease. In addition, cell viability is best evaluated *in vivo* because the only inlet for nutrients is the articular surface[6] versus *in vitro* where nutrients can be acquired via not only the articular surface but also the cut surfaces. Therefore, if the surface modification is affecting nutrient transport, cell viability and metabolism will also be affected *in vivo*. Finally, rats and rabbits are good candidate animals for the proposed *in vivo* studies since many animals will need to be used.

9.3.2 Other future (long-term) considerations

The current prodrug design may not be adequate for treatment of OA. First, the current structure of one drug molecule per tTG coupling site may not provide enough of the therapeutic agent to the joint for continuous long-term disease treatment, even if all the sites are utilized. In addition, it may not be advantageous to use all available coupling sites with one application. Healthy cartilage does not turn over collagen II very rapidly; the turnover time of the cartilage in the human femoral head is estimated at 400 years[17]. As OA remits with treatment, there may be no new collagen produced, and therefore, no new tTG coupling sites would be generated to which additional drug molecules could be attached. Therefore, in order to make multiple drug applications so as to keep OA in continuous remittance, using only a fraction of the coupling

sites at each application may be preferred. Secondly, the current use of an ester bond to link the drug to the substrate of tTG may not provide the stability needed for long-term disease treatment. Therefore, other bonds with greater aqueous stability need to be explored for delivery and retention of the drug. In the following sections, polymers for drug delivery, bonds for improving drug retention, and strategies for delivering proteins via this method will be discussed.

9.3.2.1 Polymers for drug delivery

An appropriate candidate polymer for use as a vehicle for therapeutic delivery is hyaluronic acid (HA). HA, a component of almost all ECMs[54], is a polysaccharide of glucuronic acid and N-acetyl glucosamine and biologically, has molecular weights varying from a few hundred to 10 million Da[351]. In the articular joint, high molecular weight HA already provides the synovial fluid with viscosity, which is important in the overall mechanical stability of the joint[6]. In addition, HA is currently used as a therapeutic agent for treating OA (see viscosupplementation, Chapter 2). Some of the roles of HA therapy include restoring the viscosity of the synovial fluid to normal[57, 352], renewing water homeostasis[351], providing analgesia[59], returning cellular metabolism to normal[55] and reducing inflammation[44-46, 352]. In other areas of the body, HA reduces scarring and helps to align collagen I fibers during wound healing[353], and prevents abdominal adhesions[354]. Using HA to deliver drugs could provide the native therapeutic effects of HA, which may act in concert with the drug being administered. In addition, since HA is removed from the joint via the lymphatics, the use of tTG coupling of the modified HA to ECM should increase the retention of HA in the joint.

Chondroitin sulfate (CS) is another polysaccharide that can be used for targeted drug delivery. CS is composed of glucuronic acid and N-acetyl galactosamine and has a sulfate ester at the 4^{th} or 6^{th} carbon in N-acetyl galactosamine. It is the primary glycosaminoglycan (GAG) in

cartilage and is associated with HA in aggrecan[6]. CS has been shown to reduce inflammation by inhibiting both leukocytes and chondrocytes from releasing degradative enzymes and by inhibiting the action of inflammatory cytokines like IL-1β[355]. In addition, CS stimulates proteoglycan and HA production in synoviocytes and also reduces chondrocyte apoptosis[355].

Since HA and CS have similar functional groups, they may be modified in similar ways. Traditionally, HA is modified with molecules, i.e. drugs or crosslinking agents, at the carboxylic acid group. However, excessive modification of these functional groups can result in loss of the benefits discussed previously, since the carboxylic acid groups are required for cell-HA interactions. Reactions with HA can occur in either aqueous solution or, with conversion to the tetrabutyl ammonium salt, in polar organic solvents. Other moieties on HA and CS can be modified as well. For example, the glucuronic acid can be oxidized to form aldehydes, which can be readily reacted with amines. A review by Verchuysse and Prestwich outlines many of these reactions and others that can be performed with HA[351].

There are additional synthetic polymers that can be used as drug carriers to the intra-articular space. While these may not possess any additional benefits for cartilage as HA and CS do, they have been demonstrated to be biocompatible. One such polymer is PEG, which has been used to modify proteins[119, 329] and other drugs [328] in order to minimize interactions with enzymes and cells in the circulatory system. PEGs can be purchased in a variety of molecular weights and with a variety of functionalizations so that a variety of bonds can be formed. PEG can also be combined with a number of other molecules to form multiblock polymers. In addition, PEG can potentially create a protective layer over the cartilage when coupled with peptides. This was attempted in Chapter 8 with a 5000 Da PEG. However, this may not have been long enough;

Greenwald et al indicated that PEGs of about 20 to 40 kDa may provide better protection from circulating enzyme and cells[356].

9.3.2.2 Bonds between drug and polymer for optimal retention, and controlled release

Ester bonds are frequently used in tissue engineered scaffolds because they degrade via aqueous hydrolysis, thus releasing the drugs contained within the scaffold. *In vivo*, ester bonds are hydrolyzed not only by aqueous hydrolysis but also by esterases in serum, synovial fluid, intestines, and liver[357]. It is not well understood how ester structure is related to the hydrolysis rate by either method. One trend identified demonstrates that increasing acidity of the alcohol, which also corresponds to a more stable conjugate base in water, is directly related to the rate of ester hydrolysis[357]. Similarly, a decrease in the number of carbons in an alcohol, increases the stability of the conjugate base, and will likewise increase the ester hydrolysis rate[357]. Experimentally, a prodrug of mitomycin C coupled to Poly-[N-(2-hydroxyethyl)-glutamine] via an ester bond had a faster rate of hydrolysis with a tetrapeptide spacer between the carboxyl group and the mitomycin C than when there was a tripeptide spacer in the same position[358]. This could possibly indicate that the length of the carboxylic acid is also important. As discussed in Chapter 8, the presence of amines in ester prodrugs facilitates an intramolecular cleavage of the ester and hence, structural constraints must be included in the molecule to minimize such interactions. Finally, *in vivo*, the type and concentration of esterases interacting with the ester will affect the hydrolysis rate. Foroutan *et al.* demonstrated that a hydrocortisone-PEG prodrug was hydrolyzed at a faster rate with esterases in porcine liver than those in ovine corneal homogenate[342]. Similarly, differences in ester hydrolysis rates were observed for prodrugs of acyclovir in plasma and intestinal homogenates of rats[337].

Hydrazone is a pH-sensitive functional group formed by the reaction of a ketone and an azine (hydrazine) and is cleaved at weakly acid pH. This type of bond is feasible for OA applications because inflammation causes the pH of the synovial fluid to become slightly acidic[359]. Using hydrazone bonds to couple drugs to the polymer would allow the release of the drug to be controlled by environmental cues, generating a negative feedback loop. This loop is formed by the release of the drug which would alter the environmental conditions, i.e. the pH, thereby halting the release of the drug. Finally, the stability of these bonds can be modulated by the structure of the hydrazone.[360] Therefore it is possible that this could also be used for long-term applications.

Another type of "bond" for coupling the therapeutic agent is an MMP sensitive peptide sequence. While there is just one peptide bond that is cleaved, MMPs require at least a tetrapeptide for binding to the enzyme. Each MMP has an optimal sequence for its substrates. However, there are many types of MMPs and while there is some substrate specificity, they can cleave substrates that do not have their native sequence[272]. This type of linakge would also generate a negative feedback loop: the production of MMPs would trigger the release of the drug, which would then result in a drop in MMP production. The drug would then act to decrease MMP production which would cause a decrease in the amount of drug released.

9.3.2.3 Delivery of proteins

Many new therapies include the use of proteins, such as antibodies, receptor inhibitors, or signaling molecules. The tTG coupling strategy can be used with proteins and peptides if they are not natural substrates of tTG. Sato *et al.* demonstrated that IL-2 could be modified genetically with a small glutamine peptide at the N terminus and cadaverine, a small tTG substrate, could be crosslinked to it[361]. A similar strategy could be attempted for coupling

proteins to tissue surfaces. However, at conditions of very high enzyme or prolonged reaction times, tTG crosslinking will occur with residues that have low reactivity.

If the protein or peptide contains potentially reactive lysines and glutamines, the protein would need to be entrapped in microspheres. A double wall approach may be appropriate to assure that the entrapped protein does not react with tTG[362]. This process involves the initial formation of the sphere with the protein entrapped. Then this sphere can be coated with another polymer that has been modified with the tTG peptide. This can then be used to couple the microsphere to the tissue surface. However, there is the potential for inflammation as observed in uses of other microspheres[322].

9.4 Summary

The development of short synthetic peptide substrates of tTG has made it possible to modify a variety of molecules and polymers and these conjugates have been coupled to cartilage. While in its infancy, this strategy for coupling therapeutic molecules to cartilage has great potential for improving drug retention in the joint where the drug is administered. Additional *in vitro* experiments with a more stable prodrug may reveal potential benefits of the technique and limitations that need to be addressed. This should be followed with the development of a polymer drug delivery system, which should result in a more efficient use of each tTG site since there will be more drug molecules per site. However, this technology goes beyond cartilage; since tTG substrates are located in the ECM of many different tissues, this approach can be used to modify tissue throughout the body.

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