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Restoring Joint Lubrication: The Development of a Biomimetic Lubricin Peptidoglycan to Reduce Friction at the Articular Cartilage Surface

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RESTORING JOINT LUBRICATION: THE DEVELOPMENT OF A BIOMIMETIC LUBRICIN PEPTIDOGLYCAN TO REDUCE FRICTION AT THE ARTICULAR CARILAGE SURFACE

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ABSTRACT

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Osteoarthritis (OA) is a painful and debilitating disease affecting 27 million people nationwide and is often characterized by the degradation of articular cartilage. The loss of lubrication in synovial joints is a major contributor to cartilage damage, and damage can be prevented if proper lubrication is restored through supplemental lubricant treatments. Hyaluronic acid (HA) injections are on the market today, but there is little clinical evidence to support that these products are effective in treating the long-term effects of osteoarthritis. Lubricin, a lubricating proteoglycan found in the human synovial fluid, has become a potential alternative to HA injections. Overall, lubricin has proven to be more efficient than HA as a lubricant. However, as a native molecule, it is still susceptible to enzymatic degradation and depletion factors that are over expressed within OA joints. Synthetically derived molecules show the possibility of resisting these enzymatic degradations. Here, we engineered a biomimetic lubricin proteoglycan mimic that is designed to imitate the lubrication effects of native lubricin but withstand enzymatic degradation. The lubricin mimic was synthesized using a chondroitin sulfate backbone with collagen II and HA binding peptides to promote adhesion to the cartilage surface and interaction with the synovial fluid components. Our synthesized molecule was shown to reduce the kinetic coefficient of friction at the articular surface to a level comparable to that of native synovial fluid. Confocal imaging of the articular cartilage surface after lubricin mimic treatment showed binding of the molecule at the surface even after friction testing. Overall, our biomimetic lubricin shows potential as a long-term supplemental lubrication treatment for OA patients.

CHAPTER 1. INTRODUCTION

1.1 Osteoarthritis: An Unmet Clinical Need

Osteoarthritis (OA) is a multifactorial disorder of synovial joints[1] affecting approximately 27 million Americans aged 25 years and older[2]. The most prominent and disabling symptom of OA is pain that is associated with the progressive loss of lubrication and load-bearing capacity of articular cartilage[3]. Pain is a source of high unemployment rates in sufferers, which makes OA one of the leading causes of disability nationwide[4,5]. Currently, there is no cure for OA, and existing treatments cost the US healthcare system nearly $185.5 billion annually[6]. These treatments concentrate on pain management, but do little to stop the progressive wear of the articular cartilage[7,8]. In severe cases of OA, surgery is a common solution to alleviate pain, improve function, and delay the possibility of total joint replacement[9]. But even after these procedures, the symptoms of OA are still reoccurring since the native cartilage is severely degraded if not gone, and there are no current clinical treatments for its restoration. Recently, progress has been made in engineering cartilage-like tissues that are able to withstand and support the compressive and tensile forces in weight bearing synovial joint[10,11]. Because these tissue-engineered cartilage constructs lack the lubrication properties present in native articular cartilage, they would eventually be worn down over time. Therefore, a reliable and sustainable way to lubricate synovial joints affected by OA is critical, not only to increase the functionality of future engineered cartilage constructs, but also as a preventative measure to treat cartilage degradation in the early stages of OA.

OA can have a wide variety of pathological causes that make treatment difficult, but it is usually characterized by the breakdown of articular cartilage[12]. The degradation of articular cartilage is often a direct result of the loss of lubrication. Once failure of cartilage lubrication occurs, it is believed that excessive mechanical forces applied to the joint, especially friction, accelerate cartilage wear[13]. The exact cause of lubrication loss is unclear. Mechanical and biochemical factors are often blamed, but one common hypothesis is that the over expression of matrix metalloproteinases (MMPs) leads to the degradation of the cartilage extra-cellular matrix (ECM), at which lubricating molecules such as hyaluronic acid (HA) and lubricin localize[14]. MMPs are enzymes responsible for the remodeling of cartilage tissue by degrading the ECM that is quickly restored by chondrocytes, the primary cell type found in cartilage. When there is a large

increase in MMPs, degradation occurs at a much faster rate, and the restorative abilities of the chondrocytes fail to maintain a balance[15]. Other hypotheses include mechanical factors such as excessive friction applied to the cartilage surface and misalignment of joints[16]. Altogether, in order to successfully treat OA through lubrication restoration, it is important to take into consideration both the mechanical and biochemical factors that lead to a loss of lubrication in synovial joints.

The current treatments of OA are limited and most fail to successfully reverse/stop the progress of the underlying symptoms. Mild OA symptoms are treated with acetaminophen and topical or oral non-steroidal anti-inflammatory drugs (NSAIDs)[8] but only give small to moderate success in pain relief. These drugs can also cause liver damage, gastrointestinal problems, renal toxicity, and cardiovascular problems[7]. Oral narcotics, opioids, and tramadol are also used for pain relief, but have minimal success and cause a variety of negative side effects[17]. Corticosteroid injections have a short term effect, require multiple injections, and may even cause additional damage to cartilage[18]. Non-pharmaceutical remedies including exercise, weight loss, thermal modalities (hot and cold packs), and assistive devices (canes, crutches, prosthetics) can be effective to increase mobility and temporarily alleviate pain, but fail to address the underlying causes[8].

While all these treatments work to mitigate the mild symptoms of OA, especially pain, their effectiveness often becomes situational and many of the drug-based therapies have concerning side-effects. When the progress of OA is severe, surgical procedures are used, including arthroscopy (removal of loose pieces of cartilage and damaged tissue), osteotomy (joint preserving surgery by improving joint alignment and stability), and joint fusion[9]. Most of these surgical procedures work to temporarily relieve arthritis pain, and whether they work better than medication or non-pharmaceutical remedies is debated. Joint replacement surgery is considered to be the last option for OA treatment and is only done in severe cases when the joint becomes completely non-functional. However, there are many risks associated with this procedure such as irreparable damage, infection, and persistent pain. It is common for these joint replacements to fail with time, and their functionality and life is limited compared to a healthy synovial joint[19].

Researchers have been studying and developing supplemental lubricant injections for the synovial fluid as a way to reverse the loss of lubrication[20]. This procedure is generally known as tribosupplementation or in other cases viscosupplementation, when the lubricant works to increase the viscosity of the synovial fluid[8]. One practiced treatment involves intra-articular injections (IA) of HA. Unlike many other pharmaceuticals, this approach is considered safe, but the actual long-

term benefits remain inconclusive. There is little clinical evidence to support that HA injections are effective in treating the long-term effects of OA. In fact, most current studies show that these treatments are susceptible to enzyme degradation and a low residency time, which would require frequent and repetitive injections[20].

Current research has also focused on making synthetic lubricant injections, which mimic the functions of native lubricating polymers, but are not the same in structure[21,22,23]. Because of this difference in structure, synthetic molecules show potential for resistance to degradation after injection into diseased joints. However, these engineered structures must be able to function and interact accordingly within the native environment of the synovial joint. Despite the promise of current lubricant treatments to decrease friction and protect cartilage, better solutions can be made to increase the residency and efficiency of these lubricating molecules. In order to develop a better solution, a thorough understanding of the synovial joint structure and function as well as the key players involved in boundary lubrication is needed. This will be discussed throughout the rest of the introduction as well as a review on the efficacy of current synthetic and biologically relevant lubrication treatment methods.

1.2 Synovial Joint Structure and Composition

Synovial joints, also known as diarthroidal joints, are the most common and movable type in the body. The presence of lubricating synovial fluid encapsulated by joint ligaments distinguishes this type of joint from others within the body[24]. OA most commonly occurs within the weight bearing synovial joints including the hip and the knee, which is why obesity is such a major risk factor of OA[25]. The synovial joint can be broken down into four major components: articular cartilage, synovium, joint cavity filled with synovial fluid, and joint capsule ligament (Figure 1.1a) [26,27]. Ultimately, articular cartilage and the synovial fluid are the main constituents that contribute to the proper lubrication properties of healthy and OA synovial joints.

1.2.1 Articular Cartilage

Articular cartilage is a thin layer of specialized connective tissue that provides the weight bearing and lubrication properties of synovial joints[28,29]. Overall, articular cartilage is a very dense structure, lacking blood vessels and nerves that are found in other tissues[16]. Chondrocytes are the cell types embedded within cartilage and produce the structural proteins that form the ECM. The

major components of the ECM are water, type II collagens (COL2), proteoglycans, aggrecan, and noncollagenous proteins such as fibronectins[30,27]. The resulting osmotic pressure created within the solid and fluid components of the ECM work in harmony to support the mechanical forces applied to cartilage[16]. The water is retained in the ECM by aggrecan, the primary structural proteoglycan that is composed of a core protein with glycosaminoglycan (GAG) side chains, chondroitin sulfate (CS) and keratin sulfate (KS)[31]. Altogether, these components are what create the depth dependent layers of cartilage: the superficial zone, the middle zone, the deep zone, and the calcified zone (Figure 1.1c)[32].

The superficial zone of cartilage is primarily responsible for providing resistance to shear and tensile forces through both lubrication and structural factors[32]. The fibers and chondrocytes in this area are densely packed and aligned parallel to the articular surface and in the direction of joint articulation, allowing for an efficient gliding motion during movement[33]. This zone is the major area for expression of proteoglycan 4 (PRG4), otherwise known as lubricin[34]. Here lubricin is in contact with the synovial fluid and together they provide the articular surface with constant lubrication[35]. The middle zone functions to provide the first line of resistance to compressive forces, and is composed of proteoglycans and thicker collagen fibrils. The deep zone provides the greatest resistance to compressive forces and acts as an anchor, attaching the cartilage to the subchondral bone[33]. Without the protection of the superficial zone, the deeper layers of cartilage are unable to resist the sheer and tensile forces present in a functioning joint. Studies have shown that the loss of the superficial layer of cartilage quickly accelerates cartilage deterioration, leading to the disease state of OA[36].

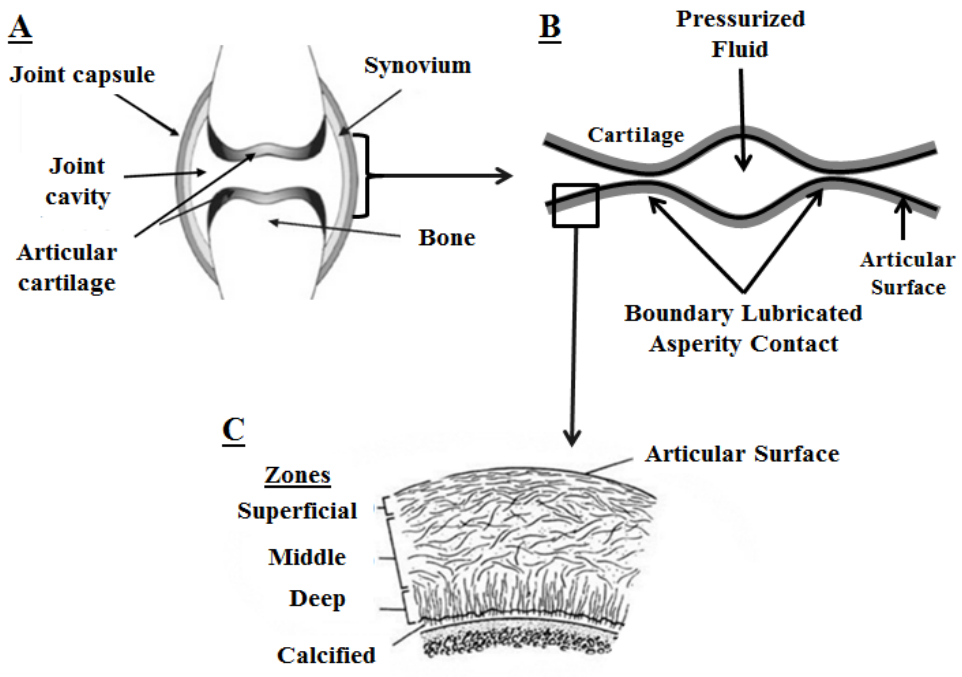

Figure 1.1 a) Structure and composition of synovial joint. b) Depicting two types of lubrication: fluid film (pressurized fluid) and boundary (points of contact). c) Depth-dependent layers of articular cartilage. Adapted from[26].

1.2.2 Synovial Fluid

The synovial fluid is an important viscous lubricant that allows nutrients and regulatory chemicals to travel across the synovial joint[27]. The main components of the synovial fluid are concentrated blood plasma and molecules that are responsible for the low friction and wear properties of the articular cartilage surface[27]. These molecules include lubricin and hyaluronic acid[37,38]. The synovial fluid is surrounded by the synovium, a thin, fibrous, vascular envelope that houses type B synoviocytes that are responsible for secreting lubricating proteins into the synovial fluid. Here, these lubricants interact with the cartilage surface and each other, allowing for low friction between surfaces[39]. In joints affected by OA, it is typical for the synovial fluid to become less viscous and lose mechanical and biochemical properties resulting in the wear and tear of cartilage.

1.3 Articular Cartilage Lubrication: Role and Function

The synovial joint withstands the applied shear and compressive forces by minimizing the coefficient of friction (COF) at the articular cartilage surface. The COF of articular cartilage interacting with human synovial fluid is usually around 0.005. In fact, the measured COFs of articular cartilage still remain the lowest out of any given material[40]. The mechanisms through which the synovial joint achieves these low friction properties involve a combination of biomechanical and biomolecular factors, which can be divided into two types of lubrication: fluid film and boundary[41].

1.3.1 Fluid Film Lubrication

Fluid film lubrication is characterized by pressurized fluid within the ECM and between the articulating cartilage surfaces (Figure 1.1b)[41], which can bear significant portions of the load[42]. Up to 90% of the applied load can be supported by ECM and synovial capsule due to the their high water and retention properties[42]. Fluid film lubrication is only active when opposing cartilage surfaces slide with respect to each other[43]. In a long term loading position, all of the fluid flows out between the surfaces and another type of lubrication, boundary lubrication, must take place at the points of contact[44].

1.3.2 Boundary Lubrication

Boundary lubrication is the most studied mechanism in the context of clinical lubrication treatments. It is characterized by low friction properties achieved through the presence of lubricant molecules located at points of surface-surface contact, referred to as asperities (Figure 1.1b)[44]. This mode of lubrication is considered important for the protection and maintenance of articular cartilage, since 10% of the total cartilage surface makes contact with the opposing layer[45]. These areas of contact are the most susceptible to wear and degradation due to the higher friction forces applied within surface-surface contact. Furthermore, the initial degradation of articular cartilage often happens in areas that use boundary lubrication as a mode of friction reduction. Once the superficial boundary layer of articular cartilage is removed at these asperities, severe symptoms of OA will occur at an increasing rate[36].

1.4 Boundary Lubrication Key Players: Proposed Mechanisms

At present, there are two key players that are considered to be involved, individually or in combination, in controlling friction of articular joints: (1) hyaluronic acid and (2) lubricin. The structure and composition of each of these lubricants has been extensively studied, but the exact functioning mechanisms are unknown. Further research is needed to determine their specific roles, which would significantly accelerate the progress of developing the most efficient and optimal lubrication treatments. Currently, there are many hypotheses as to how much of a role each plays in reducing friction at the articular cartilage surface, and to why these molecules stop functioning during OA.

1.4.1 Hyaluronic Acid

Hyaluronic acid is a major component of the ECM and is intertwined with collagen fibrils to function as a backbone for the attachment of proteoglycans, such as aggrecan and proteoglycan 4 (lubricin). It is abundant throughout the synovial fluid and contributes to the overall viscosity[46]. HA is generally found at concentrations of 3-4 mg/mL within the synovial fluid and the structure can vary in size from 0.5 MDa to 7 MDa[47], which gives rise to varying viscosities within the synovial joint.

In general, the mechanisms of HA are often debated, but a current hypothesis is that HA acts as an enhancer in lubrication, improving the function of other lubricants, rather than functioning as a boundary lubricant independently[48,49]. Researchers believe HA aids lubrication by increasing the viscosity in the synovial fluid, hence the reason HA is presently the number one component used in viscosupplementation treatments of OA[8]. This idea dates back to 1970, when Radin *et al* found that separation of HA from bovine synovial fluid reduced the viscosity, but concluded that its role was non-essential and did not affect the boundary lubrication of the treated fluid[50]. Later studies have argued that HA does play an important role in boundary lubrication and has been verified using cartilage-cartilage interfaces by Swann *et al.* and a number of other researchers[51,52,53,54]. Each of these studies acknowledged that HA depends on viscosity and concentration in order to effectively lubricate. Other studies have shown that HA does not appear to have boundary lubricating properties when studied *in vitro*, but acts synergistically with other lubricants such as lubricin[48,49]. In 2007, Schmidt *et al.* found that HA did not significantly increase the lubrication properties of a cartilage-cartilage interface on its own, and instead, only enhanced

the lubrication of PRG4[46,55]. In addition to providing viscosity to the synovial fluid, Tadmor *et al*. showed evidence that suggested chemical binding of HA to the cartilage surface is necessary to initiate a reduction in friction[56] and, in another study, wear reduction[57]. Despite contradictions within the research, there is evidence to show that HA does play a significant role in boundary lubrication, but the exact role has yet to be determined.

In joints affected by OA, the concentrations of HA have shown to decrease compared to those of healthy individuals[45]. One cause of the loss of HA is the over expression of HA degrading enzymes, which include hyaluronidase, β-D-glucuronidase, and β-Nacetyl-hexosaminidase[58]. HA is thought to play a role in preventing the development of OA, so joints with increased HA degradation are susceptible to further progression of OA symptoms, specifically, the loss of cartilage lubrication[59]. It is proposed that HA stimulates the production of tissue inhibitors of matrix metalloproteinase (TIMP-1) in chondrocytes[14], as well as inhibiting neutrophil-mediated cartilage degradation and reduces IL-1 induced ECM degeneration[60]. With this evidence, the presence of HA probably plays a role in reducing the enzymatic degradation of the ECM and other important lubricants within the joint. In fact, articular chondrocytes cultured in the presence of HA have a significantly greater rate of extracellular matrix production compared with chondrocytes cultured without HA[61]. Ultimately, the underlying causes of HA degradation are important to understand since HA injection treatments will still be susceptible to these cleaving enzymes. For this reason many current studies are focusing on creating HA injections that are resistant to degradation by enzymes such as hyaluronidase[62].

1.4.2 Lubricin/SZP/PRG4

Lubricin, also commonly known as superficial zone protein (SZP) or proteoglycan 4 (PRG4), is large glycoprotein found in the superficial zone of articular cartilage and the synovial fluid[63]. While still used interchangeably, evidence suggests that lubricin and SZP are different molecules, in which lubricin is produced by synoviocytes[64] and SZP created by articular chondrocytes at the superficial zone[65]. The difference between these two compounds is their size; the molecular weight of lubricin is 227 Da and the molecular weight of SZP is 345 Da. This variance can be explained by alterations during post-translation glycosylation[66]. Even so, SZP and lubricin are considered to be the same in functional applications. Native lubricin is encoded by PRG4 consisting of N- and C- terminal regions that are connected by a long repetitive mucin-like

domain. The N-terminal region contains somatomedin B-like (SMB) and heparin-binding domains, while the C-terminal region consists of a hemopexin-like domain[67]. The functional part of lubricin that is thought to be responsible for lubrication is the repetitive mucin-like domains. These domains attach to lubricating O-linked oligosaccharides and other ECM components including aggrecan and type VI collagen[68]. The N-terminus promotes binding between lubricin molecules, and the hemopexin domain facilitates attachment to the ECM[69]. This protein has been found to be highly conserved across species, allowing for experimental conclusions developed in *in vivo* animal studies to be directly translatable to humans.

In general, lubricin is considered to be the critical lubricant for articular cartilage and is currently the most studied among the acknowledged lubricant molecules. Lubricin was discovered by Swann *et al*. following its isolation from bovine synovial fluid[70,71]. Since then, the hypothesis that lubricin functions as the primary boundary lubricant has become increasingly popular[49]. In 2007, Schmidt *et al.* found that lubricin significantly increased the lubrication properties of a cartilage-cartilage interface, and with the addition of HA, the COF decreased even more[46]. Many mechanisms behind this function have been proposed, but most researchers believe that lubricin functions as a lubricant by adsorbing strongly to the cartilage surface, and due to its highly hydrated structure, the layers formed are sterically repulsive, lowering the COF[72,73]. Using recombinant lubricin constructs, Jones *et al.* found that the C-terminal domain controls binding and localization of lubricin to articular. This study also indicated that the N-terminal region may be responsible for lubricin-lubricin interactions[73]. A series of studies concluded that without lubricin, proper lubrication cannot occur, which is unlike the findings in HA studies. These lubricin studies include a number of rodent gene knockout models[74,75] as well as mammalian models of OA[76,77]. In 2012, Drewniak *et al.* subjected the joints of PRG4 -/- mice to 26 hours of cyclic loading. They found that the COF within these joints was significantly higher than those of PRG4 +/+ mice, concluding that the lack of lubricin reduces protection against mechanical wear[78]. Additionally, the lubricant properties of lubricin were studied *in vitro*, demonstrating boundary-lubricating ability among a number of surfaces (i.e. cartilage-glass[71] and latex-glass[35,7648,49,66]). These findings further support the hypothesis that lubricin is a highly surface active molecule which can adhere to a variety of surfaces[39,79]. Altogether, there is strong evidence to conclude that lubricin acts directly as a boundary-lubricant unlike HA, and its interaction with HA increases these lubrication properties even further[80]. However, it is unclear how these molecules specifically

interact to increase lubrication. Further research is needed to understand the exact mechanisms of interaction between lubricin and HA.

In joints affected by OA, native lubricin was found to be ineffective in reducing friction in arthritic articular cartilage due to its low concentration when compared to healthy individuals[46,81]. This could be partly explained by the lack of growth factors such as TGF-β, which is known to up regulate the expression of SZP[82,83]. Furthermore, interleukins (IL-1β and IL-6) and TNF-α have increased expression with progressing OA and are associated with reducing the synthesis of PRG4[83,84] and increasing expression of MMPs, which are major contributors to cartilage degradation[15]. For these reasons, current studies are focusing on creating lubricin treatments that are resistant to degradation enzymes such as MMP and depletion factors such as IL-1β. Ultimately, the underlying causes of lubricin degradation are important to understand in order to effectively treat the loss of lubrication in diseased joints.

1.5 Proposed Lubricant Treatment Solutions

The current progress in engineering cartilage-like tissues has revealed that the proper lubrication of these constructs is critical for their success[85,10]. Therefore, recent studies have focused on developing a reliable way to create compatible and proper lubrication as the next step towards successful tissue engineering treatments. Another major focus of these studies is to use these lubrication methods for IA supplementation at the beginning stages of OA in order to prevent further progress of the disease. The solutions for developing successful lubrication range from using purified native lubricants to synthetic molecules designed with the native lubricating abilities.

1.5.1 Native Lubricant Treatments

Hyaluronic acid injections have been used to treat osteoarthritis since 1998, when the FDA approved a treatment that was composed of HA extracted from rooster combs[86]. Since then, many HA treatments have been developed that are derived from a number of sources such as the vitreous body, umbilical cords, and bacteria[87]. The naturally occurring HA drugs on the market include Hyalgan®, Orthovisc®, and Euflexxa®.

Overall, studies show that HA IA injections may be effective, but the efficacy seems to be circumstantial. In a 6 month clinical study, Hyalgan® was administered to the knee every 5 weeks

until a final biopsy was done to analyze cartilage damage[88]. Only a third of the patients saw improvement of OA cartilage degradation, showing that the effectiveness of Hyalgan® as a lubricant treatment varies from patient to patient[88]. When compared to native synovial fluid, commercial HA treatments were limited, and despite increased viscosity, did not provide sufficient boundary lubrication[48,55]. For improved lubrication efficiency, high molecular weight HA treatments have been used (Orthovisc®, and Euflexxa®). Low molecular weight HA allows for penetration into the synovium to reduce swelling, but it does not have the elastoviscosity of higher molecular weight HA[47]. Euflexxa® showed similar benefits to Hyalgan®. It effectively reduced pain, but whether or not it is able to stop the long term progression of cartilage degeneration is inconclusive[89].

Although studies have found that HA IA injections can work to alleviate symptoms, particularly pain, it does not always benefit patients. This could be due to the varying symptoms of OA that change from individual to individual. As such, HA treatments might benefit one symptom better than another. Also, there is the likely possibility that HA does not function mainly as a lubricant, and instead plays a key role in other mechanisms of action that are involved in pain reduction, such as the inhibition of tissue nocieptors[90]. Also, HA treatment has been shown to work similar to NSAIDs by giving direct anti-inflammatory effects that suppress inflammatory mediators and proteases [60]. Further evidence shows HA injections may inhibit MMP activity, blocking the degradation of articular cartilage[17]. Even so, HA injections are probably not the most efficient method to restore the loss of lubrication, especially when in comparison to the native synovial fluid or lubricin[20].

To improve the efficiency of HA treatments, modified HA products have been developed for the market today, including Synvisc®, Synvisc-One®, and Durolane®[87]. These products are composed of synthetically-altered cross-linked (Synvisc®, Synvisc-One®) and stabilized forms (Durolane®) of HA[91,92,47]. Cross-linked HA (Hylan G-F 20) is formed using a mixture of hylan A and insoluble hylan B cross-linked by hydroxyl groups. Hylans have higher molecular weights and increased viscosity compared to HA, as well as nearly double the resident time[92,47]. Non-animal stabilized hyaluronic acid (NASHA, Durolane®) is a cross-linked form of HA produced by treatment of bacterial produced HA. NASHA is a biocompatible gel with greater viscoelastic properties, greater pain reduction, and longer persistence in synovial joints than native HA[91].

Studies have shown that synthetically derived HA-like treatments may be effective, but the significance varies between researchers. Waller *et al.* compared the function of Hylan G-F20 to that of human synovial fluid and PBS in an *in vitro* study using bovine cartilage[93]. They found that the COF for Hylan G-F 20 was significantly higher than the value measured for synovial fluid. Additionally, there was no significant difference between the COF of PBS and Hylan G-F 20. This suggests that the effectiveness of Hylan G-F 20 is minimal and lacks the ability to function independently as a lubricant. In a clinical study, IA injections of Synvisc® and a saline control were administered to the metatarsophalangeal joint (big toe) of patients. Pain was the factor measured, and there was no significance between the control and Synvisc®[94]. A long-term study was done to determine the lasting effects of Hylan G-F 20 overtime. In this experiment, multiple treatments of hylan injections were able to delay total knee replacement surgery by about 4 years, but it did not halt joint degradation[95]. Overall, these studies show no significant evidence that synthetic hylans prevent the progression of OA.

Tribosupplementation using purified human lubricin is currently being studied for its clinical use. Recently, two studies have done *in vivo* testing of lubricin IA injections. Teeple *et al.* reintroduced native lubricin (LUB) into rat joints post-traumatically and demonstrated a disease-modifying effect[20]. In this study, cartilage histology and damage in OA-induced rats (via ACL transaction) were analyzed after 4 weeks of weekly LUB injections. There was significantly less damage in OA joints treated with LUB compared to those which received none. This study also included the same test with HA-injections for a comparison and found that, independently, HA did little to stop the degradation of cartilage[20]. The effects of LUB+HA injections were also analyzed. These results supported the hypothesis given in Greene *et al*. that LUB+HA interactions *in vivo* are important for optimized lubrication abilities[80]. Jay *et al*. used lubricin derived from synoviocytes to administer IA injections into OA-induced rat joints[65]. By performing injections twice a week for 32 days, they found that there was a reduction in cartilage damage. Later, they performed a similar experiment to analyze the persistence of lubricin *in vivo* after 35 and 70 days following a single injection. The results showed that a single IA injection of concentrated lubricin reduced COL2 degradation and improved load bearing in the affected joint. Furthermore, increased immunostaining for lubricin was observed in the lubricin-treated animals after 35 and 70 days, suggesting that this method of treatment has more long-term effects than present HA treatments. While the studies mentioned above show that lubricin injections can slow the rate of cartilage

degradation, one study concluded that lubricin injections cannot reverse the damage done to the subchondral bone. This suggests that lubricin injections are most useful as a treatment in the early stages of OA when the subchondral bone has not been damaged[96].

Overall, lubricin shows great potential as a lubrication treatment for OA. Unlike HA, there is little dispute among scientists about its lubrication efficiency, and *in vivo* studies consistently show better results than HA IA injections. Regardless, there are some factors that need to be considered. As mentioned before, OA joints are often associated with an over expression of IL-1β, TNF-α, and IL-6, which decrease the native lubricin concentration leading to loss of the cartilage boundary layer[46,97]. LUB, being a native lubricant, will be exposed to these elements and as a result degradation will occur. Multiple injections and higher dosages will need to be administered to counteract the effects of IL-1β, IL-6, and TNF-α, and to sustain long-term effectiveness.

1.5.2 Recombinant Lubricant Treatments

One problem in using lubricin as a commercialized treatment is finding an abundant and available source. To address this, scientists have developed recombinant methods to produce lubricin in large enough amounts for clinical use. So far, two recombinant lubricin treatments are being studied: LUB:1 and rhPRG4.

In 2009, Flannery *et al.* produced LUB:1 by reducing the number of mucin-like repeats found in lubricin to optimize production in Chinese hamster ovary cells[98]. The removal of two-thirds of the mucin-like repeats from LUB:1 had no effect on its binding and localization to the superficial layer of cartilage. In addition, LUB:1 significantly improved boundary lubrication for cartilage explants that were subjected to friction testing, when compared with PBS treatments alone. Treatment of injured rat knee joints with LUB:1 resulted in reduced cartilage degradation and structural damage based on OA pathology scores[98]. Vugmeyster *et al.* furthered this research by using a radioactive label on LUB:1 to determine localization on the joints and residency time[68]. LUB:1 was found to specifically localize to the points of injury on the articular cartilage surface and was found to persist for about 2-4 weeks after inter-articular injections.

Researchers also studied rhPRG4, a full-length recombinant human PRG4, to test for its lubricating ability and protective qualities. In a recent study, Kwiecinski *et al.* found that rhPRG4 can bind to the cartilage surface and function as an efficient boundary lubricant, demonstrating abilities that match those of native lubricin[99]. Similar results were found by Jones *et al.*, in which

they further characterized binding interactions of lubricin and rhPRG4 to the cartilage surface[73]. According to Abubacker *et al*., the lubricating abilities of rhPRG4 were further enhanced by the addition of hyaluronic acid[100]. *In vivo* tests further support the *in vitro* studies previously mentioned. One study administered IA injections of rhPRG4 to OA-induced rat joints, and found the reduction of cartilage damage with the rhPRG4 treatment was the same as treatment with native lubricin[65]. Contrary to the results found in Abubacker *et al*., an *in vivo* study performed by Waller *et al*. concluded that the addition of HA to rhPRG4 injections did not reduce cartilage damage any more than rhPRG4 alone[101].

Altogether, research shows that recombinant lubricin treatments work just as effectively as native lubricin injections. Therefore, the same considerations should be made regarding their susceptibility to degradation in OA joints. Overall, the effects of lubricin treatments need further evaluation, specifically on how they influence the activities of cartilage-degrading enzymes. The long-term effectiveness of lubricin has yet to be evaluated, and the role lubricin plays in enzymatic resistance will significantly determine the persistence of lubricin treatments. Currently, researchers are addressing this issue by developing synthetic lubricant treatments that mimic the function of lubricin but lack the structure targeted by native degradation enzymes.

1.5.3 Synthetic Lubricant Treatments

Synthetic lubricants are a recent development in the treatment of OA. These molecules are designed to resist degradation as well as mimic the functions of native boundary lubricants[21,22,23]. Wathier *et al.* synthesized a large-molecular weight polyanion that was found to possess lubricating properties for cartilage *in vitro*[21]. The polymer was shown to not be readily degraded by hyaluronidase, due to its structure. In this *ex vivo* study, they subjected HA, Synvisc®, and their engineered lubricant to cartilage surfaces and found that the lubrication abilities of the polymer matched those of native synovial fluid and even exceeded those of Synvisc®[21]. Even so, further studies need to be done to determine what interactions the polymer might have with the multiple components of the synovial joint.

Recently, a synthetic lubricant has been developed that consists of a polyglutamic acid (PGA) backbone onto which poly(2-methyl-2-oxazoline)s (PMOXA) and hydroxybenzaldehydes (HBAs) are alternatively grafted[23]. These grafted copolymers bind to the exposed amino acids of collagen type II in degraded cartilage through the HBA domains on their molecules. Following *in*

vitro friction testing, they determined that their grafted copolymer reduced the friction of degraded cartilage to that of healthy native cartilage[23]. Even further, they showed that their molecule was able to reduce enzymatic degradation of collagen II compared to no treatment present[102].

Samaroo *et al.* has mimicked the bottle brush structure of lubricin by using polyethylene glycol (PEG) grafted onto a polyacrylic acid (pAA) core[22]. A thiol group was attached to the terminus of pAA and anchored the structure to the cartilage surface. The lubricating efficacy of this polymer was similar to that of recombinant lubricin according to *in vitro* friction testing results. They also determined that the binding ability of the polymer and lubrication were highly correlated and future experiments will focus on ways to increase the adsorption of the polymer to the cartilage surface[22].

Overall, there are a wide range of opportunities that can be found within the development of synthetic lubricants. Until recently, most research has focused on the use of native lubricants for the treatment of OA, but factors including susceptibility to degradation become problems for these types of treatments. The use of a well-designed synthetic treatment could be the solution. Recently, biomimetic polymers have been engineered to possess lubricin function as well as resistance to degradation[21,22]. If these lubricin-mimics function according to their design, they would provide greater lubrication efficacy than current HA treatments. However, research has yet to be done to establish the *in viv*o properties of these lubricin-mimics.

1.6 Motivation for Development of Biomimetic Lubricin

Current treatments for OA are limited and fail to prevent the progression of the disease. In recent years, OA-research has shifted focus from general symptom relief to a more targeted approach to prevent and even reverse symptoms of the disease, especially cartilage deterioration. The loss of lubrication in the synovial joints is a primary reason for the decrement of articular cartilage[13]. Therefore, it is believed that further damage to articular cartilage can be avoided if proper lubrication is restored through lubricant supplement treatments. HA injections and other lubricant supplements are on the market today, but there is minimal clinical evidence to support that these products are effective in treating the long-term effects of OA. Overall, lubricin proves to be more efficient than HA as a lubricant *in vivo* and *in vitro*, but it is susceptible to degradation by enzymes and depletion factors that are over expressed within joints suffering from OA. Synthetic molecules show promise of resistance to these degradation enzymes, but they must also

be designed so they are able to be biocompatible within native cartilage as well as reduce friction at the cartilage surface. Therefore, there is a significant need for the development of a synthetic lubricant treatment that can provide the efficiency of native lubricants while also being able to withstand enzymatic degradation. This resistance to degradation enzymes would improve long term effects and overall success of the treatment. To address this need, our laboratory has developed a biomimetic lubricin that is designed to mimic the lubrication effects of native lubricin, but withstand enzymatic degradation from hyaluronidase, MMPs, and aggrecanase. Our synthetic lubricin mimic is able to reduce friction at the articular cartilage surface and shows potential to be used as a lubricant treatment for patients suffering with osteoarthritis.

CHAPTER 2. SYNTHESIS AND CHARACTERIZATION OF A LUBRICIN MIMIC

Our laboratory has developed a biomimetic lubricin molecule that is designed to mimic the lubrication effects of native lubricin, while also withstanding enzymatic degradation from enzymes such as hyaluronidase, MMPs, and aggrecanase. The synthetic lubricin mimic lacks the specific cleavage sites targeted by hyaluronidase[103] and aggrecanse[104]. Furthermore, our design of biomimetic lubricin does not include the core protein present in native lubricin which is susceptible to proteolytic enzymes including MMPs. As a result, our synthesized lubricin mimic most likely can withstand degradation due to increased expression of MMPs in osteoarthritic joints[105]. The biomimetic lubricin we developed is composed of a chondroitin sulfate (CS) backbone with covalently attached HA and collagen II binding peptides resulting in the formation of a peptidoglycan (Fig. 2.1a). Peptidoglycans are molecules characterized by peptides attached to glycosaminoglycans (GAG). Chondroitin sulfate, a GAG found in lubricin and other cartilage proteoglycans, was used for this biomimetic molecule due to both its presence in cartilage and its negative charge. This negative charge is important for proper lubrication at the articular cartilage surface[106]. This lubricin mimic was designed to bind to collagen II at the articular cartilage surface through the addition of collagen II-binding peptides on the backbone. Since lubricin is known to interact with HA *in vivo,* HA-binding peptides were added to the CS backbone to further mimic lubricin's function[49]. Given that these peptidoglycans are designed to be resistant to hyaluronidase, this interaction could also protect HA from enzymatic degradation.

A lubricin mimic was developed previously in our lab that was termed mLUB due to its ability to mimic lubricin. mLUB has already demonstrated significant lubrication properties through *in vitro* tests on cartilage surfaces[107]. mLUB was also designed with a CS backbone with covalently attached collagen type II binding peptides and HA binding peptides. For a number of reasons, we decided to change the way these peptidoglycans were synthesized which resulted in slight alterations to the finalized lubricin mimic structure (Fig.2.1b). mLUB was previously synthesized through an oxidation process that included a BMPH (N-β-maleimidopropionic acid hydrazide) crosslinker that covalently attached the peptides to the backbone. This method was relatively time intensive and required opening up the rings on the CS backbone, resulting in a random coil polymer as opposed to the more rigid structure of unmodified CS. The stability of the

molecule was compromised due to the oxidation step, which reduced shelf-life of the molecules. To remedy this, our lab switched to EDC (1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride) chemistry to conjugate peptide hydrazides to the CS backbone through an amide bond (Fig.2.1b). This method was quicker, kept the structural integrity of the CS backbone, and was more stable when compared with the previous mLUB design.

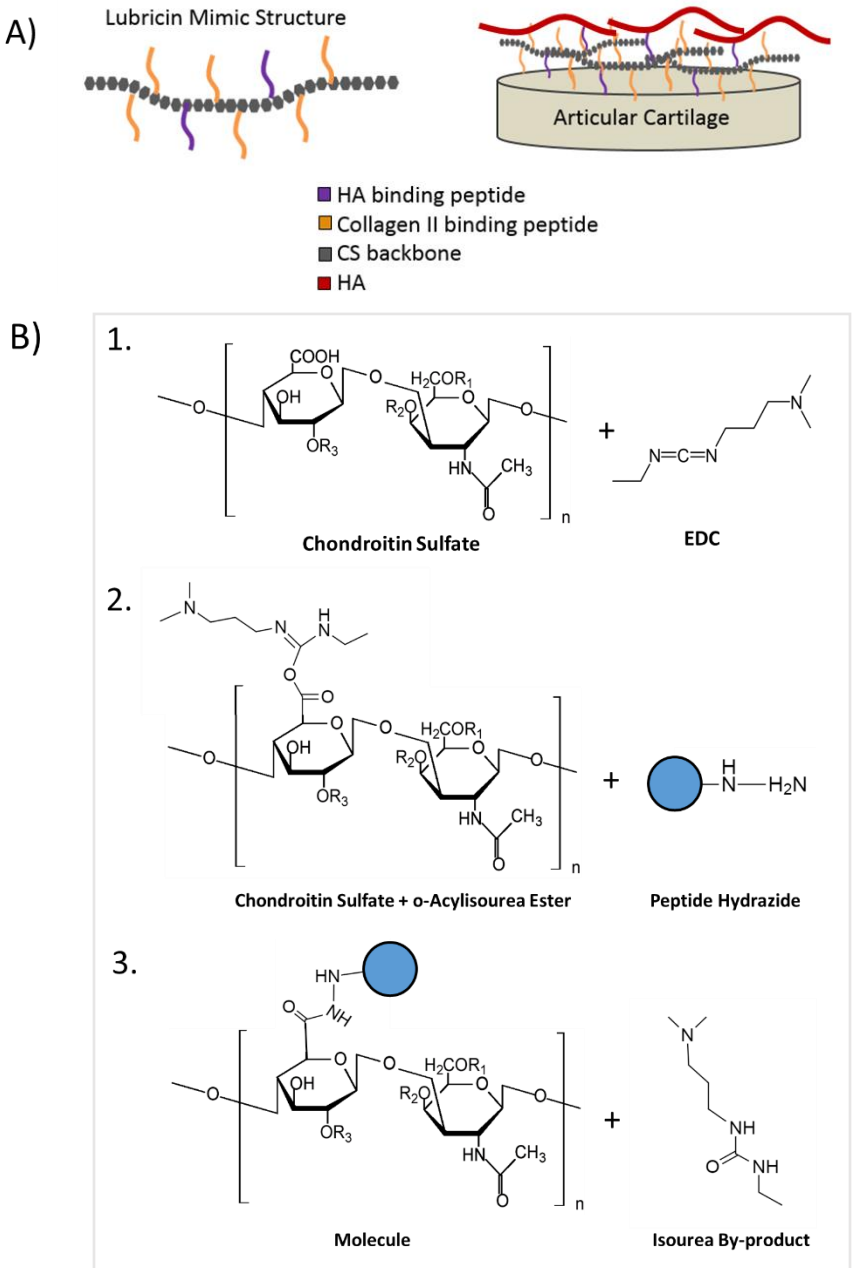

Figure 2.1. A) Design of biomimetic lubricin. B) Schematic of lubricin mimic synthesis using EDC chemistry and peptide hydrazides.

2.1 Synthesis of Peptide Hydrazides

The use of EDC chemistry to synthesize the lubricin mimic required slight alterations to the peptides attached. The peptides were synthesized in our lab to include a GSG amino acid spacer and a functional hydrazide group. These additions allowed the peptide to attach to the CS backbone. A total of four different peptides were synthesized which included the collagen II binding peptide (WYRGRLGSG), the HA binding peptide (GAHWQFNALTVRGGGSG), and biotinylated versions of each. These peptides were abbreviated as WYR, GAH, bWYR, and bGAH and had the corresponding molecular weights of 1065, 1728, 1291, and 1955 Da, respectively. The functional biotin group was added at the C-terminus to allow for future analysis and characterization of the peptidoglycans. Successful peptide synthesis was determined by mass spectroscopy in conjunction with the HPLC curves. The peptides were purified using HPLC and each major peak from the trace was collected and analyzed by mass spectroscopy to confirm the presence of the synthesized peptide. The peptides were repurified if major contaminants were indicated in the HPLC curves. Figures 2.2 and 2.3 verify that WYR and GAH were successfully made. Figure 2.4 and 2.5 also verify the synthesis of the biotinylated versions of WYR and GAH. Each HPLC trace indicates the peak collected that corresponds with the mass spectroscopy results.

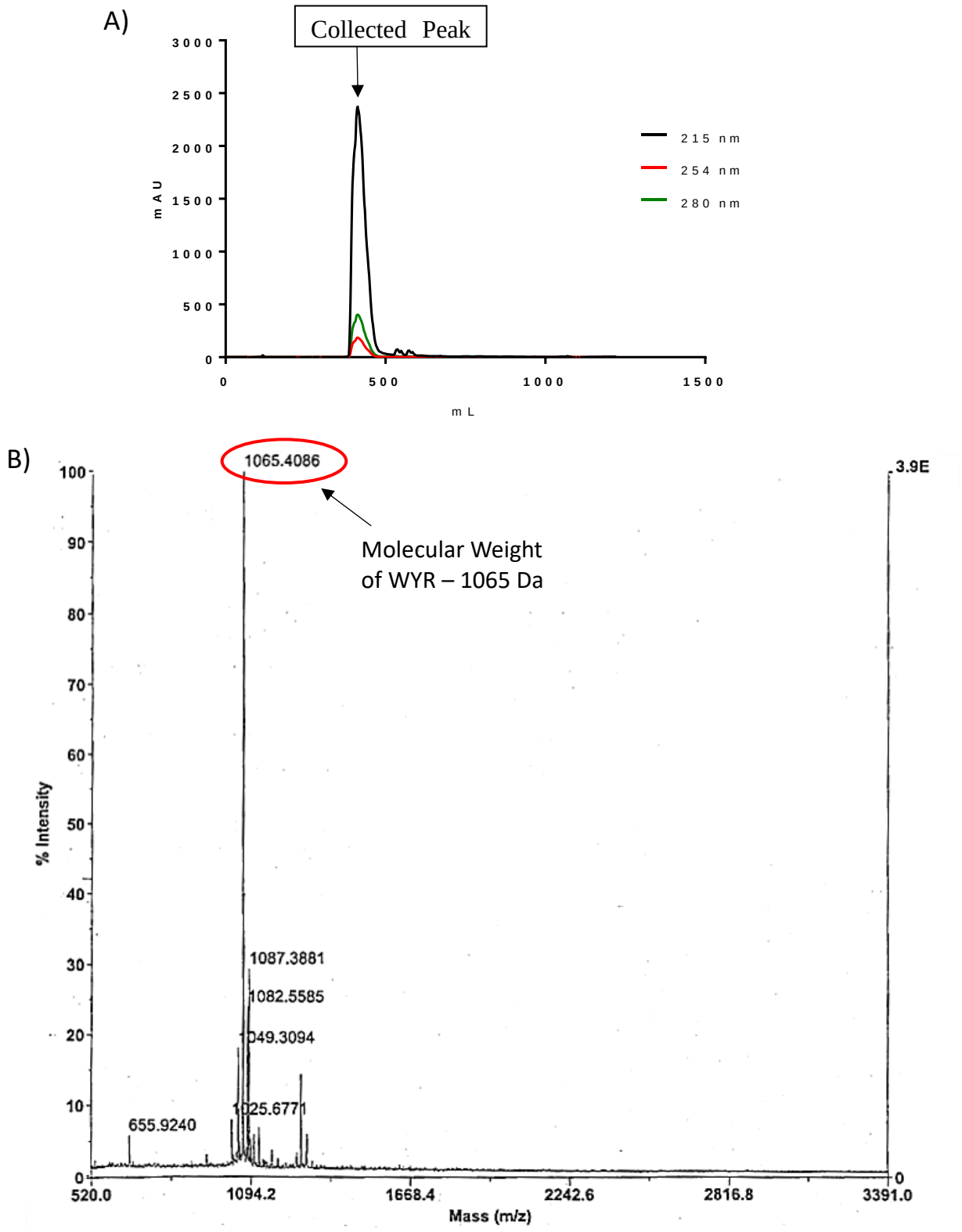

Figure 2.2. Analysis of WYRGRLGSG-hydrazide synthesis. A) HPLC curve of 2nd purification. B) Matrix-assisted laser desorption ionization time-of-flight (MALDI TOF) mass spectrometry results.

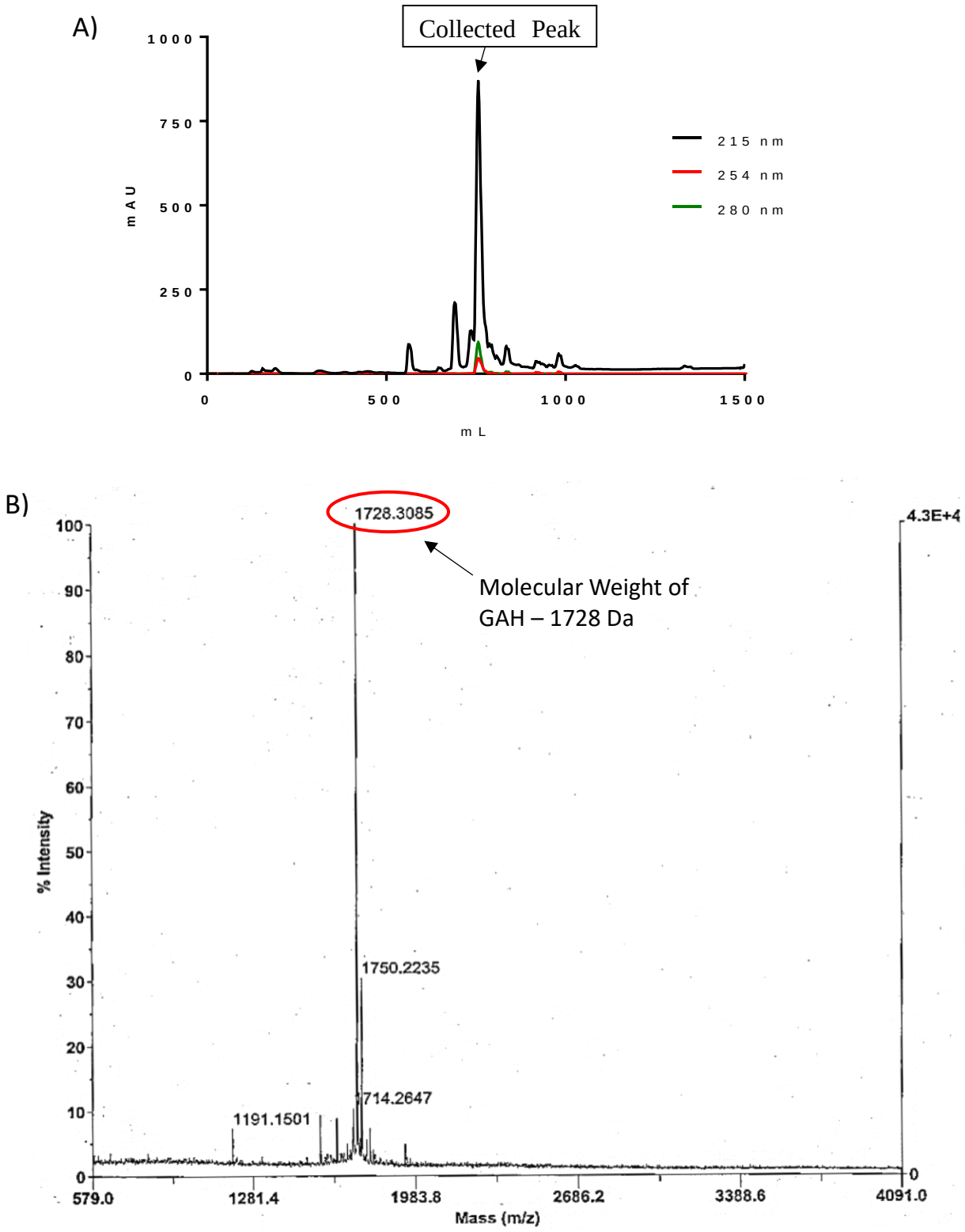

Figure 2.3. Analysis of GAHWQFNALTVRGGGSG-hydrazide synthesis. A) HPLC curve of 2nd purification. B) Matrix-assisted laser desorption ionization time-of-flight (MALDI TOF) mass spectrometry results.

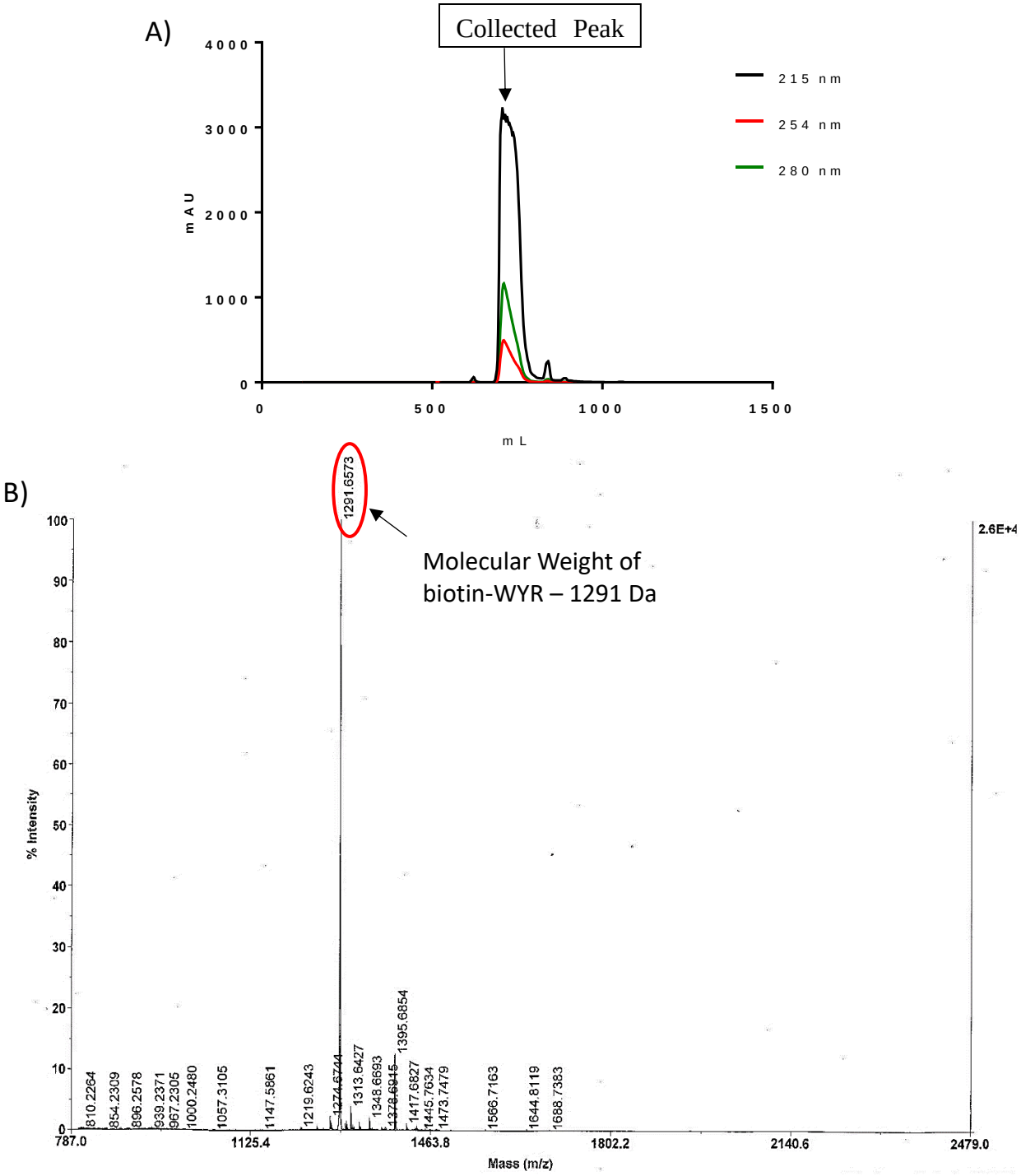

Figure 2.4. Analysis of biotin-WYRGRLGSG-hydrazide synthesis. A) HPLC curve of 2nd purification. B) Matrix-assisted laser desorption ionization time-of-flight (MALDI TOF) mass spectrometry results.

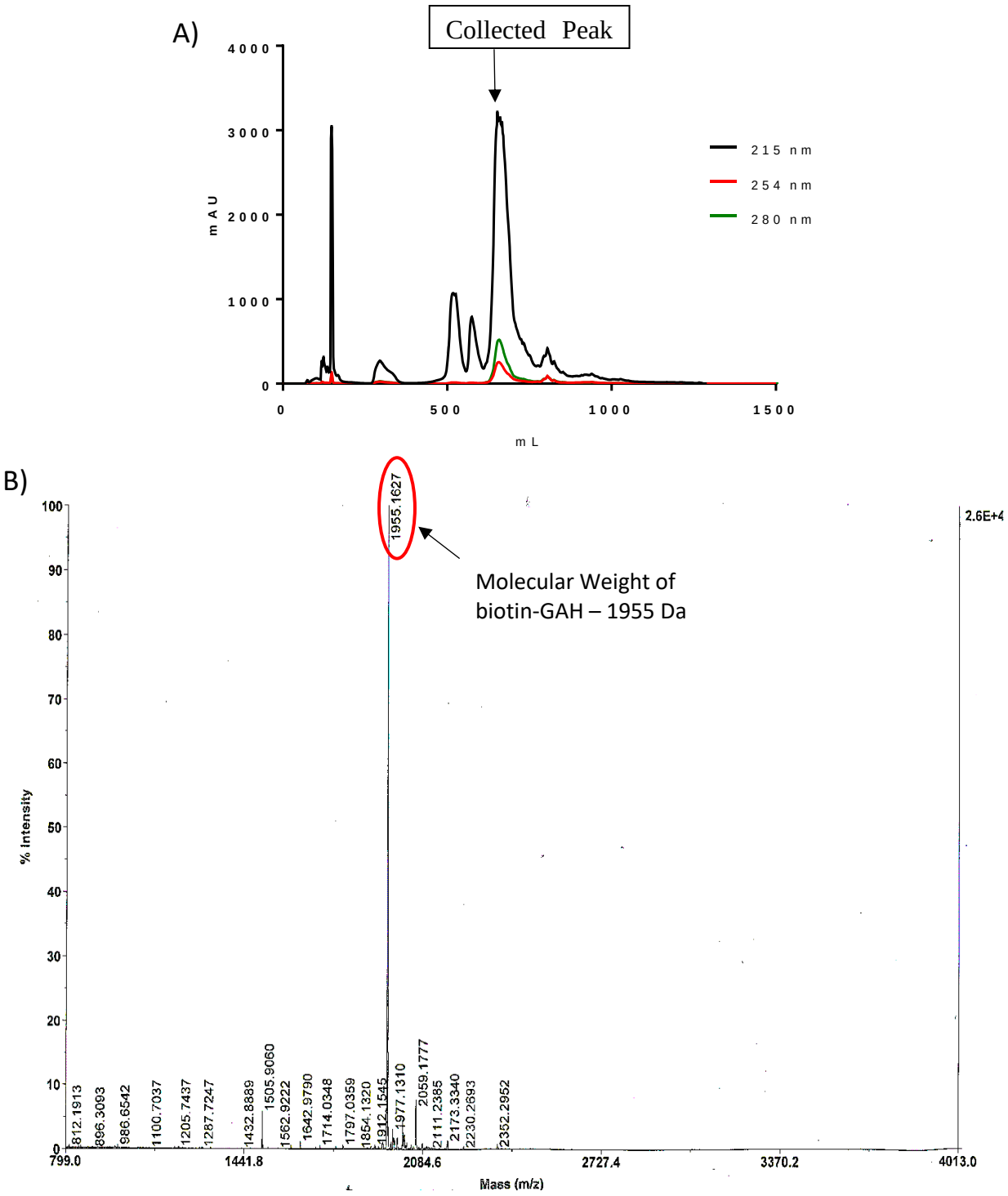

Figure 2.5. Analysis of biotin-GAHWQFNALTVRGGGSG -hydrazide synthesis. A) HPLC curve of 2nd purification. B) Matrix-assisted laser desorption ionization time-of-flight (MALDI TOF) mass spectrometry results.

2.2 EDC Chemistry: Troubleshooting Peptidoglycan Synthesis

To synthesize the lubricin mimic, a series of trouble shooting steps were taken to attach the peptides to the CS backbone using EDC chemistry. We started by determining the best way to add and quantify two different peptides to the same backbone. Since WYR and GAH had similar absorbance and fluorescence for many of the methods we investigated, it was difficult to determine how much of each peptide was on the CS backbone. Eventually, a 2-step process was developed where one type of peptide was added to the reaction, purified, and quantified. The process was then repeated with the second peptide. The bound peptides were quantified using a Nanodrop 2000 spectrophotometer using absorbance at 280 nm since the CS backbone showed no absorbance at this wavelength (Fig. 2.6a). A set of standards was made for both of the peptides. These standards were used to calculate the number of mols of peptide bound per one mol of CS (Fig. 2.6b). This method was able to quantify the number of peptides attached within ~2 to 3 mols per mol CS. Because of this uncertainty, there are cases where efficiencies are determined to be greater than 100% or less than 0%.

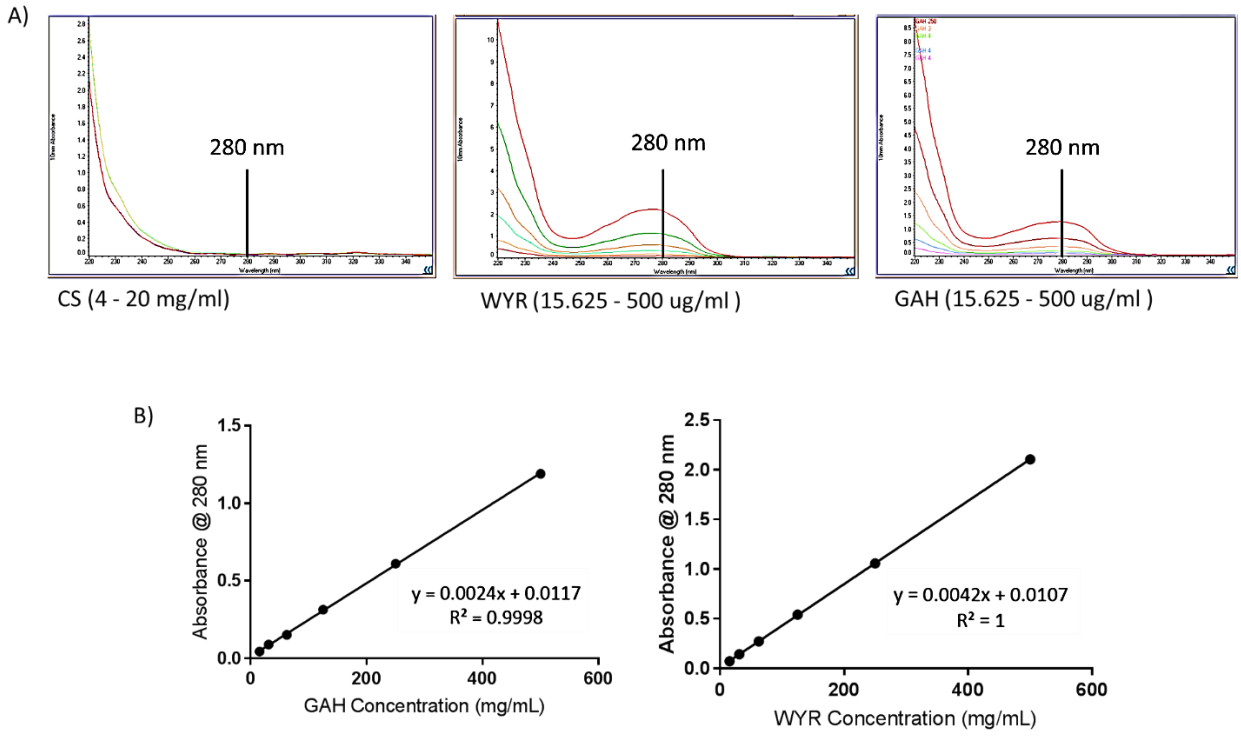

Figure 2.6. A) Absorbance curves @ 280 for CS, WYR, and GAH with varying concentrations. B) Concentration standards for WYR and GAH for peptide quantification.

The order of peptide addition during peptidoglycan synthesis was considered. A 2:6:1 ratio of GAH:WYR:CS was synthesized to determine the best peptide order. The EDC concentration was also varied for the 2nd peptide addition to determine if increasing the molar ratio of EDC would increase the efficiency of the 2nd peptide conjugation. These molecules were denoted as 2GAH_6WYR_CS or 6WYR_2GAH_CS depending on which order the peptides were added. The peptide listed first on the molecular abbreviation was the first peptide reacted and the next peptide listed was the second addition. The numbers preceding the peptide abbreviation indicate the ratios of peptide added per CS. It is important to note that our CS backbone has ~86 carboxyl groups that are activated by EDC (Fig. 2.1b). We wanted to use excess EDC to activate all carbonyl groups, in order to maximize peptide addition during each reaction. At first, 100 mols of EDC were added per mole of CS, ensuring that the ~86 carboxyl groups were activated. Later, concentrations of 200 and 400 mols of EDC per mole CS were tested as well. Overall, the coupling efficiencies were lower for the second peptide addition when compared to the first. It was determined that adding 2GAH first allowed for better coupling of the 6WYR in the second step. Increasing the molar ratio of EDC to CS decreased the coupling efficiencies for the second peptide addition.

Table 2.1. Peptidoglycan synthesis and peptide quantification of 2GAH_6WYR_CS. A range of 100-400 molar ratios of EDC to CS were used during 2nd peptide addition.

1st Peptide Addition					
Targeted Molecule	EDC Added (mols per CS)	Peptide Added (mols per CS)	Absorbance @ 280 nm	Peptide Bound (mols per CS)	Coupling Efficiency
2GAH_CS	100	2	0.364	1.81	90.29%
6WYR_CS	100	6	0.997	4.92	81.92%
2nd Peptide Addition					
Targeted Molecule	EDC Added (mols per CS)	Peptide Added (mols per CS)	Absorbance @ 280 nm	Peptide Bound (mols per CS)	Coupling Efficiency
2GAH_6WYR_CS	100	6	1.118	3.65	60.83%
6WYR_2GAH_CS	100	2	1.147	0.70	35.21%
6WYR_2GAH_CS	200	2	1.036	0.17	8.45%
6WYR_2GAH_CS	400	2	0.988	-0.01	-0.39%

Based on the results reported in Table 2.1, we continued with the addition of 2GAH first. The EDC concentration was held constant at 100 mols/mol CS for the following troubleshooting steps. Next, we analyzed the 2nd peptide coupling efficiency of WYR following the addition of

various WYR molar concentration ranging from 6-10 peptides per CS backbone. This was done to test whether increasing the molar ratio of WYR to CS would increase WYR conjugation to CS during the 2nd peptide addition step. The coupling of increased WYR per CS was hypothesized to enhance the binding of the peptidoglycan to the cartilage surface. Based on the results shown in Table 2.2, we determined that there was little increase in WYR conjugation to the CS backbone when increasing the initial reaction molar ratio from 6:1 to 10:1. The efficiency of the second peptide conjugation decreased as more peptide was added.

Table 2.2. Peptidoglycan synthesis and peptide quantification with 2GAH and varying molar ratio of WYR to CS for 2nd peptide addition.

1st Peptide Addition					
Targeted Molecule	EDC Added (mols per CS)	Peptide Added (mols per CS)	Absorbance @ 280 nm	Peptide Bound (mols per CS)	Coupling Efficiency
2GAH_CS	100	2	0.382	1.96	98.23%
2nd Peptide Addition					
Targeted Molecule	EDC Added (mols per CS)	Peptide Added (mols per CS)	Absorbance @ 280 nm	Peptide Bound (mols per CS)	Coupling Efficiency
2GAH_6WYR_CS	100	6	1.030	3.37	56.25%
2GAH_8WYR_CS	100	8	1.076	3.64	45.54%
2GAH_10WYR_CS	100	10	1.165	4.17	41.68%

We hypothesized that a side reaction was occurring in the EDC reaction for the first peptide addition which prevented effective conjugation of the second peptide to the CS backbone. The high concentration of EDC added in the first step (100 mols/mol CS) could have been altering the carboxyl groups, through an O to N-acylurea conversion, making the carbonyl groups unavailable for reaction during the second peptide addition[108]. As a remedy, the concentration of EDC was decreased during the first peptide addition to reduce the number of carboxyl groups potentially lost due to an N-acylurea conversion of the intermediate compound (Fig 2.1b). This allowed for more available carboxyl groups to be activated during the second peptide addition. Thus, the molar ratio of EDC to CS was reduced to from 100:1 to 25:1 during the first peptide addition. A 25:1 molar ratio of EDC:CS was high enough to activate the number of carboxyl groups required for peptide addition but low enough to not activate less than 1/3 of the available ~86 carboxyl groups present on each CS backbone. With the reduction in EDC for the first peptide reaction, there was

an increase in peptide coupling efficiency for the second reaction (Table 2.3). From these results, we concluded that using a 100:1 molar ratio of EDC per CS in the first reaction was impeding peptide conjugation during the second peptide addition presumably through permanent conjugation of EDC to the CS backbone. The synthesized 2GAH_15WYR_CS molecule shown in Table 2.3 was the basis for our preliminary friction experiments described later in the thesis.

Table 2.3. Peptidoglycan synthesis and peptide quantification with limited EDC during first peptide addition.

1st Peptide Addition					
Targeted Molecule	EDC Added (mols per CS)	Peptide Added (mols per CS)	Absorbance @ 280 nm	Peptide Bound (mols per CS)	Coupling Efficiency
2GAH_CS	25	2	0.412	2.13	106.74%
2GAH_CS	25	2.4	0.451	2.37	98.61%
5GAH_CS	25	5.5	1.007	6.06	110.15%
2nd Peptide Addition					
Targeted Molecule	EDC Added (mols per CS)	Peptide Added (mols per CS)	Absorbance @ 280 nm	Peptide Bound (mols per CS)	Coupling Efficiency
2GAH_10WYR_CS	100	10	2.055	10.10	101.04%
2GAH_15WYR_CS	100	15	2.311	13.20	87.97%
5GAH_20WYR_CS	100	25	3.383	20.49	81.95%

Following the successful 2nd peptide addition of WYR to the CS backbone, our goal was to make a series of peptidoglycans with varying numbers of WYR and/or GAH bound to the CS backbone to be analyzed for friction testing. A set of lubricin mimic variations were chosen for a friction testing experiment that was designed to determine which formulation resulted in achieving the lowest coefficient of friction at the cartilage surface. For this experiment set up, the selected ratios of WYR:GAH for each biotinylated molecule were 15:2, 10:2, 10:5, 10:10, and 10:15. As a starting point, a number of steps were taken to troubleshoot the synthesis of 10:5 and 10:10 WYR:GAH ratios to optimize the EDC reactions with the addition of single biotinylated peptide on each CS molecule (Tables 2.4 and 2.5).

Two different methods of peptidoglycan synthesis were tested. The first method involved adding a biotinylated GAH separately from the regular GAH peptide for separate peptide quantification. This process involved repeating the entire EDC chemistry reaction three times for each different peptide addition (Table 2.4). The second method involved adding both the

biotinylated and regular GAH at the same time which resulted in a 2-step peptide addition reaction (Table 2.5). Grouping biotin-GAH with GAH in a single EDC reaction did not allow for separate quantification of each peptide. Instead, the grouped peptides were quantified using the same standard measured for GAH on the nanodrop. The addition of biotin-GAH and GAH at the same time was done to determine if better peptide conjugation efficiency could be obtained by repeating the EDC reaction only two times instead of three. Also, the 2-step peptide addition method was quicker and more convenient than the 3-step process.

For 3-step peptide addition, Table 2.4 shows that there was sufficient coupling of the 1st and 2nd peptide. The biotinylated GAH was added first to the entire batch, which was split into groups for each additional reaction. The 3rd peptide addition resulted in lower efficiencies for all of the reactions. The highest efficiency of 40.76% for third peptide addition was seen for the bGAH_4GAH_10WYR_CS molecule. This high efficiency was due to the lower amount of peptide (5GAH) being added during the 2nd addition. When the GAH addition was before the WYR addition, there was significant conjugation for both 5 and 10 GAH. However, this led to very little WYR addition during the last peptide addition. Overall, the 3-step method did not improve the quantity of peptide addition to the CS backbone.

Table 2.4. Biotinylated peptidoglycan synthesis with a 3-step peptide addition.

1st Peptide Addition					
Targeted Molecule	EDC Added (mols per CS)	Peptide Added (mols per CS)	Absorbance @ 280 nm	Peptide Bound (mols per CS)	Coupling Efficiency
bGAH_CS	10	1.2	0.157	0.78	64.81%
2nd Peptide Addition					
Targeted Molecule	EDC Added (mols per CS)	Peptide Added (mols per CS)	Absorbance @ 280 nm	Peptide Bound (mols per CS)	Coupling Efficiency
bGAH_10WYR_CS	30	12	1.586	8.66	72.13%
bGAH_9GAH_CS	30	10.8	1.545	10.00	92.63%
bGAH_4GAH_CS	30	4.8	1.009	5.23	108.90%
3rd Peptide Addition					
Targeted Molecule	EDC Added (mols per CS)	Peptide Added (mols per CS)	Absorbance @ 280 nm	Peptide Bound (mols per CS)	Coupling Efficiency
bGAH_10WYR_9GAH_CS	100	9.9	1.723	0.43	4.39%
bGAH_10WYR_4GAH_CS	100	4.4	1.620	0.07	1.64%
bGAH_9GAH_10WYR_CS	100	11	1.861	1.92	17.41%
bGAH_4GAH_10WYR_CS	100	11	1.721	4.48	40.76%
bGAH_10WYR_EDC_CS	100	0	1.721	n/a	n/a
bGAH_9GAH_EDC_CS	100	0	1.095	n/a	n/a
bGAH_4GAH_EDC_CS	100	0	0.748	n/a	n/a

The 2-step method showed slightly higher peptide conjugation efficiency than the 3-step method. However, the calculated number of peptide bound per CS remained significantly lower than the targeted molar ratio for the final peptide addition. The best peptide conjugation was seen for 10WYR_(9GAH+bGAH) _CS, which only resulted in 5.59 GAH bound per CS vs the targeted amount of 10GAH per CS. So far, we have been unable to conjugate greater than 15 mols of peptide per CS backbone using two subsequent peptide additions.

Table 2.5. Biotinylated peptidoglycan synthesis with 2-step peptide addition.

1st Peptide Addition					
Targeted Molecule	EDC Added (mols per CS)	Peptide Added (mols per CS)	Absorbance @ 280 nm	Peptide Bound (mols per CS)	Coupling Efficiency
10WYR_CS	30	12	1.739	10.61	88.41%
(9GAH+bGAH)_CS	30	12	1.351	9.13	76.07%
(4GAH+bGAH)_CS	30	6	1.026	6.31	105.12%
2nd Peptide Addition					
Targeted Molecule	EDC Added (mols per CS)	Peptide Added (mols per CS)	Absorbance @ 280 nm	Peptide Bound (mols per CS)	Coupling Efficiency
10WYR_(9GAH+bGAH)_CS	100	11	2.481	5.59	50.86%
10WYR_(4GAH+bGAH)_CS	100	5.5	1.882	0.78	14.14%
(9GAH+bGAH)_10WYR_CS	100	11	1.886	3.49	31.76%
(4GAH+bGAH)_10WYR_CS	100	11	1.197	0.68	6.18%
10WYR_EDC_CS	100	0	1.099	n/a	n/a
(9GAH+bGAH)_EDC_CS	100	0	1.078	n/a	n/a
4GAH+bGAH)_EDC_CS	100	0	0.720	n/a	n/a

As mentioned previously, the limited amount of peptide addition seen in Table 2.4 and Table 2.5 could be caused by permanent conjugation of EDC to CS during the first reactions which subsequently blocked peptide conjugation in the in the next. Because of this concept, a group of molecules in the final peptide addition steps were reacted with EDC only without the peptide present. These molecules were listed in Tables 2.4 and 2.5 with EDC in the targeted molecule name to indicate that no peptide was added. Isolated EDC addition was tested to determine if there was a change in molecule absorbance before and after the reaction. In theory, the absorbances should remain the same, but a change in absorbance might indicate the presence of permanent EDC conjugates attached to CS due to an O to N-acylurea conversion of the intermediate active compound. Our hypothesis was that if EDC was being permanently conjugated onto the carbonyl group, there would be an increase in molecular absorbance following the EDC only reaction. According to Table 2.4, there was a slight decrease in absorbance following the addition of EDC only for bGAH_9GAH_CS and bGAH_4GAH_CS. A decrease in absorbance was also seen in Table 2.4 for all the molecules with the EDC only addition. The reduction in molecule absorbance @ 280 nm could be a result of contaminants and unbound EDC/peptide being present when the

first peptide addition was quantified. Following the subsequent EDC reaction, the unbound EDC/peptide and other contaminants were purified out of the sample before analysis.

A series of reactions were performed to determine the minimal amount of EDC that was needed for efficient peptide addition. These reactions were done to limit the amount of unwanted EDC conjugation to the CS carboxyl groups due to excess EDC present during the first peptide. Table 2.6 shows that with even a 1:1 molar ratio of EDC to peptide (12 mols per CS), there was sufficient coupling to the CS backbone. It is important to note that even though the 30:1 molar ratio of EDC resulted in better peptide coupling, the lower amounts show potential for increasing the coupling of the 2nd peptide addition. Although no further peptidoglycan synthesis troubleshooting steps were taken, the results from Table 2.6 show potential for future optimization of biomimetic lubricin synthesis using EDC chemistry.

Table 2.6. Synthesis of 10WYR_CS and 10GAH_CS with decreasing EDC.

1st Peptide Addition					
Targeted Molecule	EDC Added (mols per CS)	Peptide Added (mols per CS)	Absorbance @ 280 nm	Peptide Bound (mols per CS)	Coupling Efficiency
10WYR_CS	30	12	2.550	14.21	118.42%
10WYR_CS	20	12	1.958	11.82	98.53%
10WYR_CS	12	12	1.788	10.31	85.93%
10GAH_CS	30	12	1.664	12.28	102.32%
10GAH_CS	20	12	1.399	9.48	79.01%
10GAH_CS	12	12	1.400	9.49	79.07%

2.3 Verification of EDC Conjugates Attached to Chondroitin Sulfate

Throughout the EDC chemistry troubleshooting process there was reason to suspect the EDC was being permanently attached to the carboxyl due to an O to N-acylurea rearrangement. To verify this, FTIR and NMR analyses were done on CS only and CS +EDC dissolved in a D_2O solution. The EDC was added via a standard EDC chemistry reaction without the addition of peptide using 100 mols of EDC per CS backbone. The ^{1}H-NMR analysis was done to quantify the amount of EDC present per CS backbone (Fig. 2.7) and a ^{13}C-NMR analysis was performed as an additional verification of differences between the prepared samples (Fig. 2.8). Following the NMR

analysis, an FTIR spectrum was measured for each sample to determine if the EDC present in the CS+EDC sample was attached to the CS backbone (Fig 2.9 and 2.10).

The ^{1}H-NMR spectra of the CS and CS+EDC samples show clear differences (Fig. 2.7). Specifically, the CS+EDC spectrum shown in Figure 2.7b has a distinct peak at 1.0 ppm that is not seen in the CS only spectrum. The peak at 1.0 ppm in Fig. 2.7b is associated with the protons found in the two CH_3 groups that are attached to an amine at the end of the EDC molecules (Fig. 2.1b). With this information, we were able to calculate the number of EDC molecules present per CS backbone in the prepared sample. According to the peak values in Figure 2.7b, there were approximately 25 EDC molecules present per each CS backbone. Hence, ~25% of the initial EDC added to the CS+EDC sample remained after molecule purification. The ^{13}C-NMR also showed clear differences between the spectra (Fig. 2.8). There is a large peak at ~43 ppm in Figure 2.8b that is not present in Figure 2.8a. This further indicates that EDC is present in the CS+EDC sample. From both ^{1}H and ^{13}C NMR analysis we can conclude that there is EDC that remains with the CS following a standard EDC reaction.

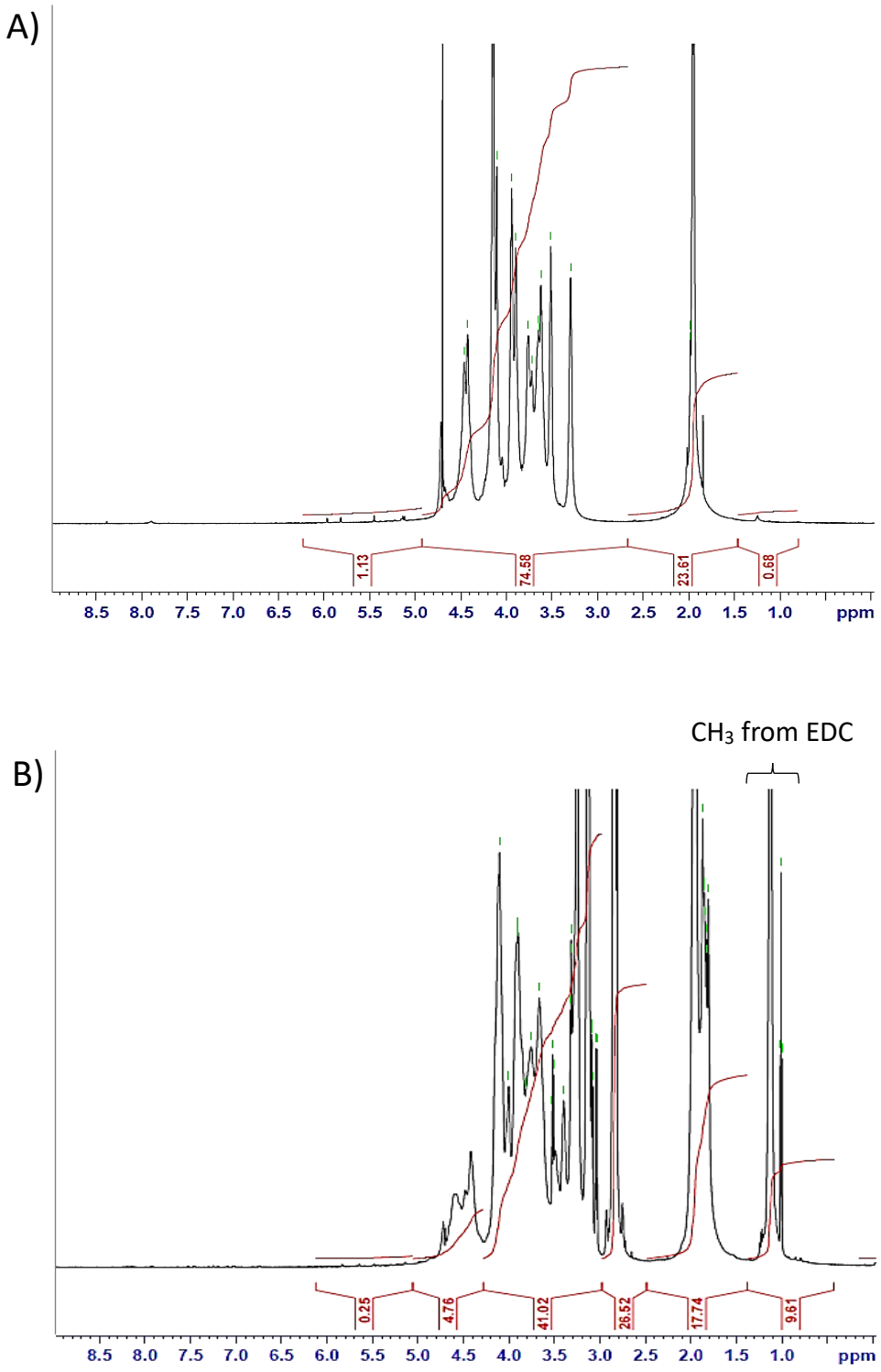

Figure 2.7. ^{1}H-nuclear magnetic resonance spectroscopy analysis of CS and CS+EDC dissolved in D_2O. A) Spectrum for CS only. B) Spectrum for CS with the addition of 100 mols of EDC per mol CS. The peak with only EDC proton contribution is indicated. According to this spectrum, approximately 25 EDC molecules were present per each CS backbone.

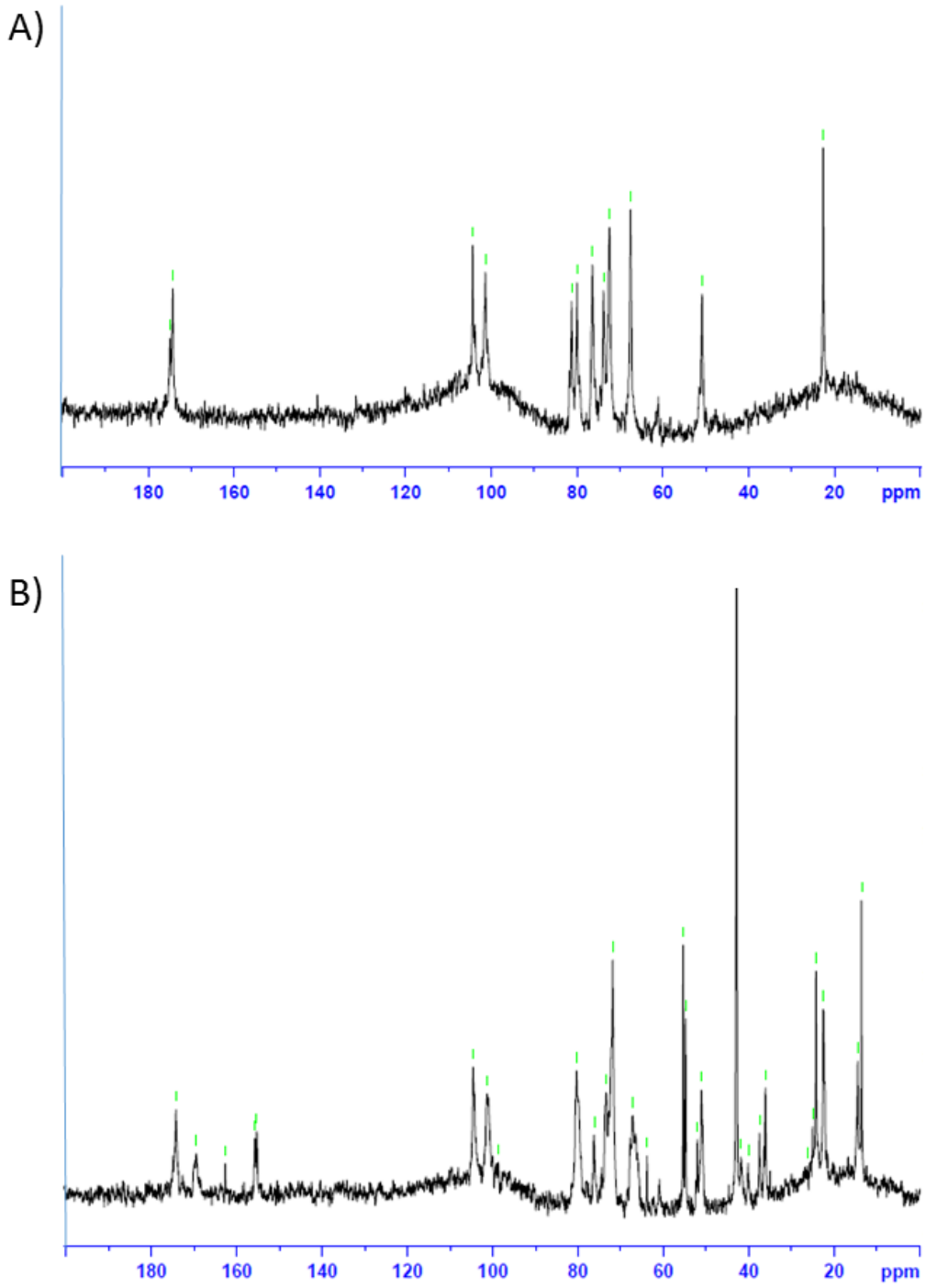

Figure 2.8. ^{13}C-nuclear magnetic resonance spectroscopy analysis of CS and CS+EDC dissolved in D_2O. A) Spectrum for CS only. B) Spectrum for CS with the addition of 100 mols of EDC per mol CS.

Although the NMR analysis showed the presence of EDC in the CS+EDC sample, this did not mean that EDC molecules were permanently conjugated to the CS backbone. There was the additional possibility that EDC was not filtered out from the CS during the purification step. To determine if EDC was attached to the CS backbone, an FTIR analysis was performed. Our area of interest on the FTIR spectrum was the peak that indicated the carboxyl group on the CS. EDC will be permanently conjugated onto the carboxyl site of CS if active O-acylisourea was altered to N-acylurea during the EDC reaction. The 1605 cm^{-1} peak from the CS only sample shown in Figure 2.10a indicates the carboxyl group found in our CS sample[109]. There is a significant reduction in this peak with the addition of EDC to the CS shown in Figure 2.10. This alteration most likely indicates the presence of permanently conjugated EDC at the carbonyl site reducing signal from the carboxyl groups in the FTIR spectra (Fig. 2.10). Additionally, the stable conjugation of EDC would create an amide bond with the CS backbone. Chondroitin sulfate already contains one amide per disaccharide and the conjugation of EDC would create two in some disaccharides since not all the disaccharides react with the EDC. According to Yan *et al.*, we determined that the amide I and II are present in our CS+EDC sample at the 1650 cm^{-1} and 1547 cm^{-1} peaks shown in Figure 2.10b[110]. Overall, the FTIR analysis indicated that there was some permanent attachment of EDC to CS backbone at the carboxyl group region, even after quenching and purifying the CS+EDC reaction.

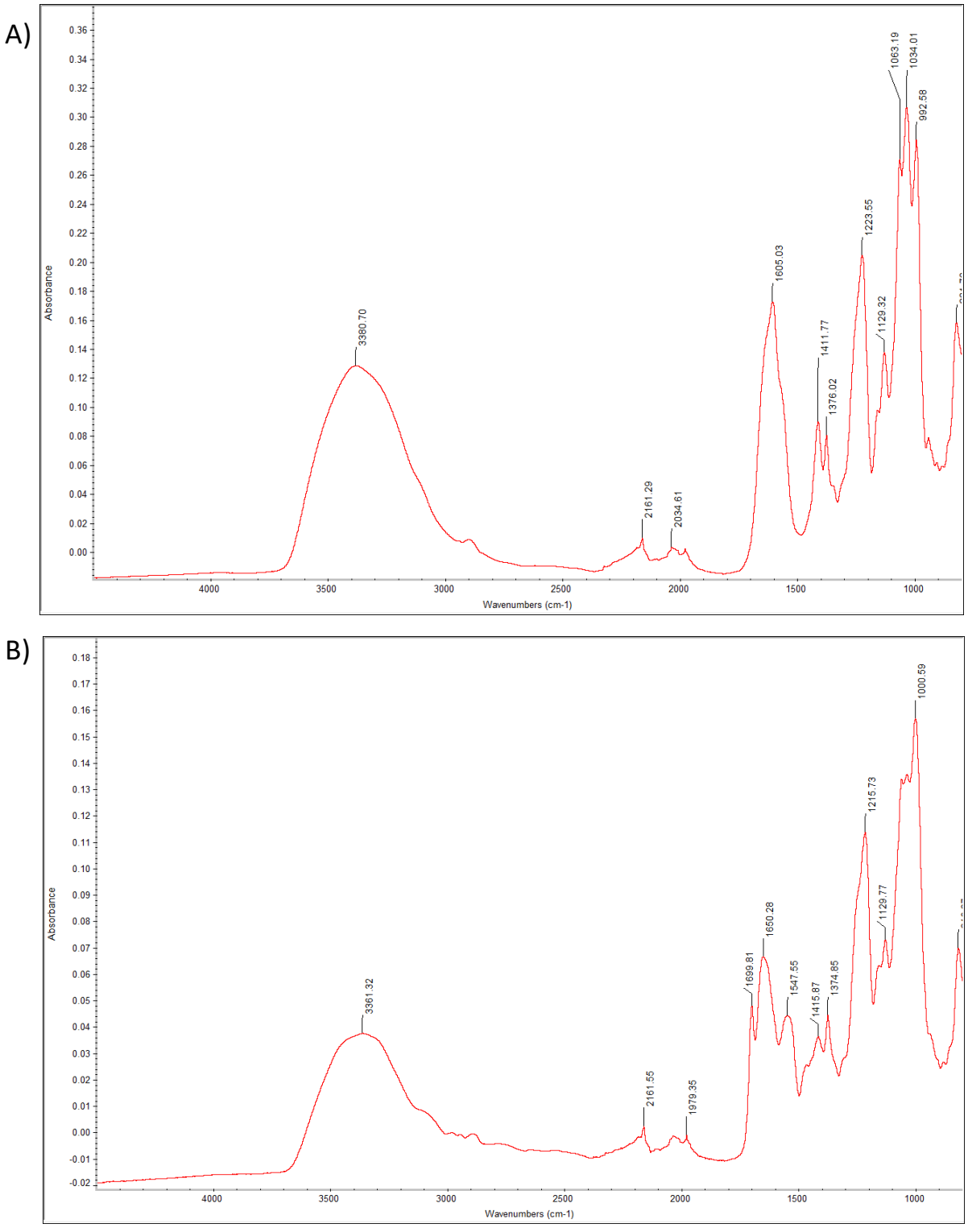

Figure 2.9. FTIR spectra of chondroitin sulfate (CS) and chondroitin sulfate reacted with EDC (CS+EDC) with peak values. A) Spectrum for CS sample. The 1605 cm^{-1} peak is associated with the carboxyl group. B) Spectrum for CS+EDC. The two amide groups are indicated by the peaks at 1650 cm^{-1} and 1547 cm^{-1}.

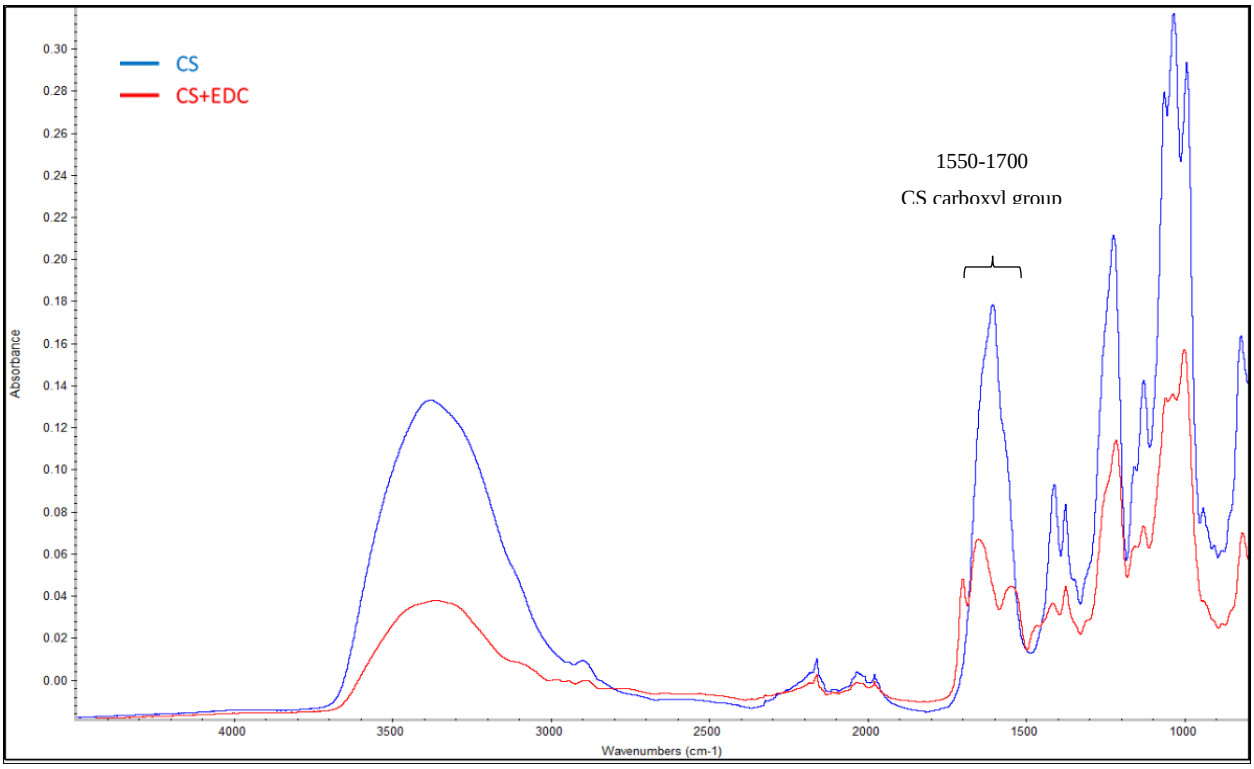

Figure 2.9. Overlay of FTIR spectra of chondroitin sulphate (CS) and chondroitin sulfate reacted with EDC (CS+EDC). The area associated with the carboxyl group located on the CS in indicated. There is significant difference in this area between the two spectra.

In conclusion, we were able to successfully synthesize a 2GAH_15WYR_CS molecule which was used for preliminary friction studies shown in the next chapter. A decrease in EDC concentration from 100 mols/mol CS to 30 mols/mol CS during the first peptide addition allowed us to synthesize the 2GAH_15WYR_CS with 70-100% peptide conjugation. However, this reduction in EDC did not seem to improve the coupling efficiency when a high molar ratio of GAH:CS was added subsequently with a high molar ratio of WYR:CS. Additional 2-peptide molecules were not able to be synthesized due to low conjugation efficiency of the second peptide addition. Adding 10GAH or more to the CS backbone significantly reduced the amount of WYR we were able to add or vice versa depending on the peptide addition order. The FTIR and NMR analysis showed that inefficient peptide coupling was caused by permanent EDC conjugation onto the CS backbone which blocked peptide attachment at the CS carboxyl site. During the EDC reaction, the active O-acylisourea intermediate formed by EDC and the CS carboxyl group was undergoing rearrangement to an inactive N-acylurea. This alteration caused EDC to remain bound

to the CS carbonyl site even after quenching and purifying the EDC reaction. For future troubleshooting, an even smaller amount of EDC can be added for each peptide addition to limit the number of inactive N-acylurea formed, which inhibits the primary anime reaction of the peptide hydrazides. We showed that even a 1:1 molar ratio of EDC and peptide added to CS resulted in sufficient peptide binding to the CS backbone.

CHAPTER 3. COEFFICIENT OF FRICTION: PRELIMINARY STUDIES

Our lubricin mimic was designed to reduce friction at the articular cartilage surface. To test the lubrication properties of the synthesized peptidoglycans, a series of macroscale friction studies were performed. The goal of these preliminary friction studies was to determine which treatments to test in comparison to the lubricin mimic and to measure the lubrication properties of our initially chosen peptidoglycan design, 2GAH_15WYR_CS (Table 2.3). Coefficient of friction (COF) measurements were obtained using a rheometer with a cartilage-on-glass setup. In the beginning, half of the harvested cartilage osteochondral plugs were treated with 0.5% w/v trypsin in PBS for three hours at room temperature. This was done to mimic the osteoarthritic condition of diseased cartilage. The trypsin treatments degraded the proteoglycans within the extracellular matrix and destroyed the structural integrity of the cartilage[111]. For the first preliminary friction study, we used 2GAH_15WYR_CS as our lubricin mimic and PBS/trypsin as our negative control (Fig. 3.1). We hypothesized that the trypsin would increase the friction compared to the non-treated cartilage (WT). We also hypothesized that the COF of the trypsin treated plugs would decrease with the addition of the lubricin mimic plus hyaluronic acid. Our initial study included treatments of PBS, 2GAH_15WYR_CS, high molecular weight HA, and low molecular weight HA to both native cartilage and trypsin treated cartilage. After testing, we did not see a significant difference in static COF between the twelve treatment groups shown in Figure 3.1. For the kinetic COF measurements, the 2GAH_15WYR_CS treatment was able to reduce the kinetic COF compared to the PBS control within the WT plug group (Fig. 3.1b). In the WT plugs, the 2GAH_15WYR_CS kinetic COF was significantly lower than both the high and low molecular weight treatments of HA. This suggests that the lubricin mimic can reduce friction at the cartilage surface more than hyaluronic acid by itself. However, it was unclear if the addition of HA to the 2GAH_15WYR_CS decreased the COF further than the lubricin mimic alone. Furthermore, the trypsin/PBS treatments did not increase the COF for either the static or the kinetic COF when compared to the WT/PBS (Fig. 3.1). Since we were using mature cartilage (18-36 months old bovine), the trypsin treatment might not have been necessary due to the cartilage degradation that was already present at harvest.

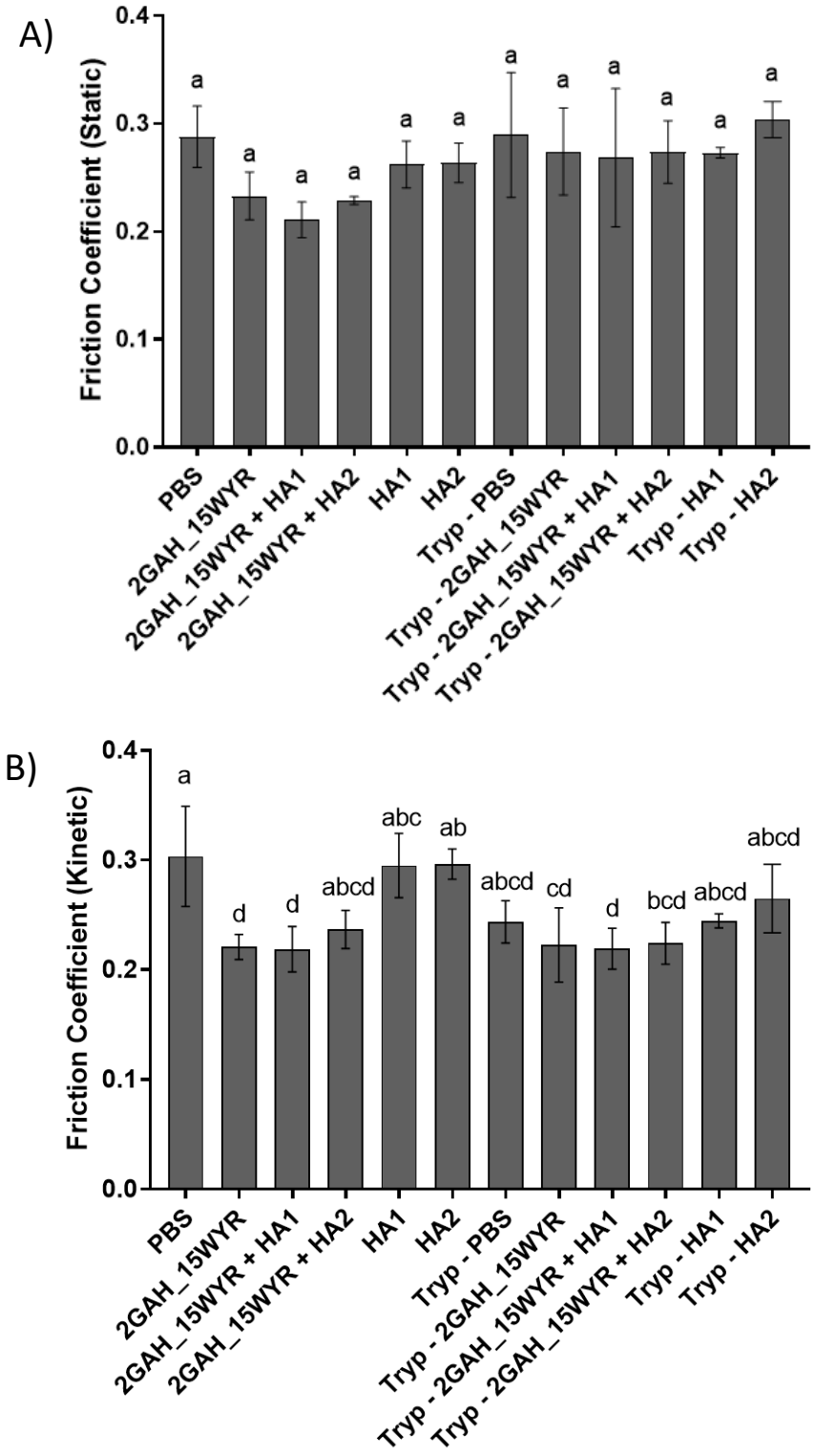

Figure 3.1. COF values measured at the cartilage surface comparing WT to trypsin treatments with the addition of 2GAH_15WYR_CS and hyaluronic acid. HA1 is high molecular weight HA and HA2 is low molecular weight HA. Statistically significant differences are represented by different letters appearing over each column. n = 3 for each treatment. A) Static COF. B) Kinetic COF.

An additional study was performed to determine if the trypsin treatment of the osteochondral plugs was necessary to induce an osteoarthritic state. In this study, the trypsin treatment time was varied (15 min, 1 hr, and 3 hr) to determine if the length of time influenced friction at the cartilage surface (Fig. 3.2). The results indicated that the trypsin treatment did not increase the static or kinetic COF (Fig. 3.2). For the kinetic COF, the friction decreased compared to the native cartilage with each of the various timed trypsin treatments to the osteochondral plugs (Fig. 3.2b). From this, we concluded that the trypsin treatment did not induce a diseased state at the articular cartilage surface. This could be due to condition of the bovine knees that the plugs were harvested from. Many of the mature bovine knees already showed signs of degradation at the cartilage surface. The decrease in kinetic friction due to the trypsin might be because the treatment did not roughen the cartilage surface; it just degraded the structure of the cartilage making it soft and sponge like. The lack of structural integrity and the soft, porous characteristics of the trypsin treated cartilage were not indicative of an increase in friction between the cartilage surface and the glass plate. From this study, we concluded that the best negative control for our next experiments would be untreated osteochondral plugs after vigorous rinsing with PBS to remove any residual synovial fluid (SF). We also determined that the best positive control would be untreated cartilage plugs with the addition of synovial fluid to mimic the friction of native cartilage.

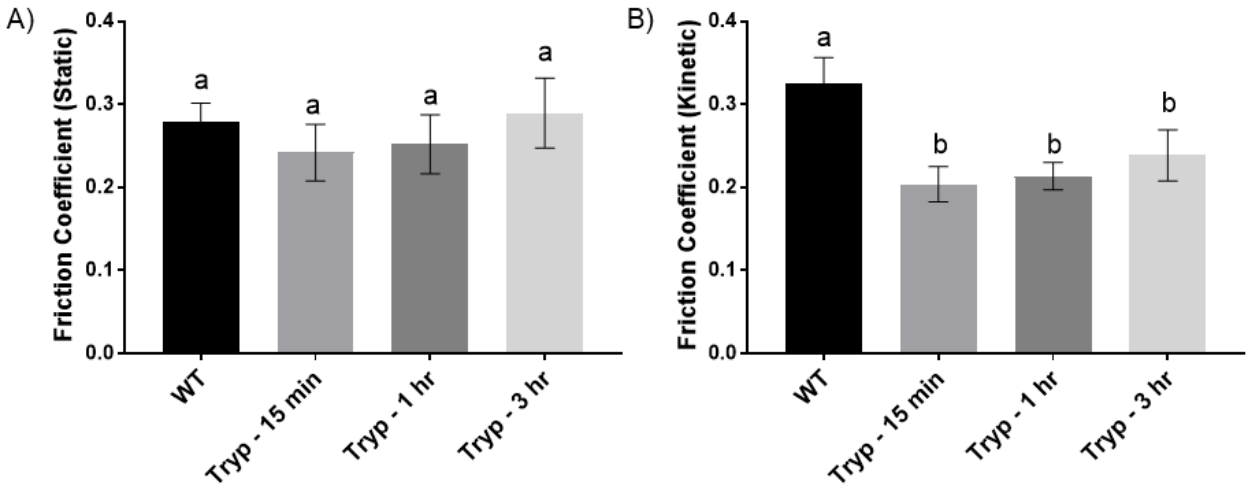

Figure 3.2. COF values measured at the cartilage surface comparing WT to 15 min, 1 hr, and 3 hr trypsin treatments. Statistically significant differences are represented by different letters appearing over each column. n = 7 for each treatment. A) Static COF. B) Kinetic COF.

From this point on, we did not use any trypsin treatment on the osteochondral plugs. For the next experiment, all of the bovine osteochondral plugs we used were harvested, rinsed overnight, and stored in treatments of either PBS, HA, SF, or 2GAH_15WYR_CS until friction testing was completed. The results in Figure 3.3a shows that the static COF of SF was significantly lower than the rest of the treatments. Aside from SF, the treatments showed the same static COF. On the other hand, the kinetic COF measurements for all the treatments with 2GAH_15WYR_CS were statistically lower than PBS and low molecular weight HA. According to the results, the peptidoglycan and peptidoglycan + HA treatments matched the kinetic COF of native synovial fluid, whereas the PBS and HA treatments did not. These findings were consistent with the data displayed in Figure 3.1. Data depicted in Figure 3.1 and Figure 3.3 suggest that 2GAH_15WYR_CS reduced the kinetic COF at the articular cartilage surface.

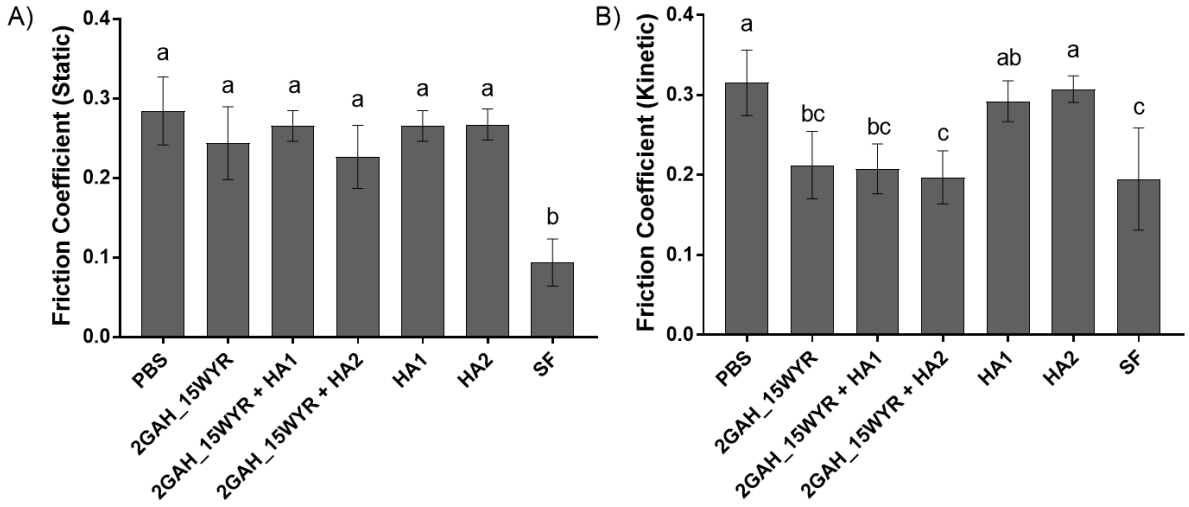

Figure 3.3. COF values measured at the cartilage surface comparing PBS, 2GAH_15WYR_CS, hyaluronic acid (HA), and synovial fluid (SF) treatments. HA1 is high molecular weight HA and HA2 is low molecular weight HA Statistically significant differences are represented by different letters appearing over each column. n = 4 for each

Our next step was to analyze which components of 2GAH_15WYR_CS were responsible for reducing the friction at the articular cartilage surface. Up to this point, 2GAH_15WYR_CS was the only peptidoglycan formulation we tested. We did not know if the addition of GAH or WYR, or both, was critical to the lubrication characteristic of the peptidoglycan. To test which components were necessary for the reduction of friction, we synthesized 2GAH_CS and 15WYR_CS in addition to 2GAH_15WYR_CS. For friction testing, we used the same positive and negative controls of SF and PBS treatments, respectively. In addition, osteochondral plugs

were treated with only CS to see if CS without the addition of peptides contributed to cartilage surface lubrication. Hyaluronic acid treatments were not included for this experiment since we saw no significant reduction of COF in the earlier studies. According to the rheology results seen in Figure 3.4, we determined that the 15WYR_CS and 2GAH_15WYR_CS treatments had a significantly lower static COF than both PBS and 2GAH_CS. The static COF of CS was statistically the same as all the treatments except synovial fluid (Fig. 3.4a). The kinetic COF of 15WYR_CS was significantly lower than PBS and 2GAH_CS (Fig. 3.4b). Based on both the static and kinetic COF, we concluded that the addition of 2GAH to the CS backbone was not a contributor to lowering the COF. The presence of 15WYR on the CS backbone has been consistent in showing a reduction in the kinetic COF (Fig. 3.1b, 3.3b and 3.4b). The kinetic COF of synovial fluid was not statistically different from the rest of the treatments for this experiment. There was quite a bit of variability in the kinetic COF for the synovial fluid sample and the 2GAH_15WYR_CS. The osteochondral plugs we used were harvested from adult bovine (18-36 months) with unknown conditions. Sometimes there was quite a bit of variability between batches of osteochondral plugs that were extracted. This could be a possible explanation for the synovial fluid treatment not having a lower kinetic COF than the rest of the treatments which was seen in previous experiments.

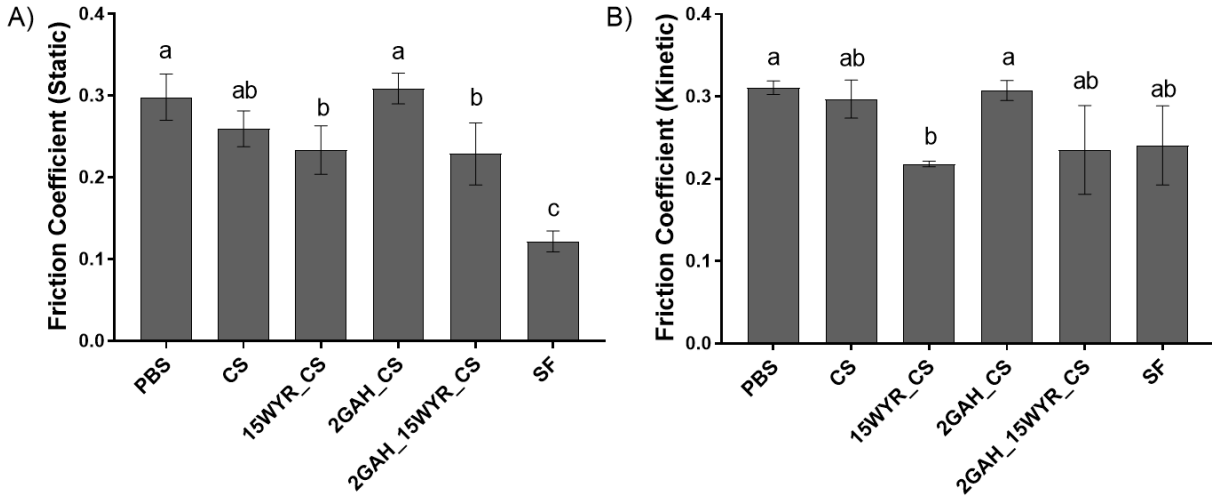

Figure 3.4 COF values measured at the cartilage surface comparing PBS, SF, and 2GAH_15WYR_CS and its separate components. Statistically significant differences are represented by different letters appearing over each column. n = 4 for each treatment. A) Static COF. B) Kinetic COF.

In conclusion, the goal of these preliminary friction studies was to determine which treatments to test in comparison to the lubricin mimic and to see the lubrication properties of our initially chosen peptidoglycan design, 2GAH_15WYR_CS (Table 2.3). We determined that the use of a trypsin treatment on the osteochondral plugs as an osteoarthritis disease model was not needed for our friction testing experiments. This treatment has generally been used on healthy cartilage, specifically on cartilage harvested from young calves aged 3-6 months. Our osteochondral plugs were harvested from older cow knees (18-36 months) that already displayed signs of wear at the articular cartilage surface. We decided the best negative control (or disease-like cartilage) was to test non-treated osteochondral plugs shaken vigorously overnight in PBS to remove residual synovial fluid. Some of these plugs were treated with synovial fluid afterwards as a positive control. Furthermore, we determined that hyaluronic acid treatments in addition to the peptidoglycan treatment did not contribute to reducing the COF. We found that the peptidoglycan alone was able to reduce the friction at the cartilage surface. One consideration we made was the amount of GAH that was added to the CS backbone. There was the possibility that only 2GAH peptides added per CS was not enough for significant HA binding to the molecule. We did not pursue this further since we were successful in reducing the COF with the 2GAH_15WYR_CS and 15WYR_CS alone. Additional studies could be done with increasing amounts of GAH peptide added to the peptidoglycan along with a corresponding HA treatment. Overall, these preliminary studies gave critical insight to the lubrication abilities of 2GAH_15WYR_CS.

CHAPTER 4. LUBRICIN MIMIC REDUCES COEFFICIENT OF FRICTION AT ARTICULAR CARTILAGE SURFACE

We designed our lubricin mimic to possess the same function as lubricin but not the same structure. The main function of lubricin is to reduce friction at the articular cartilage surface, which was our primary targeted characteristic for synthesized biomimetic lubricin. Our lubricin mimic was designed to include both collagen II binding peptides (WYR) and HA binding peptides (GAH) attached covalently to a CS backbone (Fig. 4.1a and 4.1c). The collagen II binding peptides allowed the molecule to bind at the cartilage surface creating a protective boundary layer similar to that of native lubricin. The HA binding peptides would allow the lubricin mimic to also bind to HA at the cartilage surface, imitating the lubricin-HA interactions that occur within the synovial joint. We hypothesized that the attachment of HA to the lubricin mimic at the articular cartilage surface was necessary for proper lubrication due to previous studies showing lubricin/HA interactions[48,49]. However, after the preliminary tests shown in Chapter 3, we saw that the addition of HA to the synthesized lubricin mimic did not reduce the friction at the articular cartilage surface more than the lubricin mimic alone. We also concluded that the 2GAH_15WYR_CS molecule was able to reduce friction more than PBS and HA alone. Because of this, we decided to run a series of friction tests to determine which components of the synthesized peptidoglycan were responsible for this reduction in friction. Our goal was to determine if both WYR and GAH peptides attached to the CS backbone were necessary to reduce friction at the cartilage surface. Furthermore, if only one type of peptide was needed, we wanted to determine whether varying the amount of peptide attached to the backbone affected the lubrication properties of the synthesized peptidoglycans. Ultimately, this study gave us the knowledge to formulate a biomimetic lubricin peptidoglycan with the best lubrication properties for the reduction of friction at the articular cartilage surface.

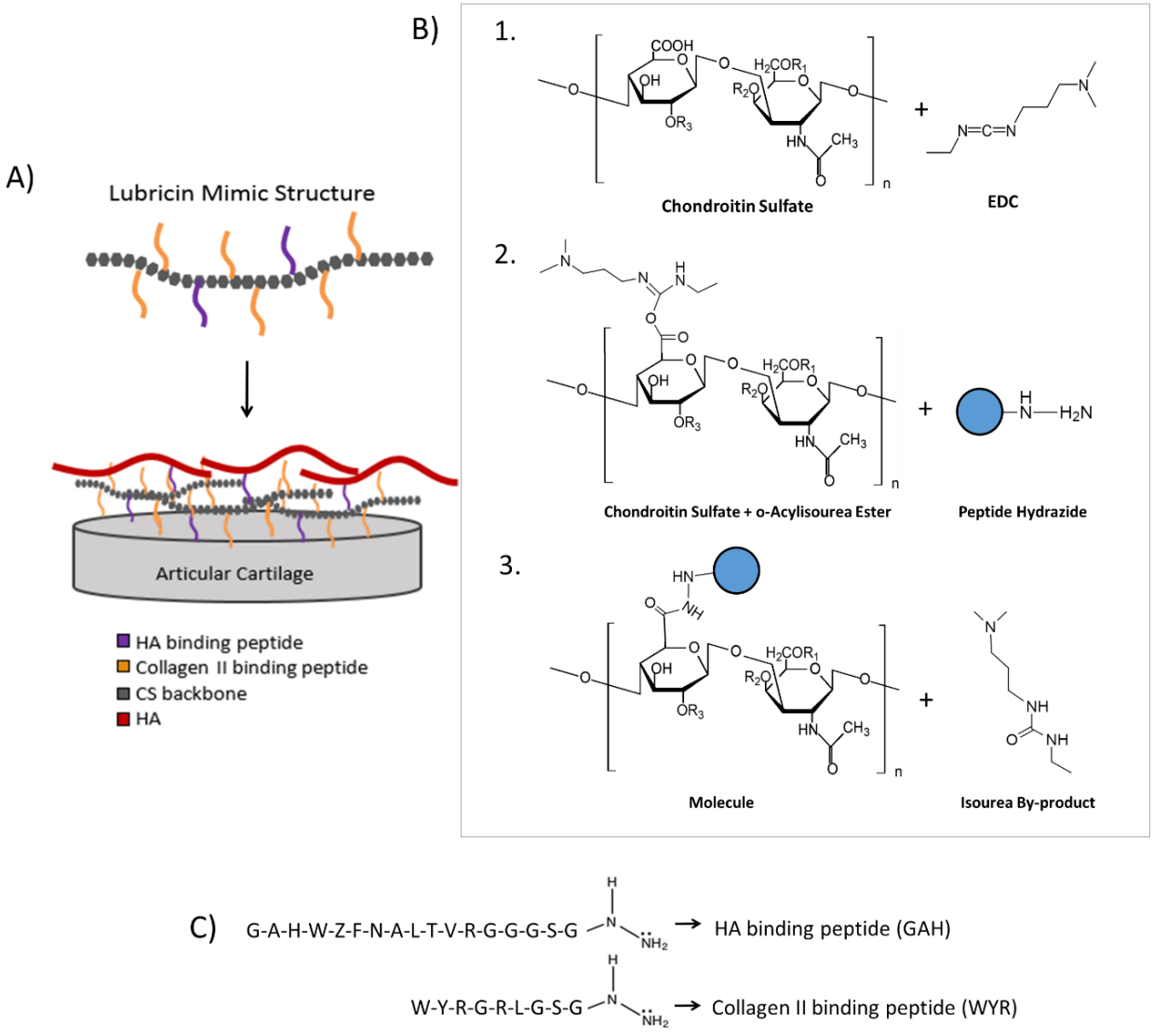

Figure 4.1. A) Design of biomimetic lubricin. B) Schematic of lubricin mimic synthesis using EDC chemistry and peptide hydrazides. C) Amino acid sequence of HA binding peptide (GAH) and collagen II binding peptide (WYR).

First, we synthesized the peptidoglycans that we planned for our friction testing experiment using the EDC chemistry method shown in Figure 4.1b. A series of WYR_CS and GAH_CS peptidoglycans with varying peptides amounts were made, as well as 2GAH_15WYR_CS (Table 4.1). All of the peptidoglycans included the addition of one biotinylated peptide per CS backbone that was included with the overall peptide quantification. Each of the WYR_CS molecules contained one biotinylated WYR and each of the GAH_CS molecules contained one biotinylated

GAH. The 2GAH_15WYR_CS was biotinylated with a biotin attached to one of the GAH peptides added to the CS backbone. The biotinylated peptide addition to the peptidoglycans allowed the molecules to be imaged at the cartilage surface using confocal microscopy for later analysis.

To synthesize each of the peptide-CS formulations, a targeted molar ratio of peptide to CS was determined for each peptidoglycan, and the corresponding amount of peptide was added to each reaction with a 10% excess. This excess served to account for loss of peptide conjugation due to incomplete reaction and manual error. The peptide coupling efficiencies were determined by dividing the amount of bound peptide by the amount of peptide added. Despite numerous efforts, the EDC chemistry process was not always consistent; therefore, peptide quantification had to be done after each reaction. Each peptidoglycan was synthesized so that the final amount of peptide conjugated was within 1-2 mols of the targeted amount. In Table 4.1 the amount of peptide conjugated for 15GAH_CS was around 12.3 mols GAH/mol CS, which is slightly lower than we targeted. However, we determined that there was enough difference in peptide bound between the 10GAH_CS (8.9 peptides conjugated) and the 15GAH_CS (12.3 peptides conjugated) to use them for friction testing.

Table 4.1. Biotinylated peptidoglycan synthesis and peptide quantification of varying peptide amounts of WYR_CS and GAH_CS. The synthesis of 2GAH_15WYR_CS was also included.

WYR Addition				
Molecule	WYR added (mols per CS)	Absorbance @ 280 nm	Peptide Bound (mols per CS)	Coupling Efficiency
2GAH_15WYR_CS	16.5	2.074	13.15	79.70%
5WYR_CS	5.5	1.076	5.528	100.51%
10WYR_CS	11	1.596	9.034	82.13%
15WYR_CS	16.5	2.236	14.627	88.65%
GAH Addition				
Molecule	GAH added (mols per CS)	Absorbance @ 280 nm	Peptide Bound (mols per CS)	Coupling Efficiency
2GAH_15WYR_CS	2.4	0.392	2.024	84.33%
2GAH_CS	2.4	0.392	2.024	84.33%
5GAH_CS	5.5	0.722	4.011	72.93%
10GAH_CS	11	1.348	8.998	81.80%
15GAH_CS	16.5	1.668	12.312	74.62%

We chose the peptidoglycan formulations shown in Table 4.1 for the friction testing experiment based on the number of peptides in the 2GAH_15WYR_CS molecule. We included a 2GAH_CS and a 15WYR_CS separately to determine which structure (or both) contributed to a reduction in friction at the articular cartilage surface. The 15GAH_CS was synthesized to match the number of peptides found in 15WYR_CS. The reduction of friction could have been due to the higher number of peptides conjugated rather than the type of peptide added. Finally, 5 and 10 ratios were made for both WYR_CS and GAH_CS to determine the minimum amount of peptide that was needed to reduce friction at the cartilage surface.

The coefficient of friction (COF) for each treatment was measured with a rheometer using a cartilage-on-glass setup. Bovine osteochondral plugs were harvested, treated, and tested within a week after slaughter. Both static and kinetic friction measurements were analyzed for each sample. A sample treatment of synovial fluid was used as a positive control to mimic the COF of articular cartilage in the synovial joint. The negative control was PBS only, which was used to dissolve each of the other treatments. The results from the static COF were measured using one single data point from the 800 data points collected during the sample rotation on the glass plate. This type of measurement might not be the best representation of static friction with the setup we used, but we did see some significant differences of the static COF between samples. The results show that none of the treatments were able to match the static COF of synovial fluid. However, 15WYR was shown to reduce the static friction more than PBS, CS, and 2GAH. The 2GAH_15WYR_CS also had a lower static COF than PBS and CS. All the other treatments, excluding synovial fluid, were not statistically different from the CS or PBS samples. This indicates that the presence of a high amount of WYR can reduce the static COF more effectively than the other peptidoglycans synthesized, including molecules with a high amount of GAH attached to the CS backbone.

The kinetic COF for both 15WYR_CS and 2GAH_15WYR CS were statistically the same as the SF treatment. None of the other treatments were able to match the kinetic COF of the synovial fluid sample. The kinetic COF for the both the 15WYR_CS and 2GAH_15WYR CS were also much lower than the rest of the treatments that included synthesized peptidoglycans. The kinetic COF of 15GAH_CS was statistically the same as the PBS control which indicates that increasing the amount of GAH bound to the CS backbone did not improve the GAH_CS ability to lubricate the cartilage. The addition of WYR to the peptidoglycan is crucial for lowering the kinetic

COF. If GAH is added to the CS backbone, the addition of WYR is needed as well. We did observe that the 10WYR_CS did not significantly reduce the kinetic COF. Based on these results, a higher collagen II binding peptide concentration of approximately 15 mols of WYR per mol CS is required to sufficiently reduce the kinetic COF. One explanation as to why such a relatively high number of WYR is needed involves how well the molecule is bound to the cartilage surface. Increasing the amount of WYR most likely increases how well the peptidoglycan can attach to the osteochondral plug surface allowing it to stay in place even during friction testing. Most likely, many of the treatments are rubbed off due to the high shear forces present during rheology testing of the osteochondral plugs. The strong attachment of the peptidoglycan creates a protective boundary layer at the articular cartilage surface. This mechanism is similar to how lubricin, a boundary lubricant, is able to reduce friction and protect the cartilage surface from degradation.

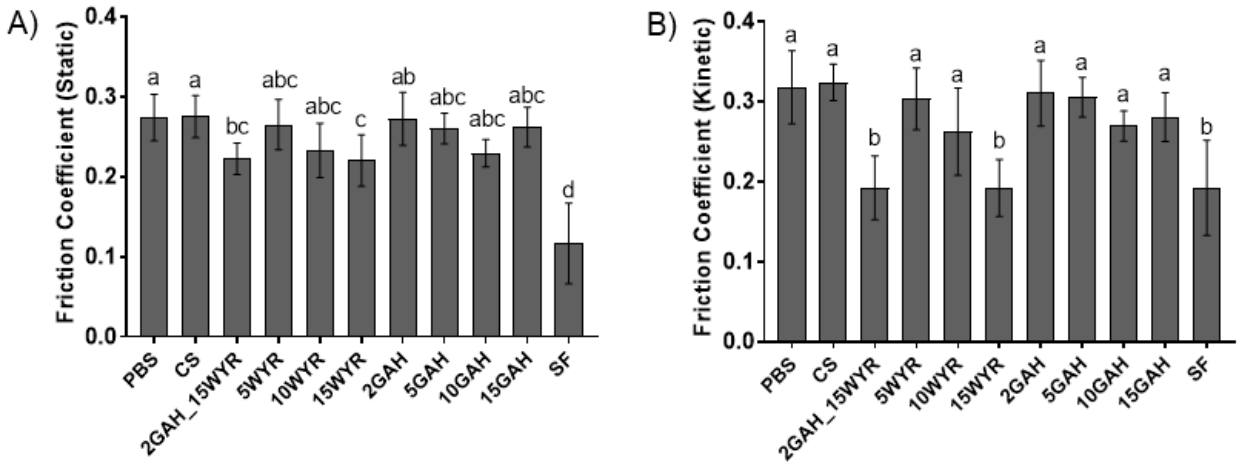

Figure 4.2. COF values measured at the cartilage surface comparing PBS, 2GAH_15WYR_CS, 5,10,15WYR, 2,5,10,15GAH and synovial fluid (SF) treatments. Statistically significant differences are represented by different letters appearing over each column. n = 8 for each treatment. A) Static COF. B) Kinetic COF.

In addition to mechanical testing, we wanted to analyze how well each of the treatments bound to the cartilage surface by using confocal imaging. The osteochondral samples were collected post-rheology, rinsed, fixed with 4% PFA and embedded in optimal cutting temperature (OCT) compound to be sectioned for imaging. Since the peptidoglycans contained a biotinylated peptide, we were able to visualize them through a microscope after staining with a streptavidin

antibody. From the confocal imaging we observed that some peptidoglycans were able to bind to the cartilage surface better than others (Figure 4.3). As expected, the images showed nothing present at the cartilage surface for the CS and PBS treated cartilage plugs. We saw more peptidoglycan bound at the plug surface for the WYR_CS formulations when compared to the GAH_CS formulations. This further supports the claim that WYR is responsible for adsorbing the peptidoglycans to the cartilage surface. The 10WYR_CS samples showed a significant amount of molecule present but 10WYR_CS was still unable to significantly reduce the friction at the articular cartilage surface. There seems to be a few slight discontinuations of the 10WYR_CS molecule attached to the cartilage surface, especially when compared to 15WYR_CS. This suggests that a complete protective boundary layer is more effective at lowering the COF as indicated in the friction testing. We also observed that there is quite a bit of 10GAH_CS and 15GAH_CS that remained on the surface despite GAH not being designed for cartilage binding. However, this is not surprising as HA is not only found in the synovial fluid but in the cartilage structure as well. Although collagen II is the most prominent component of articular cartilage, there was enough HA present within the tissue to allow GAH_CS to bind as well. Ultimately, only the 15WYR_CS and 2GAH_15WYR_CS were seen to create a full protective boundary layer at the articular cartilage surface.

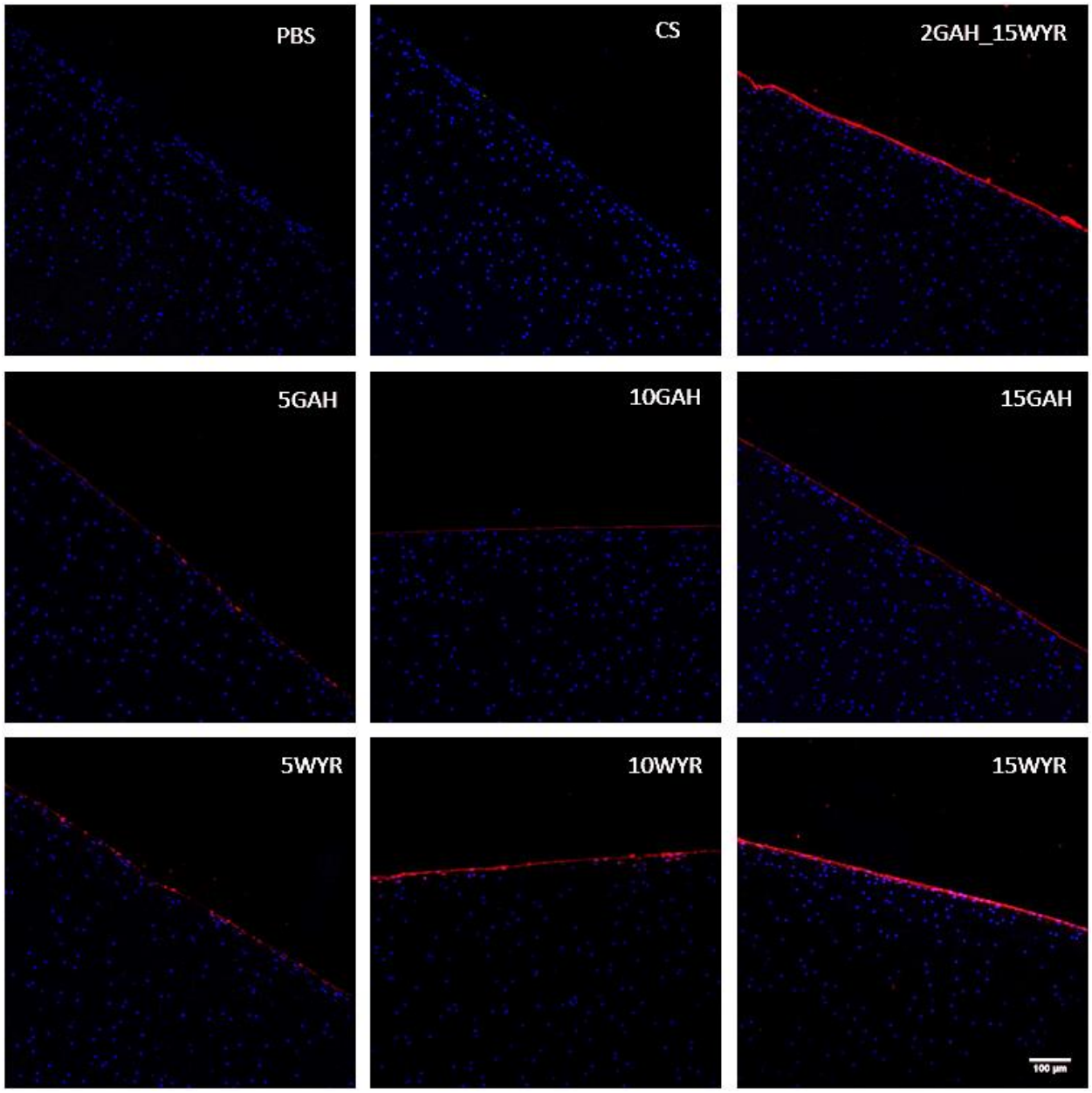

Figure 4.3. Confocal imaging of post-rheology cartilage samples. Each image shows the articular cartilage surface following sample treatment, testing, rinsing, and prepping for imaging. The chondrocytes were stained with DAPI (blue). The biotinylated peptidoglycan treatments at the cartilage surface were stained with Alexa Fluor® Streptavidin 633 (red).

In conclusion, we have successfully developed a lubricin mimic that significantly reduces the coefficient of friction at the articular cartilage surface. The addition of collagen II binding peptides to the synthesized peptidoglycan was shown to be crucial to the attachment of the molecule to the articular cartilage surface. Like lubricin, the key player in cartilage lubrication, our synthetic molecule is able to successfully create a protective boundary layer at the articular cartilage surface. Unlike previous designs, our biomimetic lubricin synthesized with only collagen II binding peptides does not require the addition of HA to successfully reduce friction at the articular cartilage surface. The benefits to this molecule formulation are not only ease of synthesis but also an increased potential for the molecule to reduce friction in the presence of degradation enzymes such as hyaluronidase that target HA, especially in an osteoarthritic condition. Furthermore, concentrations of HA are significantly reduced in joints affected by osteoarthritis. This would not hinder the effectiveness of our biomimetic lubricin since it does not require HA to successfully reduce friction at the articular cartilage surface by creating a protective boundary layer. With these considerations, our biomimetic lubricin shows potential as a long-term supplemental lubrication treatment for OA patients.

CHAPTER 5. MATERIALS AND METHODS

5.1 Peptide Synthesis

The collagen type II binding peptide WYRGRLGSG (WYR) and HA binding peptide GAHWQFNALTVRGGGSG (GAH) were synthesized using a protocol described in Coin *et al.* using standard Fmoc solid phase peptide synthesis[112]. Both the GAH and WYR were synthesized on a Cl-(Trt)-Cl resin each with a GSG spacer and an added hydrazide group. Once the last amino acid was added, the peptides were cleaved off the resin using cocktail composed of 88% trifluoroacetic acid, 5% phenol, 5% dH_2O, and 2% triisopropylsilane. Biotinylated peptides had an extra biotin group added at the C-terminus before peptide cleavage. To purify the peptides, a Vydac C18 column (Grace Davison Discovery Sciences, Deerfield, IL) was used on an ÄKTA Explorer 100 FPLC (GE Healthcare, Piscataway, NJ) with a 0.1% TFA and acetonitrile gradient. Matrix-assisted laser desorption ionization time-of-flight (MALDI TOF) mass spectrometry on a Voyager DE PRO analyzer (Applied Biosystems, Foster City, CA) was used to verify the molecular weight of the peptides.

5.2 Synthesis of Peptidoglycans

Collagen type II and HA binding peptidoglycans were synthesized using EDC (1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride) chemistry to conjugate peptide hydrazides to a chondroitin sulfate (CS) backbone with an amide bond. Using an 8M urea solution and 0.05 mM EDC, the carboxyl groups on the CS backbone were activated at a pH of 4.5 for no more than 5 minutes at room temperature. The CS backbone was then functionalized with either collagen type II binding (WYR) or HA binding (GAH) peptides to yield GAH_CS or WYR_CS. The total EDC reaction concentration for the addition of WYR to CS was 2 mg/mL. GAH was reacted with CS at a 4-5 mg/mL concentration. Biotinylated peptidoglycans were synthesized by reacting one mole of WYR-biotin or GAH-biotin per mole of CS with the addition of the non-tagged peptides. To stop the reaction, the pH was adjusted to 8 and allowed to sit at room temperature for 30 minutes before purification. The molecules were purified using size exclusion chromatography on an ÄKTA Purifier FPLC (GE Healthcare, Piscataway, NJ) with Bio-Scale Mini Bio-Gel columns packed with polyacrylamide beads (Bio-Rad Laboratories, Hercules, CA) and then lyophilized.

For molecules with both types of peptides attached (WYR and GAH), only one type of peptide was reacted at a time. After the first peptide addition, the entire process was repeated a second time once the first reaction had been purified and lyophilized. A Nanodrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA) was used to measure the absorbance @ 280 nm of the synthesized molecules. This measurement was used to quantify the final concentration of peptide attached to the CS backbone. Chondroitin sulfate was also synthesized with a hydrazide dye (Alexa Fluor® 488 hydrazide) attached for later confocal analysis. Following activation of the CS with EDC, hydrazide dye was added at a 2:1 molar ratio of dye to CS.

5.3 Tissue Harvest and Treatment

Cylindrical osteochondral explants were harvested from 18-36 month old bovine knee joints obtained 24 hours after slaughter (Lampire Biological Laboratories, Inc., Pipersville, PA). The explants were taken from the load bearing regions of the femoral condyle using a 6-mm-diameter coring reamer (Arthrex, Inc., Naples FL) and trimmed to a thickness of approximately 3-4 mm. In order to remove residual synovial fluid, samples were shaken vigorously overnight at 4°C in PBS containing a SigmaFAST protease inhibitor cocktail (PIC) tablet (Sigma-Aldrich, St. Louis, MO) and 0.5% penicillin/streptomycin. Cartilage samples were then randomly divided and completely submerged into a variety of treatment groups and stored at 4°C until mechanical testing took place. Samples were treated for at least 2 hours before mechanical testing began. Samples were not tested if stored for longer than a week in solution 4°C. All of the treatments were dissolved in PBS/PIC solution except for the bovine synovial fluid (Animal Technologies, Tyler, TX) which was aliquoted and frozen at -80°C. The chondroitin sulfate and peptidoglycan treatments were all dissolved at the same molar concentration of ~8.4, µmol which corresponded to ranges of 0.3 to 0.5 mg/ml[76]. This is consistent with the concentrations of lubricin found within the synovial fluid. High molecular weight HA (hyaluronic acid sodium salt from rooster comb, ~1-4MDa) (Sigma-Aldrich) and low molecular weight HA (hyaluronic acid sodium salt from *streptococcus equi*, ~100kDa*)* (Sigma-Aldrich) solutions were made at concentrations of 3 mg/mL, which is also similar to HA concentrations found in the synovial fluid[76]. For samples that had the treatment group of both a peptidoglycan and HA, the cartilage plugs were first stored in the peptidoglycan treatment until ~2 hours before mechanical testing when it was rinsed and then submerged in the HA treatment and stored at 4°C right up until the time of friction testing.

5.4 Measurement of Coefficient of Friction

The procedure to measure the coefficient of friction (COF) at the articular cartilage surface was based off a protocol developed by Schmidt *et al*[55]. The treated osteochondral samples (~6mm) were tested used a cartilage-on-glass setup. The underlying bone for each sample was mounted onto a piece of sanding disc with super glue to allow for a better bond between to two rough surfaces. The adhesive on the back of the sanding pieces allowed the samples to be mounted onto the center of a 20-mm flat rheometer geometry head (AR G2, TA Instruments) using a customized alignment tool. The cylindrical tool was shaped to fit around the 20 mm rheometer head by cutting a 20 mm diameter circle into the tool. A ~6mm circle was cut exactly in the center of the 20 mm embedded section where the cleaned glass microscope slide was taped to the bottom plate of the rheometer. The geometry head was lowered enough for the cartilage plug to barely touch the glass slide. The different treatment solutions were pipetted on the glass to keep the plug from drying out. The rheometer software was set to compress the samples at a rate of 0.002 mm/second until a 45 N normal force was measured to make sure that the boundary layer of the cartilage plug was in direct contact with the glass surface. The plug then was held at the corresponding strain for 30 minutes in order for the sample to equilibrate. This reduces the effects of fluid pressure from interfering with COF measurements. The time chosen for sample equilibration was based off a preliminary run that allowed the samples to equilibrate for 1, 5, 15, 30, 45, and 60 minutes (Fig. 5.1).

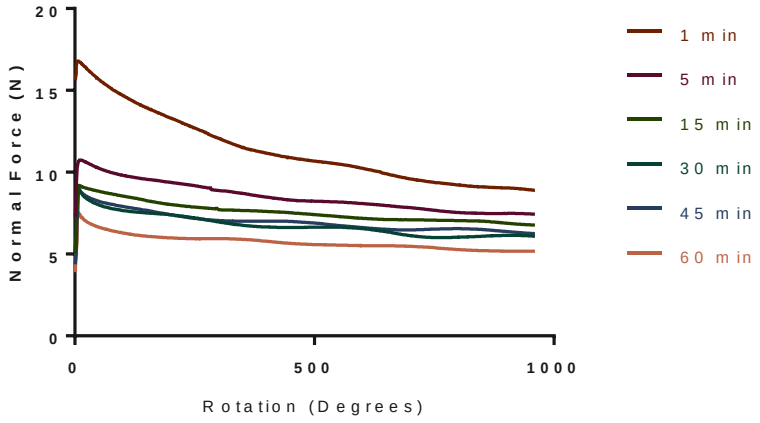

Figure 5.1. Equilibration times for friction testing.

The time of 30 minutes was determined because it seemed to be the least amount of time needed that resulted in consistent normal forces when rotational testing was performed. A lower amount of time per run meant more samples could be tested within a set amount of time, which allowed for more

treatment groups and reps for each batch of osteochondral plugs. Next, the samples were rotated with an angular velocity of 0.08726 rad/sec for 2 minutes. Torque and normal force were measured with the software and used to calculate the COF with the following equation:

$$\mu = \frac{T}{R_{eff}N}$$

Static COF was calculated by taking the maximum calculated coefficient of friction during the first 10 degrees (~2 sec). The kinetic COF was calculated by averaging the COF calculated from the second rotation. For each round of friction testing, at least three samples (n=3) were tested per treatment with one rep of each different treatment done at a time in the same order. All of the osteochondral plugs were tested within 5 days following harvest.

5.5 Immunostaining of Peptidoglycans at Cartilage Surface

After mechanical testing, the cartilage layer (~1 mm) was sliced off the osteochondral plugs and rinsed three times with PBS. The cartilage slices were fixed with 4% paraformaldehyde overnight at 4°C and then rinsed three times with PBS. A midsagittal cut was made through the cartilage samples to and the two halves were embedded in O.C.T compound (Tissue Tek) and frozen at -80°C. The frozen samples were sectioned at 7μm thickness, air dried and stored at -20°C until the immunostaining procedure was performed. For detection of biotin-labeled molecules at the cartilage surface, the sections were rinsed with PBS to remove residual O.C.T. compound and blocked with 1% BSA (lyophilized powder, ≥96% (agarose gel electrophoresis), Sigma-Aldrich) for 30 minutes at room temperature. The sections were immunostained for 15 minutes at room temperature with Alexa Fluor® Streptavidin 633 (Thermo Scientific) diluted 1:200 in 1% BSA and counterstained with DAPI diluted 1:500 in the same solution. The sections were rinsed and mounted with ProLong Gold antifade mounting media (Thermo Scientific) before they were imaged under a confocal microscope (Leica Microsystems). Chondroitin sulfate samples with no biotinylated peptides attached were analyzed on the cartilage surface using an Alexa Fluor® 488 hydrazide dye (Thermo Scientific) that was attached to the CS backbone through EDC chemistry.

CHAPTER 6. CONCLUSIONS AND FUTURE WORK

Current treatments for OA are limited and fail to prevent the progression of the disease. In recent years, OA research has shifted focus from general symptom relief to a more targeted approach centered around preventing and even reversing the symptoms of the disease, especially cartilage deterioration. The loss of lubrication in the synovial joints is a primary reason for the decrement of articular cartilage[13]. Therefore, it is believed that further damage to articular cartilage can be avoided if proper lubrication is restored through lubricant supplement treatments. HA injections and other lubricant supplements are on the market today, but there is minimal clinical evidence to support that these products are effective in treating the long-term effects of OA. Overall, lubricin proves to be more efficient than HA as a lubricant *in vivo* and *in vitro*, but it is susceptible to degradation by enzymes and depletion factors that are over expressed within joints suffering from OA. Therefore, there is a significant need for the development of a synthetic lubricant treatment that can provide the efficiency of native lubricants while also being able to withstand enzymatic degradation. This longevity will improve long term effects and overall success of the treatment. To address this need, our lab has developed a biomimetic lubricin peptidoglycan that has been designed to mimic the lubrication effects of native lubricin and reduce friction at the articular cartilage surface.

Initially, the lubricin mimic was designed with both collagen II and hyaluronic acid (HA) binding peptides attached to a chondroitin sulfate backbone. The addition of collagen II binding peptides was shown to be critical to the attachment of the molecule to the articular cartilage surface. The HA binding peptides were included to facilitate the lubrication properties of the molecule by allowing HA to bind to the lubricin mimic. However, it was shown that the addition of HA to the lubricin mimic treatment was not necessary to lower the COF. The lubricin mimic with collagen II peptides alone, without the addition of HA, was sufficient in reducing the friction, even matching the kinetic COF measured with synovial fluid. The benefits to this molecule formulation are not only ease of synthesis but also an increased potential for the molecule to reduce friction in the presence of degradation enzymes such as hyaluronidase that target HA, especially in an osteoarthritic condition. Furthermore, concentrations of HA are significantly reduced in joints affected by osteoarthritis. This would not hinder the effectiveness of our biomimetic lubricin since it does not require HA to successfully reduce friction at the articular cartilage surface by

creating a protective boundary layer. With these considerations, our biomimetic lubricin shows potential as a long-term supplemental lubrication treatment for OA patients.

Although much progress has been made with the development of our designed biomimetic lubricin, there are many steps that need to be taken in order to produce a successful treatment for osteoarthritis. We had a few problems with synthesizing a peptidoglycan that contained higher peptide amounts. The addition of both WYR and GAH to the same CS backbone often resulted in low peptide binding efficiency. Our conclusion was that EDC conjugates became permanently attached to the CS carboxyl site due to an O to N-acylurea alteration of the intermediate compound. The inactive N-acylurea formation blocked the peptides from binding to the CS backbone. We did not pursue this any further because we found that our synthesized peptidoglycan required only WYR to effectively reduce the COF at the cartilage surface. However, we have yet to test a peptidoglycan with a high amount of both WYR and GAH. There is a possibility that this formulation could reduce the friction better than the WYR_CS peptidoglycan. In addition, improving the peptidoglycan synthesis process would allow us to saturate the CS backbone with higher amounts of WYR peptide. We concluded that increasing the molar ratio of WYR:CS from 5 to 15 corresponded to an increase in lubrication ability. Molar ratios of WYR:CS higher than 15 might further improve the ability of the lubricin mimic to reduce friction at the cartilage surface. To increase peptide conjugation, one solution would be to lower the amount of EDC to a 1:1 molar ratio of peptide added to reduce the amount of unwanted interactions caused by EDC. Another solution could be to add both peptides at the same time to the coupling reaction and use a different quantification method to analyze the peptides attached.

We have stated the benefits to synthesizing a lubricin mimic that does not require the presence of HA to reduce the friction at the articular cartilage surface. However, there is a possibility that the addition of HA to a molecule with high amounts of both WYR and GAH could result in better lubrication characteristics than the peptidoglycan without the HA treatments. Future studies that focus on the effectiveness of HA addition to the lubricin mimic could include a binding analysis of HA at the cartilage surface as well as additional friction testing. Another important point to address is we only used two different types of HA in our testing. It is possible that the measured COF values for the cartilage treated with our lubricin mimic could be further reduced by using crosslinked HA and market brands such as Synvisc©.

Even though our biomimetic lubricin was designed to withstand enzymatic degradation, no tests have been done to verify this. One way to analyze the ability for the synthesized lubricin mimic to withstand degradation would be by treating the molecule with enzymes including aggrecanase, hyaluronidase, and MMPs that are common markers for OA. Following enzyme treatment, the CS-based molecules can be analyzed using gel electrophoresis comparing the untreated samples to samples that were treated with degradation enzymes.

One last important step to elucidate the potential for our synthesized lubricin mimic as a treatment of OA is to determine the residency time of the molecule *in vivo*. Current IA-injection treatments display low residency times and require multiple treatments in short spans of time. The elevated presence of degradation enzymes found in joints affected by OA contributes to the lack of time current treatments are able to remain effective. Because our lubricin mimic doesn't have the same structure as lubricin or hyaluronic acid there is potential for the molecule to withstand much of the degradation caused by the OA disease state.

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