ABSTRACT

Meniscal Tissue Bonding and Exploration of Sonochemical Tissue Modification

Drew W. Dean, M.S.

Mentor: Robert R. Kane, Ph.D.

The goal of the primary project was to explore further means of meniscal repair through application of the immobilization techniques previously developed by our research group. The menisci are susceptible to tears due to age degeneration and mechanical failure under strain of compound forces. Our focus centered primarily on the "bucket-handle" type tears which are a shearing of the meniscus along the fibers. Both reductive and oxidative methods were explored using biotin and Avidin for initial cross-linking; and later more direct means including a bis-maleimide PEG agent with reduction, and a few synthesized dihydrazide agents and some commercially acquired compounds (ex. Hydrazine) with oxidation. These direct methods, especially those with the oxidation displayed greater peak stress.

In addition the group was able to explore potential sonochemical modification in conjunction with the Seattle company Acoustx. Unfortunately there was no evidence to support that isonification had modified the tissue to facilitate immobilization.

Meniscal Tissue Bonding and Exploration of Sonochemical Tissue Modification

by

Drew W. Dean, B.S.

A Thesis

Approved by the Department of Chemistry and Biochemistry

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David E. Pennington, Ph.D., Interim Chairperson

Submitted to the Graduate Faculty of
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of
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Approved by the Thesis Committee

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Robert R. Kane, Ph.D., Chairperson

_____

Kevin G. Pinney, Ph.D.

_____

Carolyn T. Skurla, Ph.D.

_____

Mary L. Trawick, Ph.D.

Accepted by the Graduate School
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J. Larry Lyon, Ph.D., Dean

Page bearing signatures is kept on file in the Graduate School.

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LIST OF ABBREVIATIONS

ACL	anterior cruciate ligament
CL	cross-link
DI	deionized
DMSO	dimethyl sulfoxide
GAG	glycosaminoglycan
HRP	horse radish peroxidase
Hyd	hydrazide
Mal	maleimide
NHS	N-hydroxysuccinimide
PBS	phosphate buffered saline
PEG	polyethylene glycol
RT	room temperature
SMB	succinimidyl α-methylbutanoate
TCEP	tris(2-carboxyethyl) phosphine
TFA	trifluoroacetic acid
UV	ultraviolet

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I am indebted to all of my friends and family for standing by me and offering their support as I worked to finish this thesis. My parents especially have been an amazing help to me and I can't thank them enough for all their love and support.

Finally I would like to thank God for giving me the endurance needed to finish this thesis and the program, and also that my work has finally found its conclusion. Thanks all, and God bless.

CHAPTER ONE

Meniscal Bonding

Section One: Introduction

Although the meniscus was originally considered merely to be the functionless remains of leg muscle, it has come to be recognized as an integral and significant contributor to joint stability and shock absorption *(1)*. The meniscus, as part of the knee joint, continuously undergoes extreme loading forces. In an effort to alleviate some of the stress the meniscus converts axial forces into radial strain; a simple mechanism to reduce the severity by applying the force over a larger area *(2)*. It is therefore of little surprise that the most common injuries occur while there are complex external forces being applied, such as twisting of the knee joint while already under flexion strain. Injury due to acute tearing is the primary focus of most research and medical attention. This is because younger patients are better candidates for repair, as the degeneration of meniscal tissue due to age and normal wear makes for more complex injuries and exceedingly more difficult repairs *(3)*.

The menisci are described as being wedge-shaped semi-lunar or crescent-like discs that rest as a pair atop each tibia *(4)*. They are located in the knee joint between the lateral and medial condyle surfaces of the femur and tibia (Figure 1). Within the knee the menisci are attached to the transverse ligaments, to the joint capsule via the coronary ligaments, to the medial collateral ligament (medial), and to the menisco-femoral ligament (lateral) *(4)*. The menisci are connected to each other by the transverse ligament

(Figure 2) and connected to the medial condyle of the femur by the Ligament of Wrisberg that is a branch of the lateral meniscus.

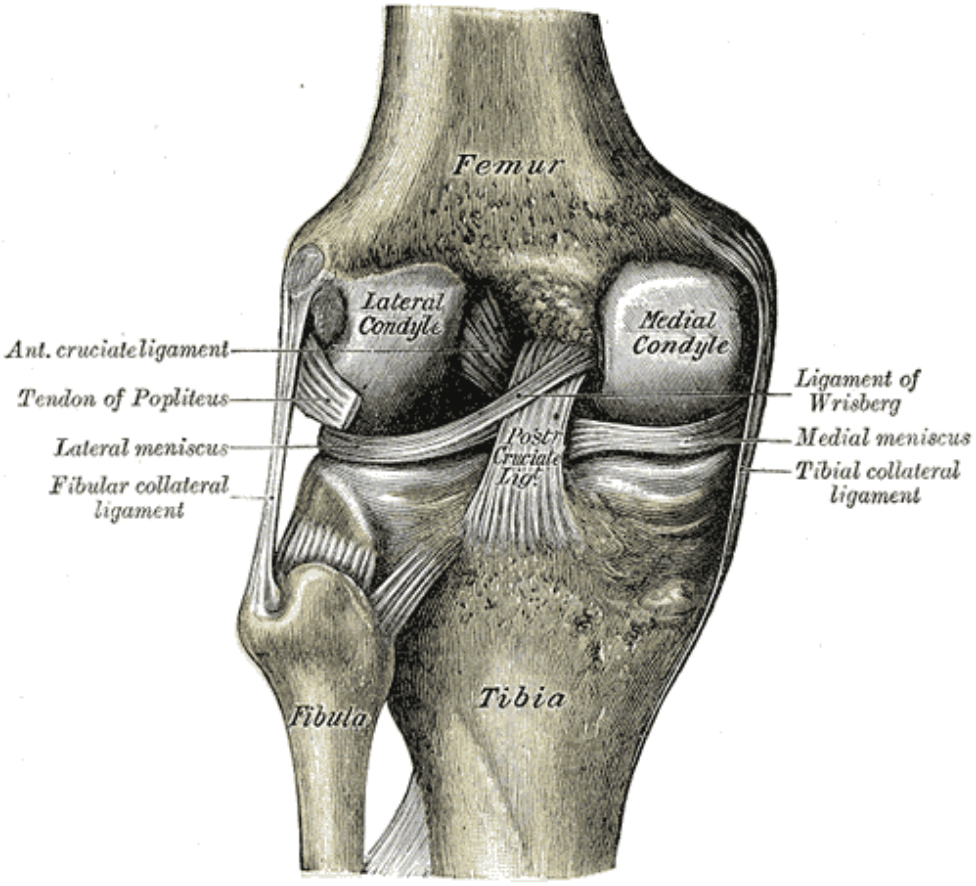


Figure 1. Posterior View of Human Left Knee-joint *(5)*

In addition to the minor connections made by the menisci, the femur and tibia are connected internally by the anterior and posterior cruciate ligaments.

The anterior cruciate ligament (ACL) is closely associated with many meniscal injuries. It is rather common that in cases of ACL rupture, there will be subsequent injury to the meniscus. These meniscal injuries can occur along with the injury of the ACL or while waiting for reconstructive surgery. Additionally, in cases of chronic ACL

injuries or in what are known as ACL deficient knees meniscal tears are virtually guaranteed *(1, 3)*.

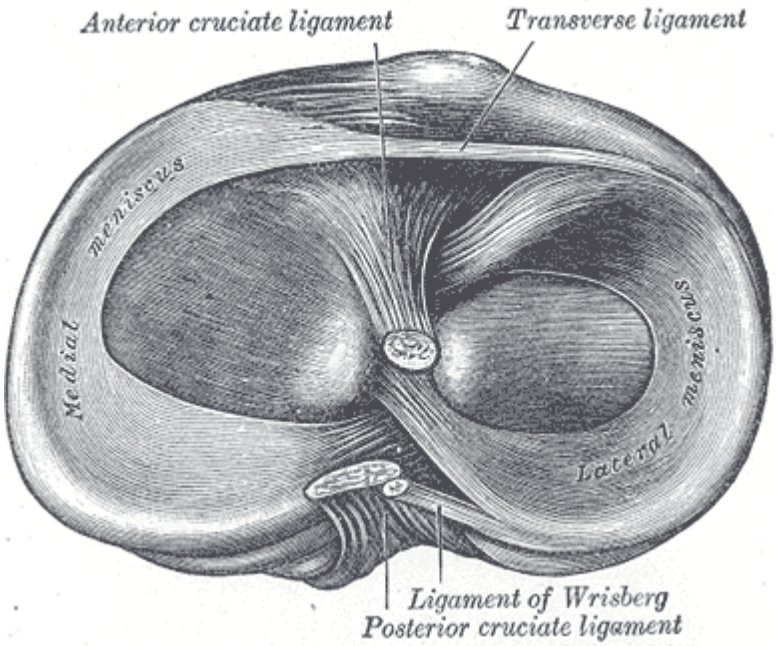


Figure 2. Tibial Plateau of the Human Right Tibia *(5)*

The meniscus itself is fibrocartilaginous and is divided into two distinct regions, the vascular outer region and the inner avascular region. Only the outer region of the meniscus is connected to blood vessels. The inner region acts more like a sponge and pumps nutrients via the synovial fluid with movement of the joint.

The meniscus is biphasic in nature. It has a solid matrix phase and a fluid phase that consists mainly of water and dissolved electrolytes *(6)*. This meniscal matrix, like that of pericardium or skin, is composed primarily of collagen (Figure 3).

Close to 98% of the collagen is type I collagen. The fibrocartilage phenotype also displays other types of collagen (II, III, and V) and glycosaminoglycans (GAG) (Figure

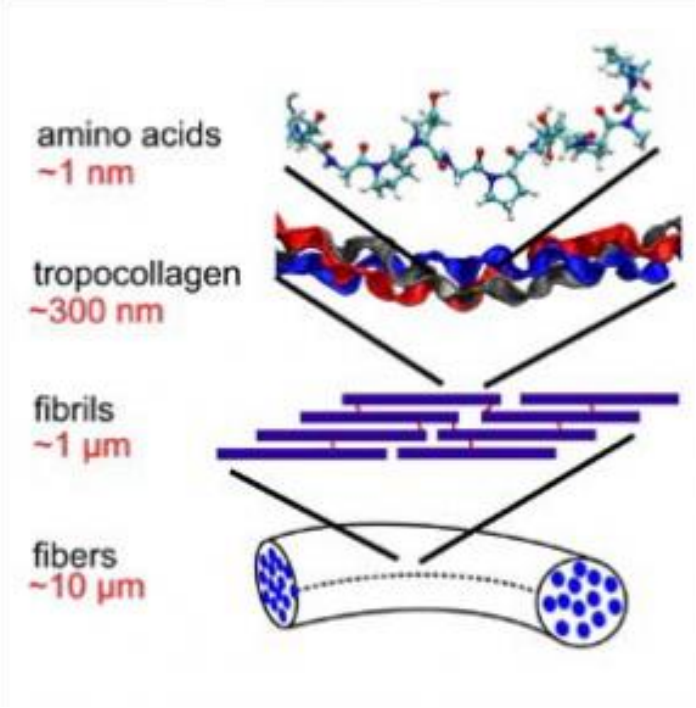


Figure 3. Collagen Structural Hierarchy *(7)*

4) in minor quantities *(8, 9)*. The example GAG in Figure 4 is one of the most prevalent and is found in connective tissues such as tendons and cartilage.

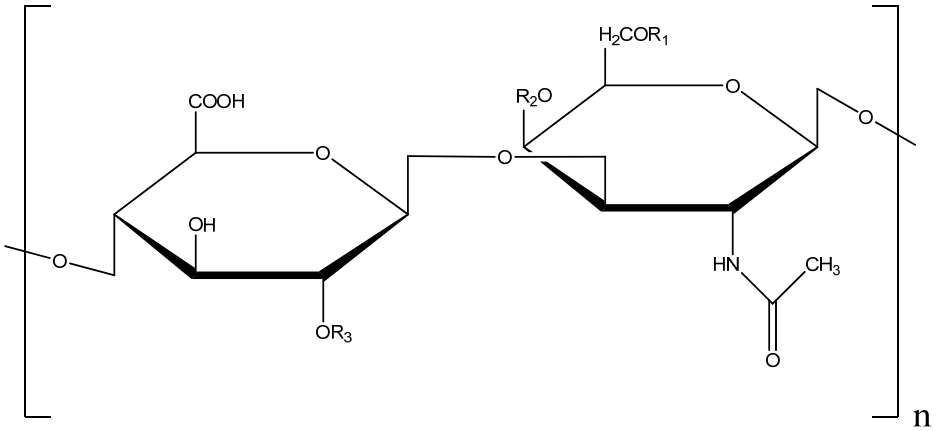


Figure 4. Generic Chondroitin Sulfate (one of the R groups is a sulfate group)

The majority of the matrix fibers are oriented circumferentially around the meniscus with radial tie fibers connecting them (Figure 5). This orientation provides tensile strength to oppose hoop stress. The fiber orientation is more random near the articular surfaces.

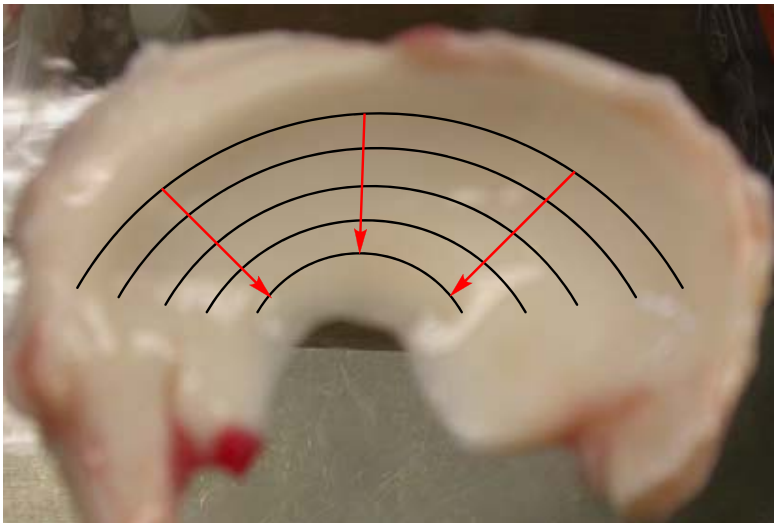


Figure 5. General Diagram of Circumferential (black) and Radial (red) Meniscal Fibers

This random orientation provides the low friction surfaces required for joint articulation. The GAGs have several roles in the meniscal matrix. They provide visco-elastic properties, maintain tissue hydration, support compression resiliency, and provide the smooth articular surfaces in contact with the tibia and femur *(9)*.

Meniscal tears generally occur as a result of complex loading, such as when the knee undergoes a sudden shift or twist while the meniscus is compressed. Typically, tears occur in the avascular region which is unable to repair itself. This is compounded by the fact that many meniscal injuries either cause or are caused by knee instability *(10)*. Without movement, fluid exchange with the avascular region is severely inhibited and natural healing cannot occur.

Originally, the treatment for a damaged meniscus was either to leave the damage untouched or to remove the meniscus itself otherwise known as meniscectomy. Meniscectomy can be performed in total or in part, however with either option the onset of osteoarthritis of the joint is hastened *(11)*. Loss of a meniscus reduces the articular contact surface area by approximately 75% and increases the peak pressure at the area of contact by as much as 235% *(1)*. Many techniques for meniscal repair, from using sutures and staples to reconnect torn segments to using screws or other means to anchor loose segments from being damaged further, have been attempted with varying but typically minimal success and common injury recurrence *(12)*.

Other methods of meniscal repair focus on trying to promote healing of the meniscus. One idea is to introduce blood into the avascular region of the meniscus to induce the natural cytokine release. Some methods use a rasping or arthroscopic "needling" to cause abrasion to the synovium and ultimately bleeding at the site of the repair. Another method utilizes a sample of pre-drawn blood that is later introduced along with the repair to induce a fibrin clot. All of these methods can however cause further problems for the damaged meniscus. The previously undamaged fibers can be damaged or separated in ways that cause them to lose some of their functionality as a result of the abrasion techniques. Furthermore, there is also the possibility that clots formed by the vascular access channels created by the "needling" may form before reaching the repair site *(13, 14, 15)*.

There has been some work done using a fibrin glue adhesive system. This adhesive was injected arthroscopically in the site of the meniscal damage as two separate solutions (simultaneously injected) that then reacted to form the adhesive system. The

first solution contained the fibrogen precursor fibrinogen, aprotinin which slowed down fibrinolysis, and factor XIII which cross-linked fibrin. The second solution contained the coagulation protein thrombin that converted fibrinogen to fibrin and calcium chloride. Researchers found that the rates of injury recurrence were within the same range as cases with meniscal suturing. Results were more positive when the fibrin adhesive mixture contained marrow cells *(16)*.

Other methods of repair focus around tissue engineering that usually involves matrixes and scaffolds that contain collagen and GAGs. Using these basic components of meniscal tissue, researchers have found ways to grow artificial matrixes that display similar characteristics to that of the natural tissue. These scaffolds are typically seeded by extracted meniscal matrix cells that allow for the proliferation of the collagen-GAG matrix. In some cases, cross-linking of the collagen and proteoglycan chains provides increased structural support and compression stiffness. One such cross-linking method is glutaraldehyde treatment which cross-links free amino functional groups. Due to the biological uniformity of extracellular matrix, collagen-GAG scaffolds have been utilized for a variety of tissue types. The biocompatibility of this system has prompted the continued interest of collagen-GAG scaffolds in tissue engineering and reconstructive research *(17, 18, 19)*.

Previous research in our group, which was documented in the doctoral dissertation of Aruna Perera *(20)*, had focused on the modification of tissue surfaces as a means to immobilize proteins or small molecules on those surfaces. Direct alkylation was achieved either by means of N-hydroxysuccinimide (NHS) conjugated compounds or photo-activated biotin compounds *(20)*. Perera's research employed the use of

chemiluminescence to assay potentially modified tissue samples. Chemiluminescence is the process by which light is released from a chemical reaction. Luminol is the substrate utilized in the system from Pierce. When luminol is reacted with peroxide in the presence of horseradish peroxidase (HRP) the result is the creation of luminol's excited state, 3-aminophthalate* (Figure 6). As this product decays back to the ground state it gives off photons that can be observed as light and captured by a sensitive camera. HRP is one of the enzymes that catalyzes the luminol reaction and is easily utilized for assay purposes as it is commercially available from Pierce conjugated to either avidin or biotin (20).

Reductive methods for tissue modification developed in our lab employed tris(2-carboxyethyl) phosphine (TCEP) as a reducing agent and maleimide conjugated compounds for alkylation of the resulting cyteine thiols. The cystine residues are found

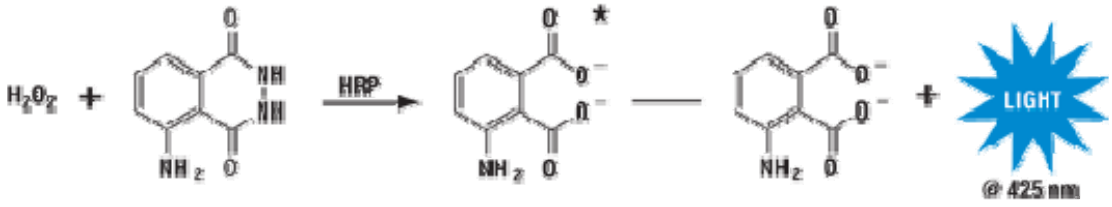


Figure 6. Luminol Chemiluminescence Reaction Using HRP and Peroxide

in natural cross-linkages in proteins. Reduction of the disulfide cross-link yields two uniquely reactive sulfhydryls (Figure 7), allowing for further modification such as immobilization of maleimide conjugated proteins or small molecules *(20)*.

Oxidative methods for tissue modification developed in our lab utilize sodium meta-periodate oxidation followed by reaction with hydrazide reagents. Oxidation using sodium meta-periodate is used to oxidize vicinal diols and especially vicinal amino

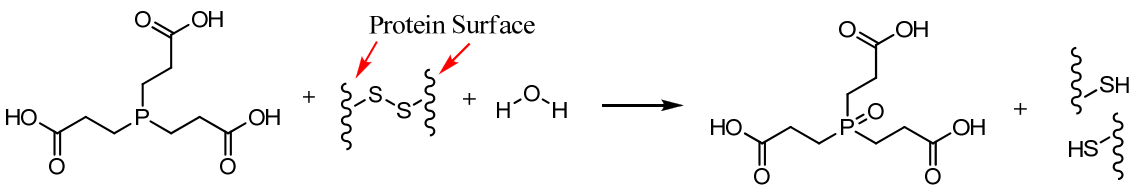


Figure 7. Reduction of Disulfide Bridge with Tris(2-carboxyethyl) phosphine (TCEP)

alcohols into aldehyde functional groups. These functional groups can be found in glycosylated amino acids of glycoproteins and ECM and in hydroxylysine, which is prevalent in collagen. Aldehydes are reactive with hydrazine and hydrazide groups, which can be derivitized for immobilization and labeling purposes as well *(20)*.

Meniscal repair has continued to be of particular interest to our research group, thus leading to further exploration of chemically bonding meniscus. The focus of this research was to apply the methods of our work in tissue modification and immobilization to further explore the potential for meniscal repair, which has been an ongoing effort by members of our group in conjunction with Dr. Carolyn Skurla's research team from the Baylor engineering department *(21)*.

We had also examined the use of naphthalimide compounds for photochemical cross-linking. Photochemical cross-linking involves the formation of cross-links between amino acids, such as tyrosine or tryptophan, when a photochemical dye is present and irradiated. This can be accomplished by the formation of singlet oxygen or some other radical species during photolysis. Though the mechanisms are often not straightforward, it is accepted that some form of photo-oxidation causes cross-linking or dimerization. This is distinguished from thermal welding, which causes tissue adhesion through protein

denaturation. The naphthalimide cross-linking has shown success in cross-linking proteins in solution as well as bonding tissue surfaces *(20, 22)*.

With the knowledge gained through the work with tissue modification, it was the goal of this project to discover a means of creating bonds that could support higher stress; and that would use chemistry that was biocompatible and potentially applicable to meniscal repair. Our previous tissue modification research provided insight into how different chemicals affected tissue surface proteins, and stimulated the adaptation of these approaches to tissue bonding.

Site specific modification of tissue surfaces on a variety of tissue types showed that proteins and small molecules could be immobilized on tissue surfaces. For the purposes of this research project, the focus was narrowed to using oxidative and reductive strategies on meniscus. Though observations during immobilization research suggested a greater effect of oxidation on meniscal surfaces over that of reduction, the cross-linking maleimide agents readily available to react with sulfhydryls provided a rapid entry into experimentation into meniscal bonding.

Section Two: Materials and Methods

Tissue modification techniques reported by Perera *(20)* and tissue bonding and mechanical testing methods reported by Skurla *(21)* formed the starting point for this research. Initially, the avidin/biotin chemistry used for assay purposes by Perera *(20)* was explored for tissue bonding potential. However, the reaction of several homo-bifunctional compounds with either oxidized or reduced sites on tissue proteins proved to be more promising, and were extensively explored.

Reduction was achieved using Tris(2-carboxyethyl) phosphine (TCEP) (compound **1**). Cross-linking for the reduction-avidin/biotin experiments used a biotin conjugated maleimide (Figure 8), hereafter compound **2**, with either an avidin conjugated maleimide (compound **3**) or pure avidin protein.

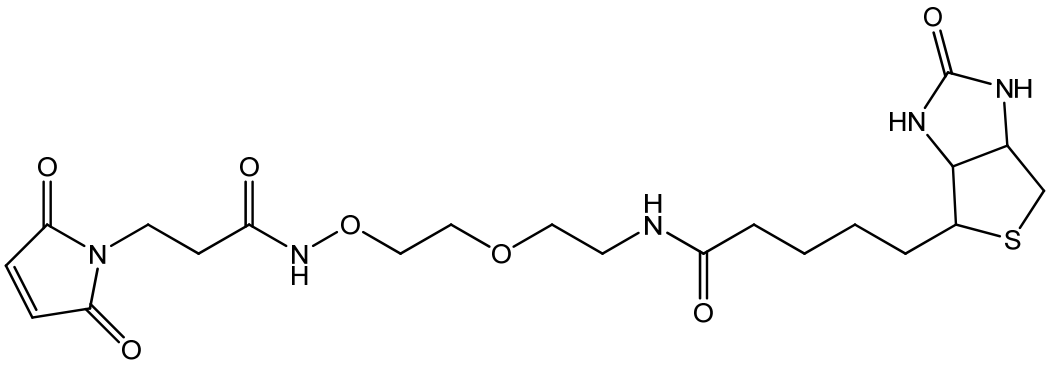


Figure 8. Structrue of Biotin-Maleimide (compound **2**) with PEO Cross-linker

Oxidation was achieved using sodium meta-periodate (compound **4**). Cross-linking for the oxidation-avidin/biotin experiments used biotin conjugated hydrazide (Figure 9), hereafter compound **5,** and pure avidin protein. All compounds for the avidin/biotin cross-linking experiments were commercially obtained from Pierce Biotechnology (Rockford, IL).

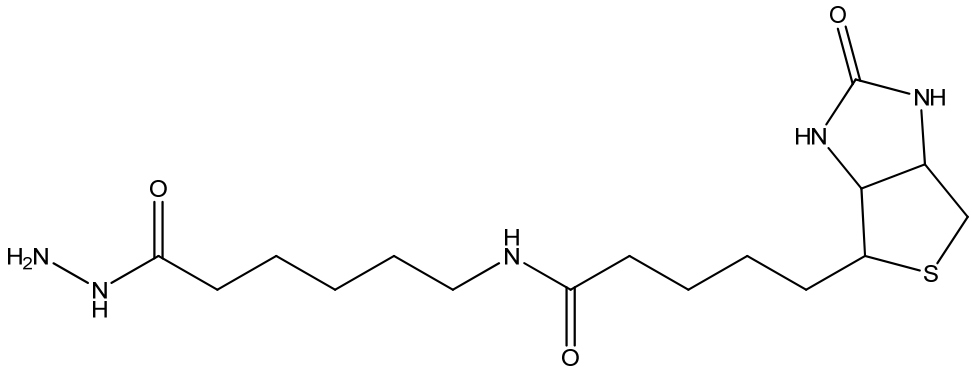


Figure 9. Structure of Biotin-hydrazide (compound **5**) with Linker Chain

Only one homo-bifunctional chain compound was used for direct cross-linking reduction experiments, a bis-maleimide PEGylated compound hereafter known as compound **6** (Figure 10). Compound **6** was obtained commercially from Nektar Therapeutics (San Carlos, CA).

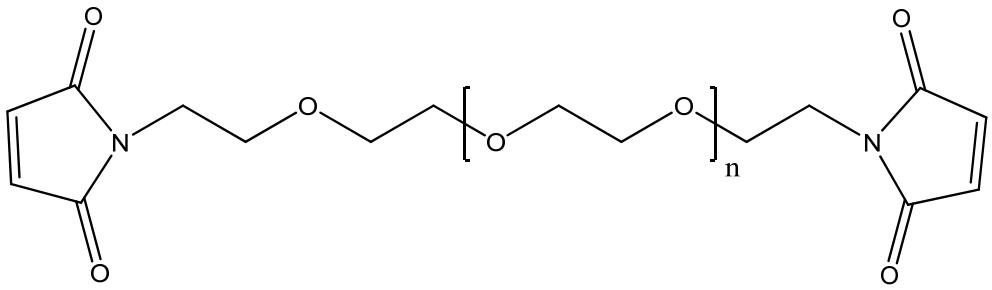
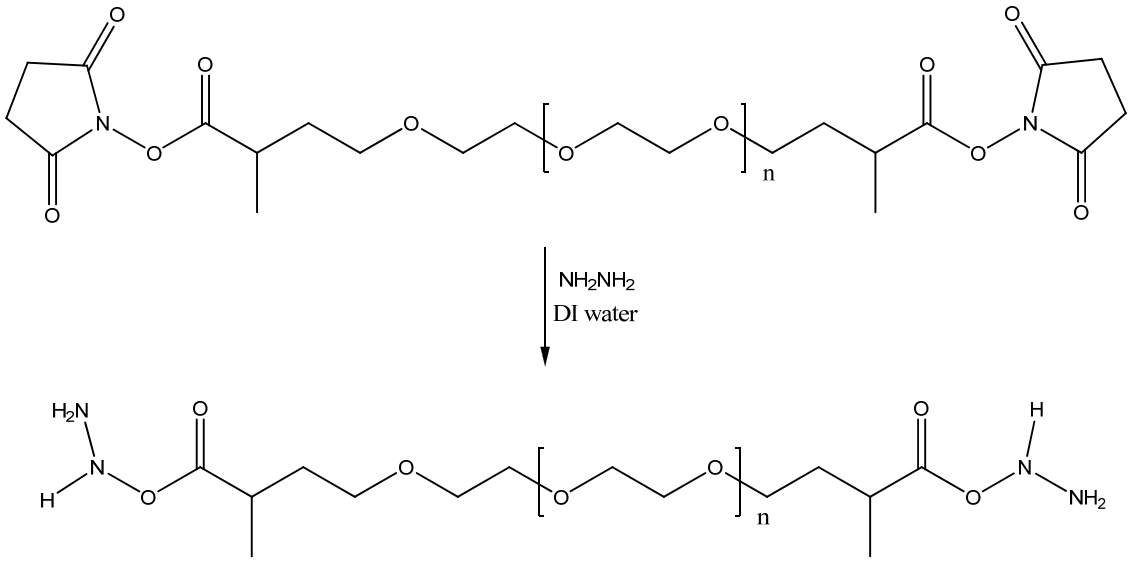


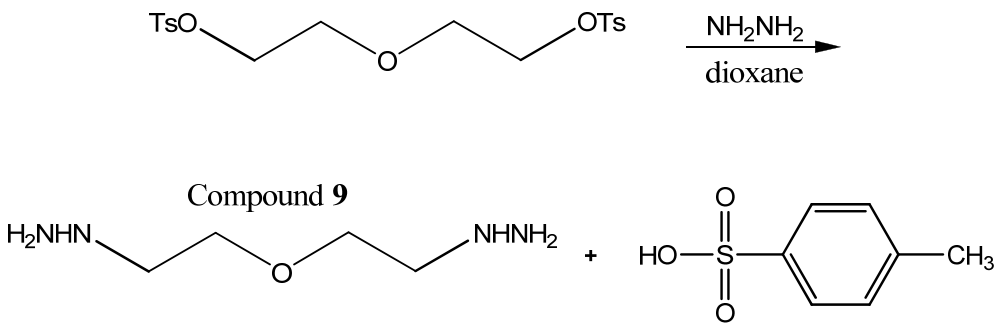
Figure 10. Structure of Mal-PEG-Mal (compound **6**)

The oxidation experiments used both commercially obtained and synthesized compounds for direct cross-linking. Compounds **7** and **8** were dihydrazide PEGylated compounds synthesized by Kang (*23*). The two were fractions separated from a mixture based on the organic solubility of compound **8** (Scheme 1).



Scheme 1. Initial Reaction of Dihydrazide PEG Synthesis (compounds **7** and **8**)

Scheme 2 depicts an initial synthetic approach to the short-chain dihydrazine compound, which was synthesized by Kang (*23*). This synthetic strategy afforded impure product that appeared to contain left over starting material as well as polymerized material *(23)*.



Scheme 2. Initial Reaction of Dihydrazine Synthesis (compound **9**)

Due to this impurity the starting materials, hydrazine obtained commercially from Sigma-Aldrich (St. Louis, MO) and bis-succinimidyl α-methylbutanoate (bis-SMB) PEGylated was obtained commercially from Nektar Therapeutics (San Carlos, CA) (Figure 11), hereafter known as compound **10**, were tested.

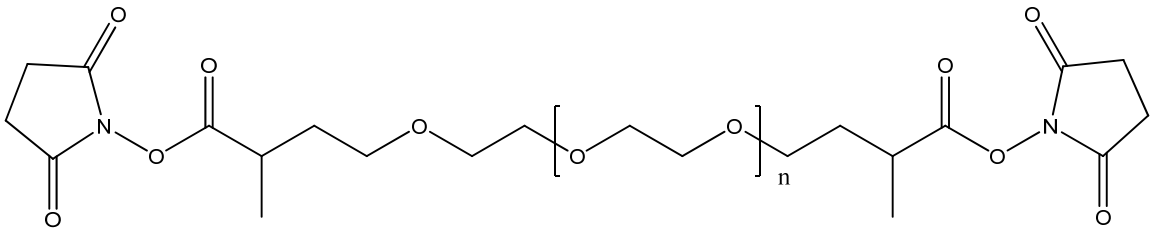


Figure 11. Structure of SMB-PEG-SMB (compound **10**)

Adipic dihydrazide (Figure 12), hereafter known as compound **11**, was obtained commercially from Alfa Aesar (Ward Hill, MA).

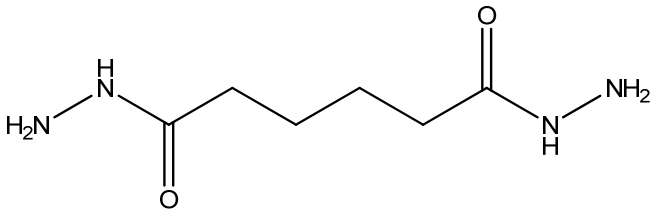
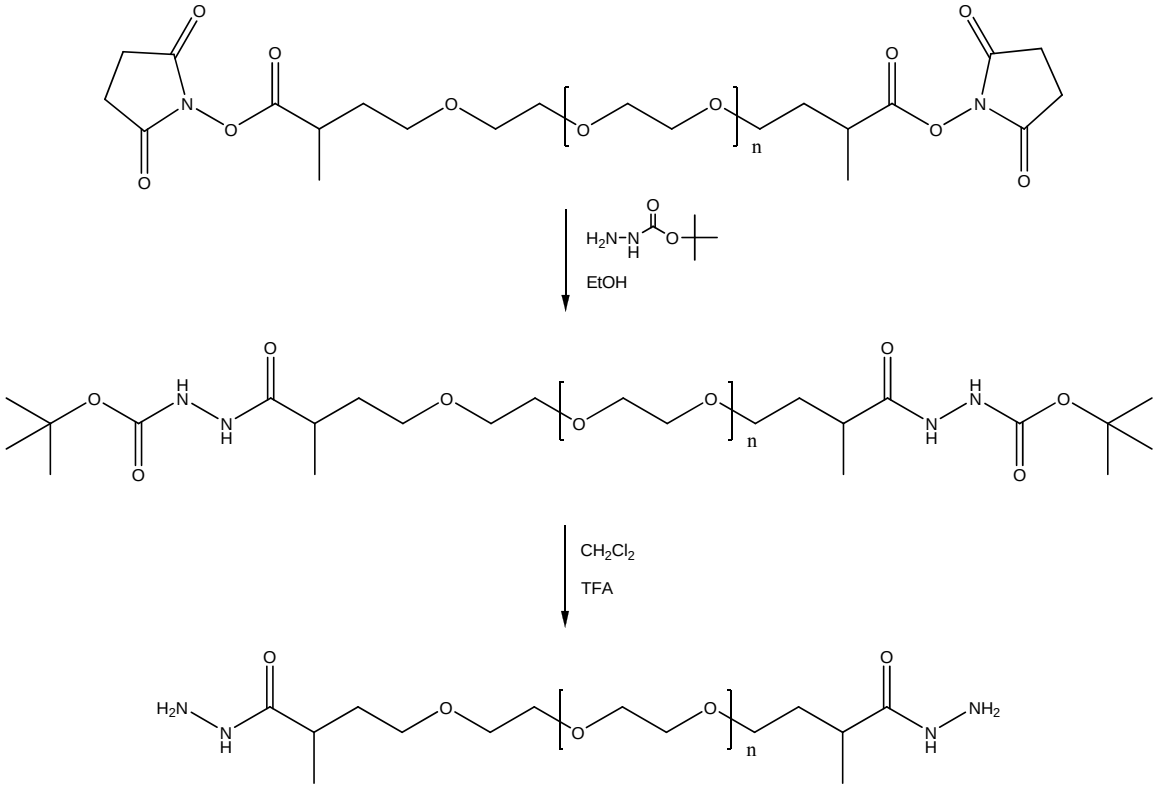


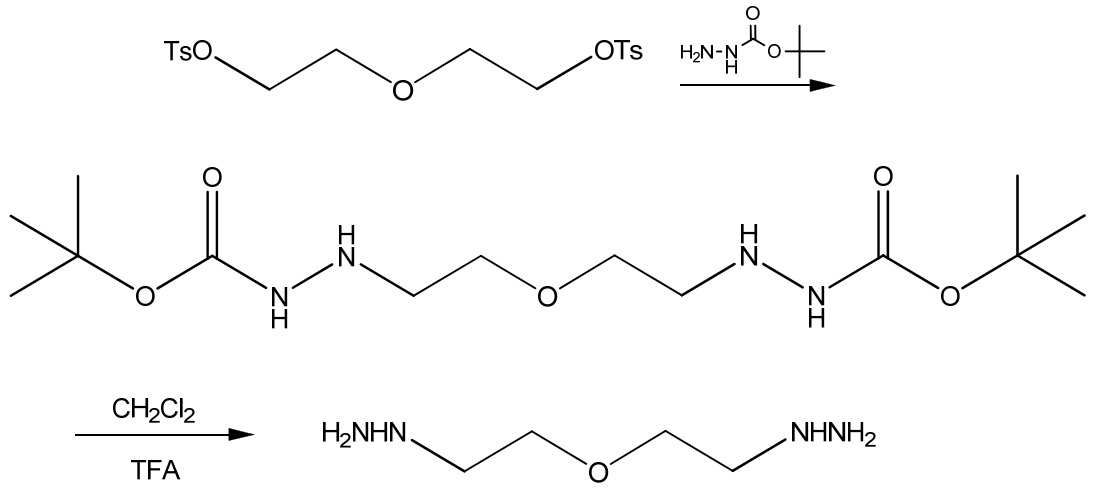
Figure 12. Structure of Adipic Dihydrazide (compound **11**)

Separate syntheses were again performed to yield dihydrazide PEGylated product (compound **12**) and short-chain dihydrazine product (compound **13**). Scheme 3 depicts the synthesis of compound **12** using the protected hydrazine, which was performed by Kang (*23*).



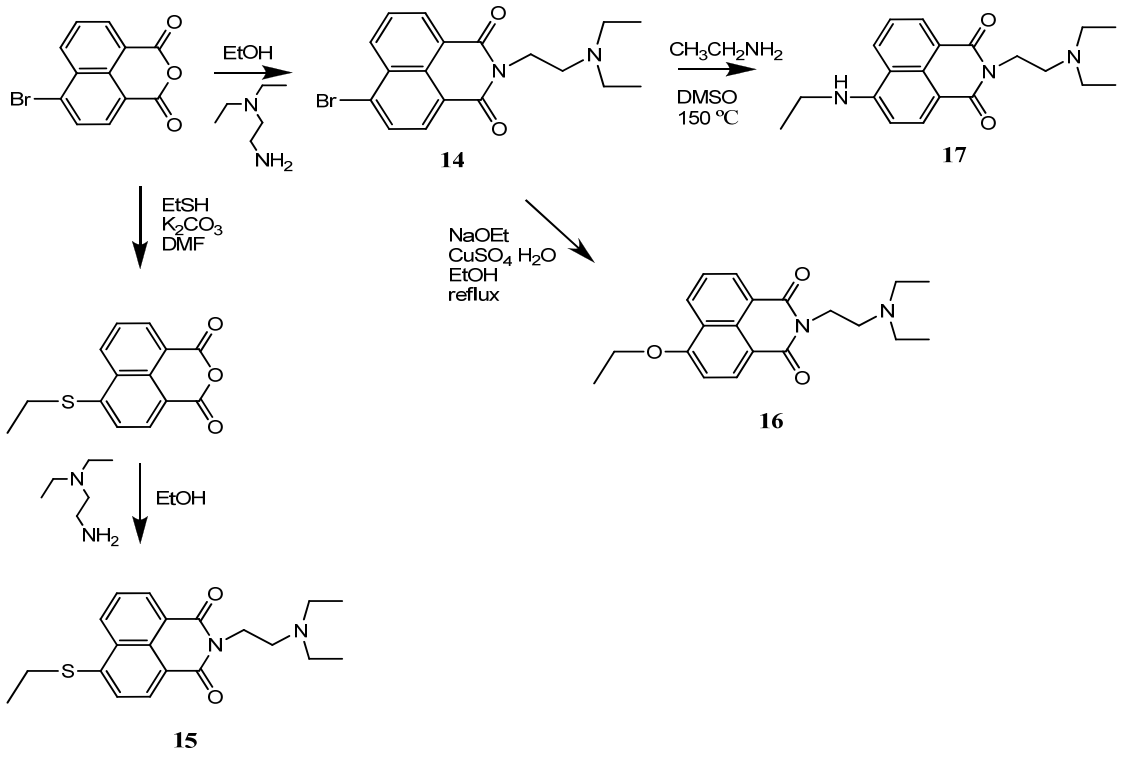
Scheme 3. Improved Reaction of Dihydrazide-PEG Sythesis (compound **12**)

A similar strategy was used for synthesis of the compound **13** depicted in Scheme 4 *(23)*.



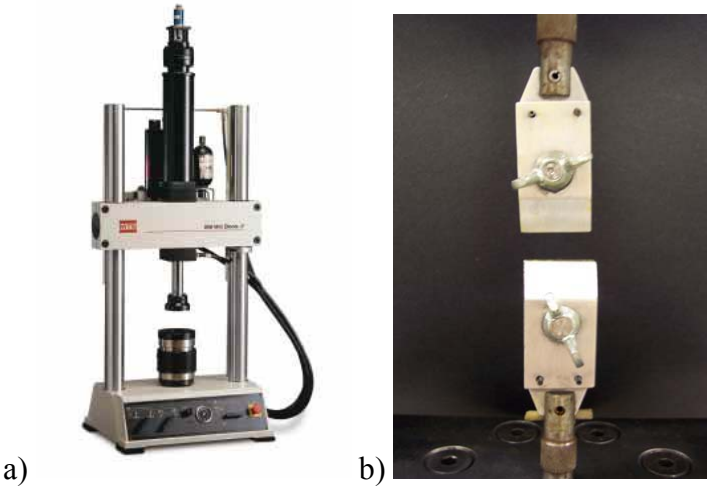
Scheme 4. Improved Reaction of Dihydrazine (compound **13**)

Photo-oxidation experiments used synthetic naphthalimide compounds studied and documented by Woods *(24)*. Scheme 5 depicts the synthetic scheme used to produce



Scheme 5. General Reaction in the Synthesis of Photo-sensitizing Naphthalimides *(24)*

Mechanical testing of bond formation was performed using an MTS 858 Mini Bionix II system (Figure 13a) that was equipped with a set of grips (Figure 13b) designed by Dr. Skurla's research group.



a) b)

Figure 13. MTS 858 Mini Bionix II (a) *(25)* and Meniscus Sample Grips (b)

Any chemicals and other materials not otherwise mentioned were obtained from VWR International (West Chester, PA). DI water is available at the Baylor Sciences Building at Baylor University (Waco, TX).

Bovine menisci (Figure 14) were obtained locally (H&B Packing, Waco, TX). Only fresh menisci or menisci stored at four degrees Celsius for no more that two weeks

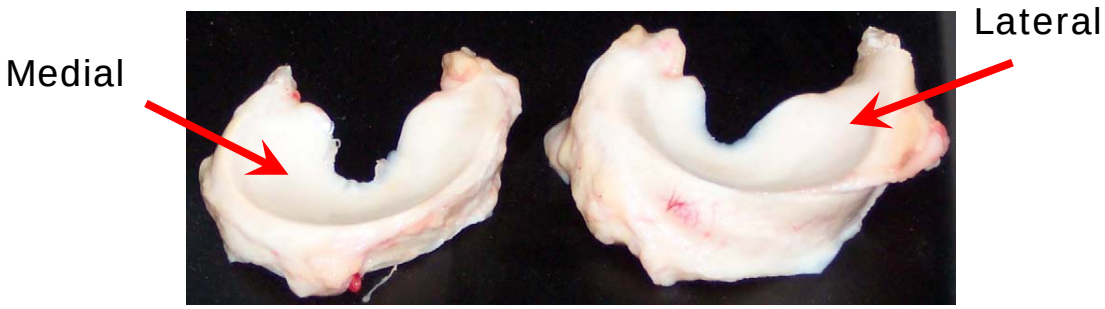


Figure 14. Freshly Extracted Bovine Menisci

were used. Menisci were soaked in PBS buffer for up to two hours before use. For experimental purposes, the meniscal horns and outer rim were removed and the menisci were sectioned into three approximately equal wedges (Figure 15) which were then sliced horizontally using a hand microtome (Figure 16). Slices were kept to a thickness of 0.25

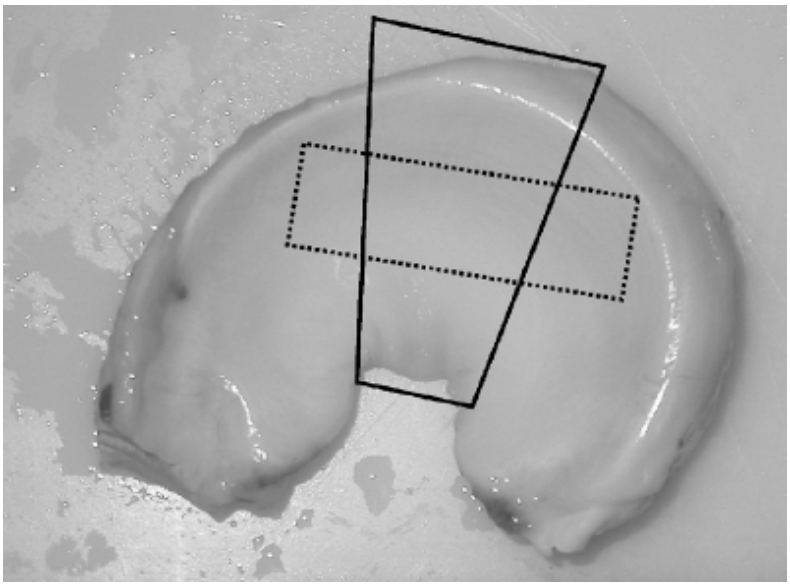


Figure 15. Approximated Slicing Diagram (solid line - wedge, dotted - slice) *(21)*

to 0.3 mm. Slices from each meniscus were kept separate and either used immediately or placed between buffer soaked strips of gauze and stored in Ziploc bags in a refrigerator for up to two weeks. For use, the slices were cut into rectangular sample pieces with dimensions of no less than 1cm by 2cm.

Bonding was done on a lap-joint model reported by Skurla (Figure 17). Repeated measures analysis was employed to ensure continuity, though only gross trends can be observed due to the inherent variability of the tissue. Averages and standard deviations were calculated for experiments with a high number of data points to allow for comparison of observed trends.

Figure 16. Hand Microtome System

Bovine pericardium was obtained from Animal Technologies Inc. in Tyler, Texas. Connective tissue and fat were carefully removed from the pericardium using a microtome blade. Pericardium was soaked in PBS buffer for up to two hours before use. Pericardium was either used fresh or stored at minus 20 degrees Celsius for future use.

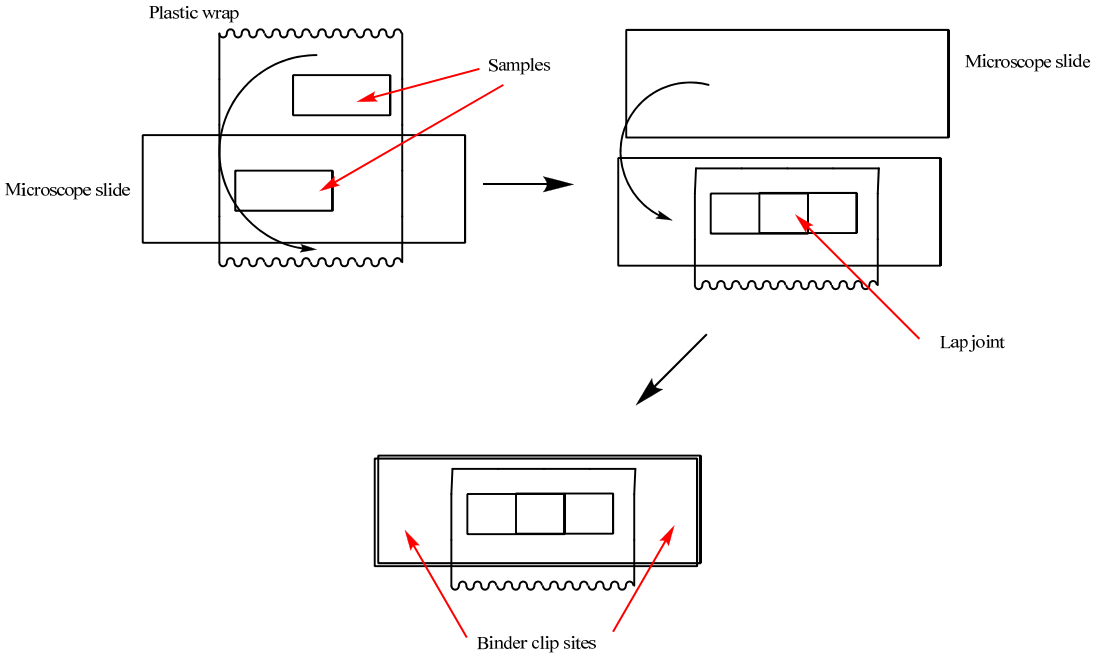


Figure 17. Lap Joint Binding Diagram

For experimental use, samples of pericardium were used as reported by Perera *(20)*. An approximately 10 by 15 cm sheet of pericardium is clamed between a glass plate and an inverted pipette tip holder. This apparatus creates open wells to provide both photo-chemicals and light irradiation to the tissue's surface.

Photo-chemical irradiation was performed using an EFOS Novacure mercury lamp spot cure system with a fiber optic light guide. The emission range was 325-500 nm to encompass the absorbance of compounds **14-17**. The intensity was periodically measured and kept at approximately 2.7 W/cm².

Pierce Biotechnologies SuperSignal West Pico Chemiluminescent Substrate system was used as the assay to explore the 2nd set of photo-oxidation experiments.

Section 3: Experimental Procedures

Experiment 1: Reduction of Bovine Meniscus and Avidin/Biotin Cross-linking (Figure 18)

Twenty two meniscal slices (prepared as described in the materials and methods) were reduced by incubation in an 87 mM solution of TCEP (compound **1**) in PBS. Ten reduced samples and one unreduced control were alkylated (for ~15 hours at 4 ºC) using a 2.5 mM solution of biotin-maleimide (compound **2**) in PBS. A second set of ten reduced samples plus an unreduced control sample were alkylated (for ~15 hours at 4 ºC) with a 0.2 mg/mL (concentration) solution of avidin-maleimide (compound **3**) in PBS. The remaining reduced samples (2) were simply incubated in PBS for ~15 hours at 4 ºC. After alkylation all samples were thoroughly washed with PBS (3 x ~7 mL, 5 min each wash). Samples were assembled for adhesive testing using the standard lap-joint assembly (materials and methods). Experimental sample pairs were comprised of one

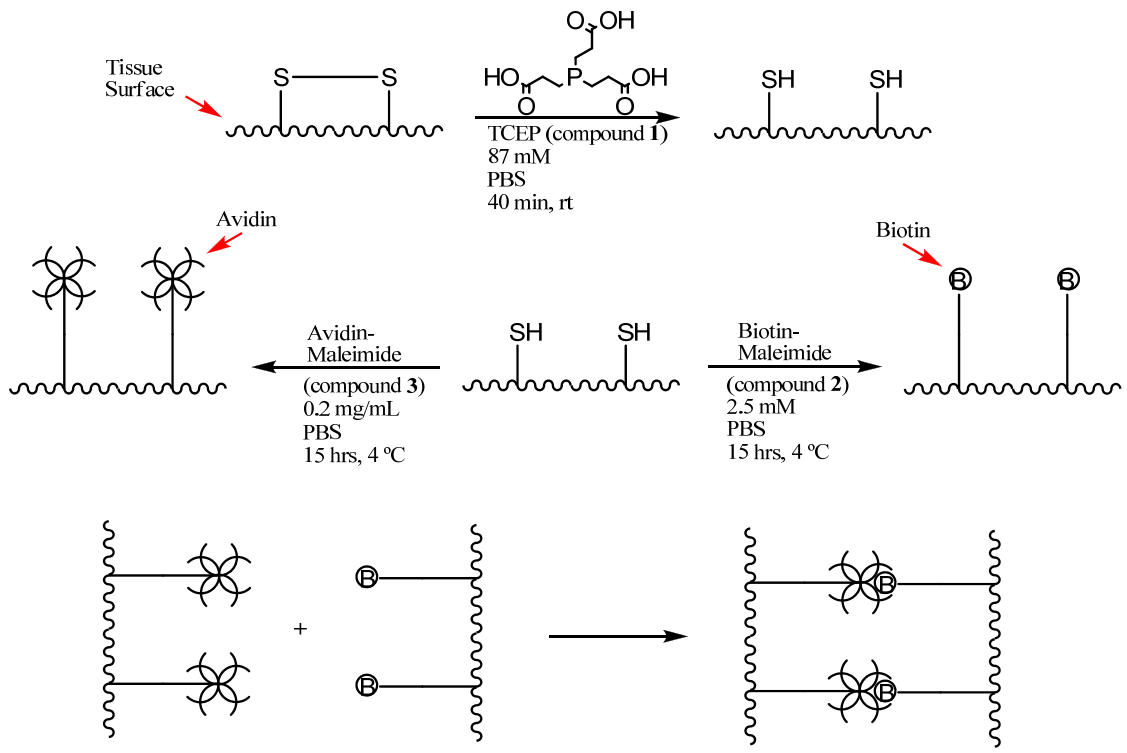


Figure 18. *Experiment 1* Reaction Diagram

biotin-alkylated slice and one avidin-alkylated slice. Control sample pairs included: two unreduced samples, two reduced samples, two biotin-alkylated samples, and two avidin-alkylated samples. All sample pairs were incubated for 12 hours at room temperature to allow adhesion. The samples were then rehydrated in PBS. Attempts to determine bond strengths did not reveal bonding above control levels.

Experiment 2: Reduction of Bovine Meniscus and Alternative Biotin/Avidin Cross-linking (Figure 19)

Twenty two meniscal slices (prepared as described in the materials and methods) were reduced by incubation in an 87 mM solution of TCEP (compound **1**) in PBS. Twenty reduced samples and two unreduced control samples were alkylated (for ~15 hours at 4 ºC) using a 2.5 mM solution of biotin-maleimide (compound **2**) in PBS. The

remaining reduced samples (2) were simply incubated in PBS for ~15 hours at 4 ºC. After alkylation all samples were thoroughly washed with PBS (3 x ~7 mL, 5 min each wash). Ten reduced samples, one unreduced control sample, and one reduced control sample were reacted (for ~15 hours at 4 ºC) using a 0.2 mg/mL solution of avidin protein in PBS. The remaining reduced samples (10), unreduced control sample, and reduced

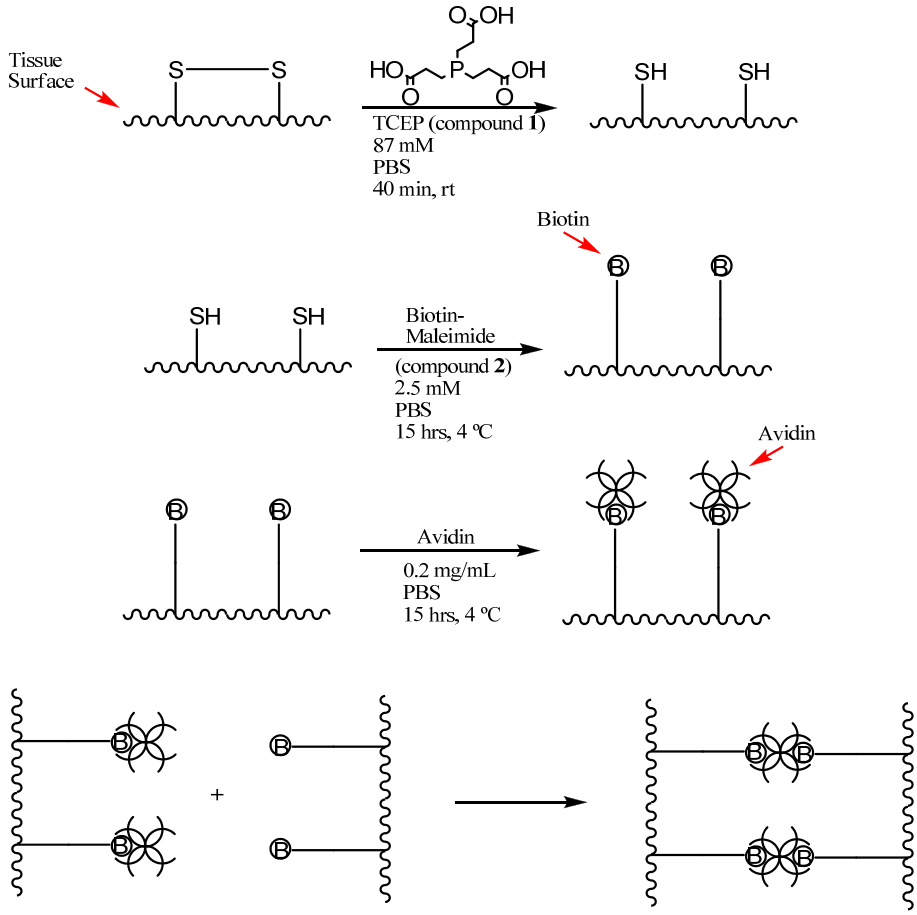


Figure 19. *Experiment 2* Reaction Diagram

control sample were simply incubated in PBS for ~15 hours at 4 ºC. After this step, all avidin reacted samples were thoroughly washed with PBS (3 x ~7 mL, 5 min each wash). Samples were assembled for adhesive testing using the standard lap-joint assembly (materials and methods). Experimental sample pairs were comprised of one biotin-

alkylated slice and one avidin-treated biotin-alkylated slice. Control sample pairs included: two unreduced samples, two reduced samples, two biotin-alkylated samples, and two avidin/biotin samples. All sample pairs were incubated for 12 hours at room temperature to allow adhesion. The samples were then rehydrated in PBS. Attempts to determine bond strengths did not reveal bonding above control levels.

Experiment 3: Oxidation of Bovine Meniscus and Biotin/Avidin Cross-linking (Figure 20)

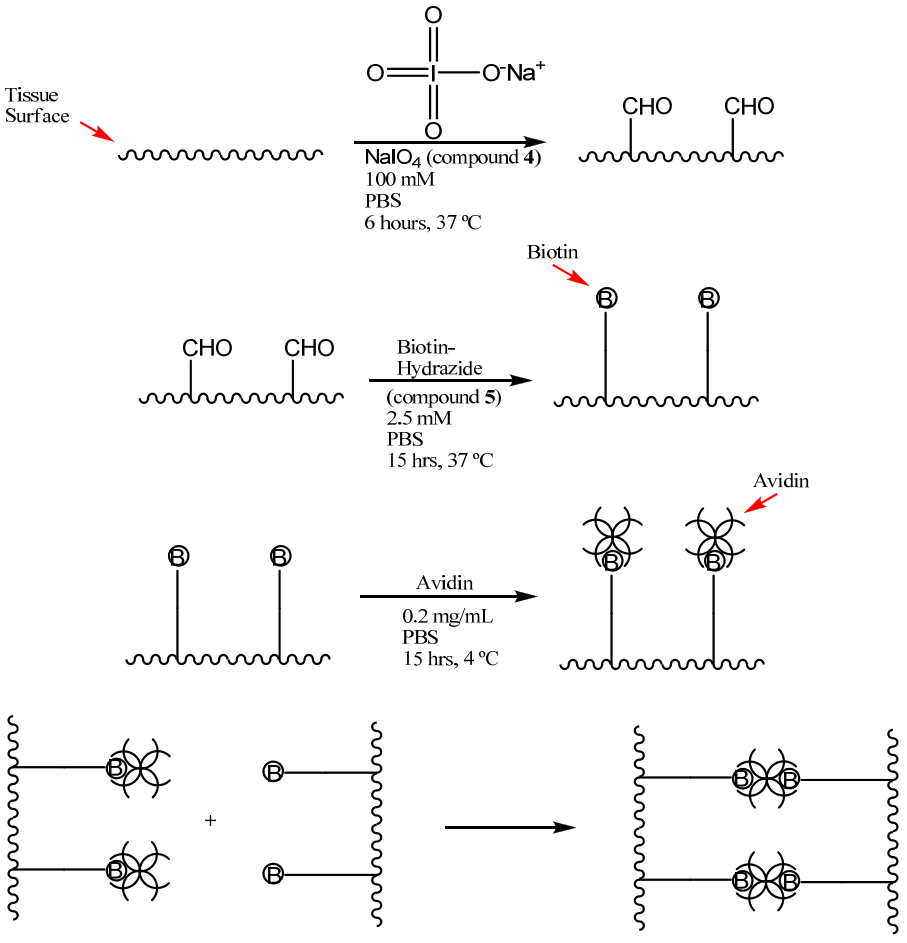


Figure 20. *Experiment 3,4* Reaction Diagram

Twenty two meniscal slices (prepared as described in the materials and methods) were oxidized by incubation in a 100 mM solution of sodium meta-periodate (compound **4**) in PBS. Twenty oxidized samples and two non-oxidized control samples were alkylated (for ~15 hours at 37 ºC) using a 2.5 mM solution of biotin-hydrazide (compound **5**) in PBS. The remaining oxidized samples (2) were simply incubated in PBS for ~15 hours at 37 ºC. After alkylation all samples were thoroughly washed with PBS (3 x ~7 mL, 5 min each wash). Ten oxidized samples, one non-oxidized control sample, and one oxidized control sample were reacted (for ~15 hours at 37 ºC) using a 0.2 mg/mL solution of avidin protein in PBS. The remaining oxidized samples (10), non-oxidized control sample, and oxidized control sample were simply incubated in PBS for ~15 hours at 37 ºC. After this step, all avidin reacted samples were thoroughly washed with PBS (3 x ~7 mL, 5 min each wash). Samples were assembled for adhesive testing using the standard lap-joint assembly (materials and methods). Experimental sample pairs were comprised of one biotin-alkylated slice and one avidin-alkylated slice. Control sample pairs included: two non-oxidized samples, two oxidized samples, two biotin-alkylated samples, and two avidin/biotin samples. All sample pairs were incubated for 12 hours at room temperature to allow adhesion. The samples were then rehydrated in PBS. Attempts to determine bond strengths did not reveal bonding above control levels.

Experiment 4: Oxidation of Bovine Meniscus and Biotin/Avidin Cross-linking Time Trial (Figure 20)

Twenty two meniscal slices (prepared as described in the materials and methods) were oxidized by incubation in a 100 mM solution of sodium meta-periodate (compound **4**) in PBS. Twenty oxidized samples and two non-oxidized control samples were

alkylated (for ~15 hours at 37 ºC) using a 2.5 mM solution of biotin-hydrazide (compound **5**) in PBS. The remaining oxidized samples (2) were simply incubated in PBS for ~15 hours at 37 ºC. After alkylation all samples were thoroughly washed with PBS (3 x ~7 mL, 5 min each wash). Ten oxidized samples, one non-oxidized control sample, and one oxidized control sample were reacted (for ~15 hours at 37 ºC) using a 0.2 mg/mL solution of avidin protein in PBS. The remaining oxidized samples (10), non-oxidized control sample, and oxidized control sample were simply incubated in PBS for ~15 hours at 37 ºC. After this step, all avidin reacted samples were thoroughly washed with PBS (3 x ~7 mL, 5 min each wash). Samples were assembled for adhesive testing using the standard lap-joint assembly (materials and methods). Experimental sample pairs were comprised of one biotin-alkylated slice and one avidin-alkylated slice. Control sample pairs included: two non-oxidized samples, two oxidized samples, two biotin-alkylated samples, and two avidin/biotin samples. Two experimental samples pairs and all control sample pairs were incubated for 12 hours at room temperature. The remaining experimental sample pairs were incubated for six hours (2 pairs), two hours (two pairs), and 30 minutes (two pairs) at room temperature. The samples were then rehydrated in PBS. Attempts to determine bond strengths did not reveal bonding above control levels.

*Experiment 5: Reduction of Bovine Meniscus and Compound **6** Cross-linking Trial (Figure 21)*

Twelve meniscal slices (prepared as described in the materials and methods) were reduced by incubation in an 87 mM solution of TCEP (compound **1**) in PBS. Seven

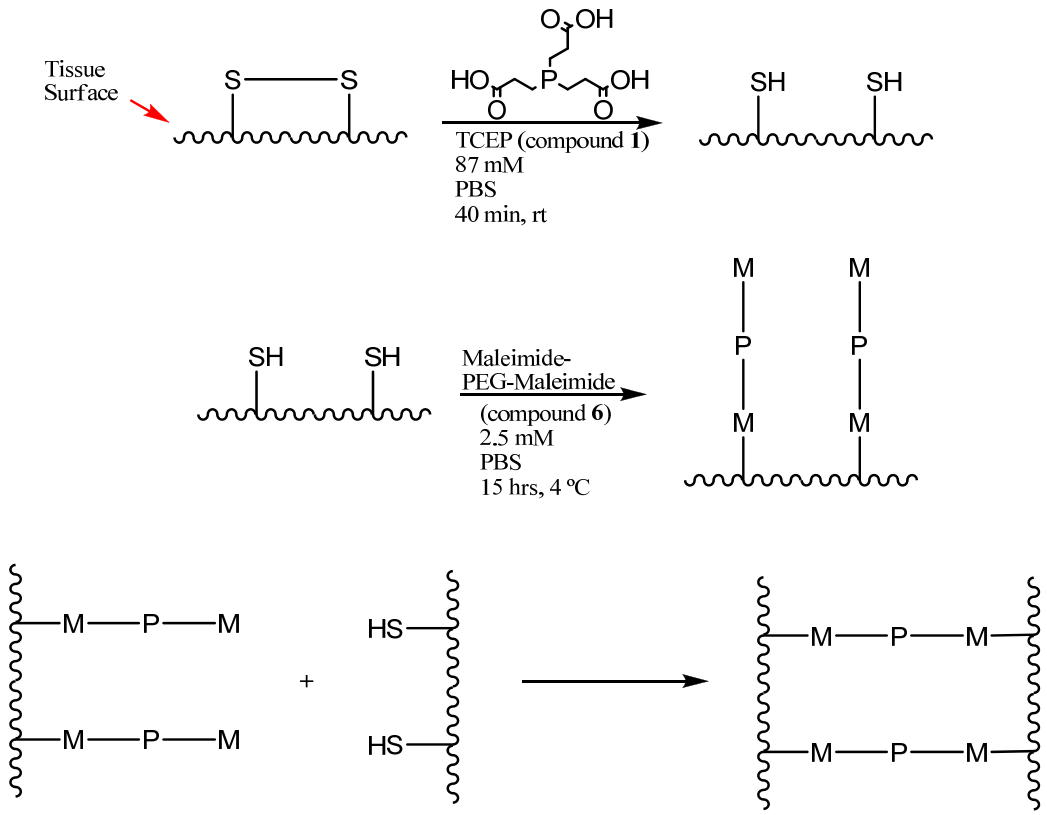


Figure 21. *Experiment 5,6,7* Reaction Diagram

reduced samples and one unreduced control were alkylated (for ~15 hours at 4 ºC) using a 2.5 mM solution of Maleimide-PEG-Maleimide (compound **6**) in PBS. The remaining reduced samples (5) and unreduced sample were simply incubated in PBS for ~15 hours at 4 ºC. After alkylation all samples were thoroughly washed with PBS (3 x ~7 mL, 5 min each wash). Samples were assembled for adhesive testing using the standard lap-joint assembly (materials and methods). Experimental sample pairs were comprised of one compound **6** alkylated slice and one unalkylated slice. Control sample pairs included two unreduced samples and two compound **6** alkylated samples. All sample pairs were incubated for 12 hours at room temperature to allow adhesion. The samples were then

rehydrated in PBS. Significant adhesion was observed and bond strengths were measured (Table 1) using the MTS (materials and methods).

Table 1. Experiment 5 MTS Data

Sample Pair	Surface Area (cm^2)	Peak Force (N)	Peak Stress (kPa)
No Reduction (control)	No bond	—	—
Both Cross-linker (control)	0.00702	0.157	2.24
1	0.00556	0.628	11.3
2	0.00410	0.186	4.54
3	0.00583	0.540	9.26
4	0.00494	0.540	10.9
5	0.00623	1.23	19.7

*Experiment 6: Reduction of Bovine Meniscus and Compound **6** Cross-linking with Four Hour Binding (Figure 21)*

Thirty eight meniscal slices (prepared as described in the materials and methods) were reduced by incubation in an 87 mM solution of TCEP (compound **1**) in PBS. Twenty reduced samples and one unreduced control sample were alkylated (for ~15 hours at 4 ºC) using a 2.5 mM solution of compound **6** in PBS. The remaining reduced samples (18) and unreduced control sample were simply incubated in PBS for ~15 hours at 4 ºC. After alkylation all samples were thoroughly washed with PBS (3 x ~7 mL, 5 min each wash). Samples were assembled for adhesive testing using the standard lap-joint assembly (materials and methods). Experimental sample pairs were comprised of one compound **6** alkylated slice and one unalkylated slice. Control sample pairs included: two unreduced samples, two reduced samples, and four compound **6** alkylated samples. All sample pairs were incubated for four hours at room temperature to allow adhesion. The samples were then rehydrated in PBS. Significant adhesion was observed and bond strengths were measured (Table 2) using the MTS (materials and methods).

Table 2. Experiment 6 MTS Data

Sample Pair	Surface Area (cm^2)	Peak Force (N)	Peak Stress (kPa)
No Reduction (control)	0.00451	0.412	9.14
No Cross-linker (control)	No bond	—	—
Both Cross-linker (control)	0.00621	0.441	7.10
Both Cross-linker (control)	Failed in setup	—	—
1	0.00526	0.667	12.7
2	0.00672	0.314	4.67
3	0.00551	0.412	7.48
4	0.00552	0.284	5.14
5	0.00515	0.206	4.00
6	0.00524	0.736	14.0
7	0.00455	0.559	3.09
8	0.00698	0.216	9.56
9	0.00616	0.589	8.31
10	0.00638	0.530	2.68
11	0.00477	0.128	3.20
12	0.00675	0.216	6.42
13	0.00810	0.520	1.16
14	0.00673	0.078	1.16
15	0.00662	0.245	3.70
16	0.00847	0.520	6.14

*Experiment 7: Reduction of Bovine Meniscus and Compound **6** Dose Response Trial (Figure 21)*

Thirty two meniscal slices (prepared as described in the materials and methods) were reduced by incubation in an 87 mM solution of TCEP (compound **1**) in PBS. Eight reduced samples and one unreduced control sample were alkylated (for ~15 hours at 4 ºC) using a 3 mM solution of compound **6** in PBS. Four reduced samples were alkylated (for ~15 hours at 4 ºC) using a 6 mM solution of compound **6** in PBS. Six reduced samples (2 per solution) were alkylated (for ~15 hours at 4 ºC) using 15 mM, 24 mM, and 30 mM solutions of compound **6** in PBS. The remaining reduced samples (14) and

unreduced control sample were simply incubated in PBS for ~15 hours at 4 ºC. After alkylation all samples were thoroughly washed with PBS (3 x ~7 mL, 5 min each wash). Samples were assembled for adhesive testing using the standard lap-joint assembly (materials and methods). Experimental sample pairs were comprised of one compound **6** alkylated slice and one unalkylated slice. Control sample pairs included two unreduced samples and four compound **6** (3 mM) alkylated samples. All sample pairs were incubated for four hours at room temperature to allow adhesion. The samples were then rehydrated in PBS. Significant adhesion was observed and bond strengths were measured (Table 3) using the MTS (materials and methods).

Table 3. Experiment 7 MTS Data

Sample Pair	Surface Area (cm^2)	Peak Force (N)	Peak Stress (kPa)
Both Cross Linker (control)	Failed in setup	—	—
3 mmol	Failed in setup	—	—
3 mmol	Failed in setup	—	—
3 mmol	0.0000477	1.23	25.8
3 mmol	0.0000477	0.245	5.14
6 mmol	Failed in setup	—	—
6 mmol	0.0000616	0.461	7.48
6 mmol	0.0000604	0.422	6.99
6 mmol	0.0000663	0.520	7.84
15 mmol	0.0000613	0.471	7.68
15 mmol	0.0000584	0.834	14.3
24 mmol	0.0000583	1.16	19.9
24 mmol	0.0000482	0.824	17.1
30 mmol	0.0000631	1.28	20.3
30 mmol	0.0000555	1.08	19.5

*Experiment 8: Reduction of Bovine Meniscus and Compound **6** Cross-linking with Combined Cross-linking and Bonding (Figure 22)*

Twenty two meniscal slices (prepared as described in the materials and methods) were reduced by incubation in an 87 mM solution of TCEP (compound **1**) in PBS.

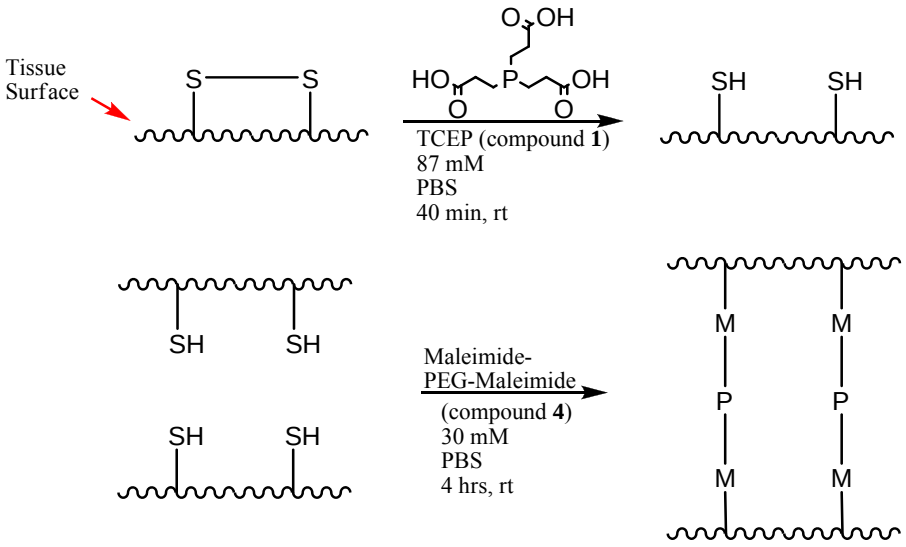


Figure 22. *Experiment 8* Reaction Diagram

Samples were assembled for adhesive testing by adding cross-linking (or buffer for control) solution prior to using the standard lap-joint assembly (materials and methods). These samples were not allowed to react as usual, but were assembled with the solution present. Experimental sample pairs were comprised of two slices with 100 µL of a 30 mM solution of compound **6** in PBS. Control sample pairs included: two unreduced samples (with 100 µL of a 30 mM solution of compound **6** in PBS) and two reduced samples. All sample pairs were incubated for four hours at room temperature to allow adhesion. The samples were then rehydrated in PBS. Minimal adhesion was observed for some experimental sample pairs.

*Experiment 9: Reduction of Bovine Meniscus and Compound **6** without Applied Pressure During Bonding (Figure 21)*

Ten meniscal slices (prepared as described in the materials and methods) were reduced by incubation in an 87 mM solution of TCEP (compound **1**) in PBS. Five

reduced samples and one unreduced control sample were alkylated (for ~15 hours at 4 ºC) using a 30 mM solution of compound **6** in PBS. The remaining reduced samples (5) and unreduced control sample were simply incubated in PBS for ~15 hours at 4 ºC. After alkylation all samples were thoroughly washed with PBS (3 x ~7 mL, 5 min each wash). Samples were assembled for adhesive testing using the standard lap-joint assembly (materials and methods), but the binder clips that are customarily used to apply pressure to the slides were not included. Experimental sample pairs were comprised of one compound **6** alkylated slice and one unalkylated slice. Control sample pairs included: two unreduced samples, two reduced samples, and two compound **6** alkylated samples. All sample pairs were incubated for four hours at room temperature to allow adhesion. The samples were then rehydrated in PBS. Attempts to determine bond strengths did not reveal bonding above control levels.

*Experiment 10: Reduction of Bovine Meniscus and Compound **6** with Reduced Applied Bonding Pressure (Figure21)*

Ten meniscal slices (prepared as described in the materials and methods) were reduced by incubation in an 87 mM solution of TCEP (compound **1**) in PBS. Five reduced samples and one unreduced control sample were alkylated (for ~15 hours at 4 ºC) using a 30 mM solution of compound **6** in PBS. The remaining reduced samples (5) and unreduced control sample were simply incubated in PBS for ~15 hours at 4 ºC. After alkylation all samples were thoroughly washed with PBS (3 x ~7 mL, 5 min each wash). Samples were assembled for adhesive testing using the standard lap-joint assembly (see materials and methods) with exceptions. Binder clips were not used (as in *Experiment 7*). Instead, pressure was applied using glass plates (~ 5.5 in by 6.5 in) which were set atop the slides (3 pairs each plate). Experimental sample pairs were comprised of one

compound **6** alkylated slice and one unalkylated slice. Control sample pairs included: two unreduced samples, two reduced samples, and two compound **6** alkylated samples. All sample pairs were incubated for four hours at room temperature to allow adhesion. The samples were then rehydrated in PBS. Attempts to determine bond strengths did not reveal bonding above control levels.

*Experiment 11: Oxidation of Bovine Meniscus and Compound **7** Cross-linking Trial (Figure 23)*

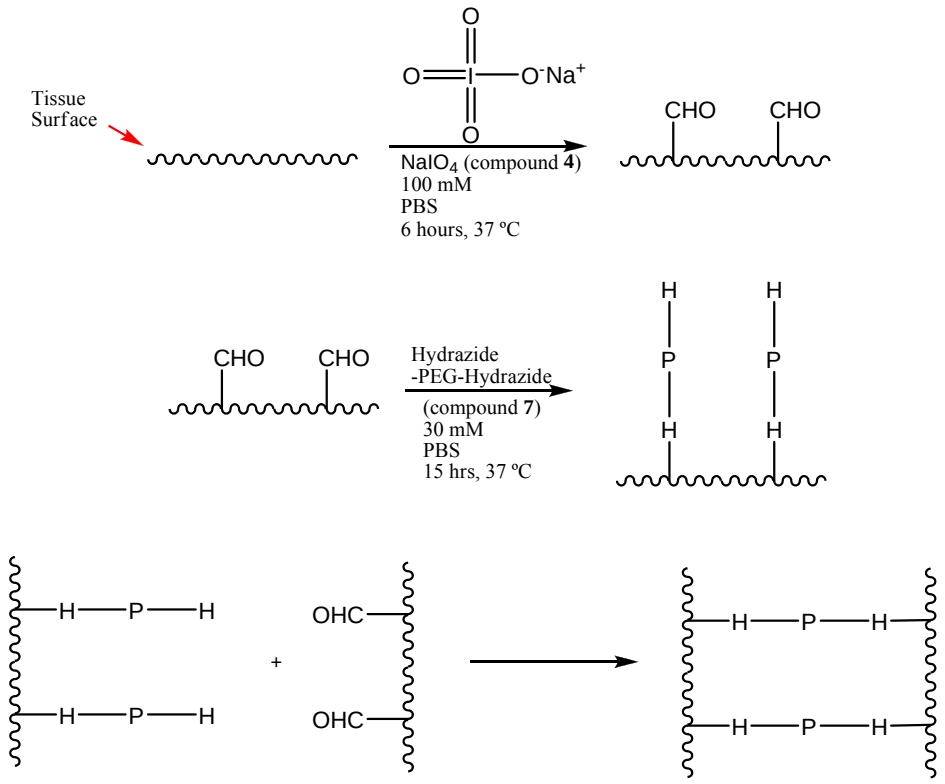


Figure 23. *Experiment 11* Reaction Diagram

Fourteen meniscal slices (prepared as described in materials and methods) were oxidized by incubation (for ~ 6 hours at 37 ºC) using a 100 mM solution of sodium meta-periodate (compound **4**) in PBS. Eight oxidized samples and one non-oxidized control

sample were alkylated (for ~15 hours at 37 ºC) using a 30 mM solution of compound **7** in PBS. The remaining oxidized samples (6) and non-oxidized sample (1) were simply incubated in PBS for ~15 hours at 37 ºC. After alkylation all samples were thoroughly washed with PBS (3 x ~7 mL, 5 min each wash). Samples were assembled for adhesive testing using the standard lap-joint assembly (materials and methods). Experimental sample pairs were comprised of one compound **7** alkylated slice and one unalkylated slice. Control sample pairs included: two non-oxidized samples and two compound **7** alkylated samples. All sample pairs were incubated for four hours at room temperature to allow adhesion. The samples were then rehydrated in PBS. Significant adhesion was observed and bond strengths were measured (Table 4, sample 5 slipped out of the grips and had to be retested) using the MTS (materials and methods).

Table 4. Experiment 11 MTS Data

Sample Pair	Surface Area (cm^2)	Peak Force (N)	Peak Stress (kPa)
No Oxidation (control)	No bond	—	—
Both Cross-linker (control)	0.00914	4.32	47.2
1	0.00409	2.45	59.9
2	0.00637	9.32	146
3	0.01020	4.66	45.7
4	0.00628	4.71	75.0
5	0.00577	2.55(1^{st} test), 5.69(2^{nd} test)	44.2(1^{st} test), 98.6(2^{nd} test)
6	0.00887	8.83	99.5

*Experiment 12: Oxidation of Bovine Meniscus and Compound **8** Cross-linking Trial (Figure 24)*

Fourteen meniscal slices (prepared as described in materials and methods) were oxidized by incubation in 100 mM solution of sodium meta-periodate (compound **4**) in

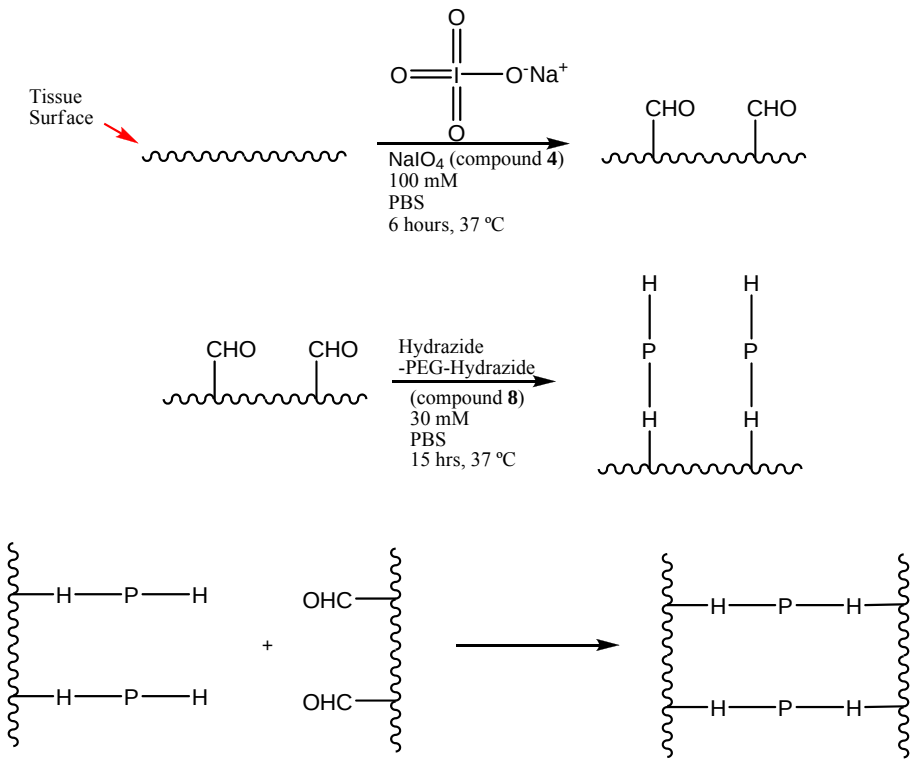


Figure 24. *Experiment 12* Reaction Diagram

PBS. Eight oxidized samples and one non-oxidized control sample were alkylated (for ~15 hours at 37 ºC) using a 30 mM solution of compound **8** in PBS. The remaining oxidized samples (6) and non-oxidized sample (1) were simply incubated in PBS for ~15 hours at 37 ºC. After alkylation all samples were thoroughly washed with PBS (3 x ~7 mL, 5 min each wash). Samples were assembled for adhesive testing using the standard lap-joint assembly (materials and methods). Experimental sample pairs were comprised of one compound **8** alkylated slice and one unalkylated slice. Control sample pairs included: two non-oxidized samples and two compound **8** alkylated samples. All sample pairs were incubated for four hours at room temperature to allow adhesion. The samples were then rehydrated in PBS. Significant adhesion was observed and bond strengths were measured (Table 5) using the MTS (materials and methods).

Table 5. Experiment 12 MTS Data

Sample Pair	Surface Area (cm²)	Peak Force (N)	Peak Stress (kPa)
No Oxidation (control)	No bond	—	—
Both Cross-linker (control)	0.00486	2.16	44.4
1	0.00590	2.80	47.5
2	0.00649	3.09	47.6
3	0.00651	2.47	37.9
4	0.00633	2.01	31.8
5	0.00642	3.83	59.7
6	0.00275	3.19	116

*Experiment 13: Oxidation of Bovine Meniscus and Compound **9** Cross-linking Trial (Figure 25)*

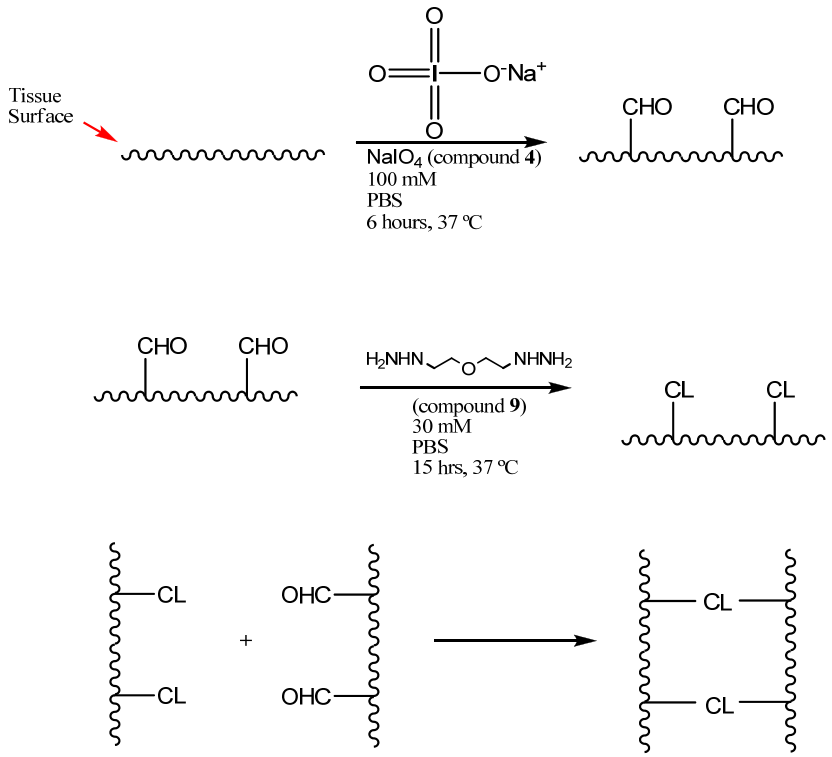


Figure 25. *Experiment 13* Reaction Diagram

Fourteen meniscal slices (prepared as described in materials and methods) were oxidized by incubation (for ~6 hours at 37 ºC) using a 100 mM solution of sodium meta-

periodate (compound **4**) in PBS. Eight oxidized samples and one non-oxidized control sample were alkylated (for ~15 hours at 37 ºC) using a 30 mM solution of compound **9** in PBS. The remaining oxidized samples (6) and non-oxidized sample (1) were simply incubated in PBS for ~15 hours at 37 ºC. After alkylation all samples were thoroughly washed with PBS (3 x ~7 mL, 5 min each wash). Samples were assembled for adhesive testing using the standard lap-joint assembly (materials and methods). Experimental sample pairs were comprised of one compound **9** alkylated slice and one unalkylated slice. Control sample pairs included: two non-oxidized samples and two compound **9** alkylated samples. All sample pairs were incubated for four hours at room temperature to allow adhesion. The samples were then rehydrated in PBS. Significant adhesion was observed and bond strengths were measured (Table 6) using the MTS (materials and methods).

Table 6. Experiment 13 MTS Data

Sample Pair	Surface Area (cm^2)	Peak Force (N)	Peak Stress (kPa)
No Oxidation (control)	No bond	—	—
Both Cross-linker (control)	0.00450	1.34	29.8
1	0.00500	3.63	72.6
2	0.00631	4.91	77.8
3	0.00632	4.71	74.5
4	0.00488	2.73	55.9
5	0.00477	4.12	86.4
6	0.00412	1.47	35.7

Experiment 14: Oxidation of Bovine Meniscus and Hydrazine Cross-linking Trial (Figure 26)

Ten meniscal slices (prepared as described in materials and methods) were oxidized by incubation (for ~6 hours at 37 ºC) using a 100 mM solution of sodium meta-

periodate (compound **4**) in PBS. Five oxidized samples and one non-oxidized control

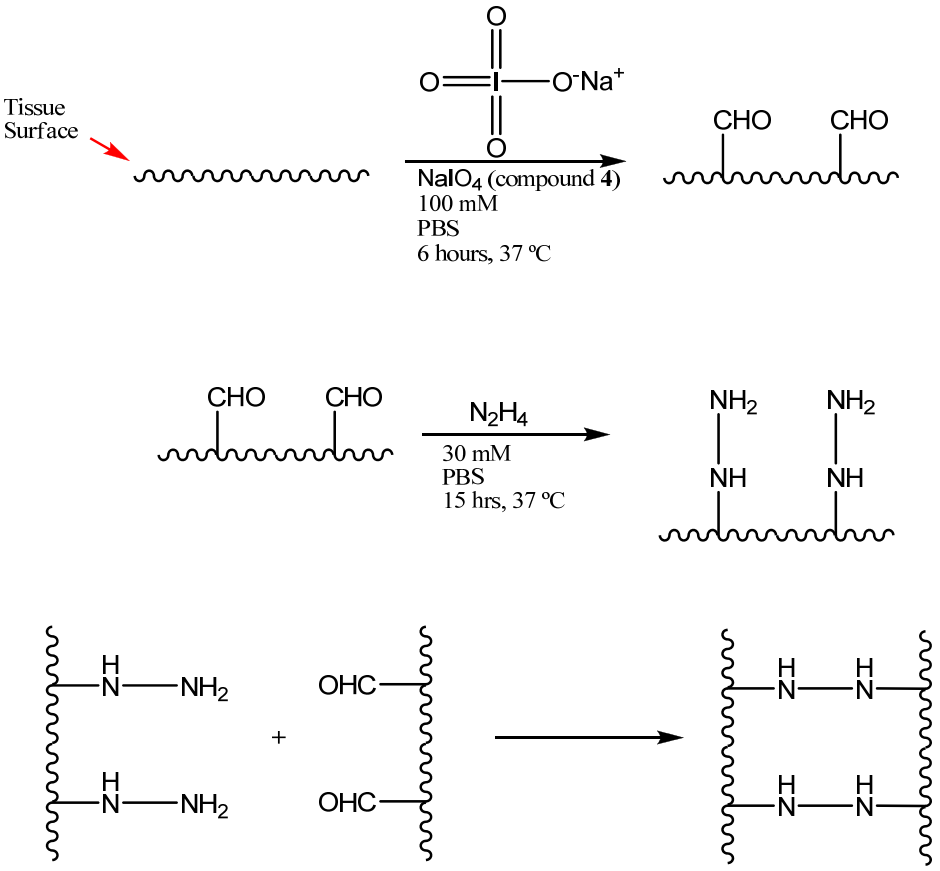


Figure 26. *Experiment 14* Reaction Diagram

PBS. The remaining oxidized samples (5) and non-oxidized sample (1) were simply incubated in PBS for ~15 hours at 37 ºC. After alkylation all samples were thoroughly washed with PBS (3 x ~7 mL, 5 min each wash). Samples were assembled for adhesive testing using the standard lap-joint assembly (materials and methods). Experimental sample pairs were comprised of one hydrazine reacted slice and one non-reacted slice. Control sample pairs included: two non-oxidized samples, two non-reacted samples and two hydrazine reacted samples. All sample pairs were incubated for four hours at room

temperature to allow adhesion. The samples were then rehydrated in PBS. Significant adhesion was observed and bond strengths were measured (Table 7) using the MTS (materials and methods).

Table 7. Experiment 14 MTS Data

Sample Pair	Surface Area (cm^2)	Peak Force (N)	Peak Stress (kPa)
No Oxidation (control)	No bond	—	—
No Cross-linker (control)	0.00454	0.147	3.24
Both Cross-linker (control)	0.00533	2.45	46.0
1	0.00417	1.33	31.9
2	0.00373	1.77	47.5
3	0.00505	2.35	46.5

*Experiment 15: Oxidation of Bovine Meniscus and Compound **10** Cross-liking Trial (Figure 27)*

Ten meniscal slices (prepared as described in materials and methods) were oxidized by incubation (for ~6 hours at 37 ºC) using a 100 mM solution of sodium meta-periodate (compound **4**) in PBS. Five oxidized samples and one non-oxidized control sample were reacted (for ~15 hours at 37 ºC) using a 30 mM solution of compound **10** in PBS. The remaining oxidized samples (5) and non-oxidized sample (1) were simply incubated in PBS for ~15 hours at 37 ºC. After alkylation all samples were thoroughly washed with PBS (3 x ~7 mL, 5 min each wash). Samples were assembled for adhesive testing using the standard lap-joint assembly (materials and methods). Experimental sample pairs were comprised of one compound **10** reacted slice and one non-reacted slice. Control sample pairs included: two non-oxidized samples, two non-reacted samples and two compound **10** reacted samples. All sample pairs were incubated for four

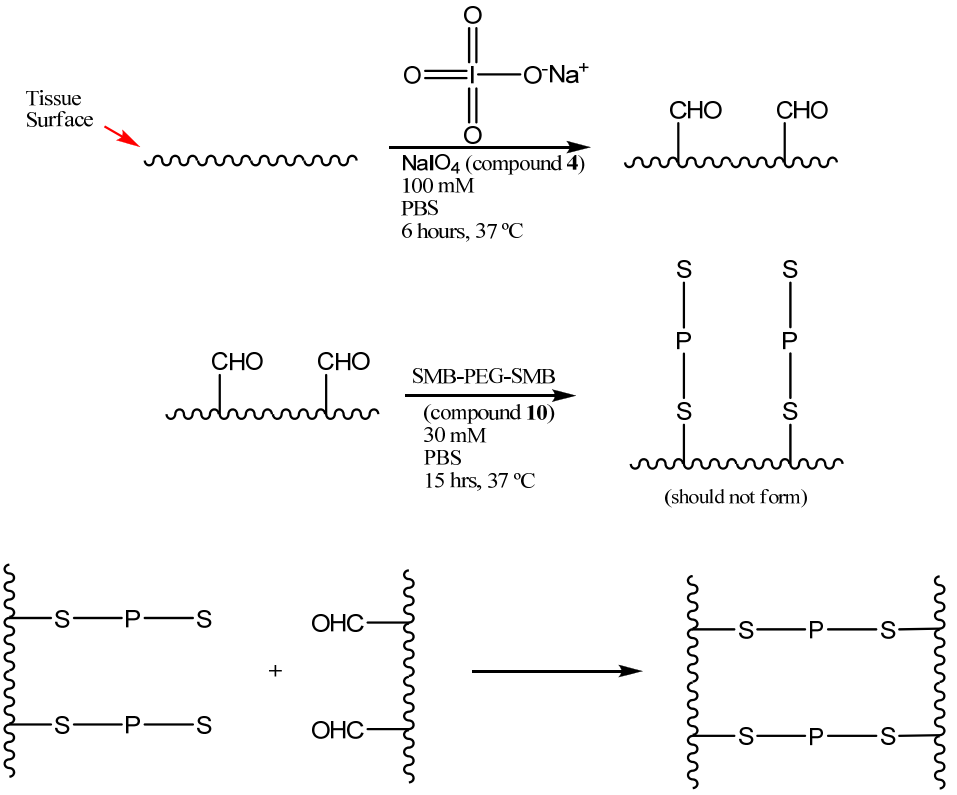


Figure 27. *Experiment 15* Reaction Diagram

hours at room temperature to allow adhesion. The samples were then rehydrated in PBS. Significant adhesion was observed and bond strengths were measured (Table 8) using the MTS (materials and methods).

Table 8. Experiment 15 MTS Data

Sample Pair	m²	N	kPa (N/ m²)
No Oxidation (control)	No bond	—	—
No Cross-linker (control)	0.0000751	0.363	4.83
Both Cross-linker (control)	Failed	—	—
1	Failed	—	—
2	0.0000771	0.284	3.68
3	No bond	—	—

Experiment 16: Oxidation of Bovine Meniscus and Hydrazine Dose-response Trials (Figure 26)

One hundred and thirty two meniscal slices (prepared as described in materials and methods) were oxidized by incubation (for ~6 hours at 37 ºC) using a 100 mM solution of sodium meta-periodate (compound **4**) in PBS. Seventy two oxidized samples and six non-oxidized control samples were reacted (for ~15 hours at 37 ºC) using 3.25 mM, 7.5 mM, 15 mM, 30 mM, 60 mM, or 90 mM solutions (12 samples and 1 control for each concentration) of hydrazine in PBS. The remaining oxidized samples (60) and non-oxidized samples (6) were simply incubated in PBS for ~15 hours at 37 ºC. After the reaction all samples were thoroughly washed with PBS (3 x ~7 mL, 5 min each wash). Samples were assembled for adhesive testing using the standard lap-joint assembly (materials and methods). Experimental sample pairs were comprised of one hydrazine reacted slice and one non-reacted slice. Control sample pairs included two non-oxidized samples and two hydrazine reacted samples. All sample pairs were incubated for four hours at room temperature to allow adhesion. The samples were then rehydrated in PBS. Significant adhesion was observed and bond strengths were measured (Appendix, Table A1) using the MTS (materials and methods).

Experiment 17: Oxidation of Bovine Meniscus and Hydrazine Time Trials (Figure 26)

One hundred and thirty two meniscal slices (prepared as described in materials and methods) were oxidized by incubation (for ~6 hours at 37 ºC) using a 100 mM solution of sodium meta-periodate (compound **4**) in PBS. Seventy two oxidized samples and six non-oxidized control samples were reacted (12 slices and one control each for ~15 hours, 12 hours, 9 hours, 6 hours, 3 hours, and 1 hour at 37 ºC) using a 30 mM solution of hydrazine in PBS. The remaining oxidized samples (60) and non-oxidized

samples (6) were simply incubated in PBS for ~15 hours at 37 ºC. After the reaction all samples were thoroughly washed with PBS (3 x ~7 mL, 5 min each wash). Samples were assembled for adhesive testing using the standard lap-joint assembly (materials and methods). Experimental sample pairs were comprised of one hydrazine reacted slice and one non-reacted slice. Control sample pairs included two non-oxidized samples and two hydrazine reacted samples. All sample pairs were incubated for four hours at room temperature to allow adhesion. The samples were then rehydrated in PBS. Significant adhesion was observed and bond strengths were measured (Appendix, Table A2) using the MTS (materials and methods).

*Experiment 18: Oxidation of Bovine Meniscus and Adipic Dihydrazine (compound **11**) Dose Response Trial (Figure 28)*

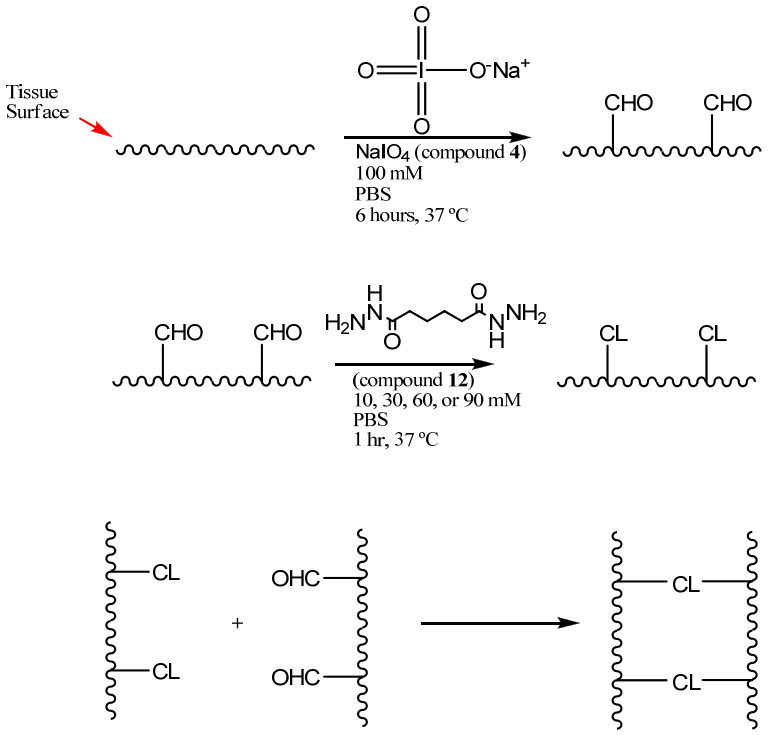


Figure 28. *Experiment 18* Reaction Diagram

Fifty four meniscal slices (prepared as described in materials and methods) were oxidized by incubation (for ~6 hours at 37 ºC) using a 100 mM solution of sodium meta-periodate (compound **4**) in PBS. Thirty four oxidized samples and four non-oxidized control samples were incubated for 1 hour at 37 ºC using a 10 mM, 30 mM, 60 mM, or 90 mM (7 samples and 1 controls for each concentration) solution of adipic dihydrazide (compound **11**) in PBS. The remaining oxidized samples (20) and non-oxidized samples (4) were simply incubated in PBS for 1 hour at 37 ºC. After the reaction all samples were thoroughly washed with PBS (3 x ~7 mL, 5 min each wash). Samples were assembled for adhesive testing using the standard lap-joint assembly (materials and methods). Experimental sample pairs were comprised of one compound **11** alkylated slice and one non-alkylated slice. Control sample pairs included two non-oxidized samples and two compound **11** alkylated samples. All sample pairs were incubated for four hours at room temperature to allow adhesion. The samples were then rehydrated in PBS. Significant adhesion was observed and bond strengths were measured (Appendix, Table A3) using the MTS (materials and methods).

Experiment 19: Oxidation of Bovine Meniscus and Compound Comparison Trials (Figures 23, 25, 26 and 28)

Two hundred and forty meniscal slices (prepared as described in materials and methods) were oxidized by incubation (for ~6 hours at 37 ºC) using a 100 mM solution of sodium meta-periodate (compound **4**) in PBS. One hundred and forty oxidized samples and twenty non-oxidized control samples were incubated for ~15 hours at 37 ºC using 30 mM solutions of hydrazine, compound **11**, compound **12**, and compound **13** (35 samples and 5 controls for each compound) in PBS. The remaining oxidized samples (100) and non-oxidized samples (20) were simply incubated in PBS for ~15 hours at 37 ºC. After

the reaction all samples were thoroughly washed with PBS (3 x ~7 mL, 5 min each wash). Samples were assembled for adhesive testing using the standard lap-joint assembly (materials and methods). Experimental sample pairs were comprised of one compound alkylated slice and one non-alkylated slice. Control sample pairs included two non-oxidized samples and two compound alkylated samples. All sample pairs were incubated for four hours at room temperature to allow adhesion. The samples were then rehydrated in PBS. Significant adhesion was observed and bond strengths were measured (Appendix, Table A4) using the MTS (materials and methods).

Experiment 20: Naphthalimide Photo-oxidation of Bovine Meniscus and Hydrazine Cross-linking Trial (Figure 29)

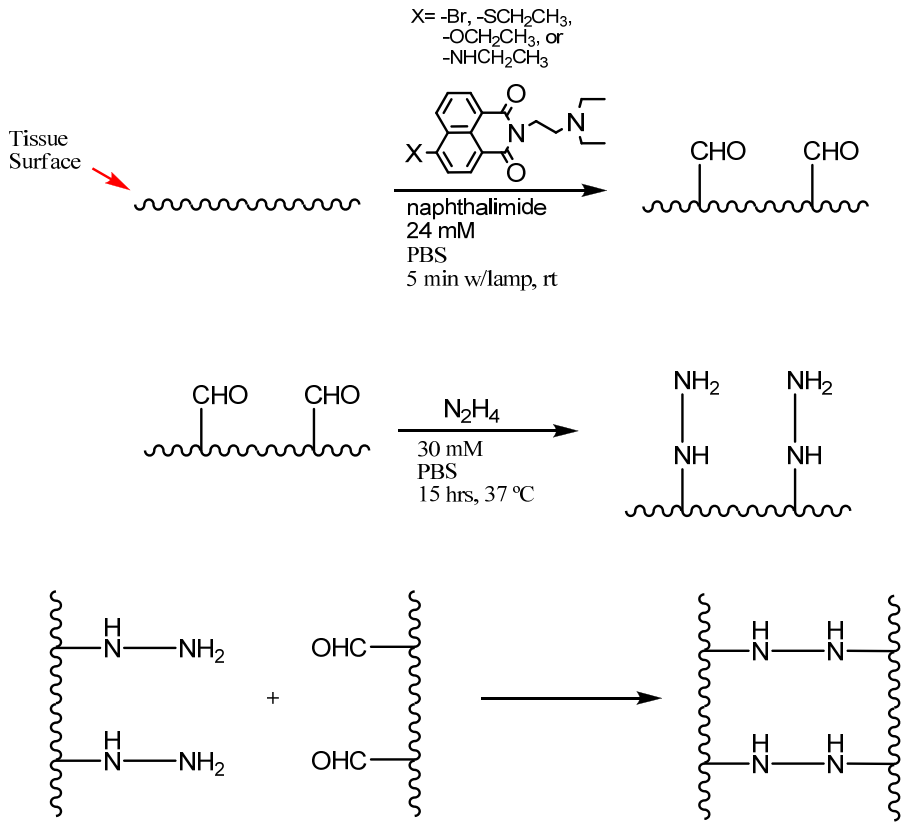


Figure 29. *Experiment 20* Reaction Diagram

Twenty two meniscal slices (prepared as described in materials and methods) were oxidized by photo-irradiation (see materials and methods) using 30 μL of a 24 mM solution of naphthalimide in PBS. Eleven oxidized samples and two control samples were incubated for ~15 hours at 37 ºC using a 30 mM solution of hydrazine in PBS. The remaining oxidized samples (11) and non-oxidized samples (2) were simply incubated in PBS for ~15 hours at 37 ºC. After the reaction all samples were thoroughly washed with PBS (3 x ~7 mL, 5 min each wash). Samples were assembled for adhesive testing using the standard lap-joint assembly (materials and methods). Experimental sample pairs were comprised of one compound reacted slice and one non-reacted slice. Control sample pairs included two non-oxidized samples, two compound reacted samples, and two non-reacted samples. All sample pairs were incubated for four hours at room temperature to allow adhesion. The samples were then rehydrated in PBS. Attempts to determine bond strengths did not reveal bonding above control levels.

Experiment 21: Naphthalimide Photo-oxidation of Bovine Pericardium (Figure 30)

Four spots on a sample of bovine pericardium (prepared as described in materials and methods) were oxidized by photo-irradiation (see material and methods) using 30 μL of a 24 mM solution of naphthalimide in PBS. Four additional wells were irradiated using just PBS and another four wells were reacted (dark) with 30 μL of a 24 mM solution of naphthalimide in PBS. The remaining four wells were incubated at room temperature in PBS. After the reaction the apparatus was disassembled and the sample was thoroughly washed with PBS (3 x ~7 mL, 5 min each wash). The sample was then incubated for ~15 at 37 ºC using ~7 mL of a 2.5 mM solution of compound **5** in PBS.

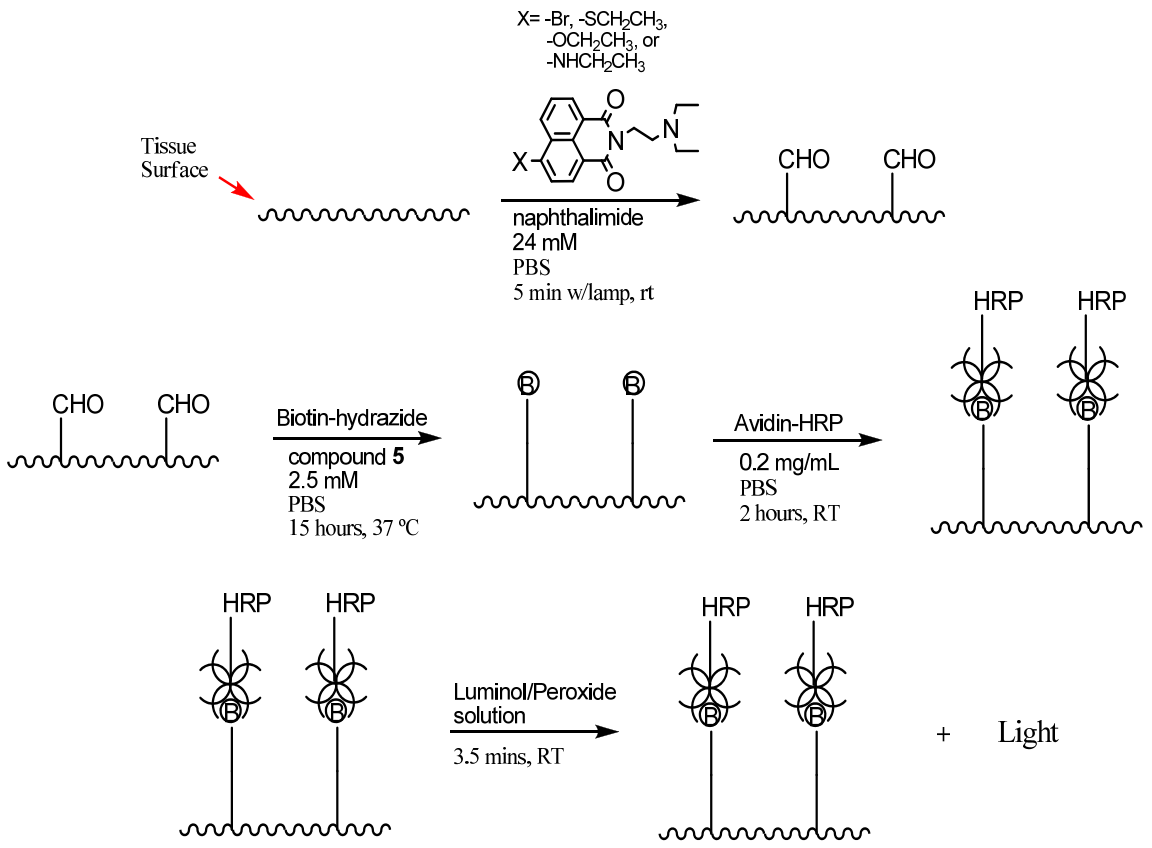


Figure 30. *Experiment 21,22* Reaction Diagram

Following alkylation the sample was washed again (3 x ~7 mL, 5 min each wash). The sample was then incubated for two hours using ~7 mL of a 0.2 mg/mL solution of avidin-HRP in PBS. After a final set of washes (3 x ~7 mL, 5 min each wash), the sample was incubated at room temperature for three minutes and thirty seconds with ~7 mL of luminol solution (materials and methods). The sample was blotted dry and imaged in the Ultra-lum Discovery Chemiluminescence system at Baylor University. There was no visible signal, and no detectable modification had occurred.

Experiment 22: Naphthalimide Photo-oxidation of Bovine Meniscus (Figure 30)

Four meniscal slices (prepared as described in materials and methods) were oxidized by photo-irradiation (see materials and methods) using a 24 mM solution of naphthalimide in PBS. Two oxidized samples and two non-oxidized control samples were incubated for ~15 hours at 37 ºC using a 2.5 mM solution of compound **5** in PBS. The remaining oxidized samples (2) and non-oxidized samples (2) were simply incubated in PBS for ~15 hours at 37 ºC. After the reaction all samples were thoroughly washed with PBS (3 x ~7 mL, 5 min each wash). All samples were then incubated for two hours using ~7 mL of a 0.2 mg/mL solution of avidin-HRP in PBS. After a final set of washes (3 x ~7 mL, 5 min each wash), the samples were incubated at room temperature for three minutes and thirty seconds with ~7 mL of luminol solution (materials and methods). The samples were blotted dry and imaged in the Ultra-lum Discovery Chemiluminescence. There was no visible signal, and no detectable modification had occurred.

*Experiment 23: Pre-oxidation (compound **4**) and Naphthalimide Photo-oxidation of Bovine Meniscus (Figure 31)*

Six meniscal slices (prepared as described in materials and methods) were oxidized by incubation using a 100 mM solution of sodium meta-periodate (compound **4**) in PBS. Three oxidized samples and three non-oxidized control samples were irradiated using 30 µL of a 24 mM solution of naphthalimide in PBS. The remaining oxidized samples (3) and non-oxidized samples (3) were simply incubated in PBS at room temperature. After the reaction all samples were thoroughly washed with PBS (3 x ~7 mL, 5 min each wash). All samples were incubated for ~15 hours at 37 ºC using a 2.5 mM solution of compound **5** in PBS. Another set of washes (3 x ~7 mL, 5 min each wash) was performed. All samples were then incubated for two hours using ~7 mL of a

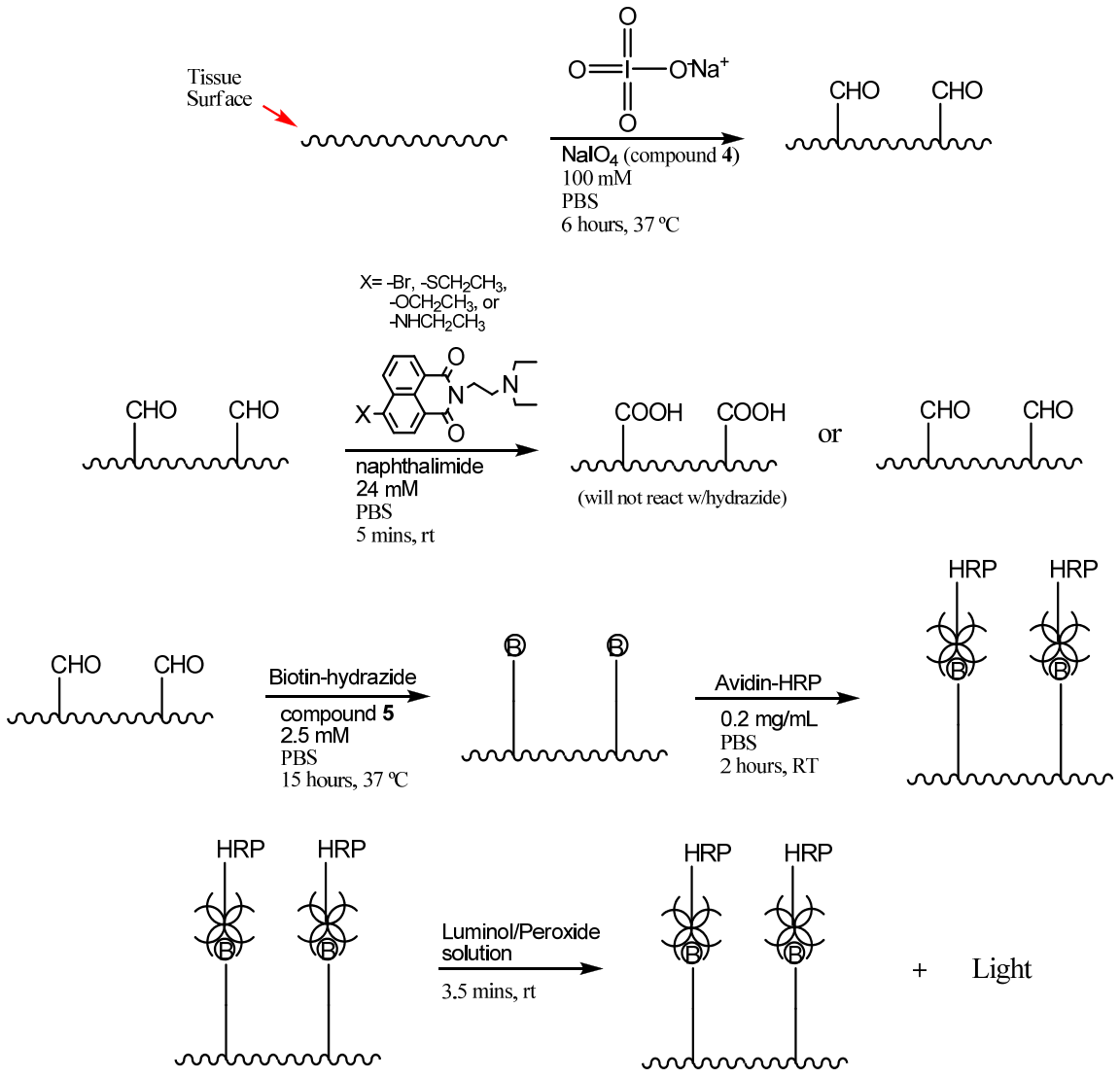


Figure 31. *Experiment 23* Reaction Diagram

0.2 mg/mL solution of avidin-HRP in PBS. After a final set of washes (3 x ~7 mL, 5 min each wash), the samples were incubated at room temperature for three minutes and thirty seconds with ~7 mL of luminol solution (materials and methods). The samples were blotted dry and imaged in the Ultra-lum Discovery Chemiluminescence system (Figure 32). The samples in columns one and two of the image were the pre-oxidizes samples.

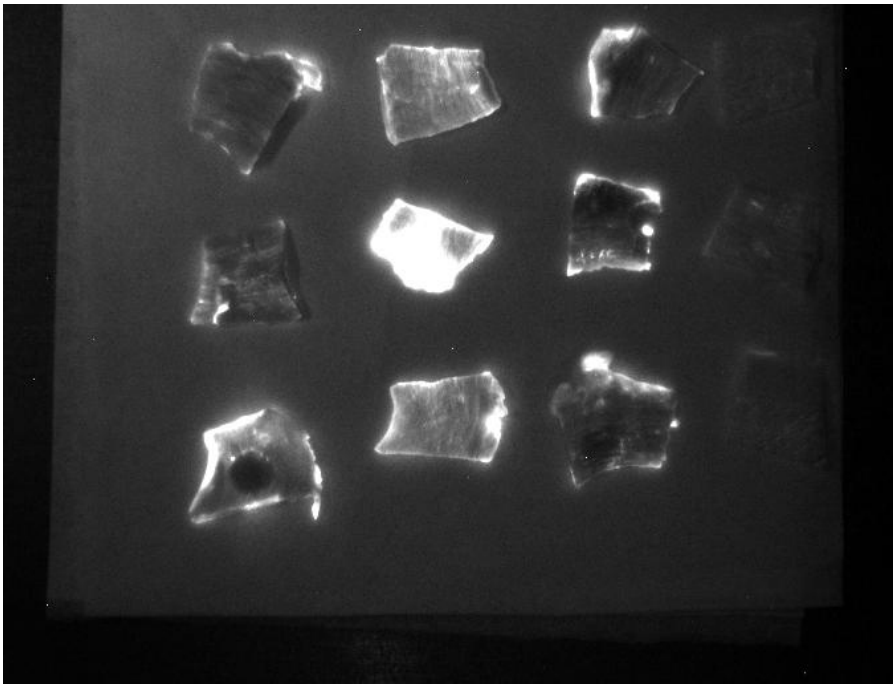


Figure 32. Chemiluminescent Image of Pre-oxidation and Compound **14**

Section Four: Meniscal Bonding Conclusions

The purpose of this project was to continue the research in wound healing and tissue repair that had been previously explored in the context of photo-chemical tissue bonding and the immobilization of proteins and small molecules on tissue surfaces. My thesis project focused on meniscal bonding using reductive and oxidative methods. In addition to the use of sodium-meta periodate as an oxidizing agent, Dr. Kane hypothesized that the naphthalimide compounds used to facilitate bonding of proteins in solution could also be used as photo-oxidation agents.

Initial experiments explored the use of the avidin/biotin interaction to afford non-covalent bonding between tissue surfaces (cross-linking) that had been appropriately modified (Figure 33).

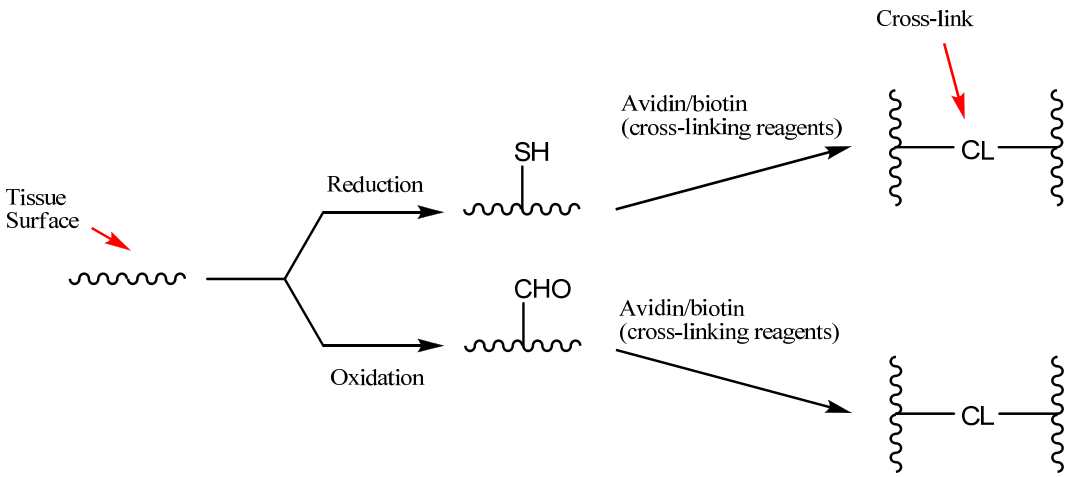


Figure 33. General Experimental Reaction Scheme

Oxidation or reduction of the tissue surfaces was followed by reaction with the appropriate functionalized biotin and avidin reagents (Experiments 1,2,3). While tissues primed by oxidation were observed to form mechanical adhesions that were longer-lived than control oxidized tissues, in no case was a bond formed that was sufficiently strong so as to allow the bond strength to be accurately measured.

Following the avidin/biotin experiments, we decided to explore the use of small organic molecules to provide direct covalent cross-linking. The first trial of compound **6** (Figure 10, page 12) provided bonds with testable strength (Experiment 5, Table 1).

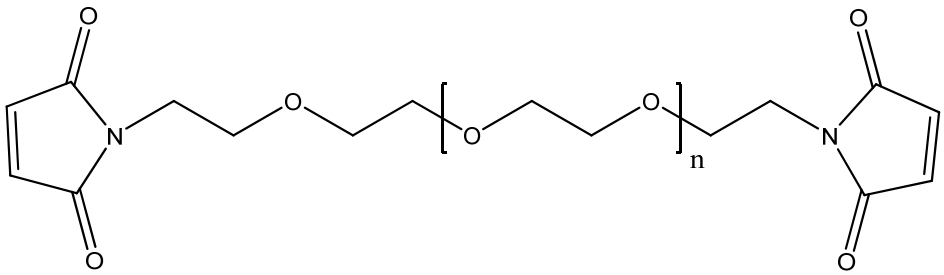


Figure 10. Structure of Mal-PEG-Mal (compound **6**)

Another trial examining the relationship of compound concentration to bond strength using compound **6** (ranging from 3 to 30 mmol) indicated a positive correlation between concentration and bond strength (Experiment 7, Table 3). This increase in bond strength began to taper off as compound **6** concentrations reached 24-30 mmol. Based on the data from Experiment 7, standard cross-linking solution concentration was set at 30 mmol for subsequent experiments. The final series of reduction experiments provided evidence that significant pressure (binder clips for our purposes) was required to achieve adhesion. Although we observed that pressure was required for bond formation, we also observed that clamping pressure from the binder clips would only facilitate bonding if the cross-linking reagents were allowed to react prior to lap-joint assembly.

Experiments involving oxidation of the tissue samples gave the most consistent bonding and the highest bond strengths. Investigation of the use of impure synthetic cross-linkers (compounds **7**, **8**, and **9)** with periodate oxidized meniscus routinely gave bond strengths exceeding 35-40 kPa. However, a probable contaminant (hydrazine) proved able to form tissue bonds with similar strengths (Table 7). While a study of concentration versus bond strength did not reveal any significant differences, we decided to use 30 mmol hydrazine concentrations in future experiments, as the data suggested a plateau of the bond strength at this concentration (Figure 34). A subsequent trial examining the relationship of hydrazine reaction time to bond strength indicated that optimal potency (average above 50 kPa) was obtained with as little as one hour of cross-linking solution reaction time (Figure 35). Although the bond strengths were encouraging, we were well aware that due to the issue with toxicity hydrazine would

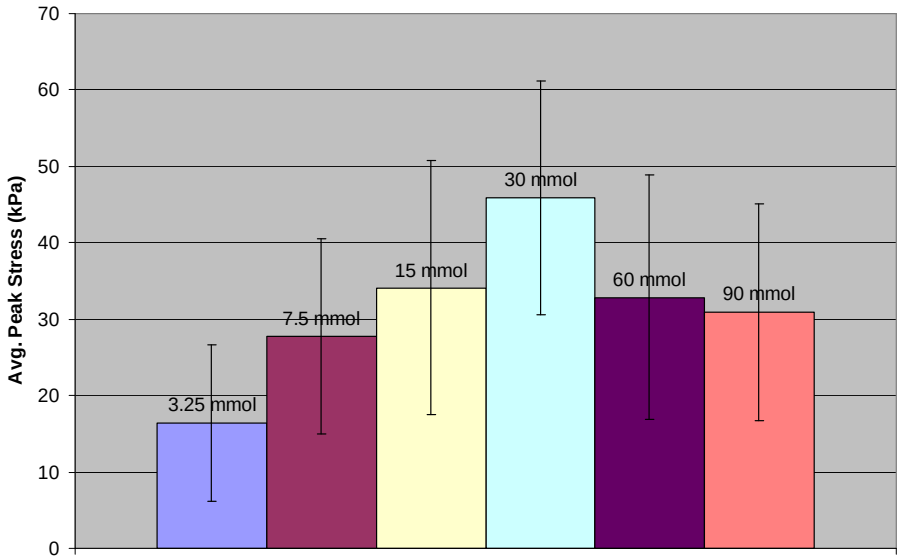


Figure 34. Average Peak Stress Chart for Hydrazine Dose Response Trial

not be a promising tissue-bonding agent for medical applications.

Compound **11** (Figure 12, page 14) was explored as another potential cross-

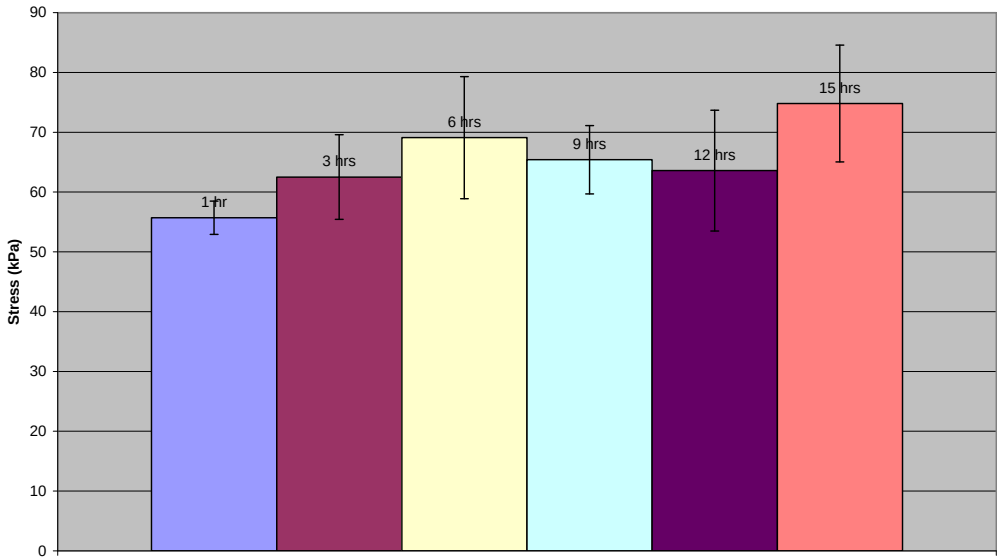


Figure 35. Average Peak Stress Chart for Hydrazine Time Trial

linking agent. A trial examining the relationship of compound concentration (ranging from 10 to 90 mmol) to bond strength using compound **11** with a cross-linker reaction

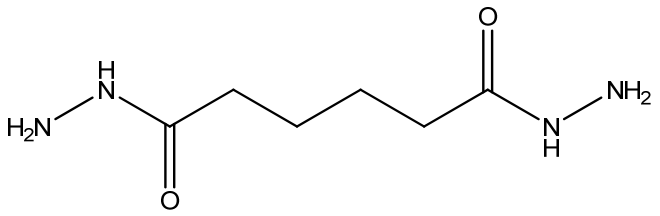


Figure 12. Structure of Adipic Dihydrazide (compound **11**)

time of one hour (based on Experiment 17) indicated significant bonding (though greatly variable) at and above 30 mmol (Figure 36).

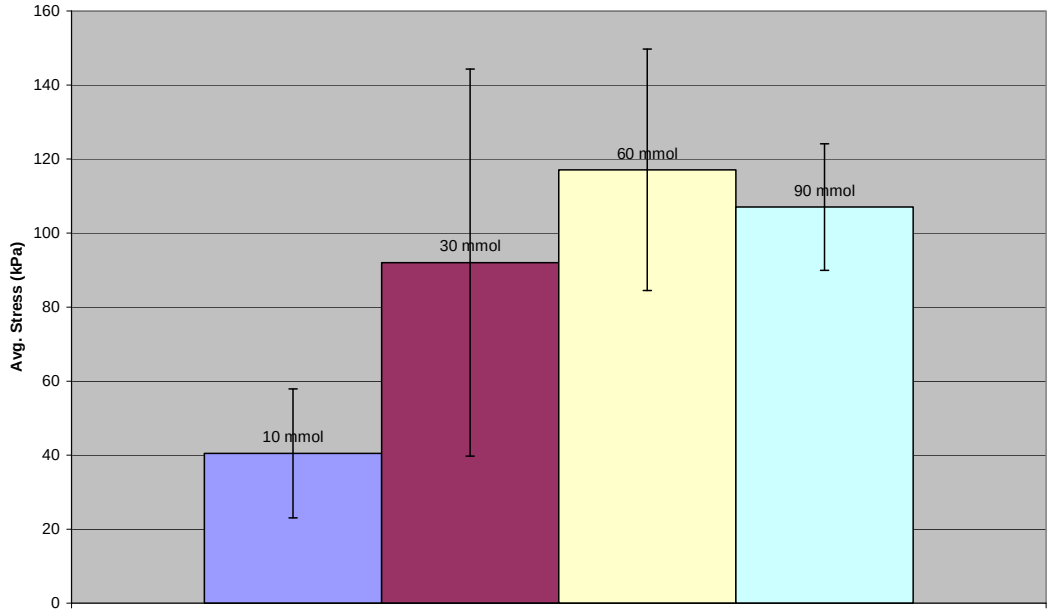
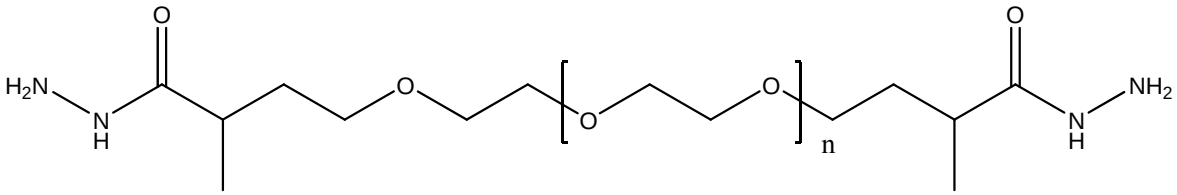


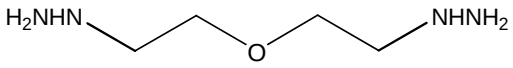
Figure 36. Average Peak Stress Chart for Compound **11** Dose Response Trial

Following experiments using compound **11**, purified versions of the previous synthetic compounds (compound **7**, **8**, and **9**) were synthesized for direct comparison

experiments involving hydrazine, compound **11**, and the new pure compounds **12** and **13** (Figure 37). Improved syntheses of the hydrazide cross-



Compound **12**



Compound **13**

Figure 37. Structure of Compounds **12** and **13**

linking agents utilized tert-butyl carbazate rather than free hydrazine, which protected the synthesized chains from polymerizing. However, both hydrazine and compound **11** displayed greater peak stress than either purified synthetic compound (Figure 38). Although compound **11** displays a greater average peak stress than hydrazine, there is no statistical evidence to support one over the other.

The final series of experiments in this study explored the use of naphthalimide compounds (compounds **14**, **15**, **16**, and **17**) as photo-oxidizing agents. Efforts to use these compounds as photo-oxidizing agents in place of periodate oxidation have thus far been unsuccessful. Any observable adhesion failed to exceed control levels, and in the case of chemiluminescence testing there was no signal detectable above background levels. Our research group continues to work with these napthalimide compounds, and has not ruled out their potential usefulness in tissue modification or bonding.

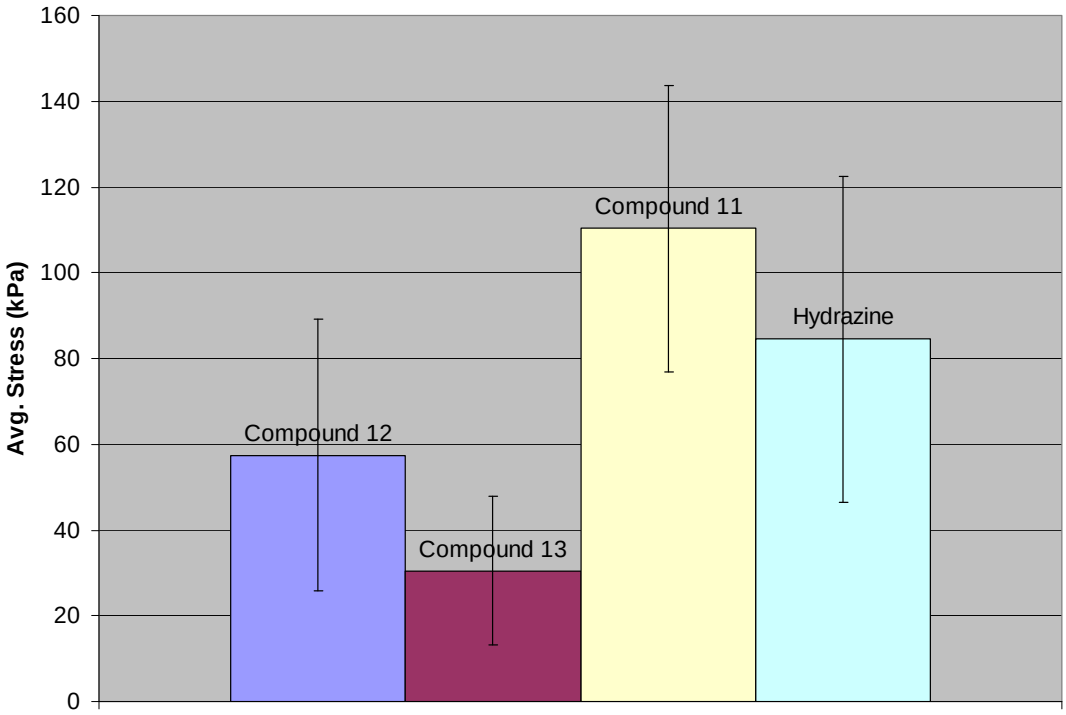


Figure 38. Average Peak Stress Chart for Experiment 19

CHAPTER TWO

Exploration of Sonochemical Modification

In addition to the ongoing research in meniscal bonding, our group was given the opportunity to collaborate with a Seattle based ultrasonic therapy research company called Acoustx. They contacted our group after reading about some of the photo-chemical cross-linking that had been done in our group and others, and the use of some photo-chemical dyes as sonochemical agents, and we agreed to work with them to investigate whether sonification would influence the reactivity of tissue surfaces.

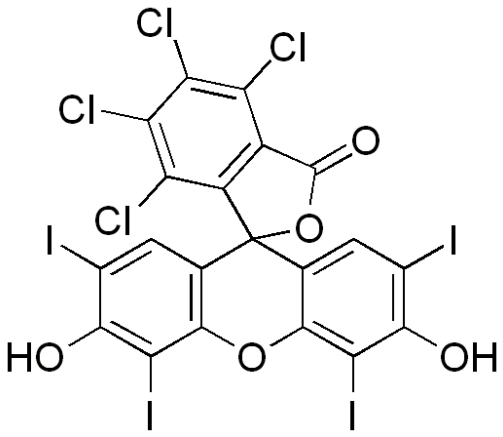


Figure 39. Structure of Rose Bengal

The dye Rose Bengal (Figure 39) was shown to enhance the cell damage induced by untrasound by Umemura while exploring its sonochemical effects on Sarcoma 180 cells *(26)*. Umemura also demonstrated the enhancement of ultrasound induced cell damage in Sarcoma 180 cells using hematoporphyrin (Figure 40) dye *(27)*. In unrelated

studies, Rose Bengal had also been reported to photo-chemically facilitate enhanced adhesion of porcine skin grafts by Redmond *(28)*. Our goal was to explore the possibility

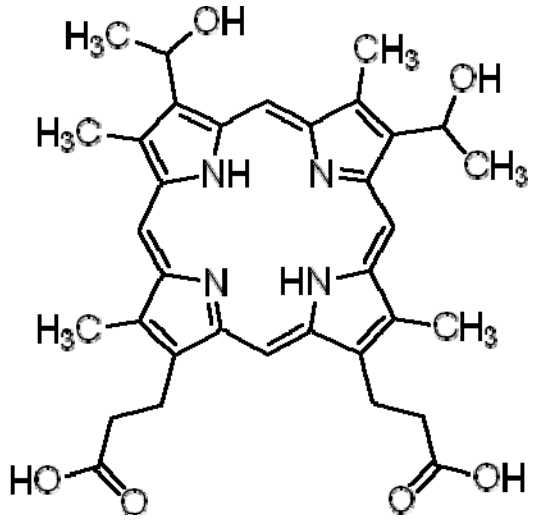


Figure 40. Structure of Hematoporphyrin

of using sonochemical excitation to enhance the chemical modification of tissue surfaces. Due to constraints on resources and time, only two reagents and two tissue types were explored.

The compounds for study dyes were obtained by Acoustx. We duplicated the solutions used in Umemura's work with Sarcoma 180 cells for our experimentation; a 195 mmol concentration of hematoporphyrin in PBS and a 160 μmol concentration of Rose Bengal in PBS. We also used a third solution which was a 10 mmol concentration of Rose Bengal in PBS, which was approximately the same as Redmond (*28*). Porcine skin and porcine aorta were obtained by Acoustx to be used in the experiment (Redmond used porcine skin in his studies). Three biotin reagents (from Pierce Biotechnology (Rockford, IL)), Compound **2** (Figure 7), compound **5** (Figure 8), and a biotinylated N-

hydroxysuccinimide (Figure 41, compound **18**), were employed to test for the chemical modification of the tissue surfaces after the sonochemistry. DMSO was used to dissolve

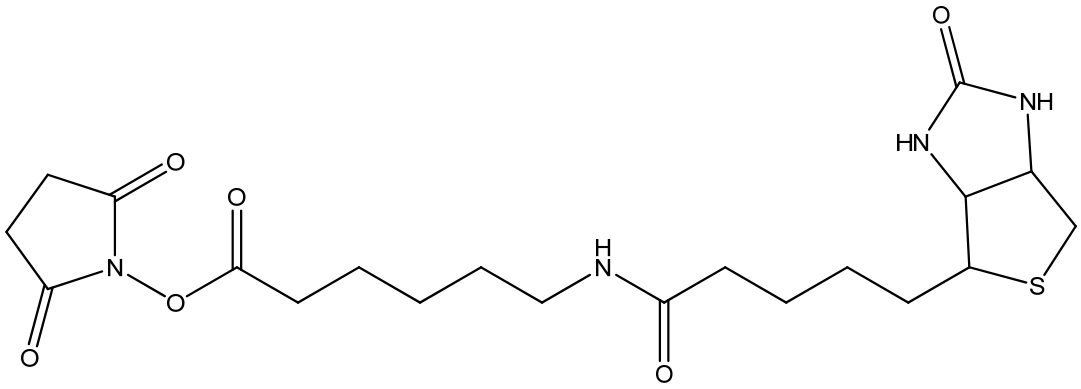


Figure 41. Structure of NHS-biotin with Linker Chain

compound 18 and was obtained commercially from Sigma-Aldrich (St. Louis, MO). The 12-well plates used for alkylation, reactions with avidin-HRP and luminol, and frozen transport were obtained commercially from VWR International (West Chester, PA). Any other materials were available on site at the Acoustx lab where the experiment was performed.

Each of the dye solutions was tested on each tissue type and then each biotinylation reagent was used to test for possible modifications: compound 2 (looking for reduction), compound 5 (looking for oxidation) and compound 18 (looking for free amine groups on surface proteins).

The experiments utilized a tank filled with water that held the ultrasound device and a holder for the samples that could be filled with a separate solution that the tank, which was custom manufactured by Acoustx. Inside the holder fit a simple apparatus the held the tissue taut and centered it in the holder. The apparatus also had a section cut out of it that allowed for an approximately 1inch by 1inch section to be direction exposed to

the solution in the holder. For the purposes of the experiment, the holder was filled with the dye solutions previously mentioned for each isonification reaction. Four samples per solution for four solutions with two different tissue types gave a told of 32 experimental samples. Figure 42 depicts the reaction process involved in this experiment.

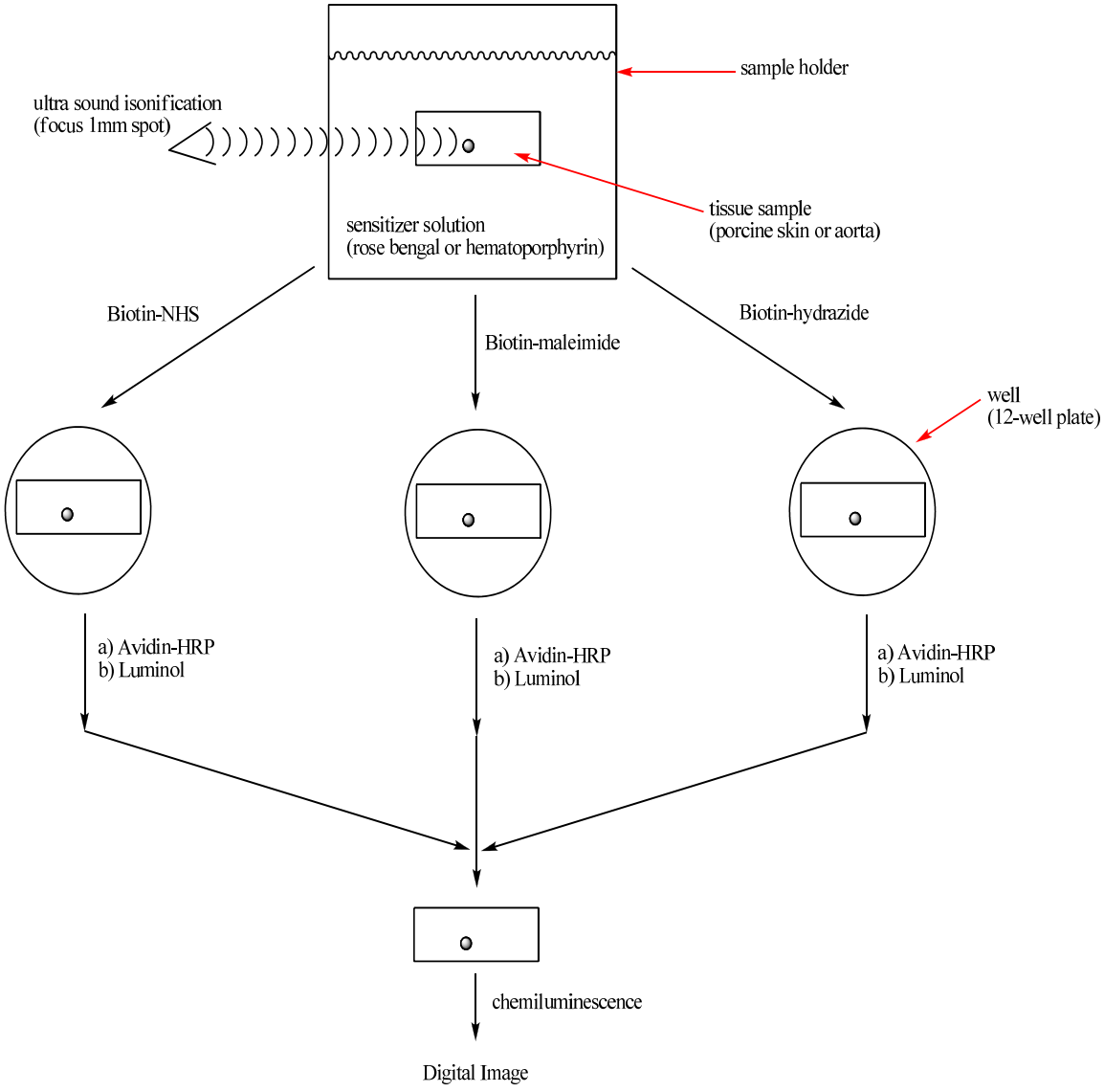


Figure 42. Sonochemistry Reaction Diagram

All spots were insonified for 5 minutes with a focused 2 MHz ultrasound transducer. The spot size was approximately 1 mm and the sample was located in the far field (beyond) the focus. The ultrasound intensity was the following: spots #1 and 6 ultrasound intensity was ~1 Watt/cm2, continuous exposure; spots #2 and 5 ultrasound intensity was ~5 Watts/cm2, continuous exposure; and spots #3 and 4 ultrasound intensity was ~50 Watts/cm2, with a 10% duty cycle (Figure 31).

After isonification the exposed section was cut out and the samples were washed in PBS buffer. Alkylating agents were added to their respective samples and all samples were incubated at 37 degrees Celsius for approximately 15 hours. After alkylation the samples were washed again and then frozen for transport.

After returning to Waco with the frozen samples, they were reacted with an approximately 0.6% solution of Avidin-HRP in PBS. The samples were allowed to react for 2 hours at room temperature. The samples were then washed with PBS and treated with a 50:50 luminol/peroxide enhancer solution for 3.5 minutes as described by the Super Signal kit from Pierce. The excess luminol was then blotted off and the samples were imaged using the Ultra-lum Discovery Chemiluminescence system at Baylor University (Figure 43).

Ultimately there was no evidence to suggest that the insonification of the tissue with or without the sonosensitzers had any effect on the tissue samples. Though a number of variables were explored and it was decided to discontinue any further exploration, there is still room for experimentation to either discover or discount fully the hypothesis that sonochemisty can modify tissue in this manner.

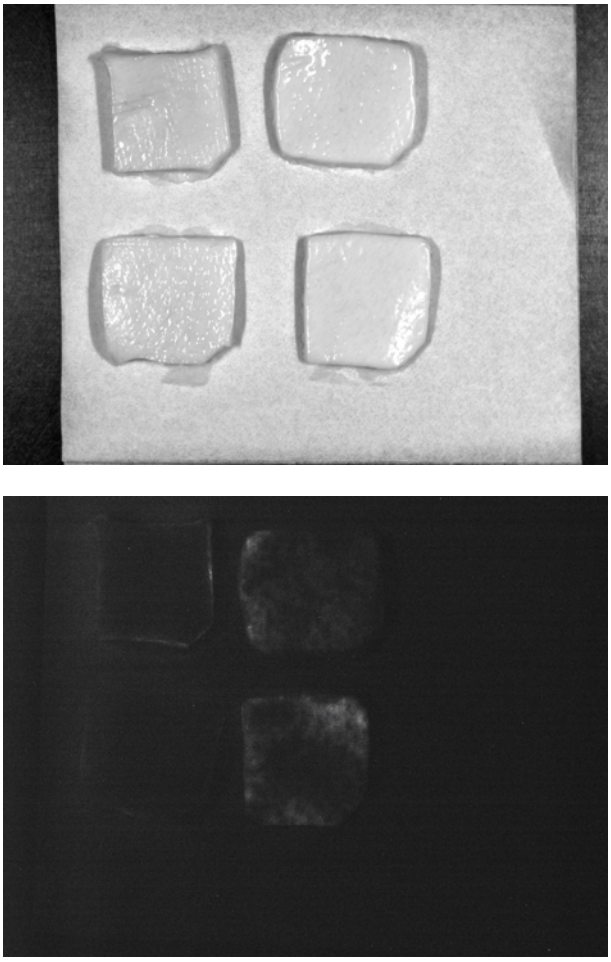


Figure 43. Photo and Chemiluminescent Image of Porcine Skin Control Samples

APPENDIX

Appendix

Data Tables

Table A1. Experiment 16 Experimental Average Peak Stress Data

Sample Pair	3.25 mmol	7.5 mmol	15 mmol	30 mmol	60 mmol	90 mmol
1	23.9 kPa	31.5 kPa	58.8 kPa	51.3 kPa	51 kPa	17.1 kPa
2	27.3 kPa	22 kPa	19.2 kPa	51.1 kPa	38.9 kPa	26.8 kPa
3	3.35 kPa	49.2 kPa	27.8 kPa	59.1 kPa	14.2 kPa	59.8 kPa
4	5.77 kPa	42 kPa	26 kPa	35 kPa	37.4 kPa	21.8 kPa
5	25.7 kPa	16.6 kPa	24.3 kPa	16.4 kPa	21.7 kPa	54.8 kPa
6	26.7 kPa	16.3 kPa	27.3 kPa	82.8 kPa	60.5 kPa	17.4 kPa
7	10.1 kPa	12.8 kPa	5.37 kPa	20.2 kPa	48.5 kPa	28 kPa
8	4.45 kPa	18.8 kPa	16.8 kPa	38.2 kPa	17.6 kPa	35.8 kPa
9	0 kPa	10.2 kPa	65.3 kPa	68.6 kPa	13.5 kPa	28.7 kPa
10	30 kPa	40.9 kPa	64.2 kPa	52.7 kPa	24.2 kPa	18.5 kPa

Table A2. Experiment 17 Experimental Average Peak Stress Data

Sample Pair	1 hour	3 hours	6 hours	9 hours	12 hours	15 hours
1	12.3 kPa	41.2 kPa	52.9 kPa	31 kPa	34.3 kPa	27.3 kPa
2	21.1 kPa	16.6 kPa	44.6 kPa	46.7 kPa	27.6 kPa	17.6 kPa
3	13.6 kPa	25.3 kPa	34.3 kPa	35.4 kPa	18.3 kPa	22.5 kPa
4	6.2 kPa	21 kPa	19.8 kPa	58.3 kPa	45.2 kPa	34.4 kPa
5	9.4 kPa	33.4 kPa	46 kPa	53.5 kPa	27.4 kPa	42.6 kPa
6	27.2 kPa	37.1 kPa	28.2 kPa	63.3 kPa	24.1 kPa	28.8 kPa
7	23.5 kPa	24.7 kPa	26.7 kPa	46.2 kPa	50.1 kPa	47.2 kPa
8	19.7 kPa	26.9 kPa	22.4 kPa	38.8 kPa	44.7 kPa	32.1 kPa
9	16.3 kPa	31 kPa	29.3 kPa	42.7 kPa	20.9 kPa	25.7 kPa
10	15 kPa	19.5 kPa	36.4 kPa	43.6 kPa	35.5 kPa	31 kPa

Table A3. Experiment 18 Experimental Average Peak Stress Data

Sample Pair	10 mmol	30 mmol	60 mmol	90 mmol
1	25 kPa	76.2 kPa	89.7 kPa	111 kPa
2	42 kPa	31.9 kPa	171 kPa	114 kPa
3	30.5 kPa	157 kPa	118 kPa	121 kPa
4	64.3 kPa	103 kPa	114 kPa	77.2 kPa
5	40.4 kPa	92 kPa	92.8 kPa	112 kPa

Table A4. Experiment 19 Experimental Average Peak Stress Data

Sample Pair	Hydrazine	Compound **11**	Compound **12**	Compound **13**
1	46.5 kPa	133 kPa	70.7 kPa	19.7 kPa
2	104 kPa	143 kPa	20.9 kPa	25.9 kPa
3	53.5 kPa	77.7 kPa	12.1 kPa	10 kPa
4	71.2 kPa	126 kPa	16 kPa	3.53 kPa
5	Lost	161 kPa	113 kPa	33.1 kPa
6	122 kPa	135 kPa	36.8 kPa	22.2 kPa
7	149 kPa	159 kPa	107 kPa	55.9 kPa
8	120 kPa	98.5 kPa	105 kPa	53.8 kPa
9	82.7 kPa	118 kPa	85.7 kPa	26.8 kPa
10	77.4 kPa	82.1 kPa	52.9 kPa	4.98 kPa
11	96.2 kPa	136 kPa	27.1 kPa	36.6 kPa
12	166 kPa	104 kPa	43.3 kPa	30.2 kPa
13	82.2 kPa	153 kPa	66.8 kPa	11.5 kPa
14	35.7 kPa	97 kPa	24.3 kPa	33.5 kPa
15	56.9 kPa	77.4 kPa	56.8 kPa	19.2 kPa
16	145 kPa	148 kPa	78.5 kPa	60.6 kPa
17	7.38 kPa	94.4 kPa	58.3 kPa	66.9 kPa
18	90.3 kPa	130 kPa	94.5 kPa	27.1 kPa
19	43.7 kPa	53.4 kPa	35.2 kPa	43.8 kPa
20	51.3 kPa	71.2 kPa	99 kPa	39.9 kPa
21	74.7 kPa	56.5 kPa	34 kPa	23.9 kPa
22	72.9 kPa	146 kPa	36 kPa	51.2 kPa
23	110 kPa	74 kPa	91.8 kPa	12.3 kPa
24	81.7 kPa	78 kPa	25 kPa	28.7 kPa
25	87.2 kPa	105 kPa	45.9 kPa	20.6 kPa

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