ABSTRACT

Modification of Fresh Tissue Surfaces; Synthesis of Labeled
L-Dopa Analogs; and Synthesis of Metoclopramide Analogs

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Mentor: Robert R. Kane, Ph.D.

One of the main goals of the primary project was to develop methods to site specifically immobilize functional proteins on a tissue surface. There are many examples of proteins that have been chemically modified and attached onto solid surfaces such as silica or gold. In those studies, proteins were chemically modified to introduce functional groups, and in most cases, the solid surface was also chemically modified to introduce functional groups onto its surface. The proteins were then immobilized onto the surface using a chemical ligation reaction. However, to the best of our knowledge there are no examples reported in literature where functional proteins have been covalently attached in a site specific fashion onto the surface of a fresh tissue. We hypothesized that the methods used for modifying and immobilizing biomolecules onto solid surfaces could be adapted for use on fresh tissue surfaces.

Several different strategies were investigated for the immobilization of the model biomolecules. A direct alkylation method involved reacting a biotin or fluorescent marker (bearing an amine reactive species such as an active ester) directly to the $-NH_2$

groups present on the tissue surface. Reductive methods involved the reduction of the tissue surface with a thiol reducing agent to give free sulfhydryl. The reduced tissue surface was then reacted with a protein or other molecule bearing a thiol reactive species. An oxidative technique used periodiate to oxidize geminal diols (such as those found in proteoglycans in the ECM of tissue) to carbonyl compounds. The oxidation was followed by the reaction of the tissue surface with nucleophilic reagents, such as hydrazides.

Aromatic L-amino acid dercarboxylase (AADC) is an enzyme that converts 3, 4-dihydroxyphenylalanine (L-dopa) to dopamine and 5-hydroxytryptophan to serotonin. Inherited deficiency of this enzyme leads to decreased levels of these two neurotransmitters, resulting in severe early onset neurological disorders. In the absence of AADC activity, L-dopa is methylted to 3-0-methyl dopa which is then accumulated in blood, urine and cerebrospinal fluid in infants and children with a deficiency of this enzyme. 3-O-methyl dopa therefore, provides a biochemical marker that can be used to screen for this disease. Thus, the goals of the second project were to synthesize a labeled analog of 3-O-methyldopa for the use in mass spectrometry for the screening of AADC deficiency.

The clinical usefulness of high dose MCA as a radio-and chemosensitizer is limited by its central nervous system (CNS) side effects. Primary side effects include drowsiness, acute extrapyramidal reactions, akathisia (generalized motor restlessness) and drug induced Parkinson. MCA has also been shown to increase irritability and anxiety. These CNS side effects are chemically related to the ortho-methoxy group in MCA, which planarizes the molecule and allows it to have a high affinity for dopamine

D_2 receptors. Since the ortho-methoxy group was suggested to be the cause of the CNS side effects, we hypothesized that modifications at the ortho-methoxy group could decrease the CNS side effects and increase cytotoxicity. Several derivatives of the O-substituted MCA were synthesized and their structure activity relationship (SAR) was studied.

Modification Of Fresh Tissue Surfaces; Synthesis Of Labeled L-Dopa Analogs; And Synthesis Of Metoclopramide Analogs

by

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AADC	aromatic L-amino acid dercarboxylase
Ala	alanine
BP	bovine pericardium
BMP	bone morphogenetic proteins
BSA	bovine serum albumin
CB	coomassie blue
CNS	central nervous system
CS	chondroitin sulfate
Cys	cystine
DD	deionized distilled
DMF	dimethyl formamide
DMSO	dimethyl sulfoxide
DTT	dithiolthreitol
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetate
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
FITC	fluorescein isothiocyanate
FRET	fluorescence resonance energy transfer
GAG	glycosaminoglycans

GlcNAc	N-acetylglucosamine
GPTS	glycidoxypropyltrimethoxysilane
HPLC	high performance liquid chromatography
HRP	horse radish peroxidase
Hyp	hydroxyproline
IC50	growth inhibitory constant 50%
IGF-I	Insulin-like growth factor
LC	long chain
Lys	lysine
mAb	monoclonal antibodies
MCA	metoclopramide
MS	mass spectrometry
NGF	nerve growth factor
NHS	N-hydroxysuccinimide
PBD	phosphate buffered sailine, pH 7.4
PEG	polyethylene glycol
PDGF	platelet derived growth factor
PG	proteoglycans
PDMS	polydimethylsiloxane
PTB	photochemical tissue bonding
PVDF	polyvinilidene fluoride
SAR	structure activity relationship
SIL	stable isotopically labeled

TEA	triethyl amine
TCEP	tris(2-carboxyethyl)phosphine
THF	tetrahydrofuran
TF	tissue factor
TFA	trifluoroacetic acid
TLC	thin layer chromatography
UV	ultraviolet

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Before the throne of God above

I have a strong and perfect plea.

A great high Priest whose Name is Love

Who ever lives and pleads for me.

My name is graven on His hands,

My name is written on His heart.

I know that while in Heaven He stands

No tongue can bid me thence depart.

Because the sinless Savior died

My sinful soul is counted free.

For God the just is satisfied

To look on Him and pardon me.

One in Himself I cannot die.

My soul is purchased by His blood,

My life is hid with Christ on high,

With Christ my Savior and my God!

To all who wander and wonder--may you find that which you seek

CHAPTER ONE

Tissue Modification

Section One: General Introduction to Protein Modification and Immobilization, Collagen, Tissue Types, and Meniscal Repair

Proteins are key players in the biological processes that make up life. They are constructed from one or more chains of amino acids; that is, they are polymers. A typical protein contains 200–300 amino acids but some are much smaller (the smallest are often called peptides) and some much larger (the largest to date is titin, a protein found in skeletal and cardiac muscle that contains 26,926 amino acids in a single chain). Proteins make up about 15% of the mass of the average person. Protein molecules are essential to us in an enormous variety of different ways.[1]

Proteins function as enzymes, which catalyze the complex set of chemical reactions that are collectively referred to as life. Proteins also serve as regulators of these reactions, both as enzymes and chemical messengers, known as hormones. They act to transport and store biologically important substances such as metal ions, oxygen, glucose, lipids and many other molecules. In the form of muscle fibers and other contractile assemblies, proteins generate coordinated mechanical motion of numerous biological processes, such as the movement of arms and eyes. The proteins of the immune system, such as the immunoglobulins, form an essential biological defense system in higher animals. Proteins on cell membranes help govern complex cellular transport mechanisms and cell division. Proteins are major active elements in, as well as the products of,

the expression of genetic information. However, proteins also have important passive roles, such as that of collagen, which provides bones, tendons and ligaments with their characteristic tensile strength. The old cliché that the proteins are the building blocks of life still hold true.[1]

The main goal of this research was to develop methods to site specifically immobilize proteins on a tissue surface. A biomolecule is a molecule which engages in biological activity or is effective in modulating biological activity. Biomolecules include proteins, enzymes, antibodies, peptides, carbohydrates, and fatty acids. More specifically, biomolecules can be growth factors, such as endothelial growth factor, and platelet derived growth factor; antimicrobial agents such as lysozyme or penicillin; antithrombic agents such as albumin; thrombic agents such as heparin; a thrombogenic agent such as collagen or hyaluronic acid.[2]

There are many examples of proteins that have been chemically modified and attached onto solid surfaces such as silica or gold. In these types of methods, proteins were chemically modified to introduce functional groups, and in most cases the solid surface was also chemically modified to introduce functional groups onto its surface. The proteins were then immobilized onto the surface using a chemical ligation reaction. However, to the best of our knowledge there are no examples reported in literature where proteins have been covalently attached in a site specific fashion onto the surface of a fresh tissue. We hypothesized that the methods used for modifying and immobilizing biomolecules onto solid surfaces could be adapted for use on fresh tissue surfaces. These methods hinged upon our ability to modify proteins and tissue surfaces to produce the desired ligation reaction, resulting in the immobilization. Therefore, we drew upon well

established methods for protein modification and protein immobilization. Discussed below are some of the more common aspects of protein modification.

Protein Modification

Background Information

The chemical modification of proteins is a common phenomenon. Proteins can be modified by biological or chemical methods. The biological modification of proteins usually occurs naturally after translation or un-naturally by using molecular biology techniques to introduce un-natural amino acids into proteins.[3] Recently developed protein engineering modifications utilize site directed mutagenesis to introduce non-coding amino acids. These proteins with un-natural amino acids can then be further modified using chemical methods.[4]

Biological Methods

Almost all proteins are post-translationally modified[7]. This ocurs when the primary structure of the protein is altered after the protein has been translated and is already folded. There are a wide range of modifications that can take place during post-translational modification. These modifications act on individual residues either by cleavage at a specific point, deletions, additions, or having the side chains converted or modified. There are, in total, over 200 amino acids that have been found in proteins after modification.[5]

There are several common post-translational modifications, including, glycosylation, acylation of the N-terminus, amidation of the C-terminus, iodination, and crosslinking.[8,9] One of the most common post-translational modifications of proteins is glycosylation, the covalent attachment of oligosaccharides. The carbohydrates are

attached to serine and thionine residues, to the N-amide atom of asparagine side chain, or in the special case of collagen, to the hydroxyl-lysine residue. There is also the acylation of the N-terminus, in which the initial methionine is hydrolyzed and an acetyl group is added to the new N-terminal amino acid by Acetyl CoA. The most common modification of the C-terminus is amidation and this occurs after the cleavage of a peptide, where the last residue is glycine.[6] The glycine residue is then broken down leaving the amino group. Iodination occurs exclusively in the tyrosine residues of the protein thyroglobulin in the thyroid gland of vertebrates. Covalent crosslinks within or between polypeptide chains is another example of post-translation modification. In globular proteins crosslinks can be formed between two cystine residues, known as disulfide bridges. Amide crosslinks are also formed between lysine and glycine residues. The amide crosslinks are present most commonly in hair, wool and the epidermis. There are many more modification of the amino acid residues such as methylation, sulfonation, phosphorylation, and cleavage of residues.[8]

Chemical Methods

Proteins and peptides are amino acid polymers containing a number of reactive side chains.[7] In addition to, or as an alternative to, these intrinsic groups, specific reactive moieties can be introduced into the polymer chain by chemical modification. These groups, whether or not they are naturally part of the proteins, or artificially introduced, serve as handles for a variety of protein modification reagents.[8] Therefore, chemistry of proteins has its origins in the chemistry of these amino acids.[9, 10]

The ability to modify proteins rests on the reactivity and availability of the reactive groups on the amino acid residues.[11] The use of formaldehyde in the tanning

industry was one of the first examples of protein modification using chemical methods.[12, 13] These reactions were carried out entirely on the basis of empirical observation, without any real understanding of the chemical nature of the reactions involved. [14] As our understanding of proteins grew and amino acid structures were deciphered, the chemistry of protein modification started to be refined as well.

There are many strategies that have emerged for the chemical modification of proteins, and more specifically the amino acid residues.[2] The amino acid residues in proteins present a host of reactive functional groups, the most common being amine groups, sulfhydryl groups, hydroxide groups and carboxylic acid groups. The functional groups can be used to selectively modify amino acid residues and hence the whole protein.[15] Site specific modification of proteins and peptides provides a powerful research tool in chemistry and biology. Site specific chemical modification has been extensively used to identify chemical groups on proteins which are involved in biological function. General applications have included the identification of amino acids that participate in catalysis, or that participates in protein-protein interactions, and the attachment of prosthetic groups which can be used to monitor conformational changes.[16, 17]

The chemical reactions to modify proteins range from the general to highly focused. At one extreme, the reactions are 'residue' or type specific in that side chains of one or more types of amino acids (e.g. the ε amino group of lysine) are modified.[18] In this situation, the reagent is usually very reactive and the ability of a given residue of the class to react with the modifying agent is largely determined by the accessibility and environment of the residue.[19, 20] Modifications of this type can be used to map protein

topography, but they also have wide applications in changing the properties of the protein, either as an analytical approach to study some feature (e.g. subunit structure) or as an aid to isolation.[21] At the other extreme, the modification of a single amino acid residue in a protein can be effected as a reflection of the unique reactivity of the residue, usually associated with some functional aspect of the protein (e.g. the modification of the active site serine residue in serine proteases and esterases).[22] Intermediate applications, where the modification of a few members of a given residue type occurs, can also provide important information about protein-protein interactions, ligand binding sites and surface distribution.[23, 14] This process is sometimes referred to as 'regiospecific' modification and is illustrated by the use of acetic anhydride to determine the reactivity of lysine residues in proteins.[24, 25, 26]

The reagents and methods to selectively chemically modify proteins are becoming increasingly important for the study and utilization of protein functions and interactions, particularly due to the explosive growth of our knowledge of protein sequences from genomic methods and the drive to conduct proteomic research.[27] The most general approach is to use electrophilic reagents that react with nucleophilic groups on the surface of the protein.[22] Discussed below are some of the more common intrinsic reactive groups of proteins and chemical reagents used to modify them.[28]

1. Amines (Lysines, α-Amino Groups

One of the most common reactive groups of proteins is the aliphatic ε-amine of the amino acid lysine. Lysines are usually present to some extent in most proteins, and are often quite abundant.[29] For example, the protein, bovine insulin, contains only a single lysine while avidin, a protein found in egg whites, contains 36 lysines. Lysine ε-

amines are reasonably good nucleophiles above pH 8.0 (pKa = 9.18) and therefore react easily and cleanly with a variety of reagents to form stable bonds.[30] Another reactive amine found in proteins is the α-amino groups of the N-terminal amino acids. The α-amino groups are less basic than lysines ε-amine and are reactive at around pH 7.0 and they can occasionally be modified in the presence of lysines. There is usually at least one α-amino acid in a protein, and in the case of proteins that have multiple peptide chains or several subunits, there can be more than one for each peptide chain or subunit. For example, bovine insulin has one N-terminal glycine residue and one N-terminal phenylalanine.[31].

There are also proteins that do not possess free α-amino groups, such as cytochrome C and ovalbumin. In these molecules, the N-terminal amino group is acylated, and therefore not reactive to the usual modification reagents[27] Since either N-terminal amines or lysines are almost always present in any given protein or peptide, and since they are easily reacted, the most commonly used method of protein modification is through these aliphatic amine groups.[27]

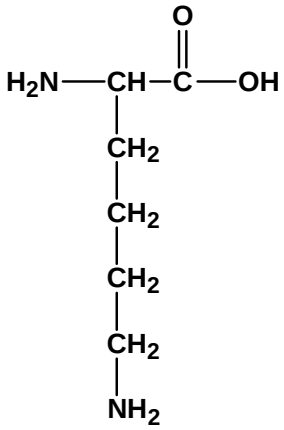


Figure 1. Structure of lysine

There are many reagents available for the modification of amine groups in proteins. These reagents will react primarily with lysines and the free amino groups in proteins and peptides under both aqueous and nonaqueous conditions. Some amine-reactive reagents are more reactive, and therefore less selective, than others, and it will be necessary to understand this property in order to choose the best reagent for modification of a specific protein.[32] The following amine-reactive reagents are a sample of the commercially available reagents for such protein modifications.

(a)*Reactive Esters (Formation of an Amide Bond)*

Reactive esters, especially N-hydroxysuccinimide (NHS) esters, are among the most commonly used reagents for modification of protein and peptide amine groups.[33] These reagents have intermediate reactivity toward amines, with high selectivity toward aliphatic amines. Their reaction rate with aromatic amines, alcohols, phenols (tyrosine), and histidine is relatively low. Reaction of NHS esters with amines under nonaqueous conditions is facile, so they are useful for derivatization of small peptides and other low molecular weight biomolecules.[34, 35]

The optimum pH for reaction in aqueous systems is 8.0-9.0. The aliphatic amide products which are formed are very stable (Figure 2). The NHS esters are slowly hydrolyzed by water at this pH, but are stable to storage if kept well desiccated.[36] Virtually any molecule that contains a carboxylic acid or that can be chemically modified to contain a carboxylic acid can be converted into its NHS ester, making these reagents among the most powerful protein-modification reagents available. Newly developed NHS esters are available with sulfonate groups that provide increased water solubility.[37]

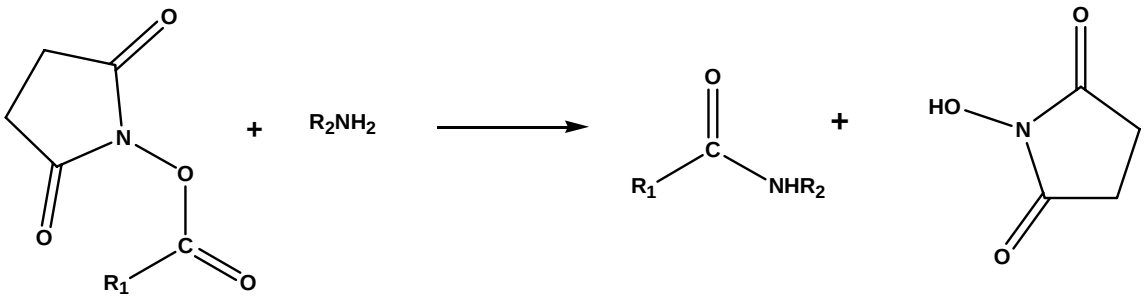


Figure 2. Reaction of NHS ester with primary amine

(b) Isothiocyanates (Formation of a thiourea bond).

Isothiocyanates, like NHS esters, are amine-modification reagents of intermediate reactivity and form thiourea bonds with proteins and peptides (Figure 3).[38] Isothiocyanates are somewhat more stable in water than the NHS esters and react with protein amines in aqueous solution optimally at pH 9.0-95. Since this is a higher pH than the optimal pH for NHS esters (which undergo competing hydrolysis at pH 9.0-9.51, isothiocyanates may not be as suitable as NHS esters when modifying proteins that are sensitive to alkaline pH conditions.[39]

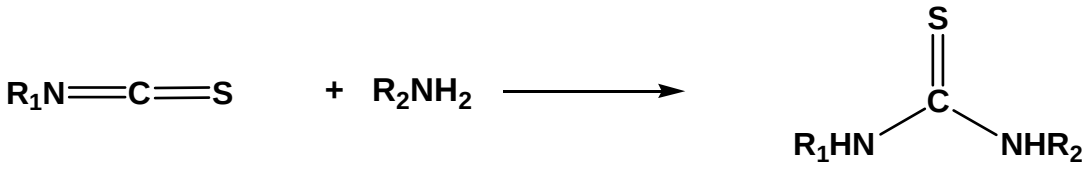


Figure 3. Reaction of an isothiocynate with an amine

One of the most commonly used fluorescent derivatization reagents for proteins is fluorescein isothiocyanate (FITC).[40] A number of other fluorescent dyes (coumarins and rhodamines) have been coupled to proteins via their reactive isothiocyanates.

(c)Aldehydes (Formation of Imine, Reduction to Alkylamine Bond)

Aldehyde groups react under mild aqueous conditions with aliphatic and aromatic amines to form an intermediate known as a Schiff base (an imine), which can be selectively reduced by the mild reducing agent sodium cyanoborohydride to give a stable alkylamine bond (Figure 4).[41]

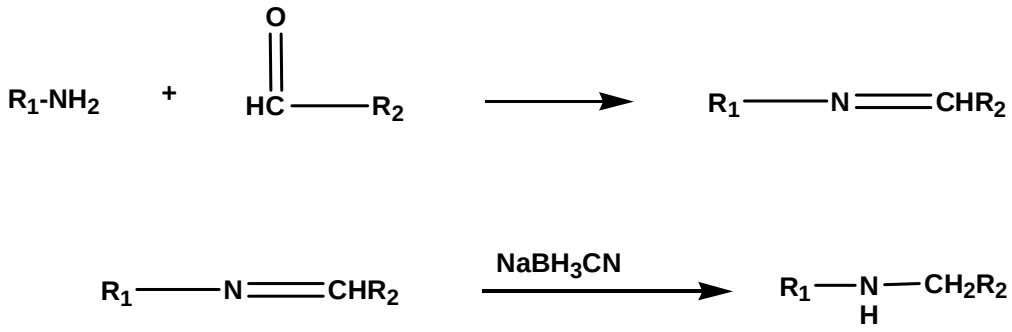


Figure 4. Reaction of an aldehyde with primary amine

This method of amine modification is not used in protein conjugations as frequently as the activated ester method, but when the molecule to be attached has an aldehyde group or can be easily converted to an aldehyde the method is mild, simple, and very effective. Aldehydes can also react with protein arginine groups[42, 43] and the nucleic acid base guanosine, making them of some use in nucleic acid modification.[44]

(d) Sulfonyl Halides (Formation of a Sulfonamide Bond)

Sulfonyl halides are highly reactive amine modifying reagents.[45] They are unstable in water, especially at the pH required for reaction with aliphatic amines[46], but they form extremely stable sulfonamide bonds which can survive even amino acid hydrolysis.[47] It is for this reason that sulfonamide conjugates are useful for amine terminus derivatization (ie, Dansyl-Edman degradation) and as tracers.[48] In addition to

amines, sulfonyl halides also react with phenols (e.g. tyrosine), thiols (e.g. cysteine), and imidazoles (histidine) on proteins; therefore, they are less selective than either NHS esters or isothiocyanates. The conjugates formed with thiols, imidazoles, and phenols are all unstable and, if not removed during purification, can lead to loss of the label from the protein during long-term storage. One of the most widely used long-wavelength fluorescent probes, Texas Red, is a sulfonyl chloride. It has the longest wavelength spectral properties of any of the common amine-reactive fluorescent labeling reagents.[49]

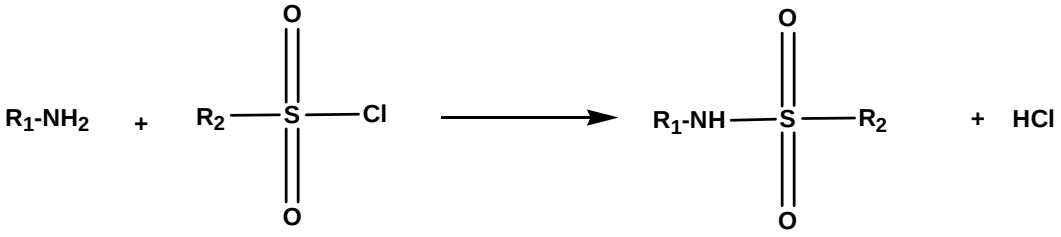


Figure 5. Reaction of a sulfonyl chloride with primary amine

(e) Miscellaneous Amine Reactive Reagents (Dichlorotriazines, Alkyl Halides, Anhydrides)

The dichlorotriazine derivative of fluorescein, known as DTAF (Figure 6), has high reactivity with protein amines and has been used to prepare fluorescein tubulin with minimal loss of activity.[50] In addition to amines, dichlorotriazines will react with alcohols at elevated temperatures (60-90 ^{0}C) and are used to prepare polysaccharide conjugates.[51] Some alkyl halides, including iodoacetamides commonly used to modify thiols, will react with amines of proteins if the pH is in the range 9.0-9.5.[52] Other reagents that have been used to modify amines of proteins are carboxylic acid anhydrides. Succinic anhydride is commonly used to succinylate amine groups of basic proteins for

the purpose of changing their isoelectric point and other charge-related properties.[53] Mixed anhydrides derived from reaction of a carboxylic acid with carbitol or 2-methylpropanol chloroformates are excellent reagents for modification of amines under mild conditions. Of these, the carbitol mixed anhydride is relatively water soluble and is the preferred reagent for modification of amines in aqueous solution. [54]

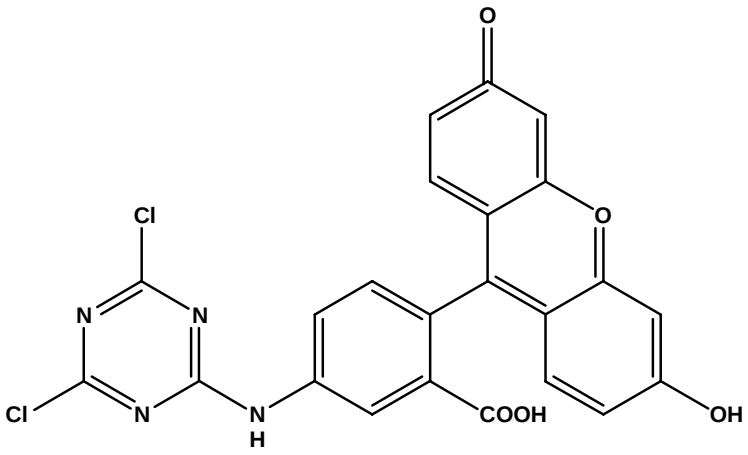


Figure 6. Structure of DTAF

(2) Thiols (Cystine, Cysteine, Methionine)

Another common reactive group in proteins is the thiol residue from the sulfur-containing amino acid cystine (Figure 7) and its reduction product cysteine (or half-cystine), which are counted together as one of the 20 amino acids.[55] Cysteine contains a free thiol group, more nucleophilic than amines, and is generally the most reactive functional group in a protein.

The cystein sulfhydryl reacts with some of the same modification reagents as do the amines discussed in the previous section, and in addition, can react with reagents that are not very reactive toward amines. Thiols, unlike most amines, are reactive at neutral

pH, and therefore they can be coupled to other molecules selectively in the presence of amines. This selectivity makes the thiol group the linker of choice for coupling two proteins together, since methods which only couple amines (e. g. glutaraldehyde, dimethyl adipimidate coupling) can result in formation of homodimers, oligomers, and other unwanted products.

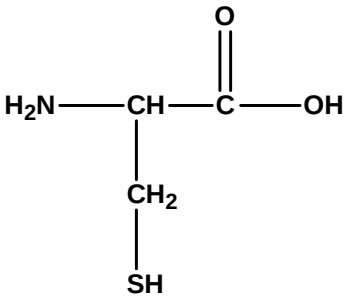


Figure 7. Structure of a cystine

Since free sulfhydryl groups are relatively reactive, proteins with these groups often exist in their oxidized form as disulfide-linked oligomers or have internally bridged disulfide groups. Immunoglobulin M is an example of a disulfide-linked pentamer, while immunoglobulin G is an example of a protein with internal disulfide bridges bonding the subunits together. In proteins such as this, reduction of the disulfide bonds with a reagent such as dithiothreitol (DTT) is required to generate the reactive free thiol.[56, 57] In addition to cystine and cysteine, some proteins also have the amino acid methionine, which contains sulfur in a thioether linkage. When cysteine is absent, methionine can sometimes react with thiol-reactive reagents such as iodoacetamides.[58] However, selective modification of methionine is difficult to achieve and therefore is seldom used as a method of attaching small molecules to proteins. Described below are some thiol reactive reagents available for modifications of SH groups on proteins.[56]

Thiol-reactive reagents are those that will couple to thiol groups on proteins to give thioether-coupled products.[59] These reagents react rapidly at neutral (physiological) pH and therefore can be reacted with thiols selectively in the presence of amine groups.[60]

(a) Haloacetyl Derivatives (Formation of a thioether bond).

These reagents (usually iodoacetamides) are among the most frequently used reagents for thiol modification (Figure 8).

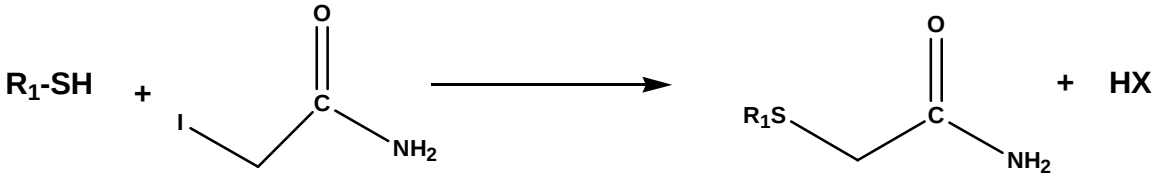


Figure 8. Reaction of an iodoacetamide with thiols

In most proteins, the site of reaction is at cysteine groups that are either intrinsically present or that result from reduction of cystines. The reaction of iodoacetate with cysteine is approximately twice as fast as that of bromoacetate and 20-100 times as rapid as that with chloroacetate[61]. As mentioned previously, in the absence of cysteines, methionines can sometimes react with haloacetamides. Reaction of haloacetamides with thiols occurs rapidly at neutral pH at room temperature or below, and under these conditions; most aliphatic amines are unreactive.[54, 55] In addition to proteins, haloacetamides have been reacted with thiolated peptides and thiolated primers for DNA sequencing, and also with RNA (on thiouridine).[62] The thioether linkages formed from reaction of haloacetamides are very stable. A potential problem in using iodoacetamides as modification reagents is their instability to light, especially in solution; therefore, they must be protected from light in storage and during reaction. The fluorescein and

rhodamine iodoacetamides are among the most intensely fluorescent sulfhydryl reagents available for protein and peptide modification.[63]

(b) Maleimides (Formation of a Thioether Bond).

Maleimides are similar to iodoacetamides in their application as reagents for thiol modification; however, they are more selective than iodoacetamides, since they do not react with histidine, methionine, or thionucleotides.[64] The optimum pH for the reaction of maleimides is near 7.0. Above pH 8.0, hydrolysis of maleimides to nonreactive maleamic acids can occur.[59]

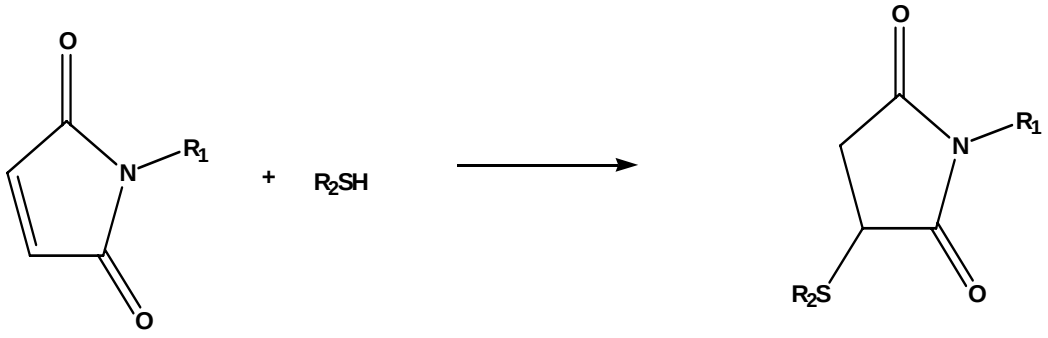
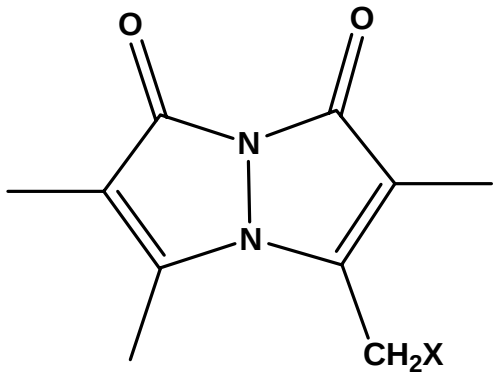


Figure 9. Reaction of a maleimide with thiol

(c) Miscellaneous Thiol-Reactive Reagents.

These reagents include bromomethyl derivatives and pyridyl disulfides. The bromomethyl derivatives are similar in reactivity to iodoacetamides. The haloalkyl derivatives monobromobimane and monochlorobimane (see Figure 10)[52] react with glutathione and other thiols in cells to give fluorescent adducts, thus providing a method of quantitation of thiols.[65] Pyridyl disulfides react in an exchange reaction with protein thiols to give mixed disulfides (see Figure 11).[52]



X = Cl, Br

Figure 10. Structures monobromobimane (X=Br) and monochlorobimane (X=Cl)

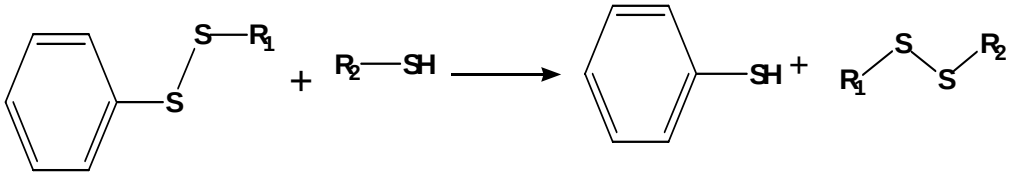


Figure 11. Pyridyl disulfide reaction with protein thiols to give mixed disulfides

(3) Phenols (Tyrosine)

The phenolic substituent of the amino acid tyrosine (Figure 12) can react in two ways. The phenolic hydroxyl group can form esters and ether bonds, and the aromatic ring can undergo nitration or coupling reactions with reagents such as diazonium salts at the position adjacent to the hydroxyl group. There is considerable literature describing the reaction of tyrosyl residues with diazonium compounds.[66] For example, a p-aminobenzoyl biocytin derivative has been diazotized and reacted with protein tyrosine groups.[67]

Proteins may be biotinylated specifically at tyrosine and histidine residues using p-diabenzoyl biocytin (DBB) (Figure 13). The biotinylation reagent is prepared immediately before use from p-aminobenzoyl biocytin, using HCl and sodium nitrite to

diazotize the precursor, yielding the reactive DBB compound. DBB reacts with the phenolic group of tyrosine and the imidazole group of histidine.[68]

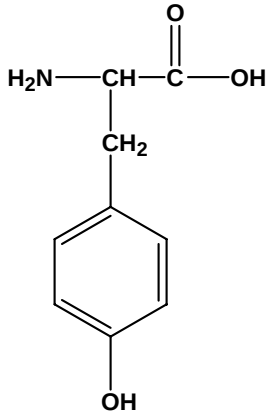


Figure 12. Structure of tyrosine

The modification of tyrosine has primarily been used in structural studies, rather than as a means for attaching specific labels, since acetylation and nitration can give useful information concerning the participation of tyrosine in the binding properties of proteins. Often, the reactivity of tyrosines with amine-selective modification reagents to form unstable carboxylic acid esters or sulfate esters is an unwanted side reaction resulting in conjugates that slowly hydrolyze during storage.

The electrophilic iodination of tyrosine can be accomplished with the use of catalysts such as chloramine T or lactoseperoxidase.[69] Tyrosine residues can also react with cyanuric fluoride via the phenolic hydroxyl group.[70]

(4) Carboxylic Acids (Aspartic Acid and Glutamic Acid)

Proteins contain carboxylic acid groups at the carboxyterminal position and within theside chains of the dicarboxylic amino acids aspartic acid and glutamic acid.

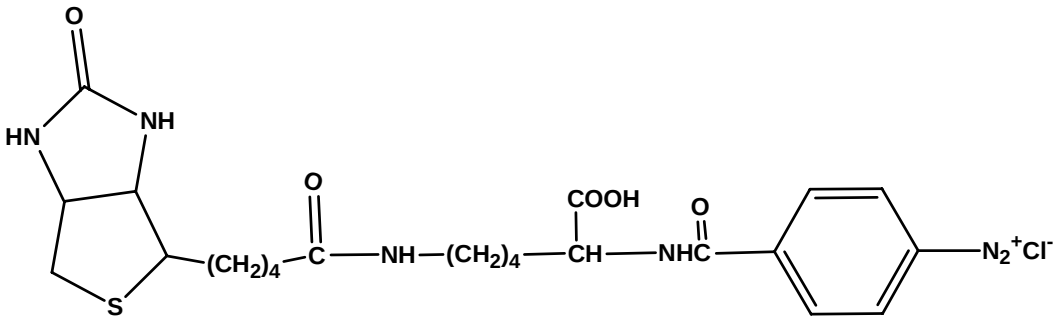


Figure 13. Structure of p-diabenzoyl biocytin (DBB).

The low reactivity of carboxylic acids in water usually makes it difficult to use these groups to selectively modify proteins and other biopolymers. In the cases where this is done, the carboxylic acid group is usually converted to a reactive ester by use of a water-soluble carbodiimide amine or a hydrazide.[71]

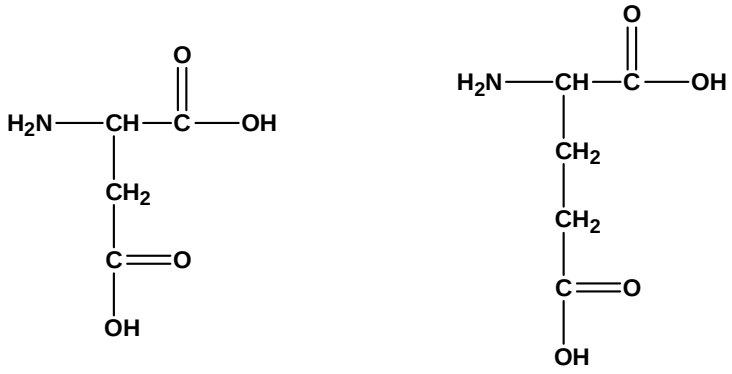


Figure 14. Structures of Aspartic acid and Glutamic acid

The amine reagent should be weakly basic in order to react specifically with the activated carboxylic acid in the presence of the other amines on the protein. This is because protein crosslinking can occur when the pH is raised to above 8.0, the range where the protein amines are partially unprotonated and reactive.[65] For this reason, hydrazides, which are weakly basic, are useful in coupling reactions with a carboxylic

acid.[72] This reaction can also be used effectively to modify the carboxy terminal group of small peptides.

There are several common carboxylic acid and aldehyde-reactive reagents.

(a) Amines and Hydrazides (formation of amide or alkylamine bonds).

Amines and hydrazides can be coupled to carboxylic acids of proteins via activation of the carboxyl group by a water-soluble carbodiimide followed by reaction with the amine or hydrazide (see Figure 15).[65]

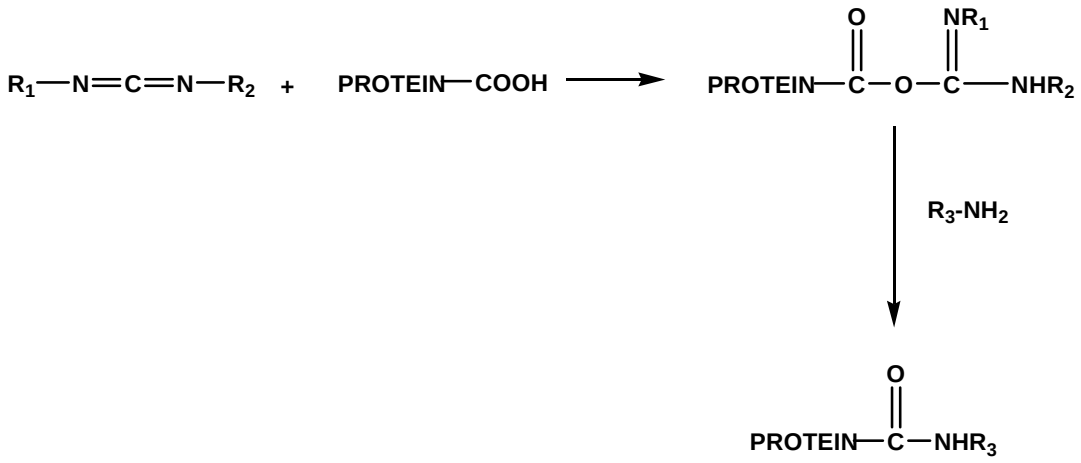


Figure 15. Reaction of carbodiimide with carboxylic acid

As mentioned previously, the amine or hydrazide reagent must be weakly basic so that it will react selectively with the carbodiimide-activated protein in the presence of the more highly basic protein amines (lysines). The reaction of these probes with carbodiimide-activated carboxyl groups leads to the formation of stable amide bonds. Amines and hydrazide reagents with react with carboxylic acid groups and are also able to react with aldehyde groups, which can be generated on proteins by the periodate oxidation of carbohydrate residues on the protein.[66] In this case, a Schiff base

intermediate is formed, which can be reduced to an alkylamine with sodium cyanoborohydride, a mild and selective water-soluble reducing agent.[73, 74] Modifications of proteins via their carbohydrate residues is discussed in more detail under oxidative modification strategy in chapter three.

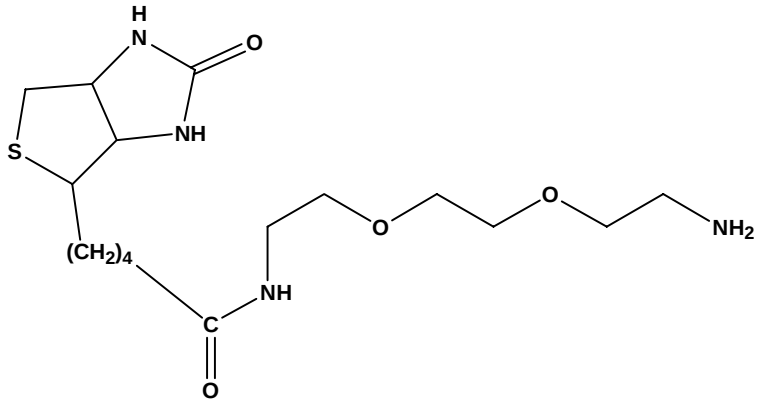


Figure 16. Structure of Biotin-peo-amine (Biotinyl-3, 6, 9-trioxaundecanediamine) for reacting with carboxylic acid activated with carbodiimide

(5) Other Amino Acid Side Chains (Arginine, Histidine and Tryptophan)(Figure 17)

The chemical modification of other amino acid side chains in proteins has not been extensive, compared to the groups discussed above. The high pK of the guanidine functional group of arginine (pK = 12-13) necessitates more drastic reaction conditions than most proteins can survive.[75] Arginine modification has been accomplished primarily with glyoxals and α-diketone reagents. 2,3-Butanedione is also another reagent that has been used to modify arginine residues. These reagents are typically used in the presence of borate buffer to enhance their reactivity.[76] The specific modifications of tryptophan is one of the most challenging problems in protein chemistry. Tryptophan modification requires harsh conditions and is seldom carried out except as a method of analysis in

structural or activity studies.[77] Treatment of the tryptophan ring with hydrogen peroxide results in the oxidation of the indole ring.

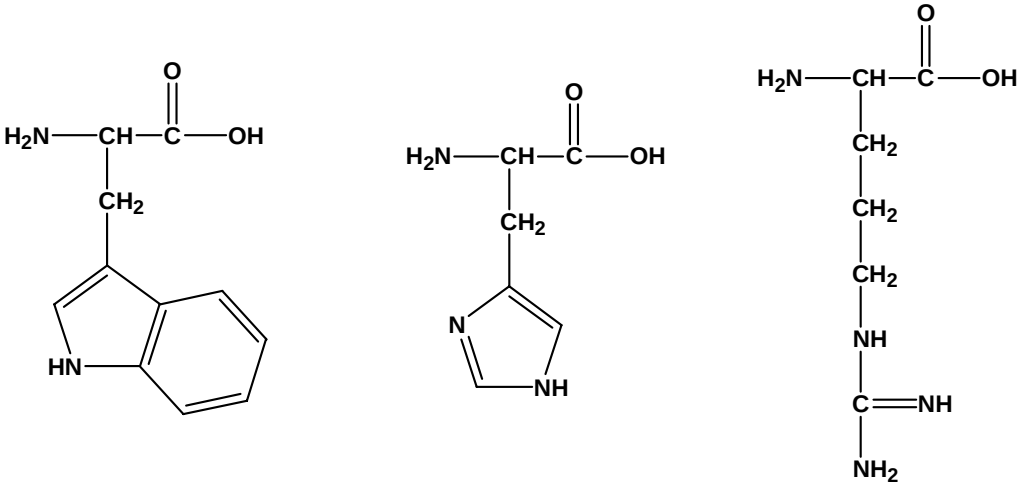


Figure 17. Structures of tryptophan, histidine, and arginine respectively.

While the reaction of NBS with tryptophan continues to be a useful method for obtaining the site specific chemical modification of tryptophan, NBS is only rarely used today for the quantitative analysis of tryptophan in proteins.[78] Histidines have also been subjected to photooxidation[79] and reaction with iodoacetates.[80]

Heterobifunctional Crosslinking Reagents for Protein Modification

Heterobifunctional crosslinkers are a specialized class of reagents used to modify, crosslink, or immobilize biomolecules. In the context of my research, a desired protein could be modified with a heterobifunctional crosslinker. This protein could then be attached to a tissue surface exhibiting the appropriate reactive group for the crosslinker. Therefore, these heterobifunctional crosslinkers can become very useful linkers between our desired protein and tissue surface. Most of the proteins or small molecules that we have immobilized were modified by us, or had been previously modified by a

heterobifunctional crosslinker to introduce functional groups to the proteins. A detailed discussion of our methods will follow in the experimental section. Discussed below is an overview of the use of heterobifunctional crosslinkers in protein modification.

Reactions of Heterobifunctional Crosslinker with Proteins

Heterobifunctional cross-linking agents are particularly useful for conjugating different proteins together.[81] By taking advantage of the different side-chain reactivities, for example, coupling can be carried out in a stepwise manner which allows, in some cases, for partial purification and, if desired, characterization of intermediates prior to the actual conjugation. Due to the hydrolytic instability of the most important groups directed at amino side chains, the first step usually involves addition of the cross-linker to the amino groups of one member of the future hybrid pair (which either has no thiol groups or where thiols, if present, are at least temporarily blocked). The removal of unreacted or hydrolyzed reagent and other unwanted substances is usually possible at this stage. The resulting derivative is then directly coupled via an introduced thiol-reactive maleimide or α-halocarbonyl group(s) to the thiol-containing member of the intended hybrid pair.[82]

Crosslinking Proteins with Heterobifunctonal Reagents

The cross-linking of proteins and their immobilization, either by attachment to an insoluble support or by various other means, has a long and important history.[75] The former is sometimes employed to increase the stability of proteins or of certain conformational relationships in proteins, to couple two or more different proteins (e.g. to join different activities into a single molecule), to identify or characterize the nature and extent of certain protein-protein interactions, and, in other cases, to determine distances

between reactive groups in or between protein subunits.[75] Proteins are sometimes immobilized to facilitate their reuse and their separation from other products and, in some cases, to increase their stability.[83] A large number of different procedures, including physical as well as chemical procedures, have been developed to immobilize proteins. These methods will be discussed in the section under protein immobilization.[77]

A large number of different types of crosslinking bifunctional reagents have been described. One class is the so-called zero-length crosslinking agents that bring about the direct formation of covalent bonds between existing amino acid side chain groups.[77] The use of water-soluble carbodiimides to bring about the formation of amide linkages between carboxyl groups of aspartate or glutamate and the ε-amino groups of lysine side chains appear to be the most prominent zero-length crosslinking strategy. Disulfide bonds obtained from existing thiol groups would also, presumably, be considered zero-length cross-links.[84] Such linkages appear to be formed only when the reacting groups are in close proximity.

Other cross-linking agents may be organized according to the type(s) of reactive groups, their side chain reactivity, their hydrophobicity or hydrophilicity, and the length or distance between the reactive groups; whether the two, or in some cases more, reactive groups are the same or different (i. e. , “homobifunctional” or ”heterobifunctional” reagents), whether the structure connecting the reactive groups is readily cleavable, and whether the groups are membrane permeable or impermeable, and according to various other criteria.[76] The reactivities of cross-linking agents, except for one or two special cases, are very similar to those of the corresponding monofunctional reagents.[85]

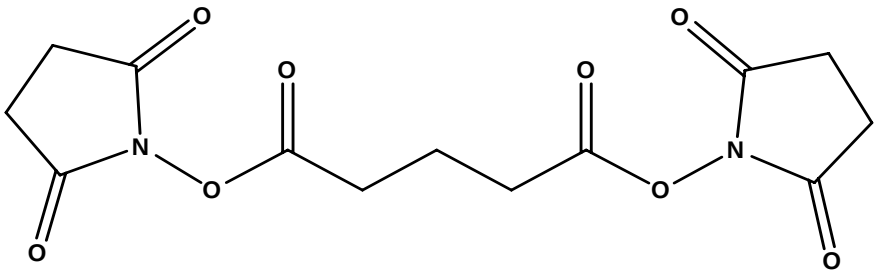


Figure 18. Homobifunctional crosslinker, disuccnimidyl glutarate (DSG) , for linking two proteins via their amine groups.

The initial reaction with a protein is presumably, in most cases, a simple second-order process, not seriously affected by the second reactive group. The latter's reaction, however, is completely dependent on the availability of a second appropriate side chain which, for fast, efficient cross-linking, must be both nearby and in an appropriate orientation. Cross-linking agents with different lengths, different stereochemical configurations (i.e. some with little and others with a great deal of conformational flexibility), and with different side-chain specificities have been developed to fulfill different needs.[86] Distances between potentially reactive side chains in the same or different subunits of some oligomeric proteins have, for example, been estimated by comparing rates and yields of cross-link formation with a series of cross-linking agents differing in length, stereochemical configuration, and side-chain reactivity.[80]

Of the 20 or so amino acid side chains normally present in proteins, ε-amino groups of lysine residues are usually among the most abundant and most accessible of the potentially reactive groups. A relatively large proportion of the most commonly used cross-linking agents are therefore amino group selective reagents (i.e. N-hydroxysuccinimide esters, isothiocynates etc). Most of them, however, also undergo fairly rapid hydrolysis in addition to their reaction with amino groups, which, except for

cases involving close proximity, seriously limits the yields that may be obtained. Glutaraldehyde, which does not hydrolyze or become otherwise inactivated over long periods of time, is widely used to immobilize enzymes by cross-linking and to stabilize their adsorption to or entrapment in various materials.[87] The nature of its reactions with proteins may involve Schiff base formation but is clearly much more complicated than that and not completely understood.[88]

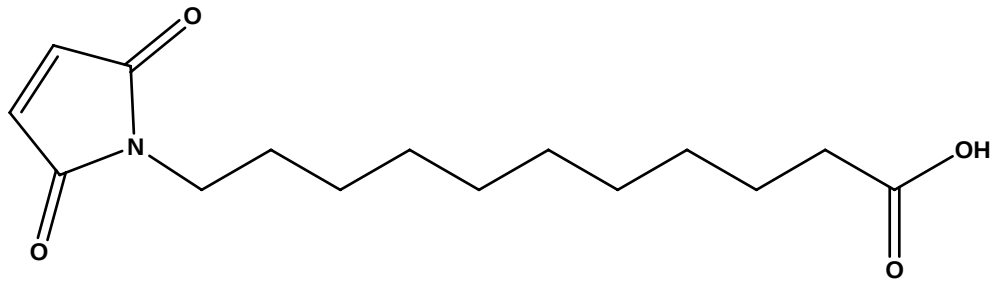


Figure 19. Heterobifuctional crosslinker, KMUA (N-κ-maleimidoundecanoic acid) with spacer arm of 15. 7 angstrom, used for linking proteins via sulfhydryl groups and amino groups (when used with EDC)

The high reactivities of thiol groups with N-ethylmaleimide, iodoacetate, and many related α-halocarbonyl compounds has led to the development of many crosslinking agents containing maleimide and α-halocarbonyl moieties. Under the conditions usually employed for cross-linking, the α -halocarbonyl moieties are much more stable to hydrolysis than the amino group reagents mentioned above and the yields of cross-linked products are, therefore, usually somewhat less dependent on side chain proximity.[89] A large number of heterobifunctional cross-linking reagents have been developed which usually contain a thiol reactive and an amino group reactive moiety. N-alkyl- or N-arylmaleimide and α-halocarbonyl groups are the most common of the

former and N-hydroxysuccinimide esters appear to be the most common of the latter. To increase aqueous solubility, sodium salts of sulfonated N-hydroxysuccinimide esters are also commonly employed.[90]

In addition to the two reactive groups, a variety of different types of connecting structures or spacer arms have been employed. The nature of the spacer arm may, of course, also have important consequences. Longer spacer arms are usually assumed to be more effective for coupling larger proteins or those where the potentially reactive side chains are sterically protected.[91] The conformational flexibility, hydrophilicity or hydrophobicity, and the "cleavability" of the spacer arm are also important considerations. N-Alkylmaleimides are also generally more stable than their aryl counterparts.[84]

Collagen

Background Information

One type of protein that is of great importance for the purpose of this research is collagen. As mentioned earlier, collagen is the primary structualcomponent in many tendons, ligaments and skin.[92] Most of the tissues that were used as a surface for the immobilization of proteins (i.e.meniscus, pericardium, aorta, skin and tendons) in these studies are primarily composed of collagen. Collagen is also modified frequently for biomedical and tissue engineering applications. Most of these modifications occur in soluble collagen, but there are still many examples where tissues composed of collagen are also chemically modified. Therefore, it is important to understand the structure, and function of collagen, as well as some common collagen modification methods.

Collagen is the primary structural material of vertebrates and is the most abundant mammalian protein.[93] It accounts for approximately 20-30% of total proteins.[94] It is present in tissue of primarily mechanical function. About one half of the total body collagen is in the skin, and approximately 70% of dry solids (i.e.other than water) present in the dermis of skin and tendon is collagen. Collagens are predominantly synthesized by fibroblasts but epithelial cells also synthesize these proteins (see Figure 20).[95]

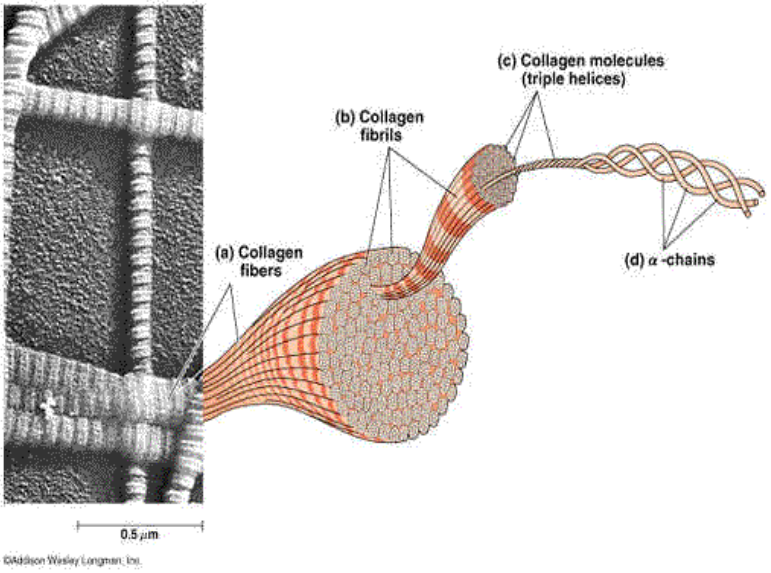


Figure 20. Hierarchical Structure of collagen fibril[96]

The molecular structure of collagen has been firmly established based on a wide body of research, including amino acid composition analysis, x-ray diffraction, electron microscopy and physiochemical examinations of solutions.[97] Nineteen chemically and genetically distinct collagen molecules and ten additional proteins containing collagen-like domains have been identified. The nineteen types of collagen are further categorized into (1) fibril containing collagens: types I, II, III, V, XI (2) network-forming collagens :

types IV, VIII, X (3) fibril-associated collagens: types IX, XII, XIV, XVI, XIX (4) beaded-filament collagen: type VI (5) collagen anchored to the basement membranes: type VII (6) collagen with transmembrane domain: types XIII and XVII and (7) newly found and partially characterized collagens: types XV and XVII. Of these, collagen type I is the most abundant and well studied collagen of them all.[89]

Collagen is used in wide range of biomedical applications due to its many interesting features including low antigenicity, low inflammatory and cytotoxic responses, haemostatic properties and promotion of cellular growth.[98] Collagen-based biomaterials are being used in many medical applications including nerve regeneration, tissue augmentation, burn and wound dressing, drug delivery system, ocular surface and urinary tract surgery.[99] These materials used for surgical applications are most often derived from porcine aortic valve, bovine pericardium, dura mater, and autologous pericardium. Among them, bovine pericardium tissue patches are widely used to close the pericardial sac after open heart surgery and to repair the vascular grafts.[100]

Collagen Crosslinking

The most common modification of collagen is crosslinking. There are several methods of artificially inducing collagen crosslinks. The main chemical reagents utilized include formaldehyde, glutaraldehyde, dialdehyde starch, and epoxy compounds. In order to control the rate of biodegration of collagen and to improve its tensile properties, these reagents are frequently used to fix tissue.[101] Glutaraldehyde treatment is the most common and the only commercially viable, widely accepted procedure. Glutaraldehyde is a bifunctional compound mainly used in the chemical modification of proteins and polymers. This bifunctional compound links covalently to the amine groups of lysine or

hydroxylysine in the protein molecules. Over the past three decades, thousands of patients have received glutaraldehyde-treated heart valves.[93, 94] The crosslinking mechanism of glutaraldehyde is not fully understood, although it is believed to crosslink at the N-terminal amino group and the ε-amino groups of lysine and hydroxyl-lysine in collagen both intra and intermolecularly.[94, 102]

Physical methods such as dehydrothermal treatment, ultraviolet and gamma radiation have also been used to introduce crosslinks to collagen bioprostheses. These methods have the advantage of not introducing chemicals protentially cytotoxic to the host, but are less versatile than their chemical counter parts and are limited by their need to balance between the desired amount of crosslinking and the degration of collagen owing to long-time exposure to the treatment.

A developing method for introducing crosslinks into collagen is photo-oxidation. It is a reaction in which certain amino acids become oxidized by an activated photosentizer. These oxidized amino acids subsequently form covalent bonds to crosslink peptides and proteins. Some examples of photochemical crosslinking reagents include methylene blue ,furocoumarin, rose Bengal, flouresceinand compounds from the benzophenone class.

One other class of compounds that have been studies for the applications for photochemical crosslinking agents is the 1,8-napthalamides. They are yellow-colored, photoactive, fluorescent compounds derived from Lucifer Yellow, another better known fluorescent dye. Our group has extensively studied the 1,8 napthalamide class of compound as protein crosslinks and expecially as photochemical tissue bonding agents. Photochemical tissue bonding (PTB) is a techinique for sealing tissue with potential

applications in many surgical specialities. In this method, the photochemically active dye is applied to the surface of the tissue to be rejoined, the tissues are intimately approximated and visible light applied to the interface. The dye forms a reactive species, which react with potential electron donors and acceptors such as amino acid (e.g. tryptophan, tyrosine, cystine) of proteins at both tissue surfaces. Strong covalent bonds are formed between the approximated surfaces, forming instantaneous protein crosslinks or nanosutures. This mechanism contrasts with laser tissue welding, where the laser energy is used to increase temperature in the tissue and form non-covalent "welds" though protein denaturation. Although the exact mechanism of PTB is not fully understood, formation of crosslinks in collagen type I molecules by photochemical methods have been reported. PTB has also been used to repair corneal lesions and skin graft adhesions. 1,8 napthalamids have also been used successfully to crosslink type I collagen from bovine Achilles tendon. Ex vivo and in vivo experiments have both show the ability of the napthamalids to photochemically bond tissue. In one such study conducted with sheep, and artificial lesion was created in the knee meniscus. Several of the animals were treated by photochemical tissue bonding of the tear with the napthalamids. These animals returned to normal activity shortly after surgery, although the untreated animals showed a substantial limp. Ex vivo experiments have also demonstrated the photochemical tissue bonding of human meniscal and articular cartilage.

Protein Immobilization

Protein immobilization is a common technique in many areas of biology and biophysics.[103] Proteins are immobilized on solid surfaces for applications in diagnosis

and drug discovery. The use of arrays of proteins attached to solid surfaces is reviving much attention as a tool for exploring function and potential relationships of all the proteins encoded in the genome.[104] Proteins arrays (or chips) are ideal reagents for such analysis in parallel fashion. Almost all these proteins are immobilized on functionalized solid surfaces such as silica, glass, gold, and various polymers.[105,106] To the best of our knowledge, there are no reports of proteins being immobilized on fresh tissue surface. Therefore, there is no background available on methods to pattern proteins on fresh tissue surfaces. We had to draw our inspiration from the methods used for protein immobilization on the previously mentioned solid surfaces such as silica. Discussed below are some of the techniques used for protein immobilization on various solid surfaces.

Methods for Protein Immobilization

Basic strategies for the immobilization of proteins include immobilization via physical adsorption, immobilization via specific surface interaction, and immobilization via covalent methods.

(a) Immobilization of Proteins via Physical Adsoprption Methods

The first, simplest, and most convenient type of immobilization of proteins is through surface adsorption. This approach is particularly useful for proteins, and it has been used in standard ELISA, dot blotting, and Western blotting for many years. The commonly used solid supports are hydrophobic plastics such as polystyrene.[107] Protein microarrays have also been generated on a cationic aminosilane surface, nitrocellulose membrane, and Hybond-P membrane (PVDF, polyvinilidene fluoride, membranes available from Amersham Biosciences).[108] Wang et al have also used hydrogel-coated

slides for arraying 43 monoclonal antibodies (mAb) against cytokines and chemokines and used fluorescence-based multiplexed immunoassays to quantify the level of these proteins.[109] The Hugan group has reported the use of hand spotting to apply 540 spots of a number of capturing antibodies onto the surface of Hybond ECL (nitrocelluose) membranes (8x8 cm) commercially available from Amersham Biotech. Fang et al have developed a membrane protein microarray by spotting cell membrane preparations containing G-protein-coupled receptors on γ-aminopropylsilane-derivatized glass or gold coated glass surface.[110]

(b) Immobilization of Proteins via Non-covalent Specific Surface Interactions

In addition to immobilization via nonspecific physical adsorption, molecules can be tagged and immobilized through specific non-covalent interactions between the tag and the already immobilized capturing molecule for the tag. A typical example is the biotin-streptavidin system for immobilizing biotinylated proteins onto streptavidin-coated surfaces such as glass slides. [111] Likewise, a small molecule can also be biotinylated and printed onto a surface that has been precoated with a monolayer of streptavidin or neutravidin. Neutravidin is sometimes preferred as it has less non-specific interaction with other proteins. Lam and coworkers were able to successfully print a microarray of biotinylated synthetic peptides onto a neutravidin-coated polystyrene microscope slide using a Wittech-03 arrayer. [112] The Lesaicherre group has also recently reported the derivatization of glass slides with avidin for immobilization of biotinylated peptides.[113]

Immobilized Metal Affinity Chromatography (IMAC) is a robust method for purifying poly-histidine-tagged recombinant proteins. This is achieved by using the natural tendency of histidine to form a complex with divalent metals (Ni^{2+}) around

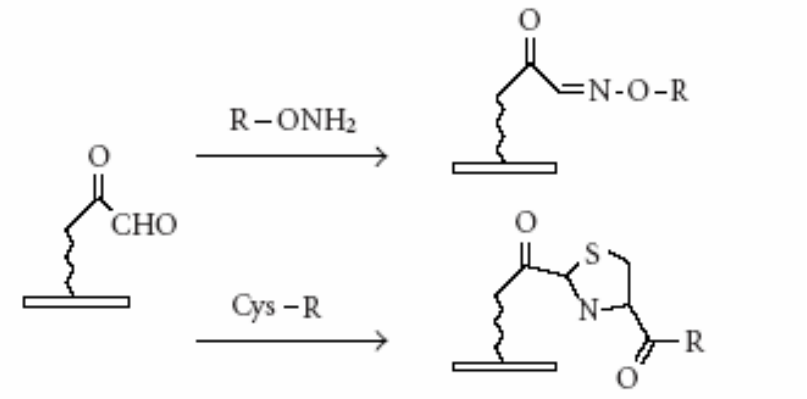
neutral pH. Fusion with ploy histidine peptides is now one of the most commonly used techniques for affinity purification. Immobilizing the metal ion on a chromatographic resin by chelation allows the separation of the histidine-tagged proteins from most untagged proteins even under denaturing conditions. The binding interaction with the tagged protein is pH dependent. The bound sample can be eluted from the resin by reducing the pH and increasing the ionic strength of the buffer or by including EDTA or imidazole in the buffer. The poly-His-Ni^{2+} system has also been reported for protein microarray generation. In this method, proteins containing poly-histidine tags were printed onto Ni^{2+}-chelating surfaces.[114] Another approach is to use anti-GST (Glutathione S Transferase) antibody or glutathione-coated slides to capture a series of GST-fusion proteins.[115] Boronic acid groups are known to form very stable complex with some moieties such as cis or coaxial 1,2-diol. Immobilized phenylboronic acids have been used in chromatography and protein immobilization on various surfaces for generation of protein arrays.[116]

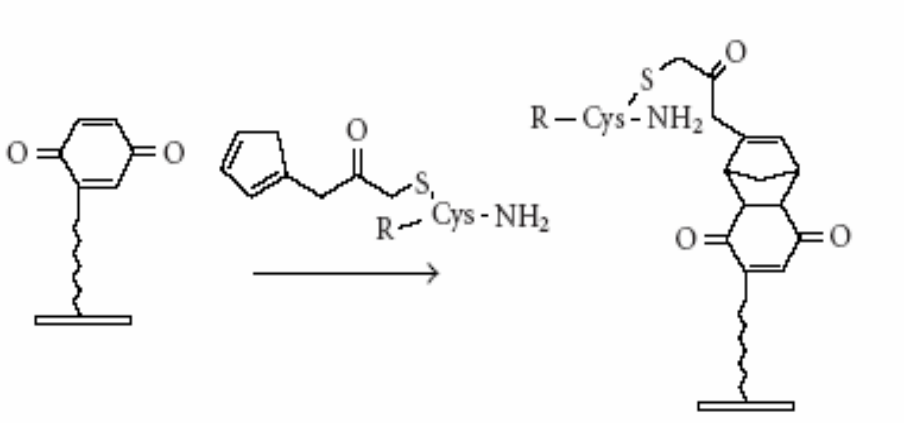
(c) Immobilization of Proteins via Covalent Attachments

Although non-specific physical adsorption has been used successfully for generating microarrays of biomolecules, this approach is less useful for the preparation of small molecule or small peptide microarrays. These small molecules can be conjugated to a tag which in turn binds to the immobilized capturing agent (see above). Alternatively, they can be immobilized via covalent attachment to a functional group on the solid surface. Figure 21 summarize some of the common chemistries used in generating microarrays by covalent attachment in addition to the types of chemistry already discussed. Chemical modification of the solid surface is often necessary to create

functional groups for covalent immobilization and to achieve homogeneous immobilization.[98]

Commercially available aldehyde-derivatized glass slides that have been used for DNA immobilization can also be used for protein microarrays.[98] The aldehyde groups on the glass surface react with primary amines on the protein to form Schiff's base linkages.[98] BSA is used to block the remaining unreacted aldehyde groups or other nonspecific binding sites. Zhu et al have described the use of a 3-glycidoxypropyltrimethoxysilane (GPTS) to activate polydimethylsiloxane (PDMS) on the slide surface prior to protein immobilization (see Figure 21).[117]





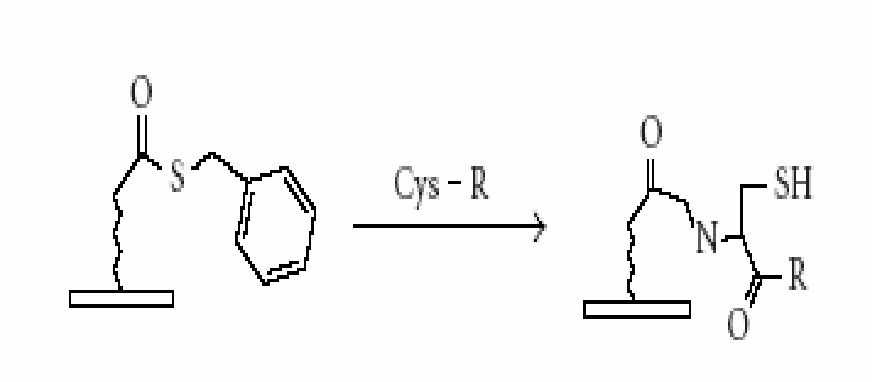


Figure 21. Some addition methods for protein immobilization (1) Aldehyde/NH_2O, N-terminal Cys, (2) Quinine/cyclodiene, (3) Thioester/ N-terminal Cys

Lin and co-wrokers have reported the printing of protein microarrays on an aminopropyltrimethoxysilane surface activated with bis-sulfosuccinimidyl suberate.[118] Furthermore, Benters et al have demonstrated the use of succinimidyl ester- or isocyanate-functionalized dendrimer on a solid surface for nucleic acid and protein microarrays.[119]

Immobilization of small molecules or short peptides often requires covalent linkage of the compounds onto the solid support. The Michael addition has been used by Schreiber's group to ligate thiol-containing compounds to maleimide-derivatized glass slides to form a microarray of small molecules.[120] They have also used covalent chemistry to attach alcohol-containing small molecules to chlorinated glass slides.[121] Lam and collaborators have recently reported a new strategy to significantly improve the loading of glyoxylyl groups onto glass surfaces by using acrylic acid as a starting material and further improvement of loading can be accomplished by adding hydrophilic linkers and bifurcating amino acids such as lysine between the glyoxylyl group and the glass surface.[122] The Lesaicherre group has recently reported the derivatization of glass slides with thioesters for chemoselective ligation of peptides with an N-terminal cysteine

(1,2 aminothiol group) via the amide bond.[105] Houseman et al have described a peptide chip prepared by the Diels-Alder-mediated immobilization of peptides on quinone-functionalized surfaces. In this method, a cyclodiene moiety is incorporated into peptides for the purpose of immobilization.[123] As an alternative to immobilizing peptide-protein conjugates onto plastic slides via physical adsorption, the Lam group has used a chemically derivatized protein scaffold (eg, glyoxylyl-functionalized BSA) to first coat the polystyrene slide via non-specific adsorption. Peptides or small molecules are then printed onto the functionalized protein-coated slides. After incubation, these compounds are immobilized onto the coating surface via a site-specific ligation reaction.[124]

The Raines group used the Staudinger ligation to site specifically immobilize proteins. Their method relied on a traceless version of the Staudinger ligation in which an azide and phosphinothioester react to form an amide. The reaction is known to occur in high yield at room temperature in aqueous or wet organic solvents and is compatible with the unprotected functional groups of proteinogenic amino acids. In some applications, immobilization of the ligand onto the glass surface via a long hydrophilic linker or a protein may be beneficial for biological activity.[125]

One important factor in protein immobilization is the retention of activity in the protein or biomolecule after immobilization. In an experiment by Ito et al., insulin was co-immobilized with growth factors on a porous poly (ethylene terepthalate) membrane. The surface of the membrane was partially hydrolyzed under alkaline conditions and the proteins were immobilized on the membrane using water soluble carbidiimide. The adhesion of STO mouse fibroblast cells onto the membrane was accelerated by the immobilization of fibronectin and polylysine.[126] The cell growth was enhanced by

immobilization on insulin.[127] Not only were the immobilized biosignal molecules active, but their activities were also higher than the diffusible ones.[128] For example, immobilized insulin had a mitogenic effect which was much higher than native insulin. Similar results were also obtained with immobilized epidermal growth factor.

When the time course of activation of cellular signaling proteins was monitored in the presence of immobilized biosignal molecules, activation by the immobilized biosignal molecules continued for a longer time than that by diffusible ones. By micro-patterned immobilization of nerve growth factor (NGF) on polystyrene film, the differentiation (neurocite formation) phaeochromacytoma PC12 cells were observed only on the immobilized regions. Therefore, neuron networks or artificial neuron tissue may be formed by culturing neuronal cells on matrix pattern-immobilized with NGF using a designed micropattern.[129, 130]

These experiments have proved that covalent chemistry could be used to attach cell adhesion factors and growth factors to solid surfaces. Furthermore, they have demonstrated that these biomolecules retained their activity after immobilization and therefore could be used in tissue engineering.

Visualization of Modified or Immobilized Proteins

Once a protein has been immobilized or modified, it must be characterized. Standard immune detection techniques, such as enzyme-linked colorimetric, fluorescent, FRET(fluorescence resonance energy transfer), chemiluminescence, or luminescence methods, are useful and usually simple methods to analyze these modifications. In our research, we have adapted several of these assays (colorimetric, fluorescent, and

chemiluminescent) to detect immobilized biomolecules on the tissue surface. Discussed below is a brief overview of these detection methods.

Chromogenic Assays

A colorimentic or chromogenic assay is usually an enzyme linked assay that can be visualized with the naked eye. Colorless, endogenous or exogenous pigment precursors are transformed by biological mechanisms into colored compounds[131]. The chromogenic assays are used in biochemistry (e.g. blotting, ELISA etc) and in diagnosis as indicators, especially in the form of enzyme mediated colourimetric assays. Glycosidase enzymes exhibit very high selectivity for hydrolysis of their preferred sugars. For example, β -galactosidase rapidly hydrolyzes β-D-galactopyranosides but usually does not hydrolyze either the anomeric α-D-galactopyranosides or the isomeric α-D-glucopyranosides.[132]

Fluorescence Based Detection Methods

Fluorescence has proven to be a versatile tool for a myriad of applications. It is a powerful technique for studying molecular interactions in analytical chemistry, biochemistry, cell biology, physiology, nephrology, cardiology, photochemistry, and environmental science.[133] Fluorescence is the phenomenon in which absorption of light of a given wavelength by a fluorescent molecule is followed by the emission of light at longer wavelengths. The distribution of wavelength-dependent intensity that causes fluorescence is known as the fluorescence excitation spectrum, and the distribution of wavelength-dependent intensity of emitted energy is known as the fluorescence emission spectrum.[134]

Fluorescence boasts phenomenal sensitivity for the analytical chemist or the life scientist working at nanomolar concentrations. But fluorescence offers much more than mere signal-gathering capability. New developments in instrumentation, software, probes, and applications have resulted in a burst of popularity for a technique that was first observed over 150 years ago.[135] Fluorescence has many practical applications in Chemistry and Biology. The binding of biochemical species can be easily studied *in situ* and distances within macromolecules may be measured. The dynamics of the folding of proteins can be studied. Concentrations of ions can be measured inside living cells and membrane structure and function may be studied with fluorescence probes. Drug interactions with cell receptors can be investigated. Minute traces of fluorescent materials can be detected and identified in mixtures. Oil samples can be fingerprinted and identified by their fluorescence spectra and the electronic structure and dynamics of an excited state of a molecule may be elucidated. These are only a few examples of the applications of modern fluorescence techniques.[122]

Fluorescence detection has two major advantages over other light-based investigation methods: high sensitivity and high speed. Sensitivity is an important issue because the fluorescence signal can be proportional to the concentration of the substance being investigated. For example, relatively small changes in ion concentrations in living cells can have significant physiological effects. Whereas absorbance measurements can reliably determine concentrations only as low as several tenths of a micromolar, fluorescence techniques can accurately measure concentrations one million times smaller -- pico- and even femtomolar. Total quantities less than an attomole (<10-18 mole) may be detected. Because it is a non-invasive technique, fluorescence does not necessarily

interfere with a sample136. The excitation light levels required to generate a fluorescence signal are often low, limiting the effects of photo-bleaching, and living tissue can be investigated with no adverse effects on natural physiological behavior137. There are many fluorescent probes and fluorescent probes conjugated to proteins commercially available. Avidin and streptavindin are two of the most common proteins conjugated with fluorescent probes and used extensively in biochemical assays such as western blots and ELISAs.[138]

Chemiluniniscence Based Detection Assays

Chemiluminescence is the generation of electromagnetic radiation as light by the release of energy from a chemical reaction. While the light can, in principle, be emitted in the ultraviolet, visible or infrared region, those emitting visible light are the most common.[139] The most common example of this process is the bioluminescence of the firefly. The firefly is by far the most efficient example of a bioluminescent system discovered to date. It approaches a theoretical limit by producing 88 photons of light for each 100 molecules of reactant yielding an astonishing 88% efficiency rate.[140]

Chemiluminescent reactions can be grouped into three types:

1. Light-emitting chemical reactions using synthetic compounds and usually involving a highly oxidized species such as a peroxide are commonly termed chemiluminescent reactions.

2. Light-emitting reactions arising from a living organism, such as the firefly or jellyfish, are commonly termed bioluminescent reactions.

3. Light-emitting reactions which take place by the use of electrical current are designated electrochemiluminescent reactions.

Chemiluminescent and bioluminescent reactions usually involve the cleavage or fragmentation of the O-O bond in an organic peroxide compound. Peroxides, especially cyclic peroxides, are prevalent in light emitting reactions because the relatively weak peroxide bond is easily cleaved and the resulting molecular reorganization can liberate a large amount of energy.[141]

Chemiluminescence as an analytical tool has three important strengths:

- Sensitivity
- Selectivity
- Wide linear range

The first often means lower detection limits for chemiluminescence when compared to absorption or fluorescence techniques in pursuit of the same analyte[142]. The second, selectivity, is derived from the fact that the analyte of interest generates its signal often in the presence of normally interfering compounds that, in this case, do not themselves produce light when the chemiluminescent reagents are mixed together. And the third is analytically useful because samples of larger concentration ranges can be analyzed without dilution. This advantage is derived from the way that chemiluminescent light is generated and measured: using no source lamp for light production and a phototransducer with an inherent wide range of response for light detection.[143]

The analytical chemiluminescent signal is produced by a chemical reaction (or reactions) and requires no light source for excitation (as in the more common fluorescence and phosphorescence).[144] Its emission appears out of an essentially black background, and therefore the only background signal is that of the photomultiplier tube's

(phototranducer's) dark current. Subsequently, light source and detector warm-up and drift and the interference from light scattering present in absorption and fluorescence methods are absent (see Figure 22).[145]

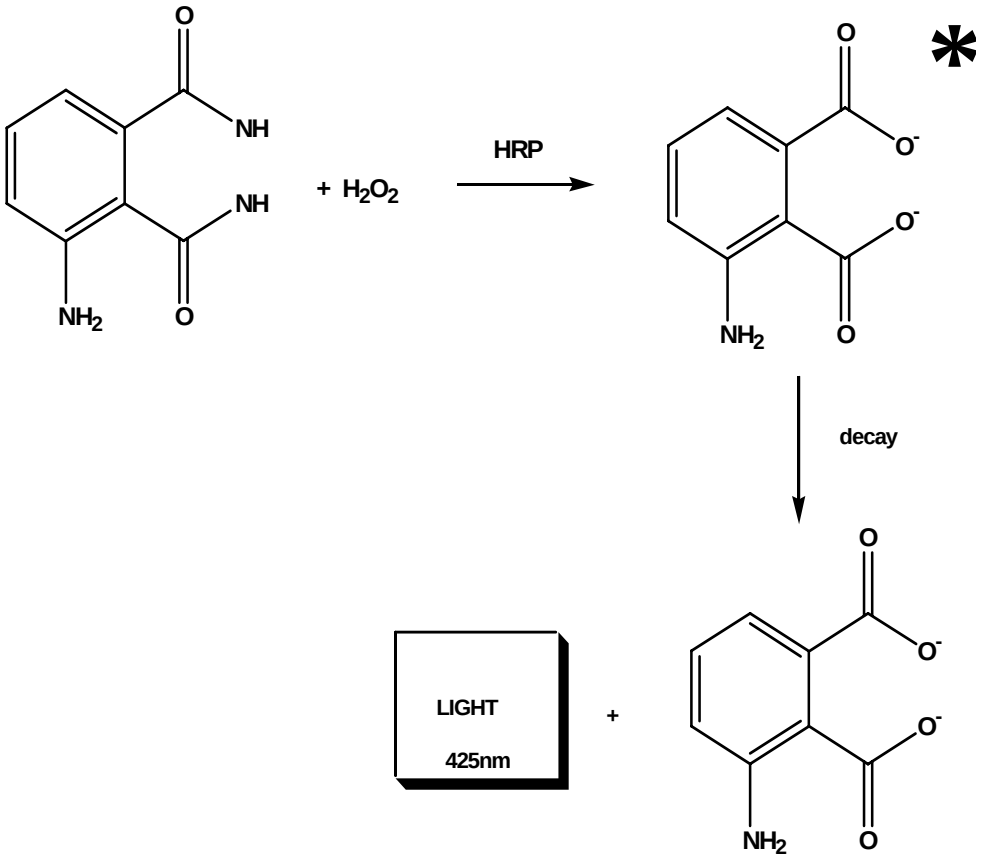


Figure 22. Reaction of luminal with peroxide and HRP (horse radish peroxidase). The luminal is oxidized in the presence of HRP and hydrogen peroxide to form an excited state product (3-aminophthalate). The 3-aminophthalate* emits light at 425 nm as it decays to the ground state.

Major Types of Tissue Used in Immobilization Methods

Several tissue types, including bovine pericardium, meniscus, aorta skeletal muscle and cornea, along with fetal pig skin and rat skin, were all investigated as possible surfaces for the immobilization of biomolecules. Most of these tissue surfaces were

primarily composed of collagen, but there was still a good degree of variability as far as the composition individual tissue surfaces were concerned. Therefore it is important to understand the nature and function of these tissues.

The Cornea

The cornea (see Figure 23) is the transparent, dome-shaped window covering the front of the eye.

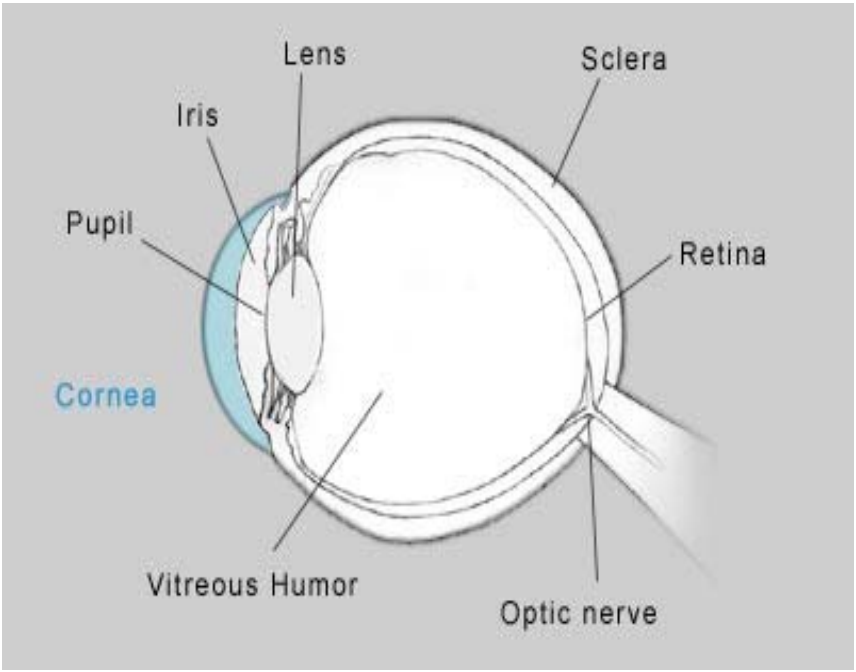


Figure 23. The eye[146]

It is a powerful refracting surface, providing 2/3 of the eye's focusing power. Like the crystal on a watch, it gives us a clear window to look through. Because there are no blood vessels in the cornea, it is normally clear with a shiny surface. The cornea is extremely sensitive--there are more nerve endings per area in the cornea than anywhere else in the body.[147]

The adult cornea is only about 1/2 millimeter thick and is comprised of 5 layers: epithelium, Bowman's membrane, Stroma, Descemet's membrane and the endothelium (see Figure 24). The epithelium is a layer of cells that cover the surface of the cornea. It is only about 5-6 cell layers thick and quickly regenerates when the cornea is injured. If an injury to the eye penetrates more deeply into the cornea, it may leave a scar. Scars leave opaque areas, causing the corneal to lose its clarity and luster. [148]

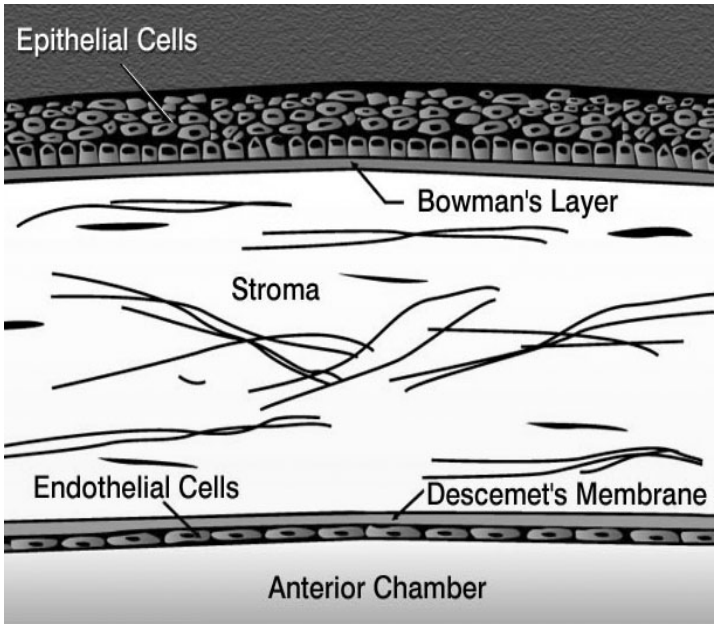


Figure 24. Layers of the cornea[149]

The Bowman's membrane is located just beneath the epithelium. Because this layer is very tough and difficult to penetrate, it protects the cornea from injury. The stroma is the thickest layer and lies just beneath Bowman's. It is composed of tiny collagen fibrils that run parallel to each other. This special formation of the collagen fibrils gives the cornea its clarity. Descemet's membrane lies between the stroma and the endothelium. The endothelium is just underneath Descemet's and is only one cell layer

thick. This layer pumps water from the cornea, keeping it clear. If damaged or diseased, these cells will not regenerate, tiny vessels at the outermost edge of the cornea provide nourishment, along with the aqueous and tear film.

Skeletal Muscle

A whole skeletal muscle is considered an organ of the muscular system. Each organ or muscle consists of skeletal muscle tissue, connective tissue, nerve tissue, and blood or vascular tissue.

Skeletal muscles vary considerably in size, shape, and arrangement of fibers. They range from extremely tiny strands such as the stapedium muscle of the middle ear to large masses such as the muscles of the thigh. Some skeletal muscles are broad in shape and some narrow. In some muscles the fibers are parallel to the long axis of the muscle, in some they converge to a narrow attachment, and in some they are oblique. Each skeletal muscle fiber is a single cylindrical muscle cell. An individual skeletal muscle may be made up of hundreds, or even thousands, of muscle fibers bundled together and wrapped in a connective tissue covering (see Figure 25). Each muscle is surrounded by a connective tissue sheath called the epimysium. Fascia, connective tissue outside the epimysium, surrounds and separates the muscles. Portions of the epimysium project inward to divide the muscle into compartments. Each compartment contains a bundle of muscle fibers. Each bundle of muscle fiber is called a fasciculus and is surrounded by a layer of connective tissue called the perimysium. Within the fasciculus, each individual muscle cell, called a muscle fiber, is surrounded by connective tissue called the endomysium [150].

Skeletal muscle cells (fibers), like other body cells, are soft and fragile. The connective tissue covering provides support and protection for the delicate cells and allow them to withstand the forces of contraction. The coverings also provide pathways for the passage of blood vessels and nerves.

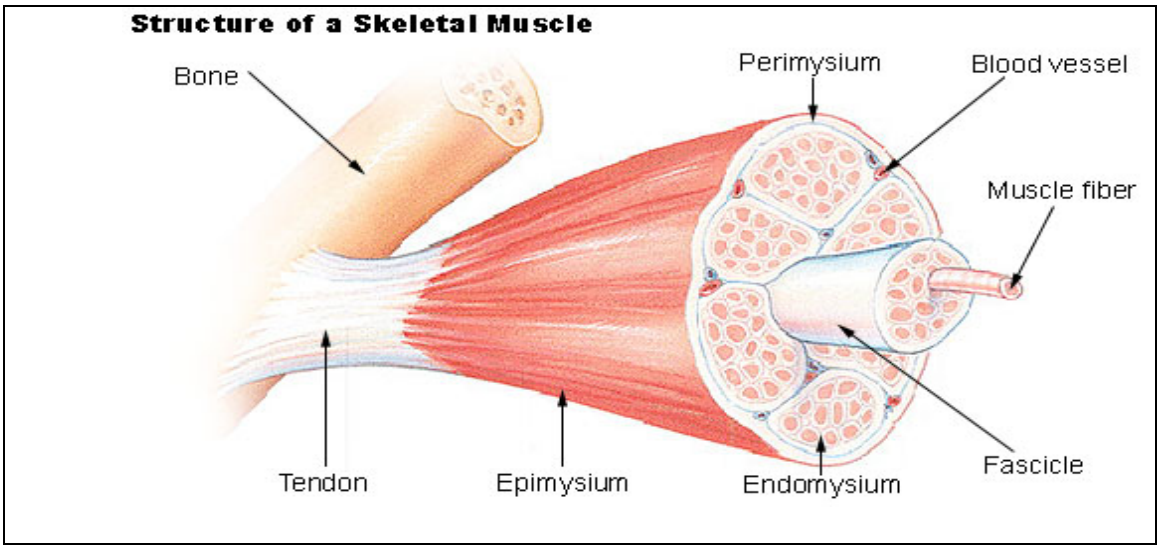


Figure 25. Structure of skeletal muscle[151]

The epimysium, perimysium, and endomysium (connective tissue composed of collagen) extend beyond the fleshy part of the muscle, called the belly or gaster, to form a thick ropelike tendon or a broad, flat sheet-like aponeurosis. The tendon and aponeurosis form indirect attachments from muscles to the periosteum of bones or to the connective tissue of other muscles. Typically a muscle spans a joint and is attached to bones by tendons at both ends. One of the bones remains relatively fixed or stable while the other end moves as a result of muscle contraction.

Skeletal muscles also have an abundant supply of blood vessels and nerves. This is directly related to the primary function of skeletal muscle, contraction. Generally, an

artery and at least one vein accompany each nerve that penetrates the epimysium of a skeletal muscle. Branches of the nerve and blood vessels follow the connective tissue components of the muscle of a nerve cell and with one or more minute capillaries.[151]

The Heart and the Pericardium

The heart is composed of three layers-the epicardium, myocardium, and the endocardium surrounding the inner chamber, and is enclosed in a fourth protective layer known as the pericardium (see Figure 26).

The pericardium is composed of two layers separated by a space called the pericardial cavity. The outer layer is called the parietal pericardium, and the inner is called the visceral pericardium. The parietal pericardium consists of an outer layer of thick, fibrous connective tissue and an inner serous layer. The serous layer, consisting largely of mesothelium together with a small amount of connective tissue, forms a simple squamous epithelium and secretes a small amount of fluid. This fluid layer helps to lubricate the surfaces to allow friction free movement of the heart within the pericardium during its muscular contraction.[151]

The fibrous layer of the parietal pericardium is attached to the diaphragm and fuses with the outer wall of the great blood vessels entering and leaving the heart. Thus, the parietal pericardium forms a strong protective sac for the heart and serves also to anchor it within the mediastinum.

The visceral pericardium is also known as the epicardium and as such comprised the outermost layer of the heart proper. The visceral pericardium forms the outer covering of the heart and has an external layer of mesothelial cells. These cells lie on a stroma of fibrocollagenous support tissue, which contains elastic fibers (elastin), as well

as the large arteries supplying blood to the heart wall, and the larger venous tributaries carrying blood from the heart wall.[152]

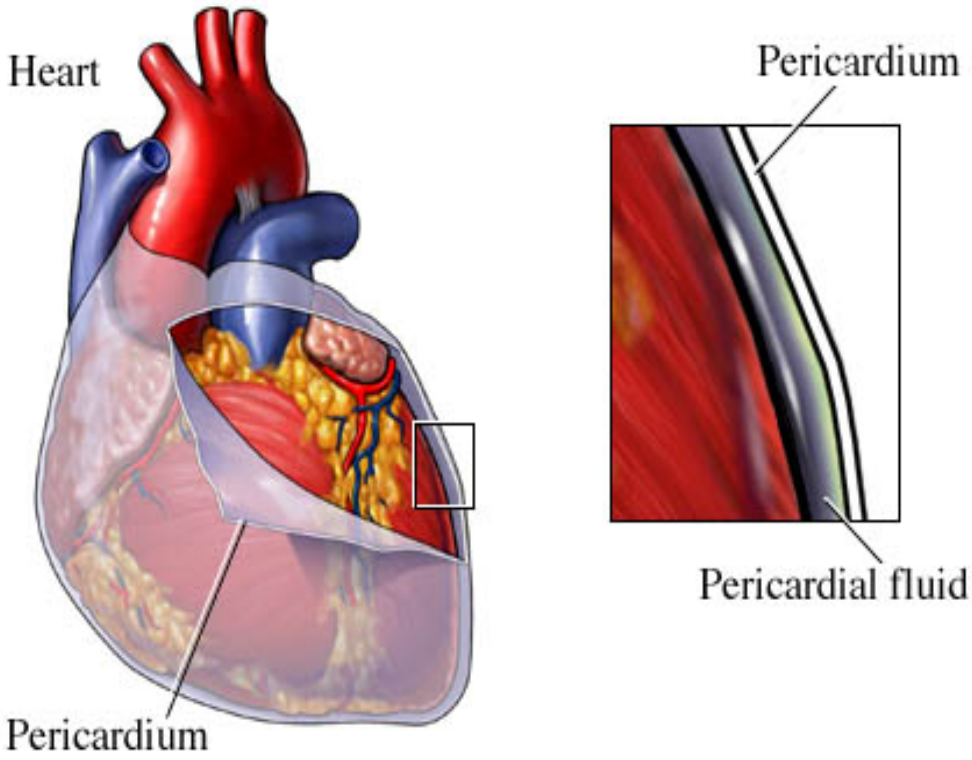


Figure 26. Heart and pericardium[153]

Bovine Pericardium

Bovine pericardium (BP) can be characterized mechanically as a non-linear, anisotropic, multilaminate composite pliable material, which is usually imposed in large deformations during its physiological function as implant material (see previous section on collagen).[154] Histologically BP is composed from a complicated network of collagenous and elastin fibers.[155] These fibers are formed into an amorphous matrix, which maintains the structural integrity and functionality of the tissue. The matrix is composed mainly of proteoglycans (PG) and glycosaminoglycans (GAGs), which engage

fibers, cells and fluid (water and soluble electrolytes). PGs which are important components of connective tissue comprise a class of polyanionic macromolecules consisting of a protein core onto which sulfated GAGs and oligosaccharide chains are covalently bound. The common GAGs include the galactosaminoglycans, chondroitin sulfate (CS) and dermatin sulfate (DS). The GAGs also include the glucosaminoglycans, hyaluronan (HA), heparin sulfate (HS), heparin, and keratin sulfate (KS).[156]

Static tensile experiments have shown that the mechanical properties of the tissue results from the fibrous components, which serve as the main load bearing elements. The GAGs contribute to the viscoleastic properties by regulating the internal friction mechanism due to a swelling effect. Chemical composition of BP using alkaline hydrolysis experiments of native BP showed that it contained 263mg of hydroxyproline (Hyp)/g and 18.4 mg/g of collagen in wet tissue.[157, 158]

The Skin

Another main type of tissue surface investigated for protein immobilization is skin. We conducted our experiments on fetal pig and rat skin. Fetal pig skin has been shown to have very similar properties to human skin, and has been used in several studies as a substitute for human skin. Discussed below is a brief overview of the structure and function of skin.[153]

The skin is the largest organ in the body. The skin is composed of two main layers, the epidermis and the dermis. Below these two layers is another layer of subcutaneous fat. The epidermis forms the external surface of the skin and is mainly composed of keratinocytes which differentiate to form 4 layers, the Stratum Basale (basal

layer), Stratum Spinosum (spinous or prickle cell layer), Stratum Granulosum (granular layer), and Stratum Corneum (surface layer).[153]

Stratum Corneum is the outer layer of the epidermis. It is the corneal layer which consists of fully keratinized, flat, fused cells bound together by lipids synthesized in the granular layer. The protein involucrin is produced in the Stratum Granulosum and corneum and forms a thickened protein layer on the inner surface of the plasma membrane.[151]

The dermis is separated from the epidermis by the basement membrane and this junction consists of the interlocking rete ridges and dermal papillae. The dermis is divided into 2 layers, the papillary dermis and the reticular dermis.

The dermis has three components, cellular, fibrous and ground substance. The cellular component includes; fibroblasts(synthesize collagen, elastin, and reticulum), histiocytes, endothelial cells, perivascular macrophages and dendritic cells, mast cells, smooth muscle, and cells of peripheral nerves and their end-organ receptors. The fibrous component consists largely of collagen (Type I collagen) and reticulum (Type III collagen), which provide tensile strength. Elastin fibers provide for restoration of shape after a deformation. The third component is the ground substance. It constantly undergoes synthesis and degradation, and consists largely of glycosaminoglycans: hyaluronic acid, chondroitin sulfate, and dermatan sulfate.[151, 153]

The Meniscus

One area of particular interest for our group is in the area of meniscal repair. Much work has been done by various members of our group to affect meniscal healing by photochemical tissue bonding. While photochemical tissue bonding remains an active

area of research within the group, this research was aimed at investigating a covalent approach to tissue bonding. The meniscus is an extremely important tissue, and any method to enhance meniscal healing is greatly beneficial. One main goal with this research was developing covalent protein modification and protein immobilization chemistry to enhance meniscal repair and therefore meniscus was one of the major types of tissue used in this research.

Background on the Meniscus

The menisci are unique wedge-shaped semi lunar discs present in duplicate in each knee joint (see Figure 27). The menisci are attached to the transverse ligaments, the joint capsule, the medial collateral ligament (medially) and the menisco-femoral ligament (laterally).[159]

Initially, the menisci were considered as functionless remains of leg muscles but are now known to be very important in load bearing, load distribution, shock absorption, joint lubrication and stabilization of the knee joint.[160,161] The function of the meniscus is reflected in its anatomy as its cells and extracellular matrix are arranged in such a way that compressive forces, shear stresses, circumferentially-directed forces, and tensile hoop stress, can be endured and redirected optimally.[162] During embryonic development non-differentiated mesenchymal fibroblasts-like progenitor cells differentiate into the highly specialized meniscus tissue. Particularly, in the highly loaded avascular, inner region of the wedge shaped meniscus, the phenotype of the tissue is fibro-cartilage like.In the peripheral, vascularized region, however, where the meniscus connects to theinternalknee joint capsule, the cells and matrix have a fibrous phenotype.

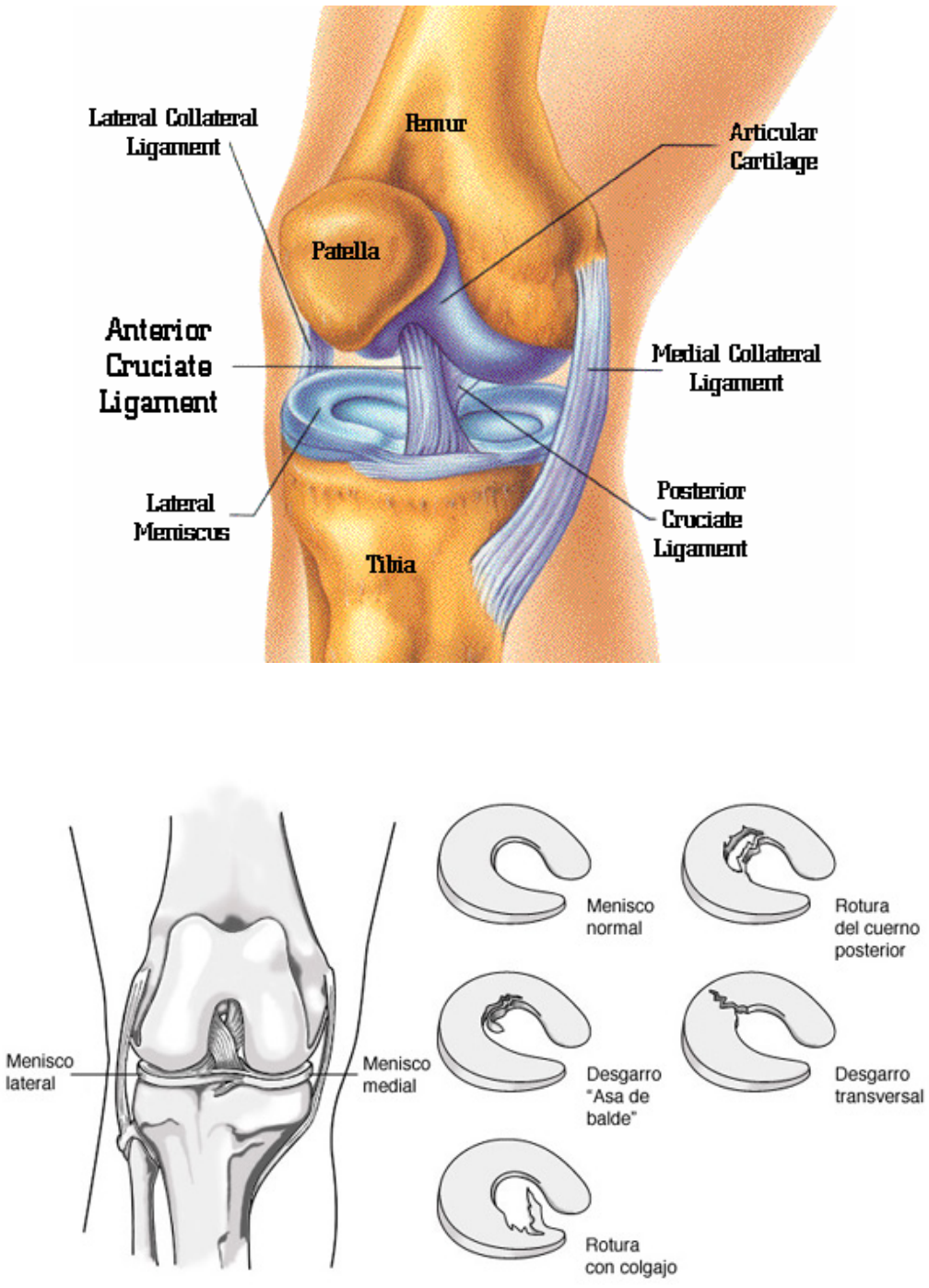


Figure 27. The meniscus and meniscal tears[163]

In general, the matrix of the meniscus is composed of type I collagen, but a number of minor collagens (for instance, type II, II and IV), and glycosaminoglycans

(GAGs) are present in lower quantities, particularly associated with the fibro-cartilaginous phenotype.[164] The numerous collagen type I bundles, which are strong in tensile stress, are oriented in a circumferential direction and are considered to be very important in preventing radial extrusion of the meniscus and maintaining the structural integrity of the meniscus during load bearing. The GAGs play an important role in the maintenance of optimal visco-elastic behavior, compressive stiffness and tissue hydration (78% of the tissue is water). Furthermore, GAGs and surface zone proteins are thought to facilitate smooth frictionless movement of the menisci over the articular surfaces on the tibia and femur.[165]

Meniscal Repair

A majority of the meniscal tears result from the fast twisting or rotational movements of the loaded knee-which frequently occur during sports or daily activity. Tears are usually located in the inner avascular part of the meniscus and consequently do not heal spontaneously. Particularly, the large more complex tears have a very limited potential for repair, especially if there is knee instability due to additional ligamentous trauma.[166]

The capacity of the meniscus to heal itself is limited, particularly in the central portions, which are largely avascular, aneural and alymphatic. However, in 1936, King showed that the meniscal healing in dogs could occur providing there was a communication with the peripheral blood supply.[167] As with other soft and bony tissue, there needs to be a balance between blood supply, and hence associated cellular and tissue repair factors and component stability to permit healing.[167]

There are several methods currently in practice to treat a torn or damaged meniscus. These techniques include "leave alone", excision and repair[168]. Partial thickness tears (i.e. <8mm) are often left alone since there is a good likelihood of spontaneous healing. Arthoscopic menisectomy (total or partial) is the most common treatment undertaken for meniscal tears and is effective in eliminating mechanical and irritative symptoms associated with a damaged meniscus. However, in the long term, with decreased amount of meniscal tissue remaining, the load bearing and load distribution capacity is still compromised.[169] The loss of meniscus reduces surface contact area across the knee joint. The knee joint force is unaltered, therefore the stress is increased. This causes the degeneration and leads to osteoarthritis.

Annandale is credited with the first meniscal repair in 1885.[148] Since then many techniques have been developed and refined for use today. Most of these techniques involve the use of sutures, staples, or fixation of the loose fragments with anchors and screws.[170] A variety of adjuncts to promote successful meniscal healing have also been undertaken. Abrasion of the synovium with a rasp or arthroscopic punch leads to bleeding in the region of the repair and it is felt that the release of tissue repair factors into the local environment would be beneficial. Several studies have shown that rasping induces the cytokine release on the meniscal surface thus leading to the recruitment and proliferation of cells and synthesis of collagen, leading to promotion of healing in the avascular zone. The use of fibrin clots has been shown to stimulate repair in dogs. The fibrin is obtained from 70 ml of blood drawn pre-operatively and is then incorporated in to the repair. This had been recommended for complex tears and those extending to the avascular zone.[171]

Vascular access channels are another means of improving the blood supply to the healing meniscus. After repair the meniscus is 'needled' arthroscopically to encourage bleeding, and hence introduce fibrin clots into the repair site. This method can have its drawbacks.[172] The creation of a large vascular access channel injures the healthy meniscus and may disrupt the normal architecture (circumferential collagen fibers), causing impairment of meniscal functions, such as load bearing. Also, in some cases, the clots are not able to reach the actual point of the tear.[173]

Tissue engineering may offer new treatment modalities for the regeneration of meniscus lesions or for the replacement of a degenerate (parts of the) meniscus by a tissue-engineered construct. Tissue engineering is based on a smart and unique combination of cells, growth factors and scaffolds to enhance healing.[174] Meniscal cells are isolated and placed in scaffolds for growth and proliferation. These scaffolds are designed to be biocompatible and biodegradable in the long term. They also allow for cell growth, serve as a carrier for stimulatory and inhibitory growth factors and provide support for the knee joint.[175] These scaffolds are usually made from fibrin, polyglycolic acid, alginate or collagen type I and II without GAGs attached. Several different types of growth factors are also used in conjunction with the scaffolds to help stimulate cells to produce collagens and inhibit degradation of extracellular matrix. Some examples include TGF-B, platelet derived growth factor (PDGF) and Insulin-like growth factor (IGF-I).[176]

Tissue engineering is also used in the case where total mesicectomy is inevitable. In this case there is a need for implants that could be used to replace the patient's own meniscus. Much work has been done to develop meniscus prostheses, which are based

on autologous, allograft or synthetic material or a combination of synthetic materials an autologous tissue.[176,177] With respect to autologous materials, a number of tissues have been used, such as infrapatellar fat tissue, perichondrium, tendon and synovial flaps. There are still many problems associated with meniscus prostheses such as poor mechanical characteristics and post surgicalcalcification.[177]

Rationale

Wound healing involves a number of complicated processes, which include inflammation, new tissue formation and tissue remodeling, all of which finally leads to at least partial reconstruction of the wounded area.[178,179] Proteins known as growth factors are key players in this process. Growth factors are proteins that bind to receptors on the cell surface, with the primary result of activating cellular proliferation and differentiation. Cytokines are a unique family of growth factors that are secreted primarily from leukocytes. Cytokines that are secreted from lymphocytes are termed lymphokines, whereas those secreted by monocytes are termed monokines.[180]

Coating implants with locally active growth factors has been shown to influence the remodeling process at the tissue-implant interface and therefore the integration of implants into healing bone. This can also hold true for tissue injuries coated with growth factors. Growth factors like PDGF, and bone morphogenetic proteins (BMPs), IGF and TGF-B facilitate the osseointegration of implants.[181] The growth factors were immobilized on collagen or demineralized bone matrix by absorption so they could be released in a controlled manner. Growth factors such as TGF-B and BMP have been shown to stimulate production of extracellular matrix proteins including collagen type I and type III, proteoglycans and fibronectin. Several other growth factors , includin EGF

(epidermal growth factor), TGF-A and PDGF-AB have been shown to enhance meniscal fibrochondrocyte proliferation.[182] These growth factors also have been found capable of increasing the collagen and non-collagen protein synthesis in a number of meniscal cells.[183]

The motivation for this work came from the existing meniscal repair method, particularly the introduction of blood clots and vascular access channels during meniscal surgery. While these clots and vasculature are introduced to produce cytokines in the tear and enhance its healing, this is a very nonspecific effect. These cytokines are not anchored at the site of injury and could easily be washed away with the body's natural fluids. Site specific immobilization through covalent bonds could result in an increased stability of the cytokines at the site of injury. Growth factor receptor proteins could also be immobilized on the meniscal tear to recruit growth factors to the site of injury. An ideal methodology would also provide the ability to carry out reactions only at desired locations giving more control over the healing process.

By attaching proteins or other molecules directly to the site of injury, a more localized healing effect can be stimulated. One such protein of interest that has played a role in motivating this research is tissue factor. Tissue factor (TF) is the cell surface integra-membrane receptor that initiates the extrinsic pathway of blood coagulation. Tissue factor is a member of the class II cytokine growth factor receptor family and is released during an injury to the tissue is a key player in the clotting cascade.[184] Soluble tissue factor could potentially be immobilized at the site of meniscal injury and should result in local thrombosis and the accompanying release of proteins that enhance healing. Other proteins, such as growth factors, which are also involved in would healing, could

potentially be modified and immobilized on the tissue surface.[185] Alternatively, anti-coagulant proteins or peptides such as tissue factor inhibitory pathway could be used to avoid clotting and again potentially enhance wound care.

Growth factors and growth factor receptors can certainly enhance meniscal healing.[186, 187] However, there is a lack of suitable methods for delivering the appropriate dose of growth factors to the meniscal lesion. Direct administration of the meniscal growth factor proteins is not effective because of the rapid clearance and relatively short biological half life of these proteins. Repeated injections of the growth factors would consequently be necessary, which is not clinically acceptable. One method that is currently being investigated for the introduction of growth factors into meniscus is gene therapy.[188] Gene therapy using viral and non-viral vectors may facilitate the delivery and expression of growth factor genes in the injured meniscus. Two different gene therapy approaches can be applicable: the direct (*in vivo*) and the indirect (*ex vivo*) methods. The direct method consists of the vector carrying the gene of interest being applied directly into the tissue. The indirect method is a process whereby cells are removed from the body, genetically manipulated in vitro, and reimplanted into target tissue.[189]

Our research could greatly impact the delivery of growth factor into meniscal lesions. Immobilized growth factor would provide a more localized response and remove the need for repeated injections. This could also be less costly than gene therapy methods.

Immobilization of proteins on tissue surfaces can also have many other potential applications other than the delivery of healing agents. Proteins or peptides could be incorporated after angioplasty to slow cell growth and avoid retonisis. Protein

immobilization techniques for tissue surfaces could also be used in tissue engineering. For example, angiogenesis could be enhanced in a spatially defined fashion in artificial organs by the use of well characterized angiogenic proteins and peptides. As a specific example, cultured skin could be modified in a spatially defined fashion with proteins aimed at enhancing the development of a capillary network.

Controlling the spatial organization of cells is vital in tissue engineering that requires defined cellular architectures. Cell adhesion factors could be directly immobilized on tissue implants which could increase the chances of the implant being incorporated into the body. Proteins directly immobilized on tissue present a far more biologically compatible environment for SAR studies than the artificial scaffold currently in use.

Tissue immunogenecity could be enhanced by modifying surfaces with immunogenic proteins or peptides. This could be used to potentially elicit an immune response to a given tissue-for example, a solid tissue that has escaped the immune system.

Another area of application could be the use of these techniques for tissue bonding. For example, the free amine groups on tissue surfaces could be utilized with a homobifunctional crosslinker to bond tissue surfaces. A biotinylated tissue surface could also be bonded to another tissue surface containing immobilized avidin, via the avidin-biotin interactions.

Some Possible Applications of Modified and Immobilized Proteins

Proteins are frequently immobilized using covalent chemistry in biomaterials research.[190] Cell adhesion to extracelluar matrices provides tissue structure and signals

critical in development, tissue remodeling and wound healing. Furthermore, cell adhesion to adsorbed proteins or biomimetric surfaces is central to biotechnological and biomedical applications, such as cell growth supports, biomaterials and tissue engineering.[191] Biomaterial plays an important role in most tissue engineering strategies. Biomaterials can serve as a substrate on which cell populations can attach, migrate and be implanted with a combination of specific cell types as a cell delivery vehicle, and be utilized as a drug carrier to activate specific cellular function in the localized region.[192, 193] Incorporation of cell binding proteins can help endow the biomaterial with bioactivity making the surface of the biomaterial cell adhesive.[194]

Research Goals

Our goal was to develop methods to site specifically immobilize molecules on fresh tissue surfaces. The molecule could be a protein (such as tissue factor) or a small organic molecule (such as biotin). The immobilization should be accomplished efficiently and quickly to avoid a prolonged surgical time (if it is to be used in tissue repair). The immobilized molecules also had to remain active for an extended period of time once immobilized on the tissue surface so as to affect the healing process (in the case of meniscal repair). Maintaining the activity of a protein is a critical issue during immobilizations techniques since the chemistry required can potentially alter the confirmation of the protein and reduce or inhibit its activity. However, as noted earlier, several groups have shown that proteins immobilized on solid surfaces do retain their activity, and in some cases, the immobilized proteins are more active than unbound proteins.

We hypothesized that the techniques used to modify and immobilize soluble proteins could be adapted to be used to modify proteins on the surface of tissue. Several different strategies were investigated for the immobilization of the desired biomolecules. The direct alkylation method involved reacting a protein or biomolecule (bearing an amine reactive species such as an active ester) directly to the $-NH_2$ groups present on the tissue surface. The reductive methods involved the reduction of the tissue surface with a thiol reducing agent to give free sulfhydryl. The reduced tissue surface was then reacted with a protein or biomolecule bearing a thiol reactive species. The oxidative technique involved the use of periodiate to oxidize the geminal diols (e.g. those found in proteoglycans in the ECM of tissue) to carbonyl compounds. The oxidation was followed by the reaction of the tissue surface with a nucleophilic reagent such as hydrazides.

Several tissue types were also investigated for the immobilization. These were mostly tissues composed of type 1 collagen such as bovine meniscus, pericardium, aorta, cornea, fetal pig skin, and rat skin. These tissue were modified in a spatially defined manner (by physically limiting the sites of exposure of tissue to the reagents), or in a uniform bulk fashion (by exposing the entire tissue to sample to the reagents). Presence of the desired surface modification has been primarily detected using biochemical detection methods (i.e. chemiluminescence, colorimetric and fluorescence).

Section Two: Direct Alkylation Method of Tissue Modification

Experimental Strategy and Results

In the most straightforward strategy, the direct alkylation method, we investigated the use of amine reactive compounds to modify free amine groups on the surface of

meniscus. As noted earlier in the introduction, utilizing amine reactive probes to modify lysine or terminal $-NH_2$ groups in soluble collagen and other proteins via amide linkages is a fairly common technique in biochemistry. Amine reactive dyes are widely used in many areas of biochemistry and molecular biology for immunochemistry assay, cell tracing, and receptor labeling. Various proteins (e.g. antibodies) are modified using their lysine residues to attach a fluorescent tag, contrast agent, or other probes of interest. These modified proteins are then used in blotting, enzyme linked immunoassays (ELISA) or structure activity relationship (SAR) studies. We hypothesized that the reagent used for the modification of free amines in soluble proteins could be easily adapted to modify proteins on tissue surfaces.

There were two main challenges for the research: the immobilization of biomolecules utilizing the free amines on the tissue surface, and the detection of the immobilized biomolecules via a convenient detection assay. Initially, we decided to use a model assay to test our methods. The model served to investigate possible pathways to immobilize a biomolecule (e.g. protein or fluorescent probe) to the tissue surface and various methods to detect its presence.

Three major classes of commonly used reagents were investigated to label amines on the tissue surfaces: succinimidyl esters (SE) (including sulfosuccinimidyl esters), isothiocynates (ITC), and sulfonyl chlorides (SC). Other possible amine reactive groups include dichlorotriazines, aryl halides and acyl halides.

Succinimidyl esters are excellent for amine modification because the amide bonds they form are as stable as peptide bonds. There are also many commercially available succinimidyl esters of fluorescent dyes and nonfluorescent biomolecules like biotin. The

great advantage of using an amide bond formation to tether a biomolecule to a surface is the fact that the chemistry is simple, straightforward, easily reproducible, and can sustain a diversity of experimental conditions which is a prerequisite for the development of a protein immobilization method.

One of the most common modifications performed on proteins is biotinylation, followed by conjugation to a labeled avidin. The avidin is usually labeled with a fluorescent tag or radioactive probe or enzyme, which can then be visualized using fluorescence microscopy, photographic film or an enzymatic assay. The avidin-biotin techniques are popular due to the high selectivity and affinity between the two reagents ($K_a = 1 \times 10^{15} M^{-1}$) and the ability of avidin to bind four biotin molecules. This egg-white protein (avidin) and its bacterial counterpart (streptavidin) have become standard reagents for many diverse detection schemes.[195] These techniques are commonly used to visualize antigens in cells and tissues and to detect biomolecules in immunoassays and DNA hybridization techniques. Labeled avidin/streptavidin conjugates and biotin products are generally less expensive than labeled antibodies. They are also easy to prepare, and many are commercially available.

For our model system, we decided to use a biotin-N-hydroxy succinimide ester for coupling with free $-NH_2$ groups, followed by chromogenic detection with avidin labeled β-galactoside. β-galactosidases are enzymes that exhibit very high selectivity for hydrolysis of their preferred sugars. Endogenous galactosidase activity is frequently used to characterize strains of microorganisms and label organelles of mammalian cells. Galactosidases are also used in conjunction with galactosidase-conjugated secondary detection reagents in immunohistochemical techniques and ELISAs. β-galactosidase

assays are also attractive due to the fact that there are many fluorogenic and chromogenic galactosidase substrates commercially available.

In our model assay, we used the chromogenic substrate for β-galactosidase, X-gal (5-bromo-4chloro-3-indoyl β-d-galactopyranoside) (Figure 28), which yields a dark blue precipitate at the site of enzymatic activity and can often be detected with the naked eye.[196]

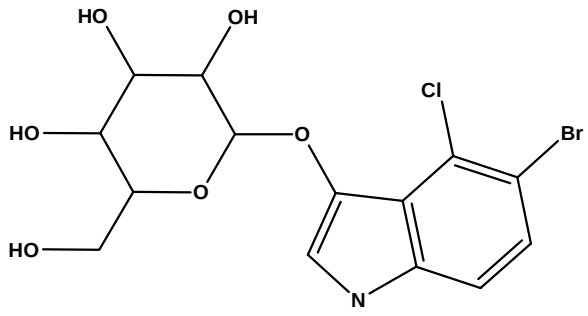


Figure 28. Structure of X-gal

We initially decided to perform a gross modification of the entire surface meniscal slices in order to gauge the availability of the free $–NH_2$ on the meniscal surface. PBS buffer was used because other types of buffers that contain free amines, such as Tris and glycine, must be avoided to prevent deactivation of the amine reactive probe. Biotin-xx-se, 6-((6-((biotinoyl) amino) hexanoyl) amine) hexanoic acid (biotin-NHS)[197], was used as the amine reactive probe because it has a 14-atom spacer between the biotin and a reactive succinimide ester. This spacer helps separate the biotin moiety from its point of attachment, potentially reducing the interaction of biotin with the tissue surface to which it is conjugated and enhancing its ability to bind to the relatively deep biotin binding sites in avidin.

The meniscus was sectioned longitudinally and the slices were exposed to the biotin-xx-se (biotin-NHS), followed by avidin-β-galactosidase, and finally x-gal to visualize the modification. A blocking step with BSA was used between each step to prevent any non-specific binding of avidin to the surface of the meniscus. A blue precipitate was formed only on the sections of tissue modified with biotin-NHS, indicating that the biotin was immobilized on the tissue (Figure 29).

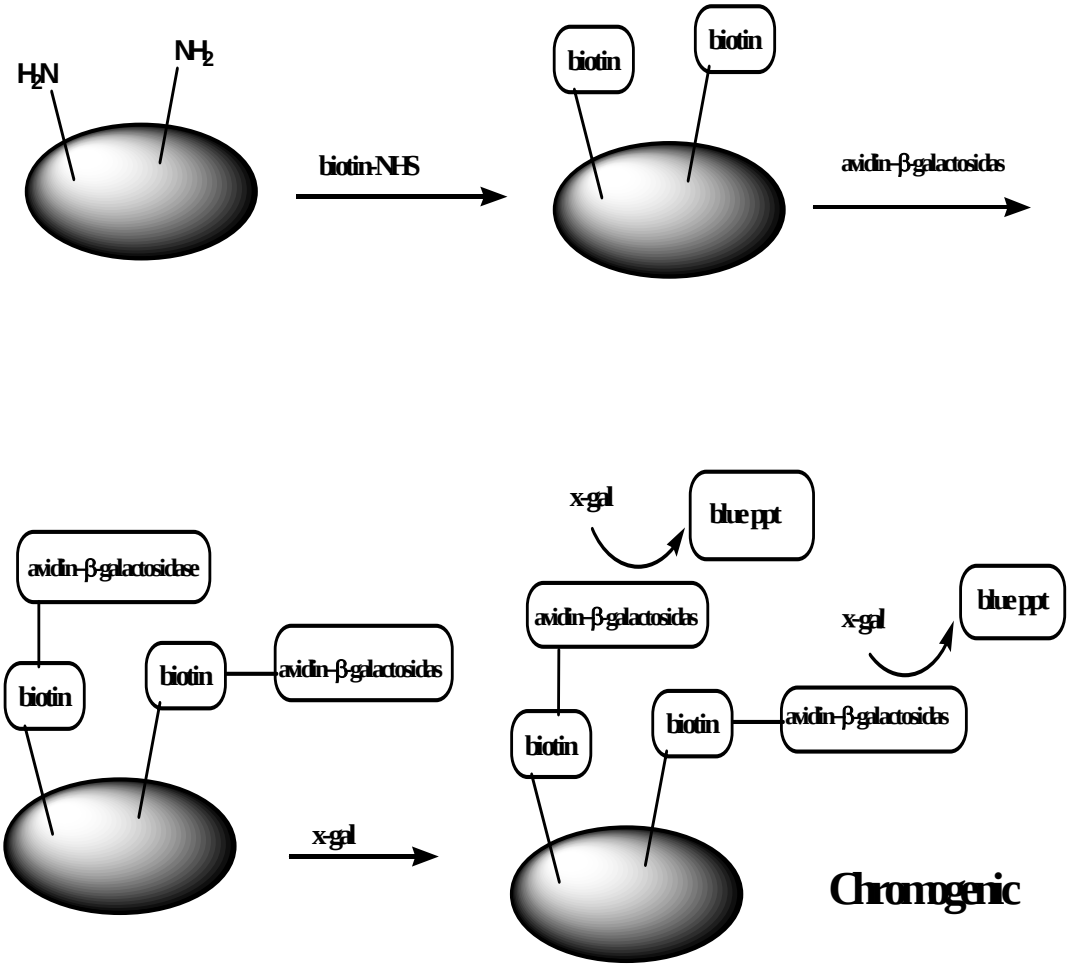


Figure 29. Modification of meniscus using direct alkylation method with chromogenic detection.

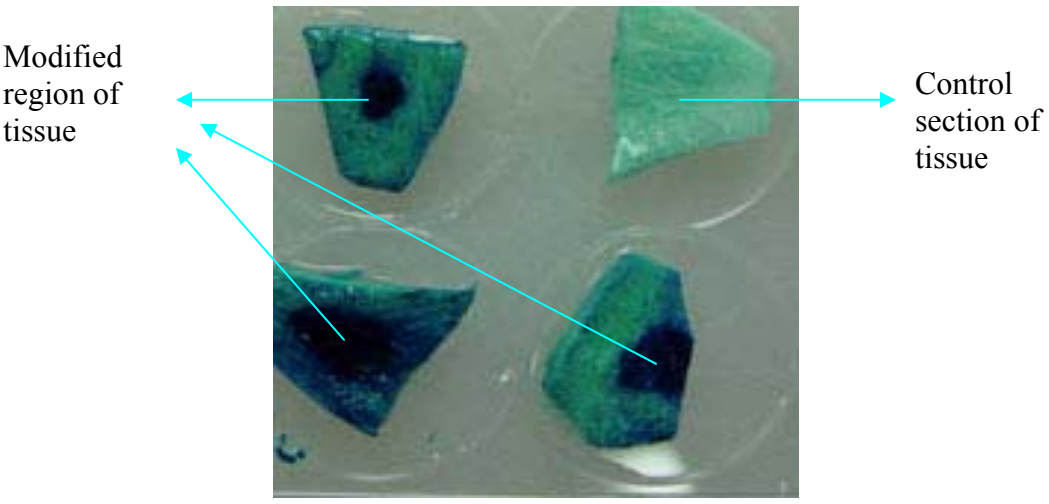


Figure 30. Site specifically modified meniscal slices using biotin-NHS and chromogenic detection with x-gal. The regions of tissue modified with the biotin-NHS display an intense blue precipitate, while the controls remain fairly clear.

This experiment demonstrated that the tissue amine groups could be modified with the biotin-xx-se ester and that the β-galactosidase/ x-gal system could successfully detect the bound biotin (see Figure 30). The next step was to test if the meniscal surface could be site-specifically modified by the biotin-NHS. A small quantity of biotin-xx-se (in DMSO) was added to the center of the meniscus by hand and allowed to remain as a drop on the surface for the desired period of time. The immobilized biotin was then detected using our chromogenic assay. We were able to see deposits of blue precipitate primarily at the site of alkylation with the biotin ester, indicating that the site specific immobilization was also successful. By increasing the concentration of the biotin ester to 150mg/ml, we were also able to reduce to the reaction time on the surface of the meniscus to about 1min.

In order to determine the homogeneity of the free amine groups on the meniscal surface, two different amine stains, coomassie[198] and silver[199], were performed on the

meniscal slices. The general coomassie stain and the more sensitive silver stain both failed to show any heterogeneity in the available amino groups on the tissue surface.

We also investigated fluorescence as an alternate type of detection method for the tissue modification to broaden the scope of our assay. As discussed earlier, there are many commercially available fluorescent probes, and it is also fairly easy to label a desired biomolecule with a florescent probe. Fluorescence is also generally a more sensitive detection method allowing for improved resolution and greater signal intensity.

Once the tissue slices were alkylated with the biotin ester, streptavidin conjugated fluorescent probe (streptavidin-fluorescein and steptavidin Alexa Fluor-430) was coupled to the bound biotin. The slices were then imaged to detect the immobilized biotin via fluorescence. Unfortunately, we were unable to see a clear difference in signal between the modified and control slices (which were not alkylated with the biotin ester) when using the streptavidin-fluorescein. The streptavidin Alexa Fluor-430 probe did enable us to distinguish between control and modified sections of meniscus, but we were unable to consistently generate positive results with this assay. We realized that the tissue itself has a high degree of background fluorescence, which makes it very difficult to differentiate between the control and modified slices. The background fluorescence is due to the natural fluorescence of the tissue and the non-specific binding of the reagents.

Natural fluorescence of tissue is due in large to substances like flavins and porphyrins. These compounds are generally extracted by solvents and are not a problem in fixed tissue, but even then there is always some background fluorescence.[200] However, we wished to avoid harsh treatments, preferring to keep the system biocompatible as possible.

One way we sought to overcome the natural fluorescence and to acquire interpretable signal was to increase the fluorescence of the modified slices above the autofluorescence of the tissue. Consequently, we decided to investigate the possibility of coupling a fluorescent probe directly on the surface of the meniscus. The direct conjugation might allow for a greater concentration of the probe to be present on the surface and hence increase the signal above the background auto fluorescence of the tissue. This could also provide a clear map of the available $-NH_2$ groups present on meniscal surface.

We also attempted to increase the fluorescent signal by using an enzymatic digestion to remove any heteropolysaccharides present on the tissue surface prior to the amine reaction. The most abundant heteropolysaccharides in the body are the glycosaminoglycans (GAGs). These molecules are long unbranched polysaccharides containing a repeating disaccharide unit. The disaccharide units contain either of two modified sugars: N-acetylgalactosamine (GalNAc) or N-acetylglucosamine (GlcNAc) and an uronic acid such as glucuronate or iduronate. GAGs are highly negatively charged molecules, with extended conformation that imparts high viscosity to solutions of these compounds. GAGs are located primarily on the surface of cells or in the extracellular matrix (ECM). Along with the high viscosity of GAGs comes low compressibility, which makes these molecules ideal for a lubricating fluid in the joints. At the same time, their rigidity provides structural integrity to cells and provides passageways between cells, allowing for cell migration. The specific GAGs of physiological significance are hyaluronic acid, dermatan sulfate, chondroitin sulfate, heparin, heparan sulfate, and keratan sulfate. The majority of GAGs in the body are

linked to core proteins, forming proteoglycans (also called mucopolysaccharides). The GAGs extend perpendicularly from the core in a brush-like structure.

Chondroitinase ABC is an enzyme that has been used to remove GAGs from the surface of cartilage or tissue, because the GAGs could potentially interfere with reactions on the surface of the meniscal slices and hence reduce signal intensity. We believed that the absence of GAGS from the tissue surface could enhance the biotin-NHS reaction and prevent any non-specific binding of avidin and other reagents. A simple chondroitinase ABC digestion was performed on the meniscal slices before conjugating with biotin-NHS or fluorescent tag. Unfortunately, this experiment did not improve our results.

The Alexa Fluor dye conjugates are fluorophores that have been used in lieu of fluorescein in a variety of fluorescein based assays (e.g. western blotting, ELISA, cell tracing etc). These dyes have a spectrum almost identical to that of fluorescein, but with far greater fluorescence and significantly better photostability. Alex Fluor 430 NHS (Molecular Probes, St Louis, MO.) was used as probe since it had an emission wave length of 530nm, which is compatible with the Bio-Rad imager available to us and it is also a very common amine reactive probe. When the Alexa Fluro-430 NHS was directly coupled to the tissue surface, we were able to detect a site specific fluorescence signal from the modified regions on the tissue.

Fluorescein isothiocynate (FITC) is another common amine reactive probe used widely in biochemical assay. Isothiocynates form thioureas upon reacting with amines and are quite stable in water and most solvents (see discussion under amine modification). We used FITC as an alternative to the Alex Fluor 430 dyes since it is a less expensive alternative to the more expensive Alex Fluor dyes. The FITC also gave a

site specific fluorescence signal from the modified regions of the tissue, and was able to alkylate the tissue surface with a reaction time of about 1min (see Figure 31).

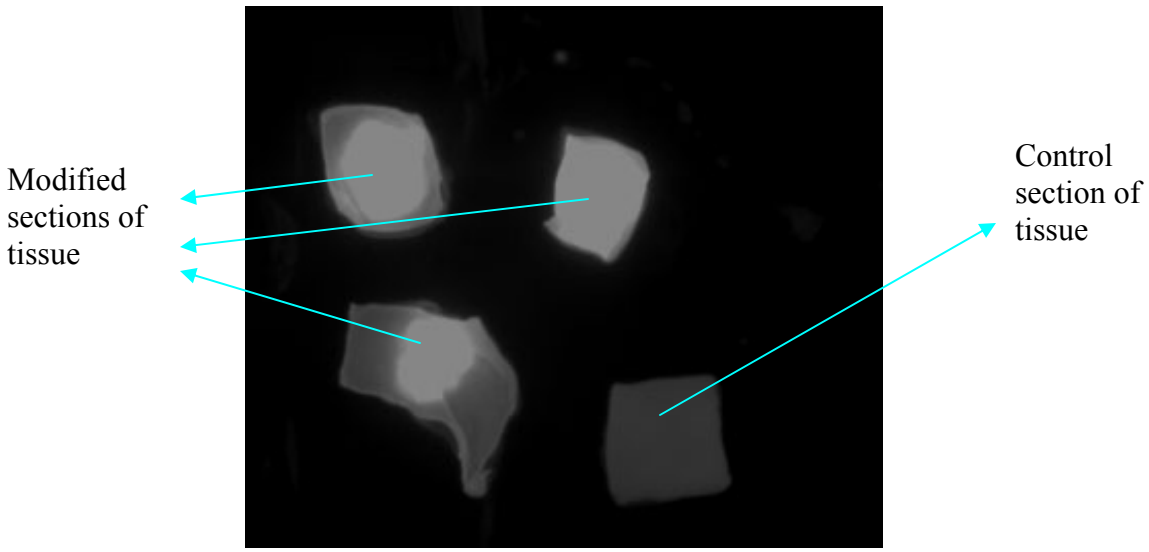


Figure 31. Fluorescence image of meniscal sections modified with Flouresceiniso thiocynate (FITC)

The chromogenic assay for the detection of bound biotin was successful, but not on a consistent basis. The results of the experiments varied depending on the batches of meniscal tissue used. Of the fluorescence based assays, only the FITC and Alexa Flour 430 gave consistent results. Most of the other fluorescence assays we tried were plagued by either background or autofluoresence or poor binding of the fluorescent probe. Even though the FITC and Alexa Fluor 430 assays were successful, our goal of attaching a protein on the tissue surface needed further investigation.

The meniscus also proved to be a fairly difficult tissue on which to work. One problem was the fact that there was too much variability between each meniscus. The color of the tissue varied from milky white to yellow. The consistency of the tissue also ranged from soft to brittle. Depending on the age of the cow, the meniscus could also be

heavily crosslinked and calcified. A heavily crosslinked meniscus probally would have very few available $-NH_2$ groups for reacting with the biotin-xx-se, and would severely decrease the signal intensity of any detection method. There was no knowledge of age, sex, breed, size, general health or type of cow from which the menisci were harvested since the slaughterhouse did not keep such records. Due to the variability of the tissue, it became increasingly difficult to pinpoint a general set of reaction conditions for the biotinylation assay.

Because the meniscus proved to be a difficult tissue to work with, we turned our attention to bovine pericardium. Dr. Les Mathews of the Baylor Medical Center, Dallas, TX, advised us that most of the problems we were facing with the inconsistency of the meniscus could be avoided by using a more consistent tissue such as bovine pericardium. Bovine pericardium is a well characterized tissue with little variability, and therefore is a much more consistent surface on which to carry out the model experiments of protein patterning.

The direct alkylation method used on the meniscus with the biotin-NHS ester and the chromogenic detection was repeated with the pericardium. Unfortunately, the pericardium also failed to give us consistent results with this method. We reasoned that the chromogenic assay was not sensitive enough to detect the amount of biotin binding to the tissue surfaces. We also attempted the florescence detection of the biotin with streptavidin Alexa Fluor 430, but when using this assay we were also unable to differentiate between control and modified slices. There was also a fair degree of non-specific fluorescence from the pericardium. Part of the background fluorescence is due to the fact that avidin fluorescent conjugates also have a tendency to bind non-specifically

to the tissue. The rest is due to the natural fluorescence of the tissue itself. FITC, which was successful at modifying the meniscal tissue, was equally successful with pericardial tissue. We were able to paint the reagent on the tissue surface in an 'x' pattern using a Pasteur pipette and detect the modification via fluorescence. The image displayed a bright 'x' in the region of tissue modified with the reagent (Figure 32).

Figure 32. Fluorescence image of pericardium section reacted with FITC in an 'X' pattern

The preferred reagents for detecting and quantifying amines in solution or on amine-containing polymers are those that are non-fluorescent but form fluorescent conjugates stoichiometrically with amines. Fluorescamine is non-fluorescent but readily reacts with primary aliphatic amines, including those in peptides and proteins, to yield a blue-green fluorescent adduct that can be excited by UV light. The amine adduct has absorption maximum at 385 nm and fluorescence maximum at 486 nm. Fluorescamine is widely used to detect amino compounds, including amino acids, peptides and proteins. It is a popular fluorogenic reagent for determining protein concentrations in solutions and

on gels. Fluorescamine is also used to analyze low molecular weight amines by TLC, HPLC and capillary electrophoresis.[201]

The homologous aromatic dialdehydes o-phthaldialdehyde (OPA), and naphthalene-2,3-dicarboxaldehyde (NDA), are essentially non-fluorescent until reacted with a primary amine in the presence of excess cyanide or a thiol, such as 2-mercaptoethanol, 3-mercaptopropionic acid, or the sulfite, which result in the formation of a fluorescent isoindole. Modified protocols that use an excess of an amine and limiting amounts of other nucleophiles permit the determination of carboxylic acids and thiols, as well as of cyanide in blood, urine and other samples. Without an additional nucleophile, NDA forms fluorescent adducts with both hydrazine and methylated hydrazines (excitation/emission maxima ~403/500 nm).[202] Amine adducts of NDA have longer-wavelength spectral characteristics and greater sensitivity than the amine adducts of OPA. The stability and detectability of the amine derivatives of NDA are also superior; the detection of glycine with NDA and cyanide is reported to be 50-fold more sensitive than with OPA and 2-mercaptoethanol.[203]

The reagents Fluorescamine, NDA and OPA are attractive for our model assay because they only fluoresce when reacted with a primary amine. This eliminates unwanted fluorescence due to non specific binding as in the case of streptavidin conjugated probes. For reactions with bovine pericardium, we focused only on NDA and fluorescamine since they are the more sensitive reagents. The direct coupling of NDA proved successful at modifying the tissue surface and emitting a fluorescence signal above the control tissue. Unfortunately, the fluorescamine assay failed to generate positive results. These results demonstrated that a fluorescent probe directly coupled to

the tissue surface is often able to emit a fluorescence signal above the natural fluorescence of the tissue. However, if the fluorescent probe is indirectly bound via biotin and streptavidin, the signal is not strong enough to overcome the natural fluorescence of the tissue. This may be related to the fact that the amount of biotin present of the tissue surface is not able to bind enough of the streptavidin conjugated probe to overcome the background fluorescence.

Based on these results, we determined that the fluorescence and chromogenic assays were not sensitive enough to be used in an indirect assay. Also, the emission wavelength of the fluorescent probes was too close to the natural fluorescence of the tissue. One way we could have overcome this was to use a probe that emitted in the IR region of the UV the spectrum. Unfortunately, this required the use of special filters which were not available to us.

Chemiluminescence, as discussed earlier, is a far more sensitive method of detection than fluorescence. Chemiluminescent substrates differ from other substrates in that the light detected is a transient product of the reaction that is only present while the enzyme substrate reaction is occurring. Using the chemiluminescence substrates also allows multiple exposures to be performed to obtain the best image, and a large linear response range allows detection and quantification of the proteins immobilized. The main advantage in the context of this research is the fact that chemiluminescence allows for the freedom from autofluorescence of the tissue (Figure 33).

For our initial chemiluminescence assays, pericardial sections were directly alkylated with a biotin ester (i.e.biotin-LC-NHS, which was essentially identical to the biotin-xx-se, was used from Pierce chemicals due to cost considerations, see Figure 50)

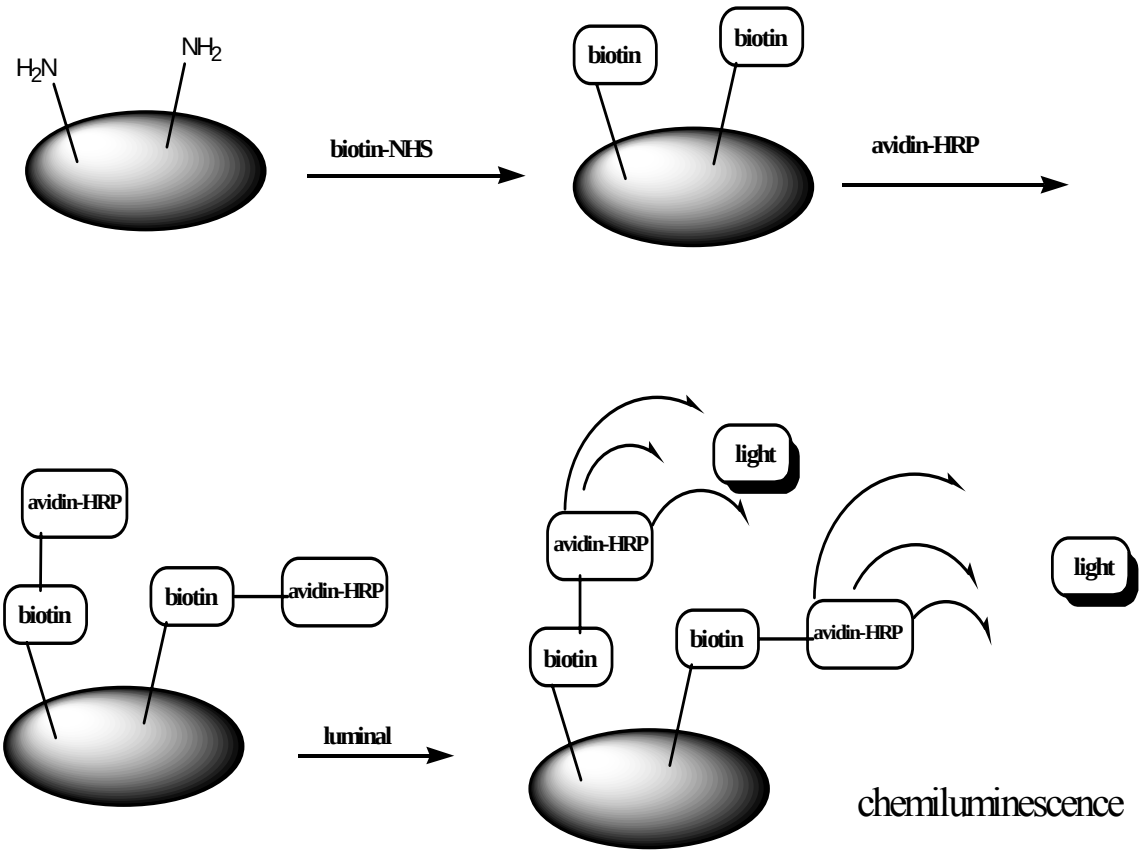


Figure 33. Direct alkylation modification of tissue with chemiluminescent detection

and then incubated with streptavidin-HRP conjugate. The modified tissue was then immersed in luminol (a substrate for HRP based chemiluminescence) and detected using a Bio-Rad detector. In this case we were successful in detecting the regions modified with biotin-LC-NHS on the tissue surface. Using a 96 well plate, a more site specific immobilization was carried out in which the tissue was alkylated in a 'BU' pattern. The resultant chemiluminescence image displayed a bright 'BU' pattern, where the reagent was contacted with the tissue surface, against an almost dark background (Figure 34). The background was not completely dark due to non specific binding between of the avidin-HRP, but this is a common phenomenon with biotin-avidin systems.

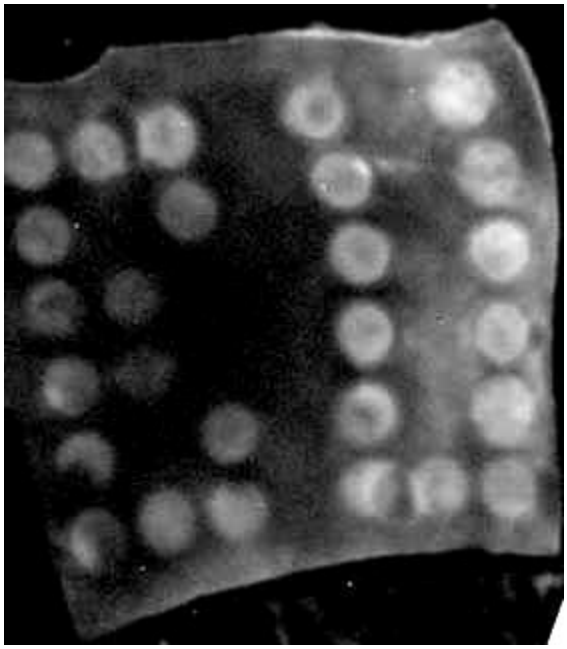


Figure 34. Chemiluminescent image of bovine pericardium site specifically alkylated with biotin-NHS in a 'BU' pattern. The tissue was modified in a way to have the modified 'pixels' light up in a 'BU' pattern for Baylor University. The pixel resolution is ~4mm in diameter.

To reduce the background signal and increase the signal of the modified region, we used a biotin-TFO-PEO probe, which has a better leaving group and a longer spacer arm than the biotin-LC-NHS. A blocking step with BSA was also done in an attempt to reduce the non-specific binding of avidin to the tissue surface. The image from this experiment had far less background when compared to the biotin-LC-NHS modified tissue. The quantity of proteins immobilized on a tissue surface was approximated by comparing the signal on the tissue to a series of serial dilutions of avidin on a nitrocellulose membrane along with modified tissue. The intensity of the signal from the known concentrations of avidin was compared to the signal intensity of the modified regions on the tissue to estimate the quantity of protein immobilized.

We also directly alkylated a sample of fresh fetal pig skin with biotin-LC-NHS using a 96 well plate method. A chemiluminescence assay with avidin-HRP and luminal was used to detect the immobilized biotin. The resultant chemiluminescence signal intensity from the image on the skin was much stronger than the pericardium. We also measured the amount of avidin immobilized in the tissue surface by using a nitrocellulose membrane with serial dilutions of avidin and the amount of protein immobilized on the skin surface was calculated to be about 1ug/mm^2.

Since the chemiluminescence based assays were very successful at detecting modification on the pericardial surfaces, we investigated the possibility of direct alkylation modification of other tissue types including meniscus, aorta, skeletal muscle and rat skin. We repeated the direct alkylation of the meniscus with biotin-NHS, but used the avidin-HRP and luminal based chemiluminescence assay for detection. In this case, there was a clear difference between the modified and control meniscal slices. Similar experiments were carried out on bovine skeletal muscle, aorta and rat skin. However, we were unable to detect any modification on these tissue types.

Material and Methods

Chemicals and Reagents

Except where otherwise noted, reagents used for the modification were obtained from Aldrich Chemical CO., Milwaukee, WI, and Fisher Scientific, Pittsburgh, PA (including Across reagents), Pierce and Molecular Probes, and used directly as purchased. Solvents such as dichloromethane, hexane, ethanol, ethyl acetate and methanol were obtained from commercial sources (via Baylor University Chemistry

Department stockroom) and were distilled prior to use. Deionized water (DI) was obtained via a US Filter system provided by Baylor Dept. of Chemistry.

Bovine pericardium was obtained from Animal Technologies Inc. in Tyler, Texas. The pericardium was cleaned of all excess fat using a microtome blade. The connective tissue was carefully removed by scraping with the blade and care was taken not to damage any of the actual pericardium. The cleaned pericardium was placed in a PBS solution at room temperature for up to 2hrs before use. Unused pericardium was stored at -20^0C and defrosted as needed.

The fetal calf skin was obtained from Animal Technologies, and the rat skin was obtained from Department of Neuroscience at Baylor University. The fetal pig skin was washed in PBS and trimmed of any excess fat using a microtome blade. It was then allowed to soak in PBS until use. Unused skin was stored at -20^0C and defrosted and hydrated in PBS before use. The rat skin was obtained fresh, and the hair was removed by shaving or with the use of Nair (Church and Dwigth Co., NJ.), a commercial hair removal product. It was then soaked in PBS for up to 2 hrs before use or stored at -20^0C.

Bovine menisci were obtained from H&B Packing, Waco, TX. The menisci were dissected from whole bovine knees, trimmed of fat and stored in PBS for use. Excess menisci were also stored at -20^0C and rehydrated in PBS when used for experiments. The meniscus was sliced into longitudinal sections of desired thickness using a microtome blade or scalpel and trimmed to fit into compartments of a 12 well plate.

The skeletal muscle and aorta were also obtained from Animal Technologies, soaked in PBS and used as obtained. The cornea was dissected out of whole bovine eyes,

soaked in PBS for use. Any unused aorta, skeletal muscle and cornea were stored at -20^0C and defrosted and hydrated in PBS before use.

Instrumentation

Proton (^{1}H) and carbon (^{13}C) NMR spectra were obtained on a Bruker AVANCE 300 NMR running XWIN-NMR 3.1 software at 300 MHz. Chemical shifts are expressed in ppm(∂), peaks are listed as singlets (s), doublets (d), triplet (t) or multiplet (m), with the coupling constant (J) expressed in Hz. High performance liquid chromatography (HPLC) was carried out with a Beckman System Gold, composed of a model 168 detector and a model 126 solvent module using, a Alltech Alltima C-18 5M 33mm*7mm "rocket" column. GC-Mass spec was obtained on a Hewlett Packard. Fluorescence and chemiluminescence were detected using the Bio-Rad Laboratories Fluor-S Multi-Imager and analyzed using Bio-Rad software. The chromogenic images were recorded using a Kodak 2 mega pixel digital camera.

Experimental Methods for the Direct Alkylation Strategy

Experiment 1: Gross Modification of the Meniscal Surface with Biotin-NHS ster and Chromogenic Detection

Fresh bovine meniscus was cleaned of any excess fat and connective tissue with a scalpel and a microtome blade. It was cut into longitudinal sections about 1cm in thickness using the microtome blade. The slices were placed in compartments of a 12 well plate and were soaked in PBS buffer for 30 min at room temperature until used for the experiment.

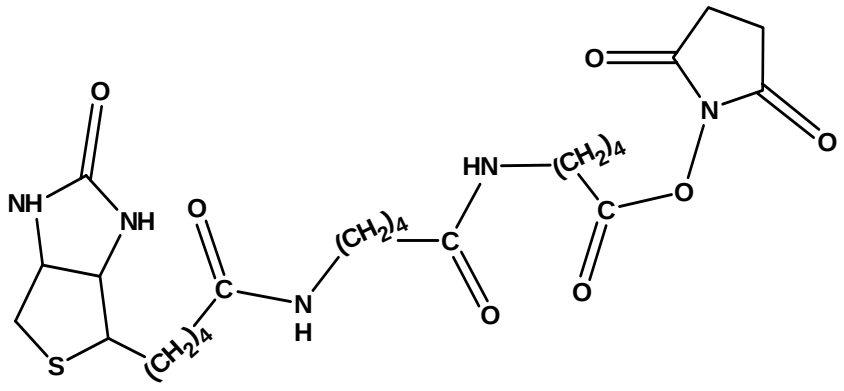


Figure 35. Structure of Biotin-xx-se (biotin-NHS)

100 μL of a 20 mg/ml solution of the biotin-xx-se, 6-((6-((biotinoyl) amino) hexanoyl) amine) hexanoic acid in PBS (with 10% DMSO v/v to assist in solubilizing the reagent) was added to each well containing the tissue slices to be modified, and allowed to react for 10min at room temperature. Additional wells were filled with 100 μL of PBS (also with 10% DMSO) to serve as controls. All the meniscal slices were washed in PBS (2x5 min), with a change of buffer between washes to remove any unreacted biotin ester. All the slices, including controls were then placed in the blocking buffer (PBS containing 2. 5% BSA w/v and 0.05% Triton X-100) for 1hr at ambient temperature. The sections, including controls, were washed in PBS (3x10 min), with a change of buffer between washes and placed in 5 ml avidin-β-galactosidase (0.5 mg/ml in PBS) solution in a Petri dish for 2 hrs at room temperature. The slices were once again washed several times with PBS and 5 ml X-gal (1 mg/ml in PBS) was added and left to react at room temperature. After about 1hr the slices that were modified with the biotin-xx-se displayed a blue precipitate, whereas the control tissue slices remained clean.

Experiment 2: Site Specific Modification of Meniscal Surface with Biotin-NHS and Chromogenic Detection

Fresh bovine meniscal sections 1 cm in thickness were soaked in PBS for 30min and then placed in individual chambers of a 12 well plate. 3 μL of a 2 mg/ml concentration biotin-xx-se in DMSO was then added carefully by hand-held micropipette to the surface of each tissue slice to be modified.

Figure 36. Site specifically modified meniscal slices with biotin-NHS and chromogenic detection with x-gal. The regions of tissue modified with the biotin-NHS display an intense blue precipitate, while the controls remain fairly clear.

The solution was allowed to remain as a deposit on the meniscal surface for about 30 min at room temperature. To serve as a negative control, 3 μL of DMSO was added to the surfaces of several meniscal slices. The tissues were then washed in PBS (2x5 min) and placed in the blocking buffer at ambient temperature. After 30 min, the slices were once again washed in PBS (2x5 min) and placed in a solution of avidin-β-

galactosidase (0.5 mg/ml in PBS) for 2 hrs at room temperature. The tissues were then removed from the avidin solution and washed thoroughly in PBS (2x5 min) with a change of buffer between washes. They were then placed in the X-gal solution (1 mg/ml in PBS) at room temperature. After 1 hr, a blue precipitate formed in the center of the meniscal slices that had been modified with the biotin-xx-se, while the control tissue sections gave no precipitate, see Figure 36.

Experiment 3: Investigation of Reaction time and Concentration of the Biotin-NHS with Meniscal Sections (dose-response studies)

We conducted several more experiments in which we varied the time of reaction of the biotin-xx-se on the surface of the meniscus. The biotin-xx-se was allowed to react on the meniscal surface for time intervals of 30 min, 20 min, 10 min, 5 min and 1min. The remainder of the experiment including reaction with avidin-β-galactosidase, tissue washes and reaction with x-gal was identical to the procedure in experiment 1. Only the specimens treated with 30 min and 20 min intervals gave positive results after incubation with the x-gal. We also varied the concentration of biotin-xx-se using, concentrations of 40 mg/ml, 80 mg/ml, 120 mg/ml and 150 mg/ml (saturated solution) and used reaction time intervals of 20 min, 10 min, 5 min and 1 min for each concentration. All the tissues sections gave positive results with the x-gal, with a five minute reaction time. The biotin-xx-se concentration of 150 mg/ml was the only concentration that gave a positive result with a reaction time of 1 min. Therefore, we used 150 mg/ml concentration and tried reactions times of 1min, 45 sec, 30 sec, 15 sec and 1 sec on the tissue surface. The minimum reaction time at this concentration was determined to be 1 min.

These experiments demonstrated that the biotin-NHS could be made to react in a site specific manner in a short period of time and easily detected using the avidin chromogenic assay. These results were exciting since in an actual surgical procedure, the delivery of the proteins would need to happen quickly to avoid long surgeries.

Experiment 4: Investigation of the Homogeneity of $-NH_2$ on Meniscal Surface (commasie and silver staining

The two previous experiments demonstrated that we were bonding the biotin-NHS with amine groups on the surface of the meniscus, but we were unsure of the homogeneity of available $-NH_2$ groups on the tissue surface.

The coomassie blue (CB) stain is a common protein stain used in blotting techniques and has become an important method of protein analysis, primarily due to its convenience and high sensitivity. CB is a triphenylmethane dye belonging to the magenta family and is also used as a soluble synthetic food color. The dye binds to the proteins, especially to protonated amine groups, and forms a blue dye-protein complex easily visible to the unaided eye.

Commassie Staining

The meniscus was cut into sections 0.5cm thick and placed in PBS buffer until use. The commasie staining solution was made by mixing 40% methanol, 10% acetic acid, 50% water and 0.1 % (w/v) coomassie brilliant blue-250. The meniscal slices were placed in individual wells of a 12 well plate and 2 ml of the stain was added to each well. The slices were allowed to stain for 1hr at room temperature with gentle agitation. The tissues were then washed in PBS (3 x 10 min) with a change of buffer between washes. We observed that the entire tissue turned light blue. The experiment was repeated as

before but this time the stain was left for 30 min, 15 min, 10 min and 5 min. It was still observed that the entire slice turned blue. The coomassie stain was not able to reveal any clear heterogeneity in the free amine groups present on the surface of the tissue.

Silver Staining

Silver staining is another common method to detect proteins on membranes and is about 100-fold more sensitive than the coomassie stain. The basic mechanism of silver staining is well understood, involving the binding of silver ions to the proteins followed by reduction to free silver. The different protocols can be divided in alkaline and acidic methods. The alkaline methods are most sensitive but require longer procedures, whereas the acidic protocols are faster but less sensitive. The silver staining method was used after the coomassie method was unable to give a picture of the homogeneity of proteins on the surface of the meniscal slices.

Silver nitrate (0.8 g) was dissolved in 4 ml of DI water to give solution A. Twenty one milliliters of 0.36% sodium hydroxide and 1. 4 ml of 14.8 M ammonium hydroxide was mixed to give solution B. Solution A was added dropwise to solution B with constant vortexing, and the total volume was increased to 100ml with DI water to give the staining solution. The meniscal slices were placed in wells on a 12 well plate and 2 ml of the staining solution was added to each well. The plate was agitated for 15 minutes. The developer solution was made by mixing 2.5 ml of 1% citric acid, 0.25 ml of 38% formaldehyde and taking the final volume to 500 ml with DI water. The slices in the staining solution were washed in PBS (2 x 5 min). They were placed in dry wells on a new 12 well plate and 2 ml of the developer solution was added to each well. The plate was gently agitated for 15 min. The slices were then removed from the developer

solution and washed in PBS and after 15 min the tissues slices turned uniformly brown. The experiment was repeated but the time of reaction with the staining solution varied for 10 min, 7 min and 5 min. Unfortunately, there was no change in the result. The silver staining of the tissue did not give any evidence for macro heterogeneity in the protein content on the surface of the bovine meniscus.

Experiment 5: Biotinylation of the Meniscus and Fluorescence Detection with Streptavidin- fluorescein

The bovine meniscus was cleaned, cut into slices 0.5 cm in thickness, soaked in PBS for 30 min, and reacted with the biotin-xx-se indicated in experiment 1. To serve as negative control, several meniscal sections were placed in PBS solution without the biotin-xx-se (Figure 37).

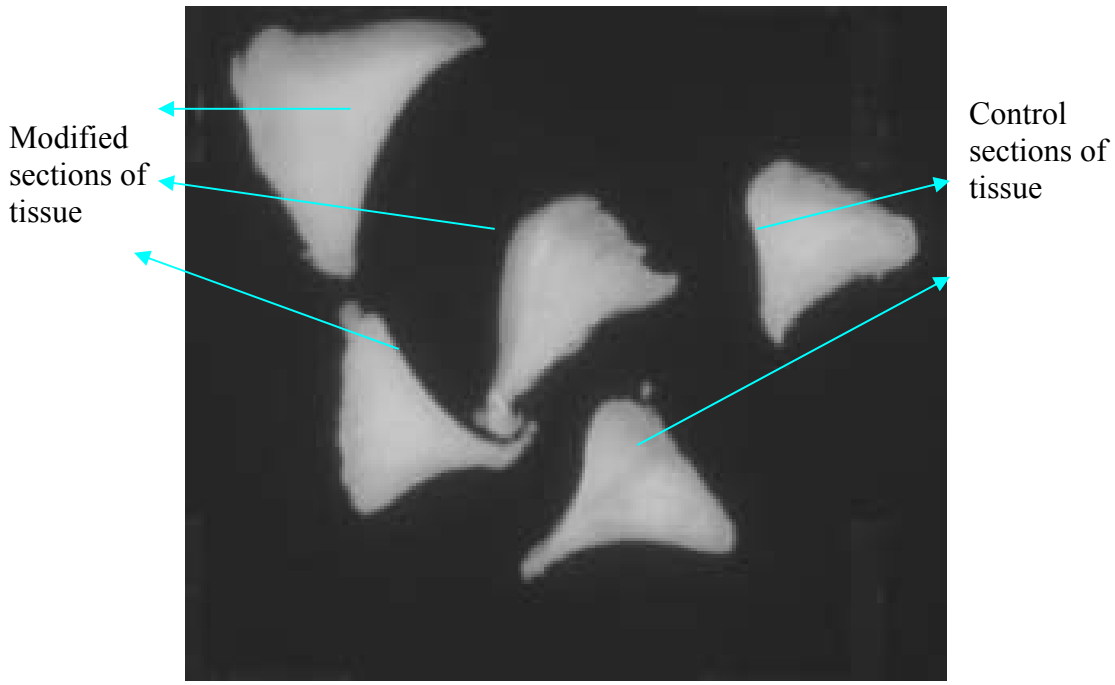


Figure 37. Meniscal slices modified with biotin-NHS and fluorescence detection with streptavidin fluorescein

The experiment was run at room temperature for two hours. The tissues were then washed 3 times in PBS and placed in clean wells on a 12 well plate. 1ml of a solution of streptavidin-fluorescein conjugate (0.02 mg/ml in PBS with 1% DMSO to aid in solubilizing the reagent) was added to each well, including controls, and the experiment was carried out for two hours at room temperature. The tissues were then washed thoroughly in PBS and imaged. The fluorescence signal was detected using a Bio-Rad multi-imager running the Quantity One software. The fluorescence was detected for 60s, with a 520 nm filter and a fully open aperture on the camera. Several time intervals and aperture settings were used to obtain the best signal, but the auto fluorescence off the tissue or the non-specific binding of reagents made it difficult to distinguish between the control and modified slices.

Experiment 6: Biotinylation of the meniscus and fluorescence detection with streptavidin-Alexa Fluor-430

The bovine meniscus was cleaned of fat, washed and sliced into sections 0.5 cm in thickness. The slices were soaked in PBS for 30 minutes and placed in individual compartments of a 12 well plate. 100 μL of a biotin-xx-se solution (20 mg/ml in PBS with 10% DMSO v/v) was added to each well. The meniscal sections which served as the controls were soaked in 100 μL of PBS (with 10% DMSO). The reaction was carried out at room temperature for 1 hr with gentle agitation. The tissues were then washed in PBS (3x5 min) and placed individually in wells on a 12 well plate. 100 μL of the streptavidin-430 (0.2 mg/ml in PBS (Molecular Probes, St Louis)) was added to each well and left to react for 2hr at room temperature in low light surroundings. The slices were then washed once in PBS, blotted to remove any excess buffer, and imaged. The

fluorescence signal was detected using the Bio-Rad- imager with the aperture fully open and acquisition time of 30 sec with a 520 nm filter. The slices reacted with biotin ester gave a much stronger fluorescence signal than the control. The experiment was repeated again, but this time 10 μL of the biotin-xx-se was added to the center of each slice to attempt a more site specific modification. In this case, we were unable to distinguish between modified and control sections. This was probably due to the low concentration of biotin present on the tissue surface when using only 10 μL of reagent, which was not sufficient to cause a fluorescence signal above the natural fluorescence of the tissue.

Experiment 7: Direct Coupling of Alexa Fluor 430 NHS to Meniscal Sections and Fluorescence detection

The meniscus was processed as in previous experiments, and the slices were placed in compartments of a 12 well plate.

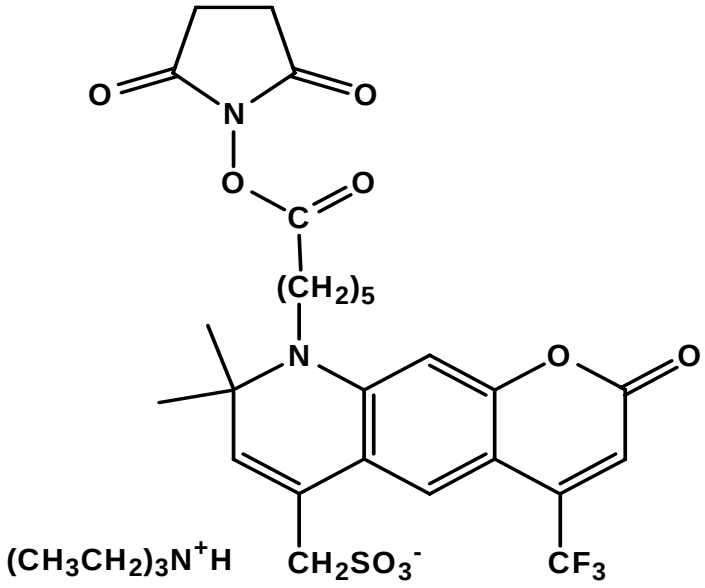


Figure 38. Structure of Alexa Fluor-430 NHS

20 µL of the Alexa Fluor 430 succinimide ester (0.02 mg/ml in PBS with 10% DMSO v/v to help solubilize the reagent) was added to the center of each section to be modified. For a negative control, the Alexa Fluor 430 NHS ester was reacted with a 100mM solution of N,N-diethyl diamine for 2 hrs at room temperature. 20 µL of this solution was then added to each control tissue slice. The reaction was run for 2 hrs at room temperature, undisturbed and in low light. All the slices were then washed once in PBS, blotted and then imaged. The fluorescence was detected with the Bio-Rad imager for 30 sec, with a 520 nm filter and the aperture fully open. The modified slices clearly gave a stronger fluorescence signal when compared with the control slice (as seen in Figure 39).

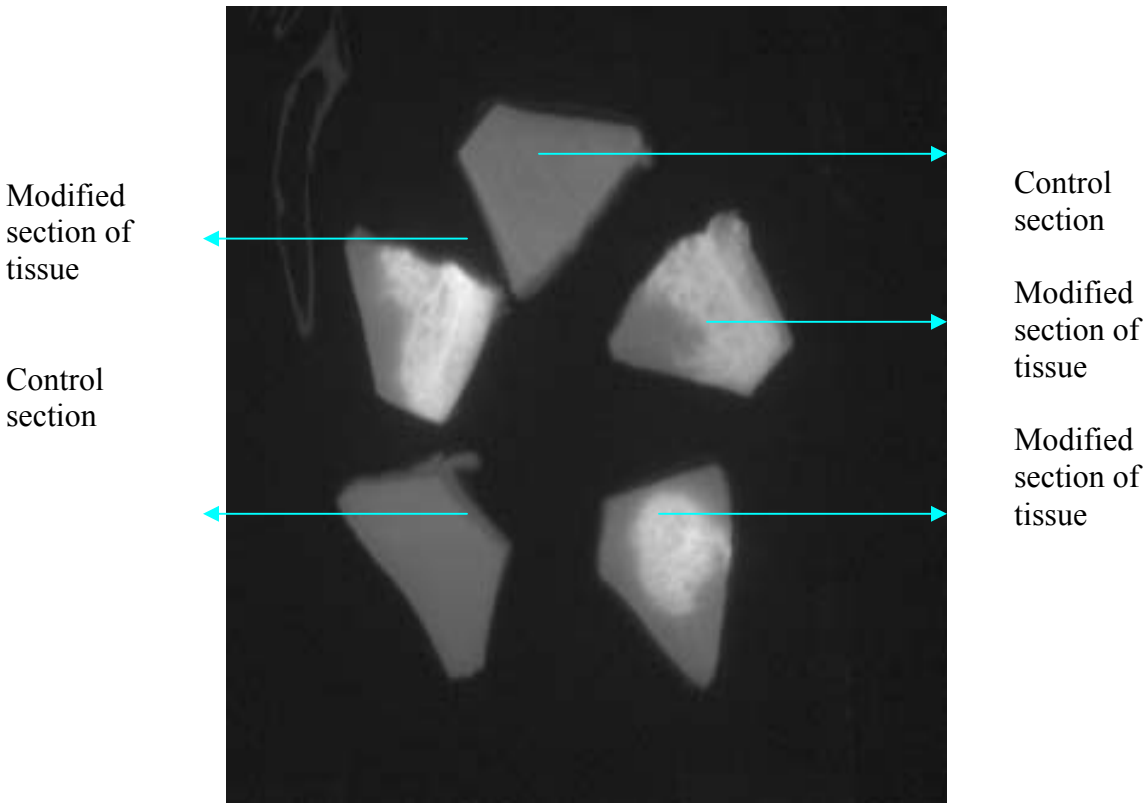


Figure 39. Fluorescence image of meniscal slices modified with Alexa Fluor 430 NHS. The region of tissue modified by the Alexa Fluor 430 NHS emit a bright fluorescence signal, while the controls and unmodified sections of tissue remain dim

Experiment 8: Direct Coupling of Fluorescence iso thiocynate (FITC) to the Meniscal Surface and Fluorescence Detection

The bovine meniscus was cleaned, washed in PBS and cut into longitudinal slices about 0.3 cm in thickness.

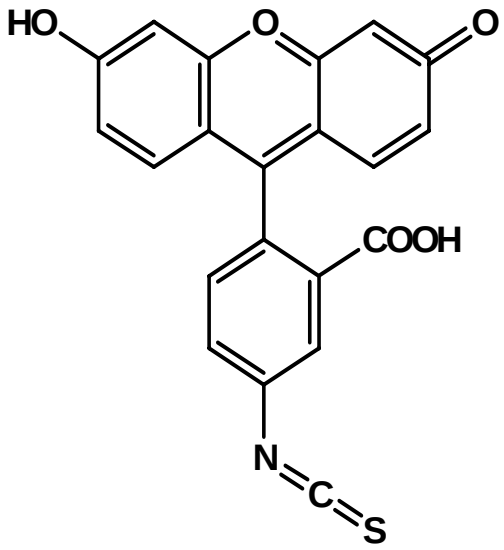


Figure 40. Structure of Fluorescein iso thiocynate (FITC)

About 3 μL of FITC (10 mg/ml in DMSO) was added to the center of each slice and allowed to react for 1hr. For the control experiment, 1ml of FITC (10mg/ml in DMSO) was reacted with 300 μL of a 100 mM solution of N, N-diethyl diamine for 1hr. 3 μL of this reaction mixture was then added to each of the control meniscal slices and allowed to react for 1 hr. All the slices were then washed in PBS (3x5 min) with a change of buffer between washes, blotted on filter paper to remove any excess buffer and then imaged using Bio-Rad multi imager. The aperture was set at the maximum opening and the signal was detected for 30 sec with the 520 nm filter.

The slices modified with FITC gave a fluorescence signal at the center, while the control slices did not emit a fluorescence signal (Figure 41).

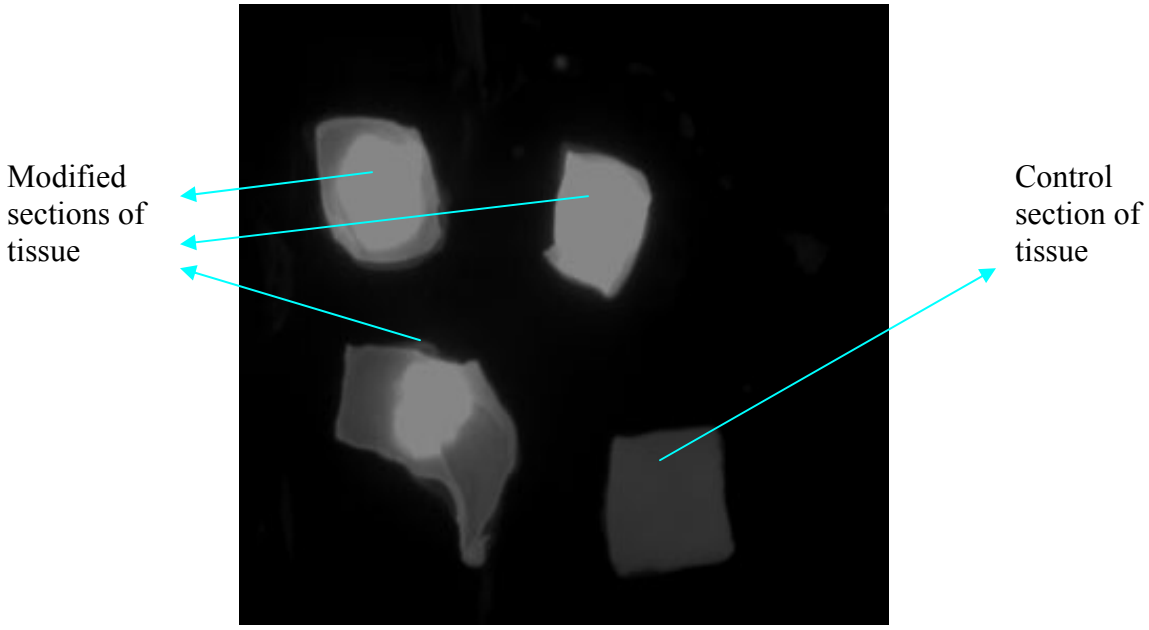


Figure 41. Fluorescence image of meniscal sections modified with Flourescein iso thiocynate (FITC)

We ran several more experiments where the time of reaction of the FITC with tissue surface was varied. We used time intervals of 1 hr, 45 min, 30 min, 15 min, 5 min and 1min. The minimum reaction time at a concentration of 20 mg/ml FITC was shown to be 15 min. We then increased the concentration of FITC to 30 mg/ml and were able to detect a signal with reaction time of 1min on the tissue surface.

Experiment 9: Incubation with Chrondroitinase ABC to Remove Proteoglycans

The meniscus was cleaned, washed and sliced as before. The slices were placed in a Petri dish and incubated in 5 ml of chondroitinase ABC (5 units/μL in PBS) for 3 hrs. The slices were then washed in PBS and experiment 1 and 3 with the biotin-xx-se and

streptavidin Alexa Fluor-430 NHS were repeated. However, we did not see an improvement in the signal intensity of the reacted slices.

Experiment 10: Modification of Bovine Pericardium with Biotin-NHS and Chromogenic Detection

The pericardium was cut into small sections 2 cm x 2 cm. Each of the sections was placed individually in compartments of a 12 well plate. 1ml of biotin-xx-se (20 mg/ml in PBS with 10% DMSO v/v to assist in solubilizing the reagent) was added to each of the reaction slices. The remaining slices served as negative controls, and each slice received 1ml of PBS (also with 10% DMSO). The reaction was carried out at room temperature for 2 hrs. The slices were all washed in PBS (2 x 5 min) with a change of solution between washes. Then they were placed in new wells on a 12 well plate, 1ml of the avidin-β-galactosidase (0.2 mg/ml in PBS) was added to each well, and the plate was gently agitated for 2 hrs at room temperature. The avidin solution was discarded out of the wells and the slices were washed with PBS (2x5 min) with a change of buffer between washes. 1 ml of the X-gal (1 mg/ml in PBS) solution was added to each of the slices and the plate was gently agitated for 2 hrs. The formation of a blue precipitate was not observed on any of the slices.

Experiment 11: Modification of Pericardium with Biotin-NHS and Fluorescence Detection with Sreptavidin Alexa Fluor- 430

Pericardium was sectioned, placed in wells and reacted with the biotin-xx-se as with the previous experiments. After two hours, the slices were all washed three times in PBS and placed in new dry wells. Then 500 μL of the streptavidin Alexa Fluor-430 (0.02 mg/ml in PBS) solution was added to each well and the plate gently agitated for two

hours. After this time, the slices were all washed in PBS and blotted to remove any excess buffer. They were then imaged using the Bio-Rad imager (530 nm filter, 30sec acquisition time, aperture at 3.0). We were unable to distinguish between modified and control slices from the resultant fluorescence image (Figure 42).

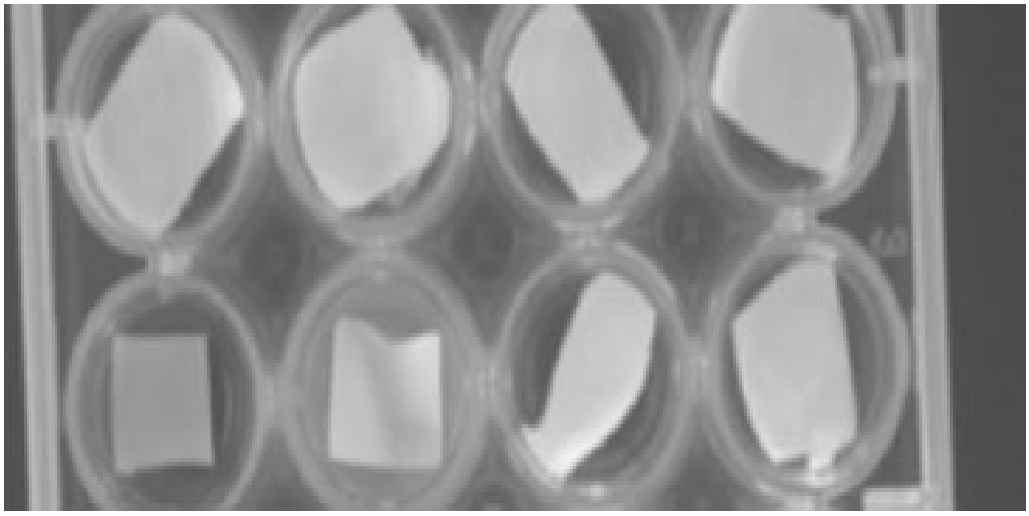


Figure 42. Fluorescence image form Alex Fluor 430 probe. The four control sections (top row) and the four modified sections of tissue (bottom row) are identical under fluorescence detection.

Experiment 12: Modification of the Pericardium with Fluorescein-NHS and Fluorescence Detection

When using the streptavidin fluorescent dye we were unable to detect any biotin on the tissue surface, we decided to conjugate a fluorescent probe directly on the $-NH_2$ on the tissue surface. The tissue slices were processed as in the previous experiments and reacted with the fluorescein NHS. 200 μl of the 2 mg/ml fluorescein ester in PBS (with 10% v/v DSMO) was added to each slice to be modified, while the control slices received 200ul of PBS (also with 10% DMSO). After 4 hrs, the slices were washed and imaged under the same conditions of the previous experiment. Unfortunately, we were still

unable to differentiate between control and modified slices from the acquired images. This was due to the interference from the auto fluorescence from the tissue.

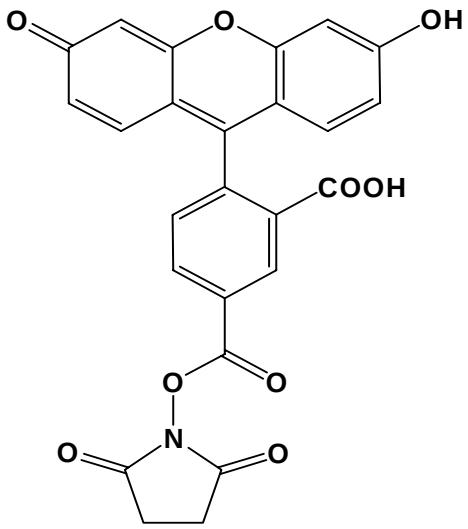


Figure 43. Structure of fluorescein- NHS

Experiment 13: Modification of the Pericardium with Fluorescamine and NDA with Fluorescence Detection

*NDA (Figure 44) reaction with pericardium. T*he pericardium was cut into 2 cm^2 slices and soaked in bicarbonate buffer for 30 min prior to use.

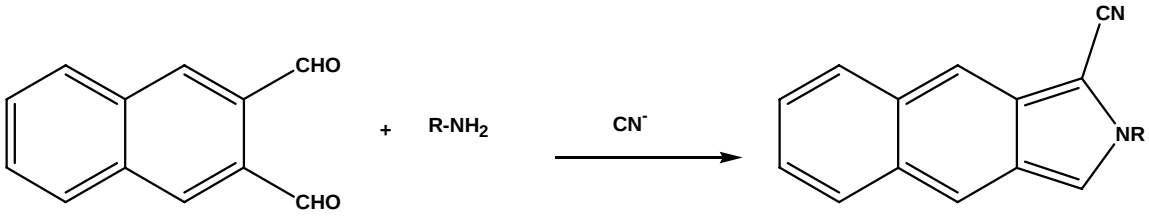


Figure 44. Reaction of NDA with primary amine

The slices were placed individually in wells on a 12 well plate and 250 µL of 0.1M borate buffer was added to each well and left to equilibrate. After 10 min, 30 µL of

NaCN was also added to each well and plate was gently agitated for 10 more minutes at room temperature. A total of 30 µL of NDA in acetonitrile (20 mM) was also added to each well and reaction was left to proceed at room temperature for another 60min. For a negative control, 30 µL of the NDA in acetonitrile was prereacted with a 50 µL of N, N-diethyl ethylene diamine in 250 µL of 0.1M borate buffer and 30 µL of NaCN. 30µL of this solution was added to each pericardium slice which served as a control. After 60min, all the slices were washed in PBS (2 x 5 min) with a change of solution between each wash. Fluorescence was recorded using the Bio Rad imager using 520 nm emission filters, 30 sec acquisition time and fully open aperture. The reacted slices gave a stronger fluorescence signal when compared to the controls (Figure 45).

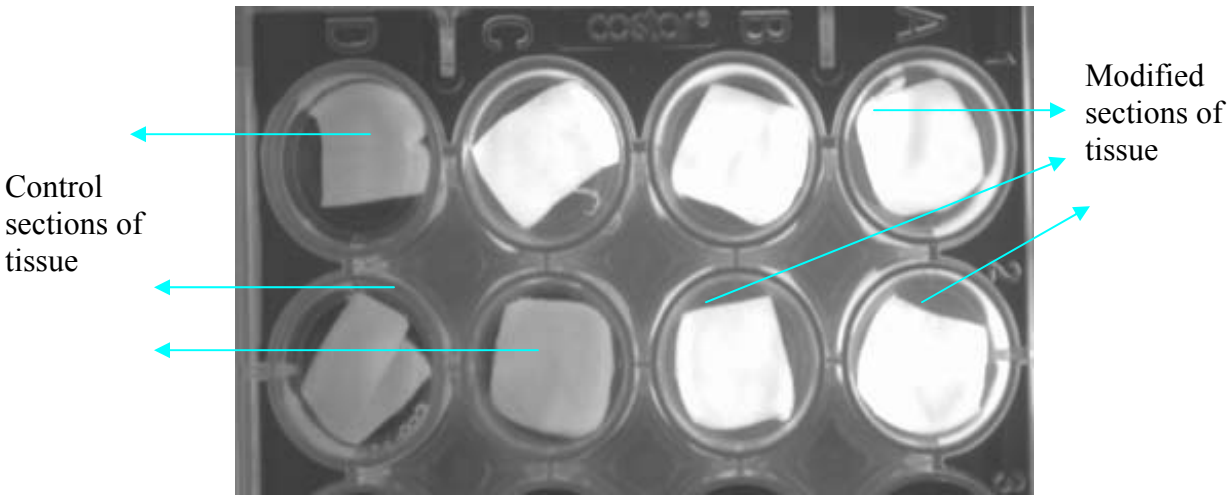


Figure 45. Fluorescence image of pericardial sections modified with NDA. The control sections appear darker (emit less fluorescence) when compared to the modified section.

*Fluorescamine Reaction with Pericardium (Figure 46).*The pericardium was cut into 2 cm^2 slices as before and soaked in bicarbonate buffer for 30 min prior to use. The slices were placed individually in wells on 12 well plate. 90 µL of 0.1% (w/v)

fluorescamine dissolved in acetonitrile, 15 μL of 0.1 M borate buffer and 190 μL of water were added to each of the wells.

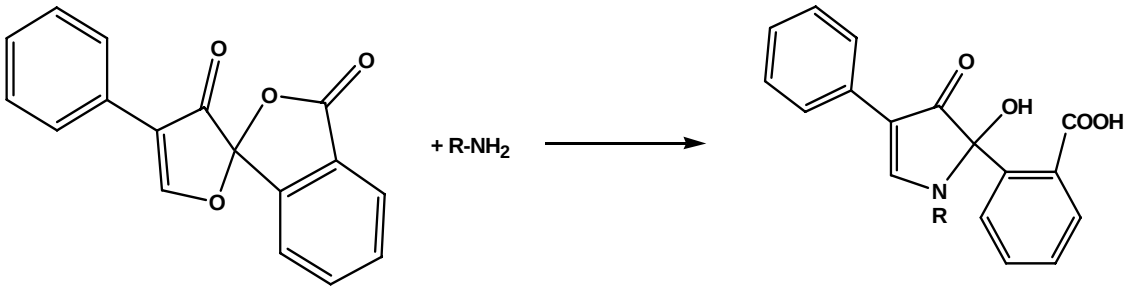


Figure 46. Reaction of fluorescamine with primary amine

The reaction was allowed to proceed at room temperature for 10 min with gentle agitation. For a negative control, 1 ml the fluorescamine was reacted with 300 μL of a 100mM solution of N, N-diethyl diamine using the above reaction conditions (Figure 47)

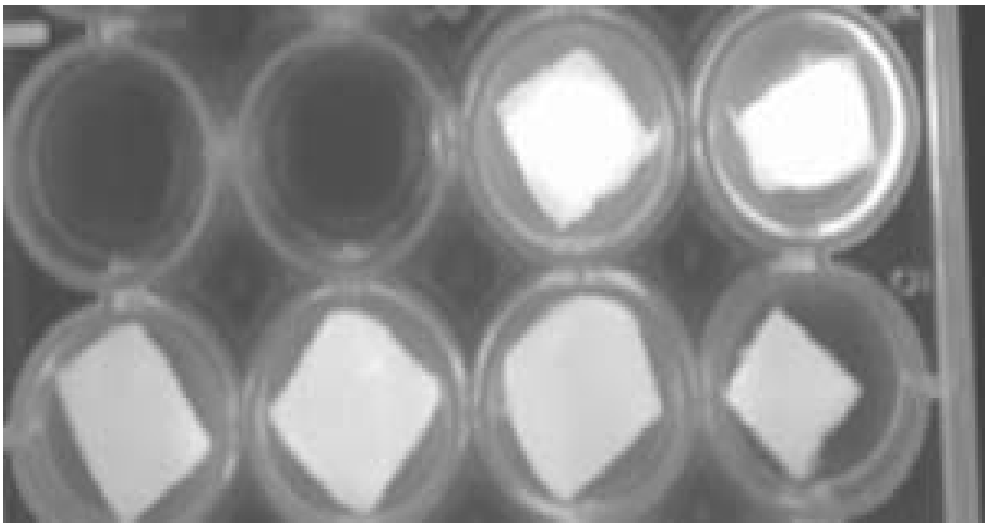


Figure 47. Fluorescence image from pericardial sections modified with fluorescamine. The control sections (top row) and modified sections of tissue (bottom row)

Experiment 14: Modification of pericardium with FITC and fluorescence detection.

90 μL of this solution was added to each of the control slices along with the rest of the reagents as above. After 10 min, all the slices were washed in PBS (2x5 min) with a change of solution between each wash. The fluorescence was detected using the conditions for the NDA experiments. The sections modified with the fluorescamine gave a stronger fluorescence signal when compared to the control sections. The unconjugated FITC was used for directly reacting with the primary amines on the bovine pericardium. The pericardium was cleaned and hydrated for 30 min in PBS and cut into a slice about 4 cm^2.

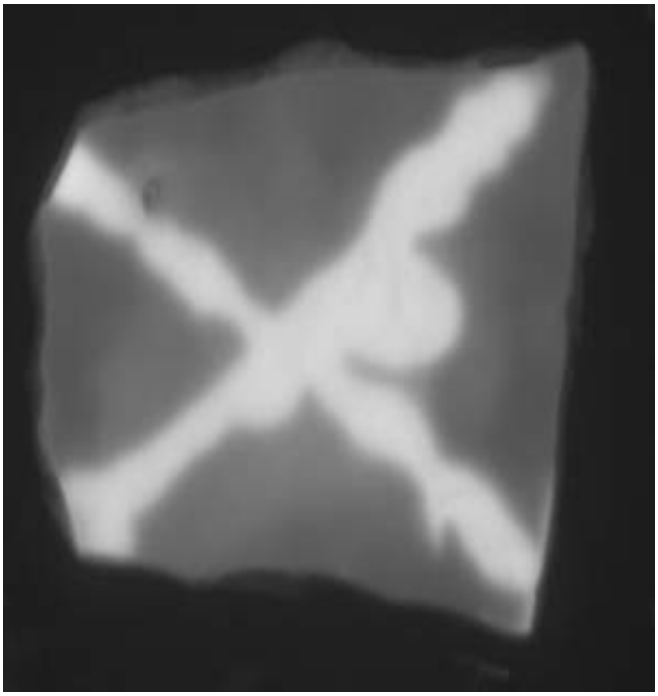


Figure 48. Fluorescence image of pericardium section reacted with FITC in an 'X' pattern

The pericardium was removed from the buffer, drained of excess buffer and placed on several wet paper towels to keep the tissue hydrated. 0.8 μL of the FITC (10 mg/ml in

DMSO) solution was then added to the surface of the pericardium by hand, using a glass pipette in the shape of an 'x'. The FITC was then left to react for 30min at room temperature in low light. After this time, the pericardium was then washed in PBS (2x5 min) with a change of buffer between each wash. The fluorescence was detected using the Bio-Rad imager, with a 520 nm filter, aperture fully open, and 30 sec exposure time. The 'x' pattern was clearly visible on the pericardium. In a similar experiment, additional washing steps with methanol and hexane was performed after reacting the pericardium with FITC. The organic wash was done to definitively remove any FITC non-specifically bound on the tissue surface. The tissue became fairly stiff after the wash, but the 'x' pattern was still clearly visible when imaged under the previous conditions.

Experiment 15: Modification of Pericardium with Alexa Fluor-430 NHS and Fluorescence Detection

Since the direct conjugation of FITC with the bovine pericardium proved to be successful, the Alex Fluor-430 NHS reagent was investigated as another reagent for direct modification of the pericardium.

The pericardium was cut into 2cm^2 slices and soaked in bicarbonate buffer for 1hr prior to use. In this case, we used a bicarbonate buffer as opposed to PBS. The slices were placed in wells on a 12 well plate, and 200 μL of the Alexa-Fluro-430 (0.02 mg/ml in bicarbonate buffer) was added to each well. The reaction was allowed to proceed for 2 hrs at room temperature with gentle agitation. For a control experiment, 200 μL of bicarbonate buffer was added to several slices. After 2 hrs, all of the slices, including

controls, were washed three times in PBS, with a change of buffer between washes. The slices were then imaged on the Bio-Rad imager using previous conditions.

In this case, we were unable to distinguish the modified from the control slices.(Figure 49)

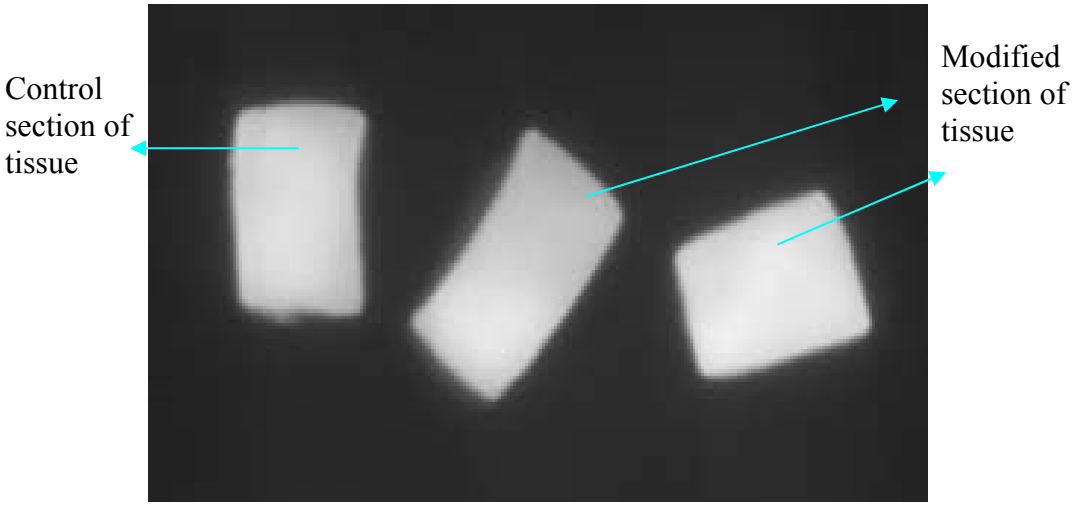


Figure 49. Fluorescence image of pericardial sections modified with Alex Fluor 430 NHS. The control section (on the right) and the two modified section appear identical under fluorescence detection.

Experiment 16: Gross Alkylation of Bovine Pericardium and Chemiluminescent Detection

The pericardium was processed as in experiment 1 and alkylated with the biotin-LC-NHS ester (the N-hydroxy-succinimide ester of the 6-aminohexanoic acid amide of biotin, which was purchased from Pierce). The tissue section, including controls which were not reacted with the biotin ester, were then washed and incubated with a 0.2 mg/ml solution in avidin-HRP in PBS for 2 hrs. The sections were then washed several times in PBS, immersed in luminal for 4min and blotted to remove any excess reagent. They were then placed into the Bio-Rad imager for detection of chemiluminescence. The

signal was detected for 100 sec, high sensitivity and a fully open aperture. The resultant imaged showed a stronger chemiluminescent signal from modified slices, when compared to controls (Figure 50).

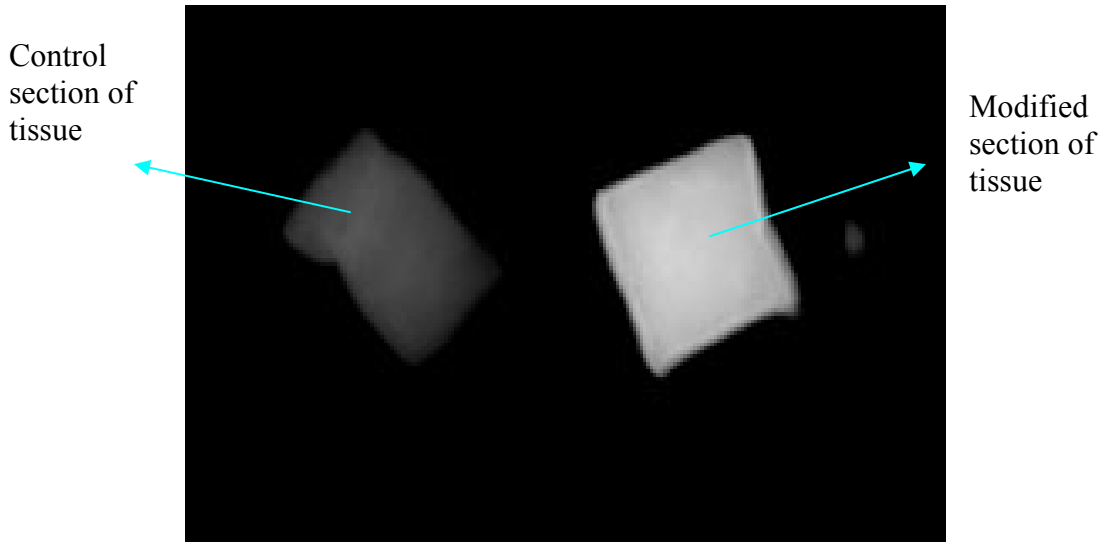
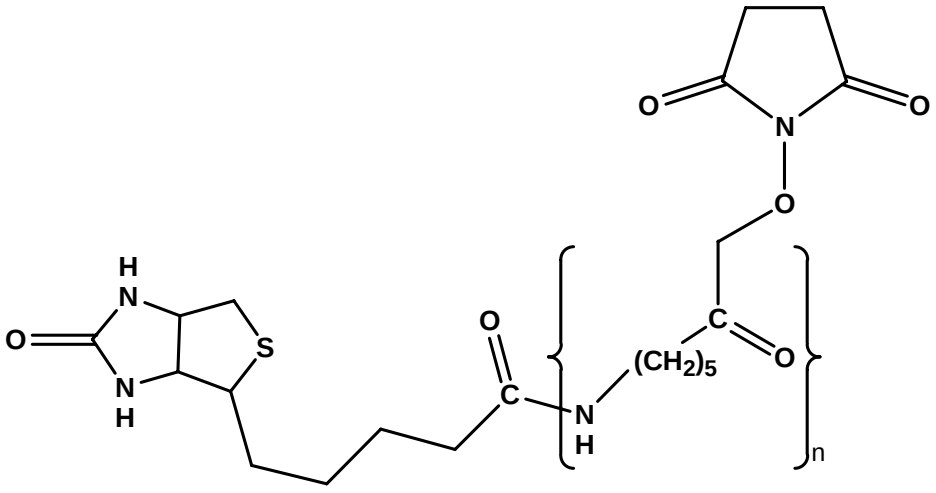


Figure 50. Chemiluminescent image from reduction of BP. The control on the left appears dimmer when compared to the modified section on the right.

Experiment 17: Site Specific alkylation of Bovine Pericardium using Direct Alkylation Methods and Chemiluminescent Detection

A sample of fresh bovine pericardium was cleared of fat and cut to fit on a 96-well plate. 210 µL of a 0.1 M solution of NHS-LC-biotin in PBS (10% v/v DMSO added for solubility) was added to wells to be modified and 210 µL PBS (also 10% DMSO) was added to all other wells to serve as a control. The tissue sample was placed over the filled wells, a solid glass cover was clamped into place using spring clamps, and the entire apparatus was flipped over so that the reagents contacted the tissue surface. After the tissue had been exposed for about 12 hours at room temperature, the apparatus was dissembled and the tissue was transferred to a small Petri dish and thoroughly washed with PBS three times for ten minutes with a change of solution between each wash.



n = 1, Biotin-LC-NHS
n = 2, Biotin-LC-LC-NHS

Figure 51. Structure of biotin-LC-NHS and biotin-LC-LC-NHS

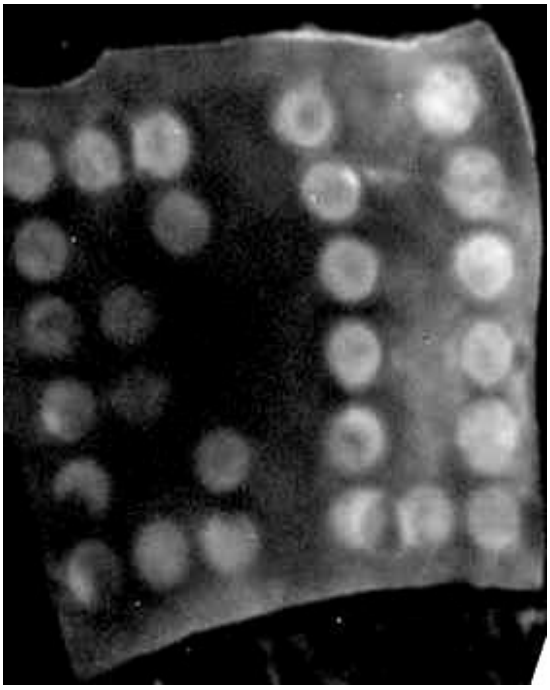


Figure 52. Chemiluminescent image of bovine pericardium site specifically alkylated with biotin-NHS in a 'BU' pattern. The tissue was modified in a way to have the modified 'pixels' light up in a 'BU' pattern for Baylor university. The pixel resolution is ~4mm in diameter.

The final wash solution was replaced by a PBS solution of avidin-horseradish peroxidase (0.03 mg/ml in PBS) and the tissue was incubated for 2 hours at room temperature. The tissue samples were once again washed with PBS three times for ten minutes with a change of solution between each wash and then immersed in the chemiluminescence substrate luminol (Supersignal® West Pico, Pierce) at room temperature for 3-5 minutes. The tissue was then removed from the luminal solution, blotted with paper towels to remove excess solution, placed on a sheet of plastic film, and placed into the imager for detection of the chemiluminescence using ultrasensitive chemiluminescence settings with the aperture completely open and 100 second acquisition.

The positive signal appeared as a series of bright dots (BU) corresponding to regions of the tissue which had been contacted with the wells containing NHS-LC-biotin. Though the BU pattern is visible, there was still some background signal associated with non-specific binding of the reagents.

Experiment 18: Site Specific Alkylation of Bovine Pericardium with Biotin-TFO-PEO ester and Chemiluminescent Dtection

The pericardium was processed as in experiment 1 and reacted with 0.1 M solution of the biotin-TFO-PEO (Pierce Biotechnology) in PBS (with 10% DMSO to assist in solubilizing the reagent) for 12 hrs at 37^0C, using a 96 well plate as in experiment 19. The tissue was then washed and the remainder of the procedure (including the tissue washes, incubation with avidin-HRP, further tissue washes, and incubation with luminal) was identical to experiment 1. However, the use of biotin-TFO-PEO to modify pericardium did not improve signal intensity, or decrease background when compared to results from using biotin-LC-NHS to modify similar tissue.

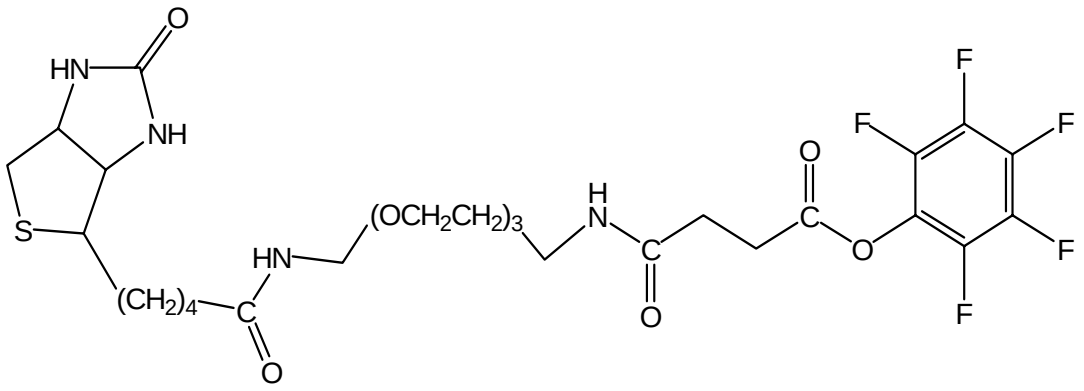


Figure 53. Structure of biotin-TFO-PEO

Experiment 19: Site specific alkylation of bovine pericardium with biotin-Sulfo-NHS and chemiluminescent detection

The pericardium was processed as in experiment 1 and reacted with 0.1M solution of the biotin-sulfo-NHS in PBS for 15 hrs at 37^0C.

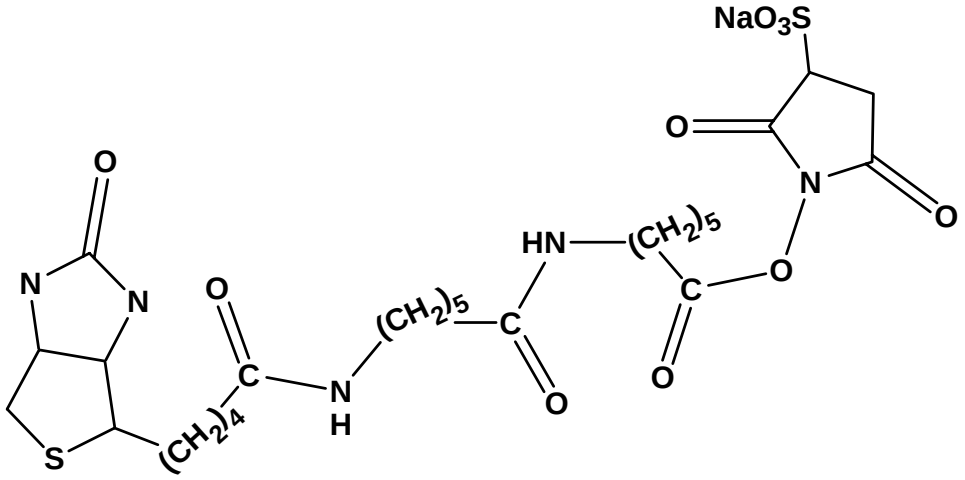


Figure 54. Structure of biotin-sulfo-NHS

The tissue was then washed and the remainder of the procedure (including the tissue washes, incubation with avidin-HRP, further tissue washes, and incubation with luminol)

was identical to experiment 1. Unfortunately, we did not see any significant improvement in signal intensity from the reacted regions of tissue when compared to intensities from experiment 18.

Experiment 20: Site specific Alkylation of Fetal pig Skin with Biotin-LC-NHS and Chemiluminescent Detection

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A sample of fresh fetal pig skin was cleared of fat and cut to fit on a 96-well plate. 210 µL of a 0.1 M solution of NHS-LC-biotin (the N-hydroxysuccinimide ester of the 6-aminohexanoic acid amide of biotin) in PBS (10% v/v DMSO added for solubility) was added to wells to be modified and 210 µL PBS (also 10% DMSO) was added to all other wells to serve as a control. The tissue sample was placed over the filled wells, a solid glass cover was clamped into place using spring clamps, and the entire apparatus was flipped over so that the reagents contacted the tissue surface. After the tissue had been exposed for about 12 hours at room temperature, the apparatus was dissembled and the tissue was transferred to a small Petri dish and thoroughly washed with PBS three times for ten minutes with a change of solution between each wash.

The final wash solution was replaced by a PBS solution of avidin-horseradish peroxidase (0.03 mg/ml) and the tissue was incubated for 2 hours at room temperature. The tissue samples were once again washed with PBS three times for ten minutes with a change of solution between each wash and then immersed in the chemiluminescence substrate (Supersignal® West Pico, Pierce) at room temperature for 3-5 minutes. The tissue was then removed from the luminal solution, blotted with paper towels to remove excess solution, placed on a sheet of plastic film, and placed into the imager for detection of the chemiluminescence using ultrasensitive chemiluminescence settings with the aperture completely open and 100 second acquisition. The positive signal appeared as a

series of bright dots corresponding to regions of the tissue which had been contacted with the wells containing biotin-LC-NHS.

At the same time, a slot blot was prepared with serial dilutions of avidin (20, 10, 5, 2.5, 1.25, and 0.625 μg/mL) blotted on the nitrocellulose membrane, corresponding to 4, 2, 1, 0.5, 0.25, and 0.125 μg protein applied to each slot. The membrane was placed into the Petri dish with the tissue sample and processed alongside it. The tissue and the nitrocellulose membrane were then immersed in a PBS solution of biotin-horseradish peroxidase (0.03 mg/ml) for 2 hours at room temperature, then washed with PBS three times for ten minutes with a change of solution between each wash, and finally immersed in the chemiluminescence substrate liminol (Supersignal® West Pico, Pierce) at room temperature for 3 – 5 minutes. The tissue and membrane were then removed from the luminal solution, blotted with paper towels to remove excess solution, placed on a sheet of plastic film, and placed into the imager for detection of the chemiluminescence using ultrasensitive chemiluminescence settings with the aperture completely open for a 100 second acquisition. The positive signal appeared as a bright signal against a dark background.

Only the four highest concentrations of avidin on the slot blot were evident under these conditions. The strongest signal came from the application of 4 μg of avidin to the slot, which corresponds to about 1 $\mu g/mm^2$, if quantitative immobilization is assumed. Since the maximum signal intensity on the tissue was approximately the same as the strongest signal from the slot blot (determined using freely available NIH Image J 1. 32j imaging software), it was determined that the amount of protein immobilized on the tissue was at least 1-4 $\mu g/mm^2$.

Experiment 21: Gross Biotinylation of Bovine Meniscus and Chemiluminescent Detection

The meniscus was sectioned as before and alkylated with biotin-xx-se as in the previous procedures from experiment 1.

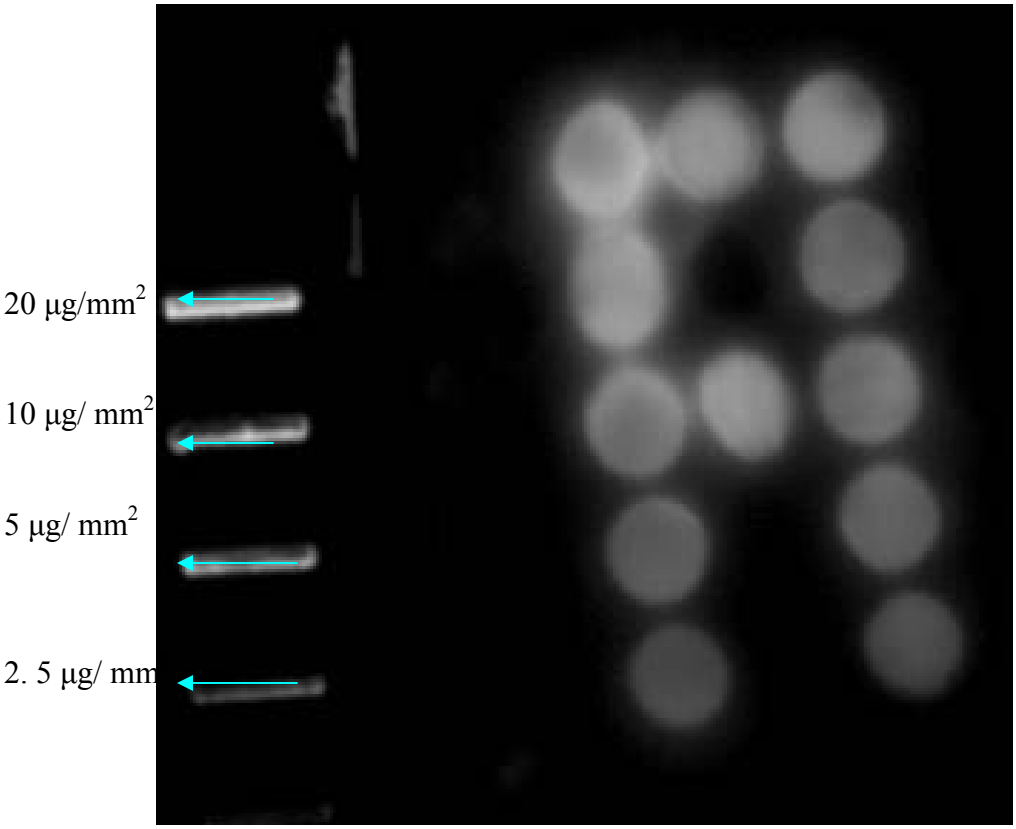

Figure 55. Chemiluminescent image from the direct alkylation of pig skin with slot blot strip for quantification. The tissue was modified in a way to have the modified 'pixels' light up in an 'A' pattern. The pixel resolution is ~4 mm in diameter.

All the tissue sections, including controls which are not reacted with biotin-xx-se, were then washed and incubated with a 0.02 mg/ml avidin-HRP solution for 4 hrs. After this time, the tissues were washed in PBS, incubated in luminal for 4 min, blotted and imaged (80 sec acquisition times, aperture at 3.0, ultra sensitivity setting). A very weak chemiluminescent signal was detected from the modified sections, while the controls were dark.

Experiment 22: Gross Biotinylation of Bovine Meniscus and Chromogenic Detection with HRP Substrate TMB

Once imaged, the slices from the previous experiment were thoroughly washed in PBS and placed in a solution of TMB (TMB is a chromogen that yields a blue color when oxidized with hydrogen peroxide (catalyzed by HRP) (Pierce Biotechnology). After about 1hr, a blue precipitate was seen on the slices alkylated with biotin-xx-se, while the controls were mostly clear.

Experiment 23: Gross Biotinylation of Bovine Skeletal Muscle and Chemiluminescent Detection

The skeletal muscle was cleaned of any connective tissue and longitudinal sections were made using a microtome blade. The chunks were placed in compartments of a 12 well plate and reacted with 0.1 M biotin-LC-NHS in PBS (with 10% DMSO v/v for solubility of the reagent), while the controls were left in PBS. After 45 min, the chunks were washed and placed in new wells and the remainder of the experiments (including the tissue washes, alkylation with avidin-maleimide, further tissue washes, incubation with biotin-HRP, further tissue washes, and incubation with luminal) were identical to experiment 1. However, there was no noticeable difference between the controls and modified tissues.

Section Three: Oxidative Method of Tissue Modification

Experimental Strategy

The most common method for introducing aldehydes and ketones into polysaccharides and glycoproteins (including antibodies) is by periodate-mediated oxidation of vicinal diols. Sodium meta-periodate is a mild oxidant for converting cis-

glycol groups in carbohydrates to reactive aldehyde groups, which may then be targeted in detection or chemical conjugation procedures.[204] Carbohydrate groups in glycoproteins are excellent sites for modification or cross-linking reactions because they allow the conjugation reaction to be directed away from amino acids in the polypeptide chain that may be critical for protein activity. Sodium meta-periodate cleaves bonds between adjacent carbon atoms that contain hydroxyl groups (cis-glycols), creating two aldehyde groups that are spontaneously reactive to amine- and hydrazide-activated labeling or cross-linking reagents (Figure 56).[205]

Certain sugar groups are more susceptible to oxidation (cleavage) by periodate, affording the possibility of adjusting the amount of periodate to cleave particular sugars in the polysaccharide chains. For example, 1 mM sodium meta periodate will oxidize only the bond between adjacent hydroxyls of sialic acid, a common terminal sugar residue in glycoprotein polysaccharides. On the other hand, treatment with >10 mM sodium meta-periodate ensures oxidation of many sugar residues, including galactose and mannose.[206]

In addition, alkenes from unsaturated fatty acids and ceramides can be converted to glycols by osmium tetroxide and then oxidized by periodate to aldehydes. Periodate will also oxidize certain β-aminoethanol derivatives such as the hydroxylysine residues in collagen, as well as methionine (to its sulfoxide) and certain thiols (usually to disulfides). These other reactions, however, usually occur at a slower rate than oxidation of vicinal diols. Periodate oxidation of the 3'-terminal ribose provides one of the few methods of selectively modifying RNA.[207] Periodate-oxidized ribonucleotides can subsequently be converted to fluorescent nucleic acid probes by reaction with fluorescent hydrazines,

hydroxylamines and amines. Furthermore, N-terminal serine and threonine residues of peptides and proteins can be selectively oxidized by periodate to aldehyde groups thus allowing highly selective modification of certain proteins such as corticotrophin and β-lactamase.[208] Moreover, because antibodies are glycosylated at sites distant from the antigen-binding region, modification of periodate-oxidized antibodies by hydrazines and hydroxylamines usually does not inactivate the antibody, as sometimes occurs with FITC, TRITC and Texas Red sulfonyl chloride labeling. Researchers have also used some of the hydrazine derivatives described in this section to detect periodate-oxidized glycoproteins in gels.[209, 210]

Aldehydes generated as described above can be used in two basic types of coupling reactions.[211] Primary amines will react to form Schiff bases with aldehydes, and the Schiff bases can be stabilized to secondary amine bonds by reduction with sodium cyanoborohydride. Alternatively, hydrazide-activated molecules will spontaneously react with aldehydes to form fairly stable hydrazone linkages; addition of sodium cyanoborohydride will increase reaction efficiency and bond stability.[212, 213]

As with the direct alkylation and reductive strategies, when we were first investigating the use of oxidation as a method for tissue modification, our primary means of detection of any modifications on tissue surfaces was fluorescence and chromogenic based assays.

In early experiments with the oxidative strategy, bovine pericardial sections were oxidized with periodiate, and then reacted with biotin-hydrazide, followed by avidin-β-galactosidase and x-gal, for chromogenic based detection. In the fluorescence based assay, the oxidized tissue was reacted with a fluorescent-hydrazide probe, or a

strepavidin-hydrazide, followed by biotin-fluorescein. Despite our best efforts, both the chromogenic and fluorescence assays were not successful at detecting any tissue modifications.

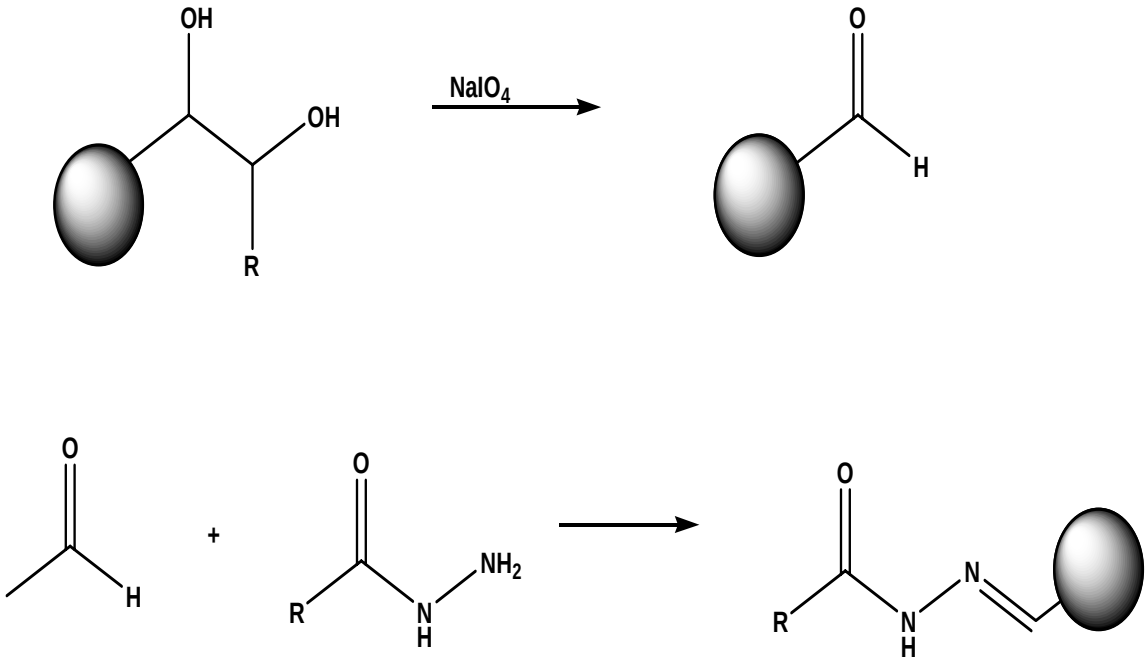

Figure 56. Oxidation of diols with sodium periodiate, followed by alkylation with biotin hydrazide

Since chemiluminescence was successful with the direct alkylation and reductive strategies and due to the lack of results with chromogenic and florescence detection, we decided to adapt the oxidative method for chemiluminescence based detection. The tissue was oxidized with the periodiate and was then reacted with the biotin-LC-hydrazide, followed by avidin-HRP and luminal for chemiluminescence detection. Once again we used the biotin-LC-hydrazide, a biotin with a long spacer, because the spacer arm makes the biotin more accessible to the avidin-HRP, thus providing better avidin

binding. With the chemiluminescence assay, the pericardium modified via oxidation was easily detectable when compared to unoxidized controls (Figure 57).

We then attempted a site specific modification to demonstrate spatial resolution obtainable on the pericardium using the chemiluminescence based detection.

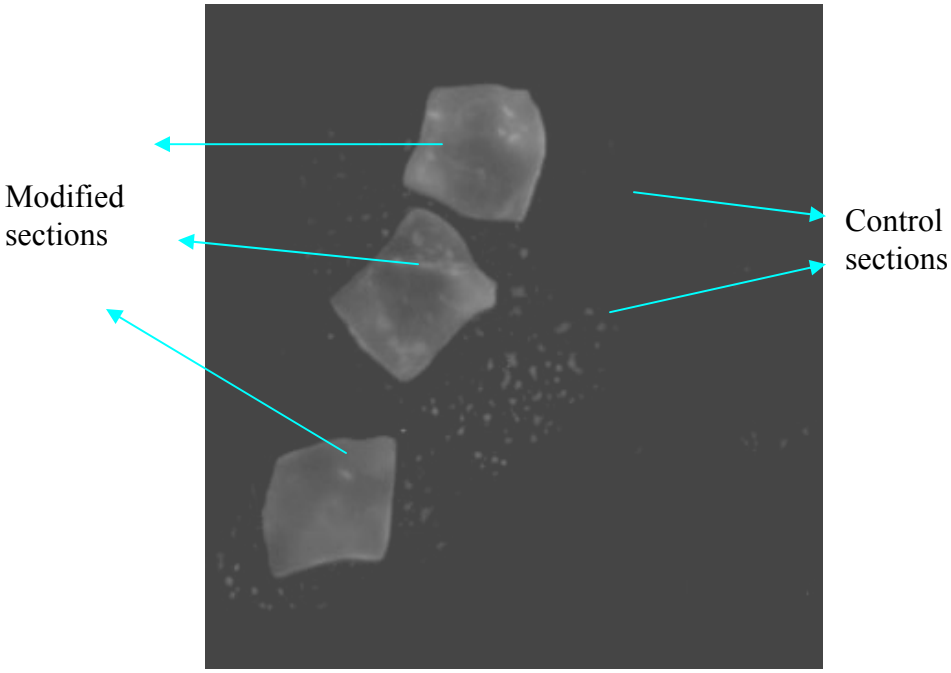

Figure 57. Chemiluminescent image from the oxidation of fetal pig skin sections. The modified sections (on the left of image) emit a much brighter chemiluminescent signal, while controls (on the right of image) are dark, and not visible.

The pericardial tissue was oxidized in a 'BU' pattern and reacted with the biotin-LC-hydrazide, followed by the avidin-HRP and luminal. The resultant chemiluminescence image showed bright dots which formed a 'BU' pattern on the tissue surface. Since chemiluminescence was a superior method of detection to the fluorescence and chromogenic assays, we decided to re-investigate the use of oxidation to modify other types of tissue, especially meniscus. Gross modification of the fetal pig

skin and meniscus was carried using the oxidative strategy with identical reagents and conditions as with the pericardium modifications. The results from both experiments also showed that we were able to modify skin and meniscus using an oxidative method. We also performed a site-specific oxidation of the fetal pig skin with the above reagents and were once again able to detect the bound protein in a 'BU' pattern. Unfortunately, oxidative modification of the bovine aorta and skeletal muscle was unsuccessful. We never attempted any oxidative modification of the bovine corneal tissue.

Material and Methods

Chemicals and Reagents

Except where otherwise noted, reagents used for the modification were obtained from Aldrich Chemical CO., Milwaukee, WI, and Fisher Scientific, Pittsburgh, PA (including Across reagents), Pierce and Molecular Probes, and used directly as purchased. Solvent such as dichloromethane, hexane, ethanol, ethyl acetate and methanol were obtained from commercial sources (via Baylor University Chemistry Department stockroom) and were distilled prior to use. Deionized water (DI) was obtained via a US Filter system provided by Baylor Dept. of Chemistry.

Bovine pericardium was obtained from Animal Technologies Inc., Tyler, Texas. The pericardium was cleaned of all excess fat using a microtome blade. The connective tissue was carefully removed by scraping with the blade and care was taken not to damage any of the actual pericardium. The cleaned pericardium was placed in a PBS solution at room temperature for up to 2hrs before use. Unused pericardium was stored at -20^0C and defrosted as needed.

The fetal calf skin was obtained from Animal Technologies and the rat skin was obtained from Department of Neuroscience at Baylor University. The fetal pig skin was washed in PBS and trimmed of any excess fat using a microtome blade. It was then allowed to soak in PBS until use. Unused skin was stored at -20^{0}C and defrosted and hydrated in PBS before use. The rat skin was obtained fresh, and the hair was removed by shaving or with the use of Nair. It was then soaked in PBS for up to 2 hrs before use or stored at -20^{0}C.

The meniscus was obtained from H&B Packing, Waco, TX. The menisci were dissected from whole bovine knees, trimmed of fat and stored in PBS for use. Excess menisci were also stored at -20^{0}C and rehydrated in PBS when used for experiments. The meniscus was made into longitudinal sections of desired thickness using microtome blade or scalpel and trimmed to fit into compartments of a 12 well plate.

The skeletal muscle and aorta were also obtained from Animal Technologies, soaked in PBS and used as obtained. The cornea was dissected out of whole bovine eyes, soaked in PBS for use. Any unused aorta, skeletal muscle and cornea were stored at -20^{0}C and defrosted and hydrated in PBS before use.

Instrumentation

Proton (^{1}H) and carbon (^{13}C) NMR spectra were obtained on a Bruker AVANCE 300 NMR running XWIN-NMR 3.1 software at 300 MHz. Chemical shifts are expressed in ppm(∂), peaks are listed as singlets (s), doublets (d), triplet (t) or multiplet (m), with the coupling constant (J) expressed in Hz. HPLC was carried out with a Beckman System Gold, composed of a model 168 detector and a model 126 solvent module using a Alltech Alltima C-18 5M 33mm*7mm “rocket” column. GC-Mass spec

was obtained on a Hewlett Packard. Fluorescence and chemiluminescence was detected using the Bio-Rad Laboratories Fluor-S Multi-Imager and analyzed using Bio-Rad software. The chromogenic images were recorded using a Kodak 2 mega pixel digital camera.

Experimental Methods for Oxidative Tissue Modification

Experiment 1: Oxidative Modification of Bovine Pericardium and Chromogenic Detection

The bovine pericardium was cleaned of excess fat, sectioned into approximately 2cm/side squares and placed in compartments of a 12 well plate. 1 ml of a 50 mM sodium periodiate solution was added to each slice that was to be modified, while the control slices each received 1 ml of PBS.

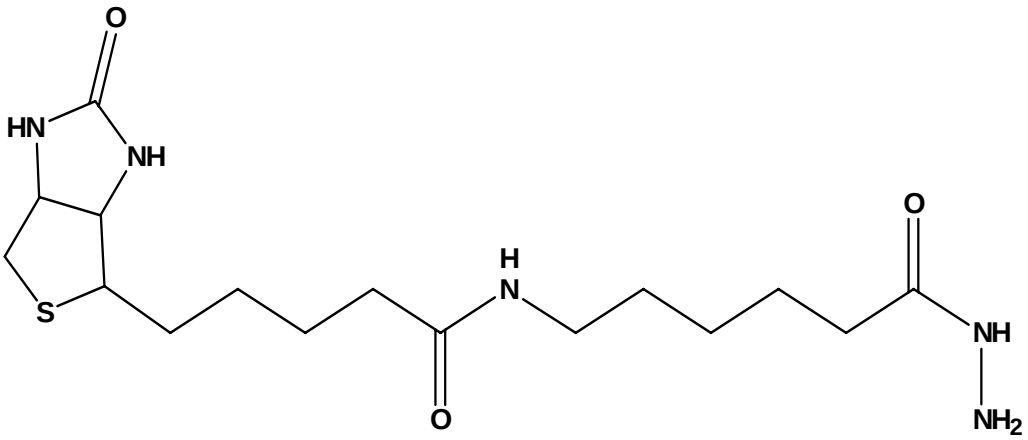

Figure 58. Structure of biotin-LC-hydrazide, biotinamidocaproyl hydrazide

The oxidation was carried out for 2 hours at room temperature. The slices were then washed in PBS (3x5 min) with a change of buffer between each wash, and placed in new wells. 200 μL of a biotin-hydrazide (biotinamidocaproyl hydrazide, Pierce

Biotechnology) solution (in PBS with 10% DMSO to assist in solubilizing the reagent) was added to each well and left to react for 2 hrs.

After this time, the slices were washed several times in PBS, and once again placed in new wells. 200 μL of an avidin-β-galactosidase (0.02 mg/ml in PBS) solution was added to each well and the reaction was allowed to proceed at room temperature. After two hours, the slices were thoroughly washed in PBS, placed in new wells, and 500 μL of x-gal solution (1 mg/ml in PBS) was added to each well.

Unfortunately, we did not see a blue precipitate in any of the slices. We also repeated the experiments with sodium periodiate concentrations of 75 mM and 100 mM, but there was no improvement in the results.

Experiment 2: Oxidative modification of bovine pericardium and fluorescence detection with Alexa Fluor-430.

The pericardial sections were reduced and reacted with biotin-hydrazide as in experiment 1. The sections were then placed in a solution of Alexa Fluor-430 (0.02 mg/ml in PBS) for 2 hrs. The slices were then washed in PBS, blotted to remove excess reagent and placed into the imager for fluorescence detection (520 nm filter, 30 sec acquisition time, aperture 3.0 setting). However, with this fluorescence detection, we failed to show a difference between the control and modified sections.

Experiment 3: Oxidative Modification of Bovine Pericardium and Fluorescence Detection with Fuorescein Hydrazide.

The pericardial sections were reduced and reacted with biotin-hydrazide as in experiment 1. The sections were then placed in a solution of fluorescein-hydrazide[214] (20 mM in PBS with 10% DMSO v/v to assist in solubilizing the reagent), a fluorescent

probe which reacts with aldehydes. After two hours, the sections were washed in PBS and imaged to detect any modification by fluorescence (520 nm filter, 30 sec acquisition time, aperture 3.0 setting). We reasoned that directly coupling a fluorescent probe to any aldehyde on the tissue surface might increase the fluorescence of the modified sections above any background autofluorescence. However, we were still unable to observe a difference between control and modified slices.

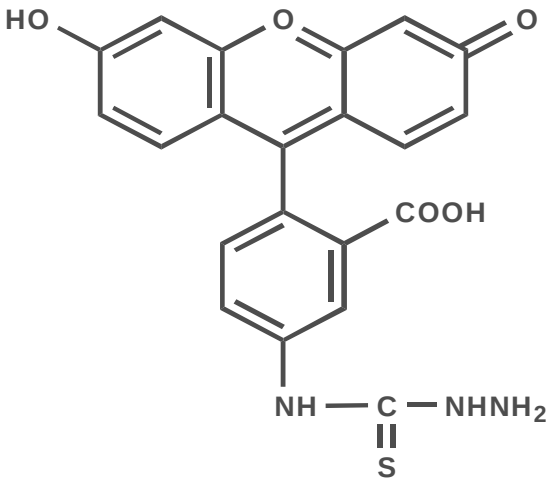

Figure 59. Structure of fluorescein hydrazide

Experiment 4: Oxidative Modification of Bovine Pericardium and Fluorescence Detection with Biotin-fluorescein.

We also conducted an experiment where after the slices were modified via oxidation and biotin-hydrazide as in experiment 1, they were reacted with streptavidin hydrazide. The slices were each reacted with 200 μL PBS solution of steptavidin hydrazide (1 mg/ml in PBS) for two hours at room temperature. The control slices were not oxidized with the periodiate and directly received the streptavidin hydrazide. After this time, all the slices were washed several times in PBS and placed in new wells.

200 μL of a biotin-4-fluorescein[215] (20 mM, 10% DMSO v/v in PBS) was added to each slice and the reaction was allowed to proceed at room temperature. After two hours, the slices were washed in PBS and imaged for fluorescence (520nm filter, 30 sec acquisition time, aperture 3.0 setting). We thought that using this combination of reagents might increase the fluorescence signal of the modified tissue over the background autofluorescence. However, the images also displayed no difference in signal intensity between control and modified slices.

Experiment 5: Gross oxidative Modification Fetal Pig skin with Chemiluminescence Detection

A sample of fetal pig skin was cleared of fat and cut into approximate squares 1 cm/side. Skin samples were placed into the compartments of a 12 well plate and approximately 1.5 mL of a 0.08 M solution of sodium periodate in PBS was added to half of the wells. Additional wells were filled with 1.5 mL of PBS to serve as control samples. The samples were incubated for 8 hours at room temperature and then the solutions decanted and the tissue samples washed thoroughly (PBS; 3x ten minutes with a change of solution between each wash). After washing, all of the tissue samples were incubated in a 12 μM PBS solution of biotin-hydrazide (with 10% v/v DMSO to assist in solubilizing the reagent) overnight (approximately 15 hours) at 4ºC. The tissue was then thoroughly washed with PBS (3x ten minutes with a change of solution between each wash) and the final wash solution replaced by a PBS solution of avidin-horseradish peroxidase (0.03 mg/ml). After incubation for 2 hours at room temperature, the tissue samples were once again washed with PBS (3x ten minutes with a change of solution between each wash) and then immersed in the chemiluminescence substrate (Supersignal

West Pico luminol (Pierce)) at room temperature for 4.5 minutes. The tissue is then removed from the luminol solution, blotted with paper towels to remove excess solution, placed on a sheet of plastic film, and placed into the imager for detection of the chemiluminescence (ultrasensitive chemiluminescence settings; aperture completely open; 100 second acquisition). The positive signal appears as a relatively bright tissue sample while the control samples are dark and almost undetectable, Figure 60.

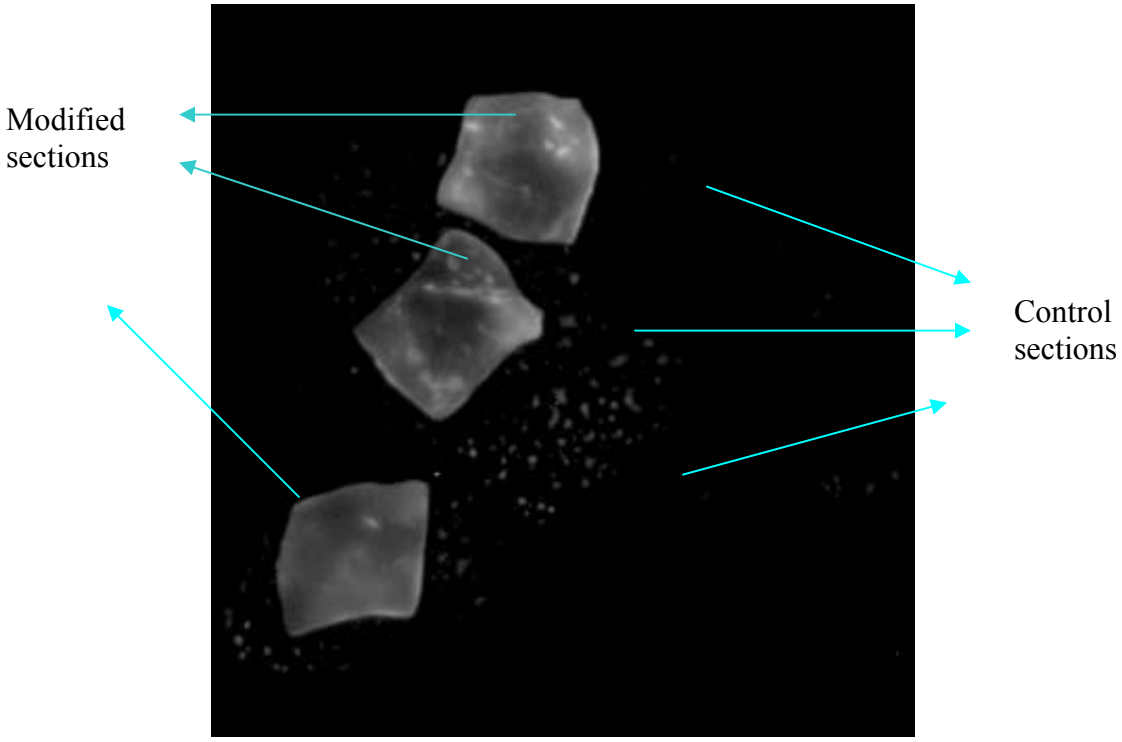

Figure 61. Chemiluminescent image from the oxidation of fetal pig skin sections. The modified sections (on the left of image) emit a much brighter chemiluminescent signal, while controls (on the right of image) are dark, and not visible.

Experiment 6: Site specific Oxidation of l pig Skin and Chemiluminescence Detection.

The fetal pig skin was cleared of fat and cut to fit a 96 well plate. The entire slice was placed in a Petri dish and oxidized with 100 mM solution of sodium periodiate for 2

hrs at room temperature. After this time the tissue was thoroughly washed in PBS for 10 minutes with several changes of buffer. 100 μL of a biotin hydrazide (20 mM in PBS with 10% DMSO v/v) solution was added to each of the wells to form a 'BU' pattern, and the remaining wells were filled with 100 μL of PBS (also with 10% DMSO). The reaction was carried out for 15 hrs at 37^{O}C. After this time, the tissue was washed in PBS (3x5min which several changes of buffer), and placed in a 0.02 mg/ml solution of avidin-HRP for 3 hrs and incubated at room temperature. The tissue was then washed, blotted, reacted with the chemiluminescent substrate for 4 min and imaged (ultrasensitive chemiluminescence settings; aperture completely open; 80 second acquisition). A pattern of bright spots in the shape of a 'BU' was visible where the reagent was contacted with the skin.

Experiment 7: Gross Oxidative Modification of Bovine Pericardium and Chemiluminescence Detection

A sample of bovine pericardium was cleared of fat and cut into squares approximately 2 cm/side. The tissue samples are placed into the compartments of a 12 well plate and approximately 1.5 mL of a 0.1 M solution of sodium periodate in PBS is added to half of the wells. Additional wells are filled with 1.5 mL of PBS to serve as control samples. The samples are incubated for 8 hours at 37^{0}C and then the solutions decanted and the tissue samples washed thoroughly (PBS; 3x ten minutes with a change of solution between each wash). After washing all of the tissue samples are incubated in a 20 mM PBS solution of biotin-hydrazide (with 10% v/v DMSO to assist in solubilizing the reagent) overnight (approximately 10 hours) at 37ºC. The tissue is then thoroughly washed with PBS (3x ten minutes with a change of solution between each wash) and the

final wash solution replaced by a PBS solution of avidin-HRP (0.03mg/ml). After incubation for 2 hours at room temperature, the tissue samples are once again washed with PBS (3x ten minutes with a change of solution between each wash) and then immersed in the chemiluminescence substrate luminol (Supersignal West Pico luminal) at room temperature for 4.5 minutes. The tissue is then removed from the luminal solution, blotted with paper towels to remove excess solution, placed on a sheet of plastic film, and placed into the imager for detection of the chemiluminescence (ultrasensitive chemiluminescence settings; aperture completely open; 50 second acquisition). The modified tissue sections appear slightly brighter than the control tissue sections under chemiluminescence detection (see figure 61).

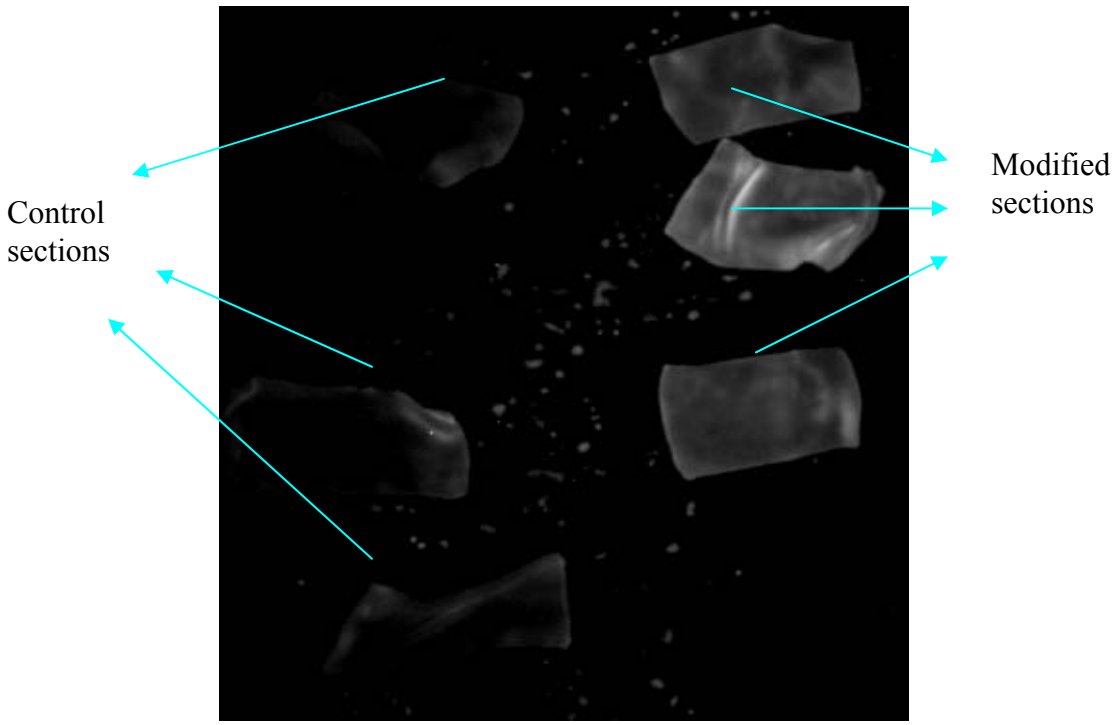

Figure 62. Chemiluminescent image from the oxidation of pericardial sections. The control sections on the left appear dimmer when compared to the modified sections on the right.

Experiment 8: Site specific oxidative modification of bovine pericardium and chemiluminescence detection

The pericardium was cleared of fat and cut to fit a 96 well plate. The entire slice was placed in a Petri dish and oxidized with 100mM solution of sodium periodiate for 2 hrs at room temperature.

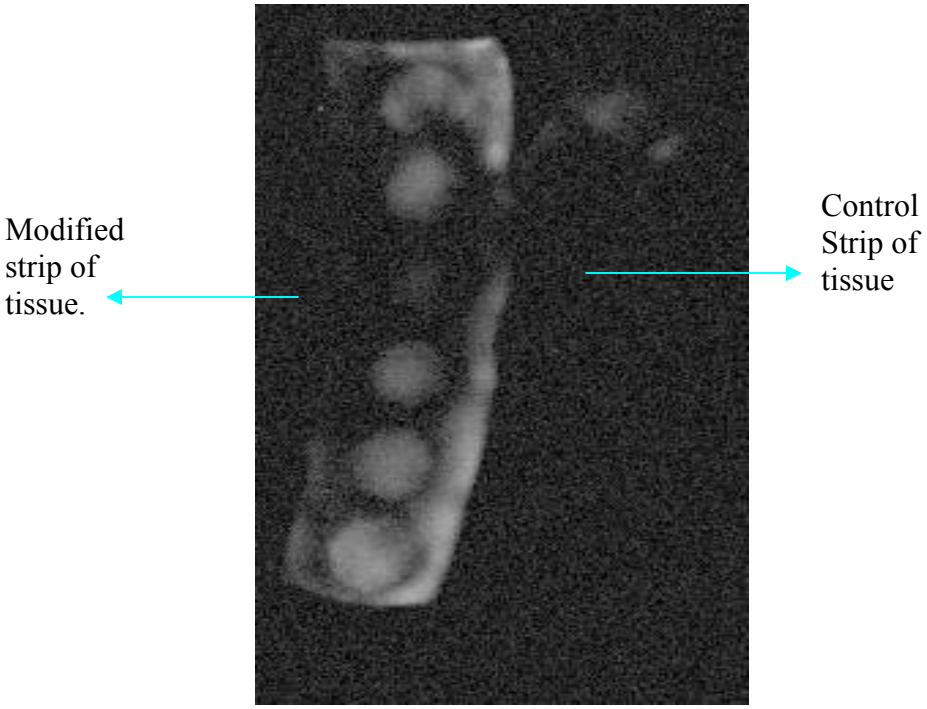

Figure 63. Chemiluminescent image from the site specific oxidation of pericardium. The modified regions appear brighter when compared to the control regions.

After this time, the tissue was thoroughly washed in PBS for 10 minutes with several changes of buffer. 100 µL of a biotin hydrazide (20 mM in PBS with 10% DMSO v/v) solution was added to the wells of the plate, and the remaining wells (controls) were filled with 100 µL of PBS (also with 10% DMSO). The reaction was carried out for 15 hrs at 37^0C. After this time, the tissue was washed in PBS, and placed in a 0.02 mg/ml solution of avidin-HRP for 2 hrs and incubated at room temperature.

The tissue was then washed, blotted, reacted with the chemiluminescent substrate for 4 min and imaged (ultrasensitive chemiluminescence settings; aperture completely open; 100 second acquisition).

The sections of tissue that were modified displayed a fluorescent signal, while the controls remained dark. However, the modified strip had a considerable amount of background signal as well. We also tried repeated this experiment to obtain a "BU" pattern on the tissue. Unfortunately, in this case there was too much background to be able to visualize the modification.

Experiment 9: Oxidative Modification of Bovine Meniscus and Chemiluminescence Detection

A sample of bovine meniscus is cleared of fat and cut into approximate longitudinal section about 2cm/side. The tissue samples are placed into the compartments of a 12 well plate and approximately 1.5 mL of a 0.1 M solution of sodium periodate in PBS is added to half of the wells. Additional wells are filled with 1. 5 mL of PBS to serve as control samples. The samples are incubated for 15 hours at room temperature and then the solutions decanted and the tissue samples washed thoroughly (PBS; 3x ten minutes with a change of solution between each wash). After washing, all of the tissue samples are incubated in a 12 μM PBS solution of biotin-hydrazide (with 10% v/v DMSO to assist in solubilizing the reagent) overnight (approximately 15 hours) at 37ºC. The tissue is then thoroughly washed with PBS (3x ten minutes with a change of solution between each wash) and the final wash solution replaced by a PBS solution of avidin-HRP (0.03mg/ml). After incubation for 3 hours at room temperature, the tissue samples are once again washed with PBS (3x ten minutes with a change of solution

between each wash) and then immersed in the chemiluminescence substrate luminol (Supersignal West Pico (Pierce)) at room temperature for 4.5 minutes. The tissue is then removed from the luminol solution, blotted with paper towels to remove excess solution, placed on a sheet of plastic film, and placed into the imager for detection of the chemiluminescence (ultrasensitive chemiluminescence settings; aperture completely open; 50 second acquisition). The positive signal from the modified sections appears to emit a brighter chemiluminescence signal when compared to the control sections. In this experiment, the control section seemed to have non-specific binding along their periphery (.Figure 64)

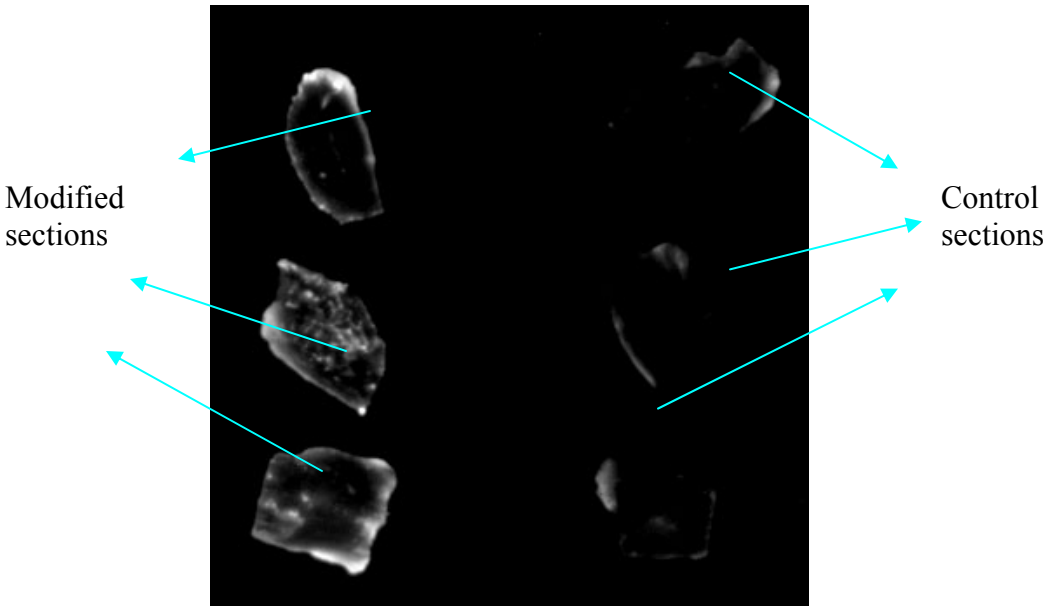

Figure 65. Chemiluminescence image from the oxidative modification of bovine meniscus. The modified section (on the left) emit greater chemiluminescent signal when compared to control section (on the right)

Experiment 10: Oxidative Modification of Aorta and Chemiluminescence Detection

The bovine aorta was washed and sectioned into chunks 2 cm^2 and about 1cm in thickness. The chunks were placed in wells of a 12 well plate and reduced in 0.1 M

solution of sodium periodiate in PBS for 2 hrs. The remainder of the experiment (including the tissue washes, alkylation with biotin-hydrazide, further tissue washes, incubation with avidin-HRP, further tissue washes, incubation with luminol and chemiluminescence conditions) was identical to experiment 9. Unfortunately, there was no chemiluminescent signal from either the control or modified slices. We repeated the experiment for periodiate concentration of 0.2 M and doubled concentrations of the rest of the reagents, but there was no difference in the results.

Experiment 11: Oxidative Modification of Skeletal Muscle and Chemiluminescence Detection

The skeletal muscle was cleaned of connective tissue and sliced into sections about 1 cm in thickness. The sections were placed in compartments of a 12 well plate and oxidized with a 50mM solution of sodium periodiate. After 2 hours, the sections were washed and the remainder of the experiment (including the tissue washes, alkylation with biotin-hydrazide, further tissue washes, incubation with avidin-HRP, further tissue washes, and incubation with luminal chemiluminescence conditions) was identical to experiment 9. Unfortunately, the skeletal muscle did not give any positive signals with the chemiluminescent detection methods. We repeated the experiment for periodiate concentration of 0.2 M and doubled concentrations of the rest of the reagents, but there was no difference in the results.

Section Four: Reductive Strategy for Tissue Modification

Experimental Strategy

As discussed in the introduction, one functional group that has proven extremely useful in the site-specific modification of proteins is the free sulfhydryl. Since free

sulfhydryls are rarely found in proteins, they usually need to be introduced by reaction with a reagent such as Trauts reagent or by the reduction of a disulfide bond using reducing agents such as TCEP or DTT (dithiothreitol). These free sulfhydryls can then be alkylated using a reagent such as a maleimide. The reaction of free sulfhydryls with maleimide is very chemoselective and this chemistry is considered to be more precise and controllable than that of the amine alkylation methodology.

The first reagent we investigated to introduce free sulfhydryl bonds is TCEP, tris(2-carboxyethyl)phosphine.[216] TCEP is a potent, versatile, odorless, thiol-free reducing agent with broad application in the reduction of disulfide bonds in proteins and other molecules (Figure 64). This unique compound is easily soluble and very stable in many aqueous solutions. TCEP reduces disulfide bonds as effectively as dithiothreitol (DTT), but unlike DTT and other thiol-containing reducing agents, TCEP does not have to be removed before certain sulfhydryl-reactive cross-linking reactions (e.g. reaction with maleimides and haloacetyl derivatives **(**Figure 66).[217]

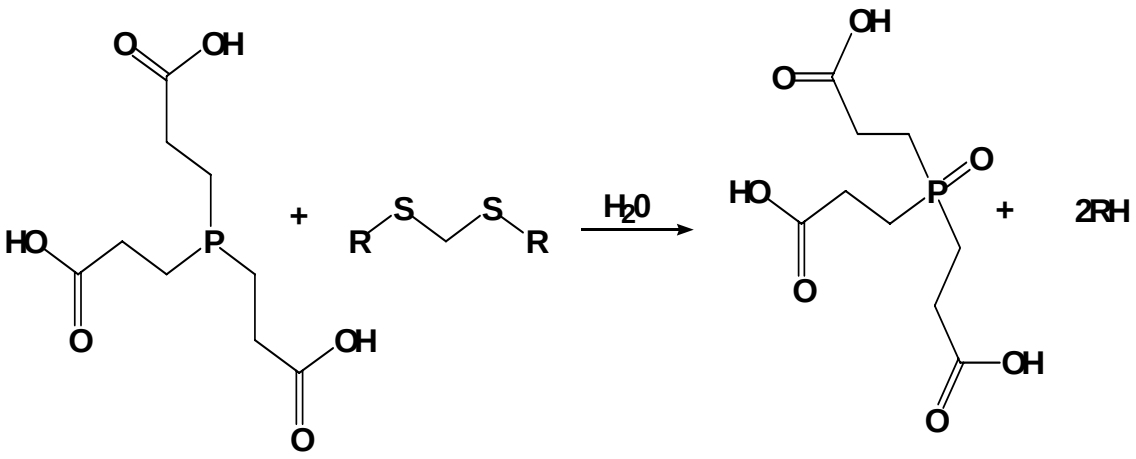

Figure 67. Reduction of disulfide bond with TCEP

The ability and virtues of trialkylphosphine compounds for the reduction of reduce protein disulfide bonds have been known for many years.[218] Phosphines are stable in aqueous solution, selectively reduce disulfide bonds, and are essentially unreactive toward other functional groups commonly found in proteins. However, widespread adoption of trialkylphosphines as reductants for protein research was hindered by their disagreeable odor and poor water solubility. These obstacles were overcome by discovery of tris (2-carboxyethyl)phosphine (TCEP). TCEP selectively and completely reduces even the most stable water-soluble alkyl disulfides over a wide pH range. Reductions of proteins frequently require less than 5 minutes at room temperature. TCEP is non-volatile, odorless, and unlike most other reducing agents, is resistant to air oxidation. Compared to DTT, TCEP is more stable, more effective, and able to reduce disulfide bonds at lower pHs.[219]

Initially, when we began our investigation of the reductive strategy to modify tissue, we were still attempting to use fluorescent and chromogenic assays for the detection of any modification on tissue surfaces. We also primarily used pericardium for this methodology because of the experimental problems that were associated with using meniscus. We first attempted a gross modification in which the pericardial sections were reduced with TCEP and reacted with a maleimide-fluorescent probe. However, we were unable to see any modification on this tissue even with the direct coupling of the fluorescent probe on to the reduced tissue, presumably due to the autofluorescence from the tissue. We then moved on to the chemiluminescent detection for tissues modified with the reductive strategy, since chemiluminescence is a far more sensitive method of

detection, and we could also avoid any interference from the natural fluorescence from the tissues.

Heterobifunctional crosslinkers are extremely useful compounds in the context of this research. As discussed in the introduction, they can be used to modify a protein to present a desired functional group. This modified protein can then be coupled to a tissue surface which presents its reactive partner. For example, avidin can be modified with a heterobifunctional crosslinker to introduce maleimide groups, which can then be used immobilize the avidin on a tissue surface expressing free sulfhydryls.

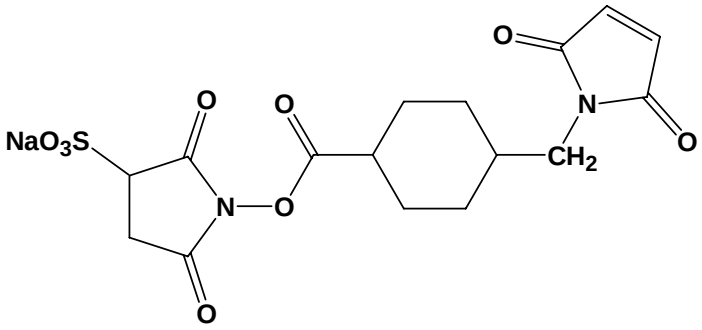

Figure 68. Structure of Sulfo-SMCC

LC-SMCC (Succinimidyl-4-[N-Maleimidomethyl]cyclohexane-1-carboxy-[6-amidocaproate]) and its water-soluble analog Sulfo-SMCC (Sulfosuccinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate) are heterobifunctional cross-linkers that contain N-hydroxysuccinimide (NHS) ester and maleimide groups (Figures 65). NHS esters react with primary amines at pH 7-9 to form covalent amide bonds. Hydrolysis of the NHS ester, which is a competing reaction, increases with increasing pH and decreasing protein concentrations. Maleimides react with sulfhydryl groups at pH 6.5-7.5 to form stable thioether bonds. At pH values >7.5, reactivity toward primary amines and

hydrolysis of the maleimide group can occur; however the maleimide groups of Sulfo-SMCC and LC-SMCC are unusually stable up to pH 7.5.[220]

For conjugation, the NHS ester is reacted first, excess reagent removed and then the sulfhydryl-containing molecule is added. Sulfo-SMCC is soluble up to ~10 mM in water and many commonly used buffers; however solubility decreases with increasing salt concentration. LC-SMCC is dissolved in DMSO or DMF and added to the reaction mixture at a final solvent concentration of 10-20% to minimize detrimental affects to the protein.

We used the heterobifunctional crosslinker to investigate our ability to modify a protein, immobilize it onto the tissue surface and detect its presence using chemiluminescence. We modified horseradish peroxidase (HRP) with a heterobifunctional probe to react with the reduced tissue. Unfortunately, the initial set of experiments carried out with the HRP-maleimide was unsuccessful at detecting any tissue modification. In subsequent experiments, we reduced the concentration of the HRP-maleimide by ten fold and were successful in visualizing modification on the tissue surface with chemiluminescence. Since we were successful at immobilizing HRP-maleimide on the tissue surface, we also attempted to immobilize alkaline phosphatase (AP)-maleimide for colorimetric AP detection. Unfortunately, this colorimetric assay failed to reveal any modification on the tissue surface.

We also investigated an indirect assay for tissue modification and detection with chemiluminescence. The general procedure for this reductive strategy with chemiluminescence detection was as follows; the tissue was reduced with TCEP, and then reacted with avidin-maleimide, followed by biotin-HRP and the chemiluminescent

substrate luminal. The reduced tissue could also be reacted with biotin-maleimide, followed by avidin-HRP and luminal. The modified tissues were then placed into a Bio-Rad imager to detect any chemiluminescent signal emitted from the modified regions on the tissue surface.

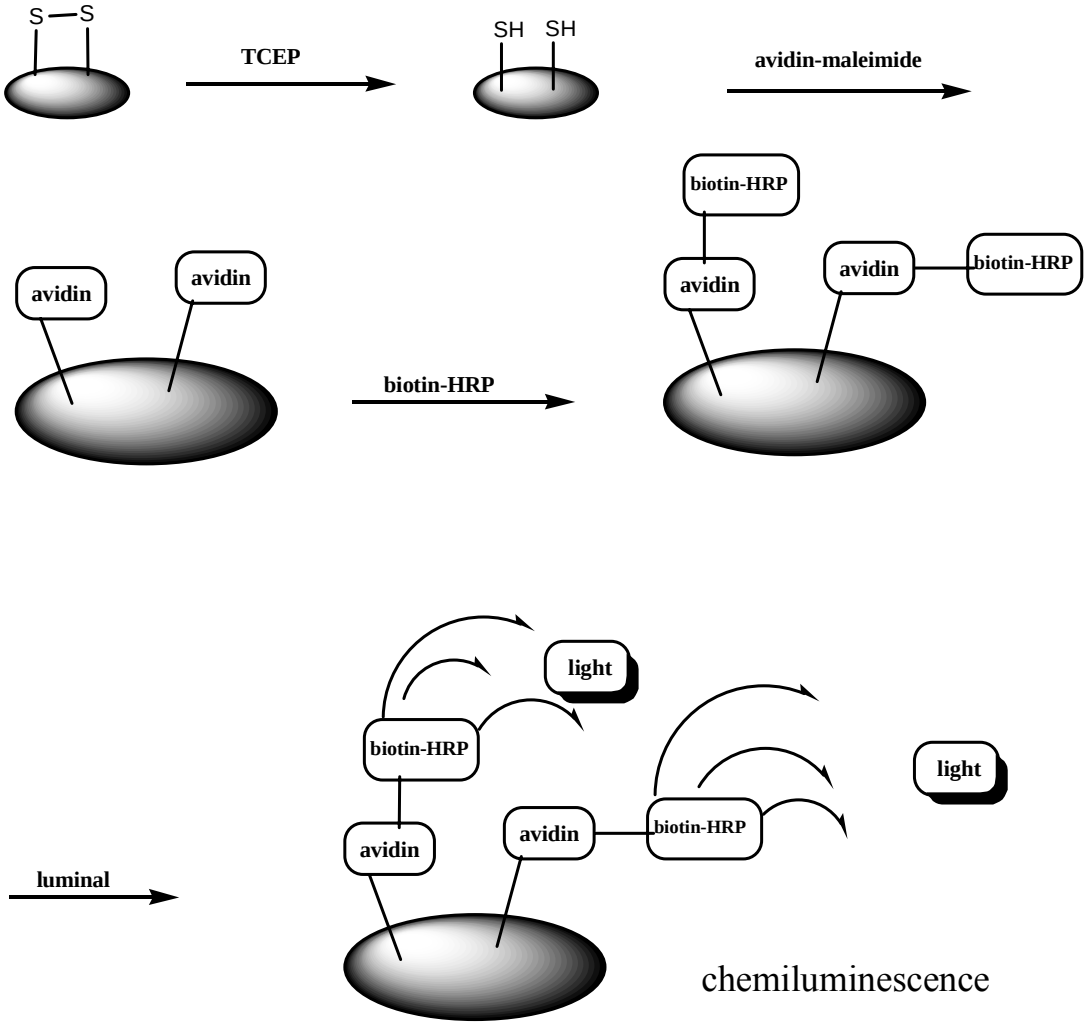

Figure 69. General scheme for modification of tissue with reductive strategy, followed by chemiluminescent detection.

Initially, we attempted to modify entire sections of the tissue in which the pericardium sections were reduced with TCEP, alkylated with biotin-maleimide, reacted

with avidin-HRP and luminal for chemiluminescence. The controls were set up where the tissue sections were not reduced with TCEP. The resultant images detected strong chemiluminescent signals from the modified tissue sections when compared to the controls.

Since the gross modification assay was successful, we moved to an investigation the possibility of site-specifically immobilizing a biomolecule on the tissue surface, in order to demonstrate spatial control of the technique. The pericardium was site-specifically reduced in a 'BU' pattern using reagents in compartments of a 96 well plate, followed by reaction with avidin-maleimide, and then with biotin-HRP and luminal. We also performed a similar experiment where biotin-maleimide was initially reacted with the reduced tissue, followed by avidin-HRP and luminal. Both resultant images showed a bright 'BU' pattern against a dark background, indicating that we were successfully able to immobilize the avidin or biotin at our desired location on the tissue surface.

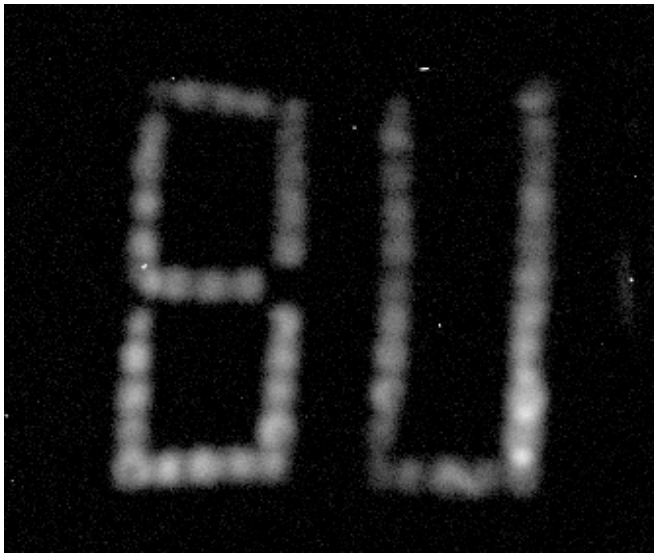

Figure 70. Chemiluminescent image of pericardium showing 'BU' after site specific protein immobilization using a 384 well plate. The 'pixel' resolution on the 'BU' is ~2mm in diameter.

In subsequent experiments, we also sought to improve the spatial resolution by using 384 and 1536 well plates and using the Supersignal West Pico® luminal (Pierce), which is more sensitive than the luminal used in previous experiments (Figure 68).

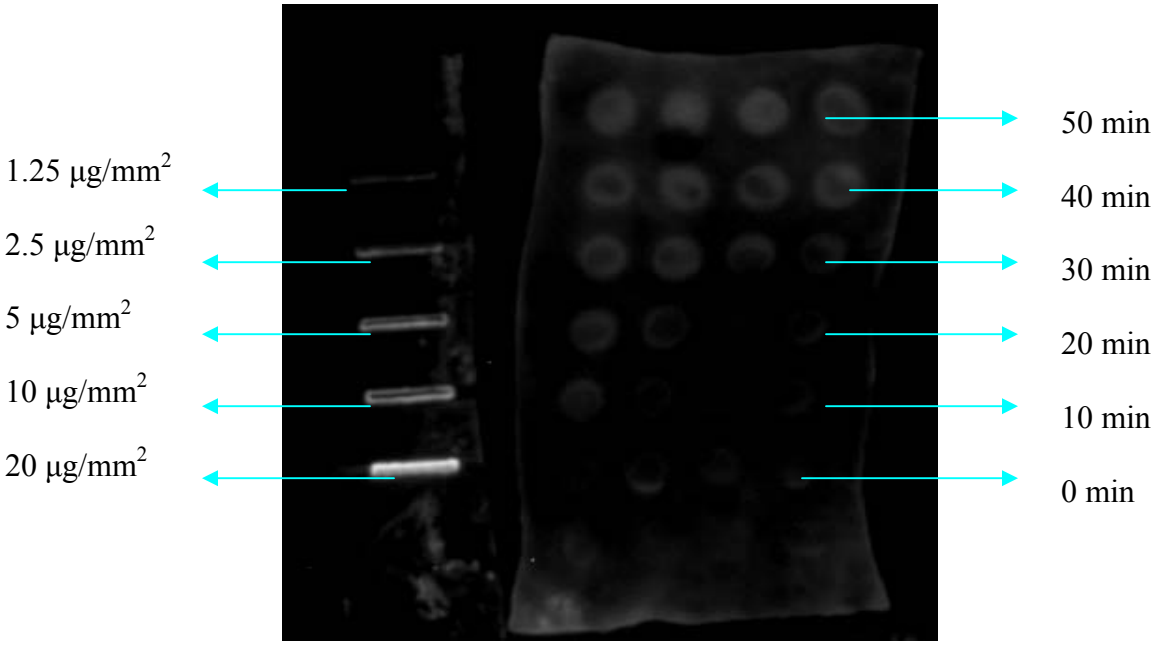

Figure 71. Chemiluminescent image from variable exposures of TCEP with pericardium and slot blot strip with serial dilutions of avidin for quantification. The brightness of the dots is proportional to the length of exposure of the tissue to TCEP. Greater the length of exposure to TCEP, the brighter the dots appear.

The images from both these experiments indicated that we were able to increase our resolution to about 1mm (with the 1536 well plate). As with the directly alkylation, we used a slot blot membrane with serial dilutions of avidin to quantify the amount of protein being immobilized on the tissue surface. This value was calculated to be between 1-4ug/mm^2, the range being presumably due to lack of homogeneity of the tissue surface.

An experiment was also performed to determine the effect of TCEP concentration on the pericardium. The pericardium was exposed to various TCEP concentrations (100

mM-10 mM range). Under these reaction conditions, minimum concentration of TCEP required to reduce the tissue to obtain a chemiluminescent signal was approximately 25mM. We also performed a time exposure experiment to determine the minimum time of reaction necessary between TCEP and the tissue to emit a detectable chemiluminescent signal, which was observed to be approximately 30 min.

Even though the reductive strategy with chemiluminescence was successful at immobilizing a biomolecule and detecting its presence on the tissue surface, we still had some background associated with our images. Background is common when using an avidin-biotin system due to non-specific binding, so we investigated several methods to reduce the non-specific binding. These methods included longer washes in-between steps, or the use of a BSA blocking step, or increased detergent in the PBS buffer. However, none of these steps helped reduce the background signal from the tissue. We then turned our attention to the use of water as opposed to PBS as our solvent for reagents and tissue washes. The resultant image from this experiment had a greater signal- to-noise ratio when compared to reactions run with PBS. Further experimentation is necessary to determine the causes of this effect with water.

Due to our success with the pericardium using the reductive/chemiluminescent assay, we then moved to investigate the possibility of using this method to modify other types of tissue. The meniscus was also reduced with TCEP, alkylated with avidin-maleimide, reacted with biotin-HRP and luminal. The resultant image also proved that we were successful at using this strategy to modify the meniscal tissue. The fetal pig skin was also site specifically reduced with TCEP in a ‘BU’ pattern, alkylated with avidin-maleimide, biotin-HRP and luminal. The bright ‘BU’ image on the tissue surface once

again showed that we were successfully able to reduce the tissue in a spatially defined pattern, and the amount of protein immobilized was about 1 ug/mm^2 using a slot blot strip with serial dilutions of avidin.

The bovine cornea was also reduced with TCEP, and we were able to distinguish between modified and control slices, but there was significant background signal due to non-specific binding of reagents. The skeletal muscle, however, failed to give us any positive results with the reductive strategy.

Since the chemiluminescence proved to be an excellent detection system for our immobilized proteins, we also investigated the use of DTT and BME as alternative reducing agents to TCEP. Pericardium sections were reduced with DTT or BME, reacted with avidin-maleimide, followed by biotin-HRP and luminal. The resulting image did indicate that these reagents could also be used in the reductive method, but there was high background associated in both cases.

The chromogenic assay, though less sensitive than fluorescence or chemiluminescence, has the advantage that the modifications can be visualized with the naked eye. Therefore, we investigated the use of an HRP based chromogenic assay with TMB as a substrate to detect any modification. After the tissues were reacted with luminal and imaged for chemiluminescence, they were washed and then incubated with TBM. The HRP present on the tissue converts the TMB to a blue precipitate which can then be seen with the naked eye. In this case, we were able to see a slight blue precipitate on the modified pericardium and meniscal sections, though the modifications were not as clear or dramatic when compared to chemiluminescence detection.

We also investigated the stability of the biotin once immobilized onto the free sulfhydryl on the tissue surface by incubating reduced tissue alkylated with biotin-maleimide with trypsin, pepsin and calf serum (cocktail of enzymes). These are some of the more common protease enzymes that are found in the body. These experiments were carried out to investigate the stability of the immobilized biotin and avidin on the tissue surface in the presence of protese enzymes. These studies demonstrated that the biotin was detectable via the chemiluminescence assay for up to 18 hrs.

Material and Methods

Chemicals and Reagents

Except where otherwise noted, reagents used for the modification were obtained from Aldrich Chemical CO., Milwaukee, WI, and Fisher Scientific, Pittsburgh, PA (including Across reagents), Pierce and Molecular Probes, and used directly as purchased. Solvent such as dichloromethane, hexane, ethanol, ethyl acetate and methanol were obtained from commercial sources (via Baylor University Chemistry Department stockroom) and were distilled prior to use. Deionized water (DI) was obtained via a US Filter system provided by Baylor Dept. of Chemistry.

Bovine pericardium was obtained from Animal Technologies IINC in Tyler, Texas. The pericardium was cleaned of all excess fat using a microtome blade. The connective tissue was carefully removed by scraping with the blade and care was taken not to damage any of the actual pericardium. The cleaned pericardium was placed in a PBS solution at room temperature for up to 2 hrs before use. Unused pericardium was stored at -20^0C and defrosted as needed.

The fetal calf skin was obtained from Animal Technologies and the rat skin was obtained from department of neuroscience at Baylor University. The fetal pig skin was washed in PBS and trimmed of any excess fat using a microtome blade. It was then allowed to soak in PBS until use. Unused skin was stored at -20^{0}C and defrosted and hydrated in PBS before use. The rat skin was obtained fresh, and the hair was removed by shaving or with the use of Nair. It was then soaked in PBS for up to 2 hrs before use or stored at -20^{0}C.

The meniscus was obtained from H&B Packing, Waco, TX. The menisci were dissected from whole bovine knees, trimmed of fat and stored in PBS for use. Excess menisci were also stored at -20^{0}C and rehydrated in PBS when used for experiments. The meniscus was made into longitudinal sections of desired thickness using microtome blade or scalpel and trimmed to fit into compartments of a 12 well plate.
The skeletal muscle and aorta were also obtained from Animal Technologies, soaked in PBS and used as obtained. The cornea was dissected out of whole bovine eyes, soaked in PBS for use. Any unused aorta, skeletal muscle and cornea were stored at -20^{0}C and defrosted and hydrated in PBS before use.

Instrumentation

Proton (^{1}H) and carbon (^{13}C) NMR spectra were obtained on a Bruker AVANCE 300 NMR running XWIN-NMR 3.1 software at 300 MHz. Chemical shifts are expressed in ppm (∂), peaks are listed as singlets (s), doublets (d), triplet (t) or multiplet (m), with the coupling constant (J) expressed in Hz. HPLC was carried out with a Beckman System Gold, composed of a model 168 detector and a model 126 solvent module using a Alltech Alltima C-18 5M 33mm*7mm “rocket” column. GC-Mass spec

was obtained on a Hewllet Packard. Fluorescence and chemiluminescence was detected using the Bio-Rad Laboratories Inc Fluor-S Multi-Imager and analyzed using Bio-Rad software. The chromogenic images were recorded using a Kodak 2 mega pixel digital camera.

Experimental Methods for Reductive Tissue Modification Strategy

Experiment 1: Reduction ofBbovine Pericardium, Alkylation with Fluorescein Maleimide and Fluorescence Detection

The bovine pericardium was washed, sectioned into 2x2 cm slices and placed in individual compartments of a 12 well plate. 14.5 mg of TCEP was dissolved in PBS to give a concentration of 50 mM. 200 μL of this solution was added to each slice that was to be reduced, while the control slices each received 200 μL of PBS. The reduction was carried out for 45min at room temperature with gentle agitation.

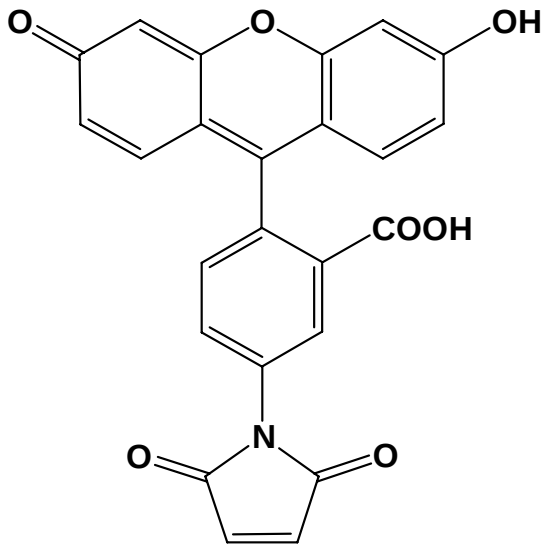

Figure 72. Structure of fluorescein maleimide

After this time, the slices were washed once in PBS to remove any excess TCEP and placed in clean wells. 200 μL of a 20 mM solution of fluorescein-maleimide[221] in PBS (with 10% DMSO v/v for solubility of reagent), a fluorescent probe which reacts primarily with sulfhydryl groups, was added to each well. The reaction was carried out for 2 hours in the dark at room temperature. After this time, the slices were washed in PBS and blotted to remove any excess buffer. They were then placed into the imager for detection of fluorescence (530nm filter, 30sec acquisition time, aperture 3.0). Unfortunately, we were unable to see a clear difference between modified and control sections.

Experiment 2: Reduction with DTT or BME, Reaction with Fluorescein Maleimide and Fluorescence Detection

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Cleland's Reagent, also known as dithiothreitol (DTT), is a water soluble disulfide reducing reagent and is widely used for the study of disulfide exchange reactions of protein disulfides. DTT readily permeates into cell membranes and hence can protect protein sulfhydryls from oxidation and restore enzyme activity lost by the oxidation of sulfhydryl groups. BME (beta-mercapto ethanol) is also another common reducing agent used for reducing disulfide bonds and is used extensively in protein biochemistry.

Two set of experiments were carried out to investigate the use of DTT and BME as reducing agents for the pericardium. The reactions were identical to that of the TCEP experiment (including tissue washes, alkykation with fluorescein-maleimide, further tissue washes and imaging) with the exception that DTT or BME was used to reduce the disulfide bonds on the pericardium. Unfortunately, we did not see any modification of

the tissue by fluorescence after reduction BME or DTT and alkylation with fluorescein-maleimide. One set of problems associated with using DTT and BME are the presence of excess free sulfhydryl groups from the reagents after the reaction. Unlike TCEP, when using DTT or BME the tissue needs to be washed thoroughly to remove any excess reagent.

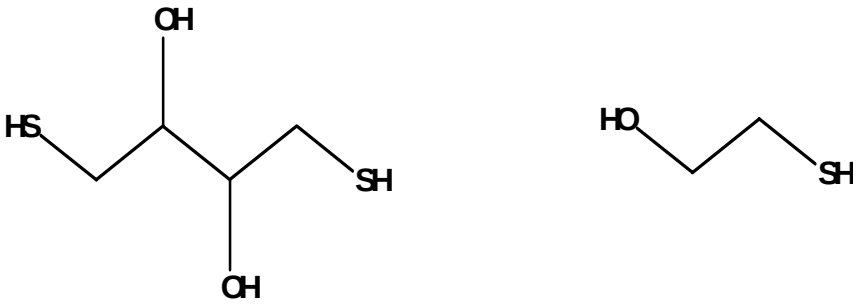

Figure 73. Structure of DTT (left) and BME (right)

Experiment 3: Site Specific Modification of Pericardium with Fluorescence Detection

The pericardium was cut into several strips about 4 cmx2cm. 20 μL of a 100 mM solution of TCEP was then added to the center of each strip by hand and allowed to remain as a spot on the surface, while the control slices each received 20 μL of PBS. The slices were covered with a plastic lid and the reaction was allowed to proceed for 45 min. After this time, the strips were washed in three times in PBS and they were all placed in a Petri dish. 1 ml of 40 mg/ml fluorescein maleimide solution in PBS (20% DMSO v/v was added to help solubilize the reagent) was added to the dish. After two hours, the strips were thoroughly washed in PBS and fluorescence was detected using the imager. However, the fluorescence assay was not able to detect any site specific modification on the tissue surface.

Experiment 4: Protein Modification via Heterobifunctional Crosslinker for Reaction with Sulfhydryls

Horseradish peroxidase modification and immobilization on fresh bovine pericardial tissue surfaces. The first protein we modified with the HBC was horseradish peroxidase, a common protein used in immunochemistry. HRP is a 40kDa protein that catalyses the oxidation of substrates by hydrogen peroxide, resulting in a colored or fluorescent product or the release of light as a byproduct. HRP was attractive since it provided an alternative means of detection (chemiluminescence) as opposed to the colorimetric and fluorescence assays that we had been using to detect the immobilized proteins.

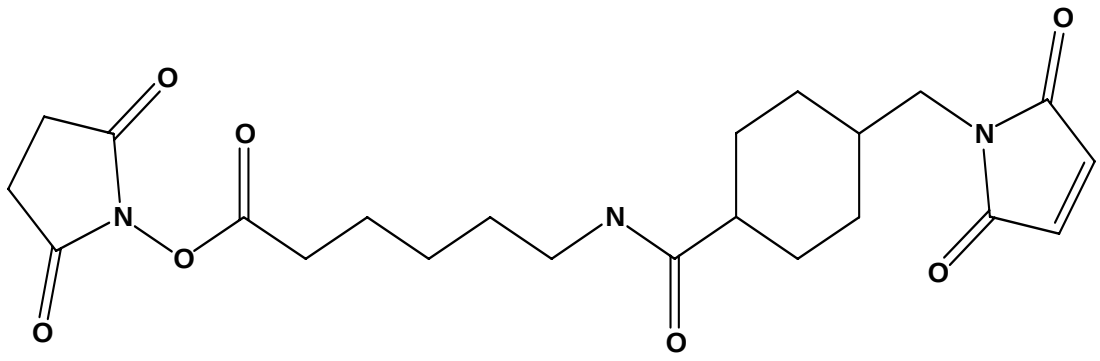

Figure 74. Structure of SMCC-LC-LC

5 mg of the HRP was dissolved in 500 μL of PBS buffer. 1 mg of the LC-SMCC or Sulfo-SMCC in 50 μL of DMSO was added to the protein. The reaction mixture was incubated for 1hr at room temperature. After this time, the HRP-SMCC conjugate was separated using gel filtration chromatography.

Gel filtration columns were inverted several times to resuspend the gel and to remove any air bubbles. The tips were snapped off the columns and then placed in 2 ml

microcentrifuge tubes. The caps were removed and the columns were allowed to drain by gravity to the top of the gel bed. The collected buffer was then discarded, the columns were placed back into the centrifuge tubes and the tubes were centrifuged for 2 min at 1,000 x g to remove excess buffer. The dry columns were placed back into clean microcentrifuge columns and the 75 μL of the biotinylated HRP-SMCC was added to each column. The columns were centrifuged for 4 min at 1,000 x g and the fractions were pooled to give a total sample volume of about 400 μL. This method was repeated to obtain the HRP-Sulfo-SMCC conjugate.

The pericardium was sectioned, placed into wells and reduced with TCEP as in the previous experiments. The slices were then washed in PBS, placed into new wells, and 300 μL of PBS, along with 50 μL of the HRP-SMCC conjugate, was added to each well. The control experiments received only PBS and the reaction was carried out for 2hrs at room temperature. The slices were then washed in PBS (3 x 5 min) and blotted to remove any excess buffer. They were then reacted with the luminal for 4min, blotted once again and placed into the imager for the detection of chemiluminescence (ultrasensitive chemiluminescence settings; aperture completely open; 100 second acquisition). Unfortunately, the resultant display was mostly dark indicating a negative chemiluminescent signal. One reason for the lack of signal might have been due to the high concentration of HRP in the system, which can too quickly consume the luminal and hence the emitted light before it is detected. We also obtained similar results with the HRP-Sulfo-SMCC conjugate. In subsequent experiments, we lowered the concentration of HRP-maleimide by 10 fold and successful at detecting modification on the tissue surface.

Alkaline phosphatase modification and immobilization. Alkaline phosphatase (AP) is a 140kDa protein that is generally isolated from calf intestine and catalyses the hydrolysis of phosphate groups from a substrate molecule as shown in Figure 70. We modified and immobilized alkaline phosphatase since it has several available chromogenic substrates, and could provide an alternate means for detecting immobilized proteins.

5 mg of the alkaline phosphatase was dissolved in 500 μL of PBS buffer. 1mg of the LC-SMCC or Sulfo-SMCC in 50 μL of DMSO was then added to the protein and the reaction mixture was incubated for 1hr at room temperature. After this time, the alkaline phosphatase-SMCC conjugate was separated using gel filtration chromatography, as with the previous experiment. The purified fractions were pooled to give a total sample volume of 450 μL.

Pericardium was sectioned, placed into wells, and reduced with TCEP as in the previous experiments. The slices were then all washed in PBS and placed into new wells. 300 μL of PBS along with 50 μL of the alkaline phosphatase-SMCC conjugate was added to each well. The control experiments received only PBS. The reaction was carried out for 2 hrs at room temperature. The slices were then washed in PBS (3 x 5 min) and blotted to remove any excess buffer. They were then incubated with a BCIP, 5-bromo-4-chloro-3-indoxyl phosphate disodium salt (chromogenic substrate for alkaline phosphatase), solution for 1hr. Unfortunately, we did not see any color development in any of the slices. The AP assays are not as sensitive when compared to fluorescence or chemiluminescent assays, and in this case the quantity of enzyme immobilized on the surface might have been below the sensitivity limits of the assay.

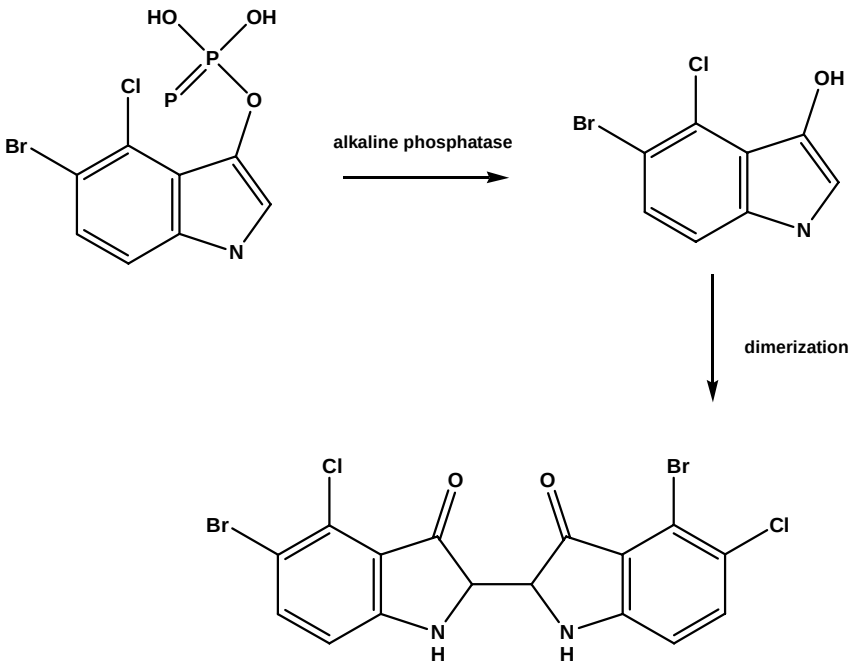

Figure 75. Reaction of alkaline phosphatase with BICP for chromogenic detection

Experiment 5: Gross Modification of Pericardial Sections with Chemiluminescent Detection

The pericardium was cleaned and sectioned into strips about 6 cm x 2 cm. The ends of each strip were placed into wells containing TCEP, while the center of the strip was placed in PBS. The strips were then reduced as in the previous experiments. After 45 min, the strips were then rinsed with PBS, placed in new wells and reacted with 200 μL of avidin-maleimide[222] in PBS (0.2 mg/ml) for 15hrs at 4^0C. After this time, the strips were washed in PBS, 500 μL of biotin-HRP in PBS (0.3 mg/ml) was added to each strip, and the reaction was allowed to proceed for 2 hrs at room temperature. The strips were then washed three times in PBS, reacted with luminal for 4min, and finally blotted to remove any excess buffer. They were then placed in the imager for the detection of chemiluminescence[223](high sensitivity, aperture fully open, 100sec acquisition time).

However, we were unable to detect any chemiluminescence signals from any of the slices Once again; reason for the lack of signal might be due to the high concentration of biotin-HRP. Therefore, we repeated the experiment as above, but in this case we lowered the concentration of biotin-HRP[224] to 0.03 mg/ml. This time we were successfully able to detect a chemiluminescence signal from the modified slices, while the controls remained dark.

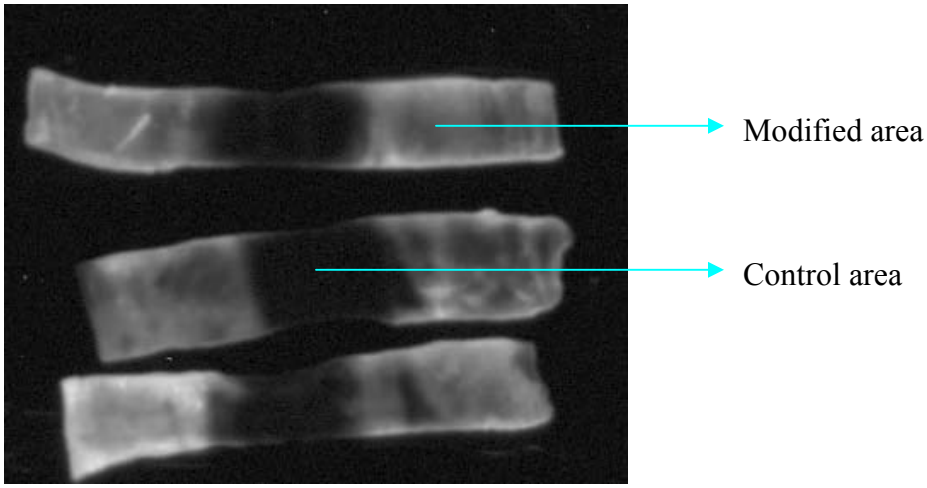

Figure 76. Gross modification using reductive strategy and chemiluminescent detection

Experiment 6: Site Specific Modification of Pericardium with Chemiluminescence Detection

With the gross modification being successful, the next step was to try a more site-specific modifications of the tissue surface. For this experiment, a specially made aluminum block with several holes, 3mm in diameter, was custom made by the Baylor machine shop. The pericardium was cut to fit the dimensions of the aluminum block, about 5 cm^2. The block was then placed on the surface of the tissue slice and 50 µl of the TCEP was added to each hole, while being firmly held down. One opening served as a

control and it received 50 µl of PBS. After the reagent was added, several heavy books were placed on top of the block to prevent any spreading out of the reagent, and the reaction was run for one hour at room temperature. After this time, the pericardium was rinsed in PBS, placed in a Petri dish and was reacted with 0.2 mg/ml solution of avidin-maleimide in PBS at 4^0C for 15 hrs. The tissue was then washed thoroughly and reacted with 0.03 mg/ml biotin-HRP in PBS in a Petri dish for a further 2 hrs at room temperature. After this time, the tissue was rinsed several times with PBS, reacted with luminal for 4 min and blotted to remove excess reagent. It was then placed in the imager for chemiluminescence detection (high sensitivity, 80 sec acquisition time, aperture fully open). Several bright dots were observed on the surface of the tissue, indicating that the tissue was specifically reduced in the desired areas where it was in contact with the reagent.

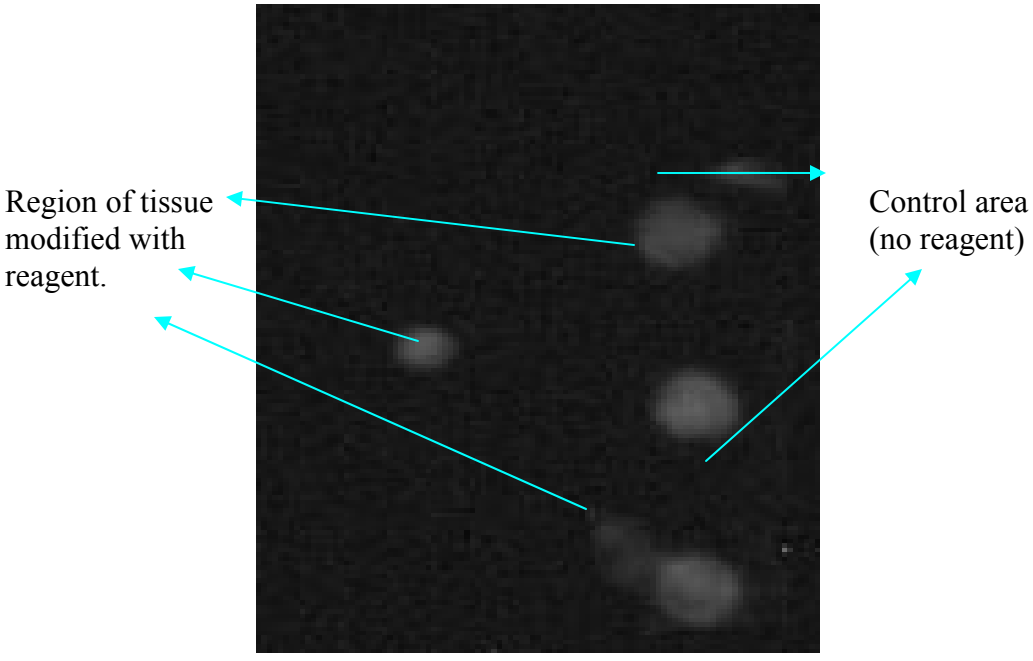

Figure 77. Chemiluminescent image of pericardium after site specific modification. The positive signal appears as a series of bright dots on the tissue surface.

Experiment 7: Site specific Modification of Pericardium Using VWR Pipette tip Holder, and Chemiluminescent Detection

The pericardium was cut to fit on the plastic pipette tip holder (VWR, 200 μL tip size) using a scalpel blade. The tissue section was then placed on the holder and stretched to fit the flat side of the plate. A solid glass plate was then placed over the tissue and clamped down using spring clamps. The whole set up is turned upside down, and 200 μl of a 0.1 M solution of TCEP in PBS is added to the wells to be modified, while the controls received only PBS.

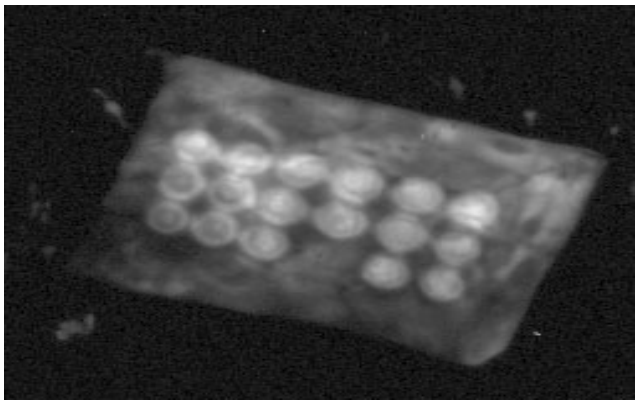

Figure 78. Chemiluminescent image of pericardium after site specific modification (avidin-maleimide, biotin-HRP, luminal). The positive signal appears as series of bright dots, while the controls remain relatively dim.

The reaction was allowed to proceed for 45 min at room temperature. After this time, the apparatus was dissembled and the pericardium was rinsed in PBS and placed in a Petri dish. 0.2 mg/ml solution of avidin-maleimide in PBS was added to the dish and it was incubated at 4^0C for 15 hrs. The tissue was then washed in PBS 3x5 min, and the final wash solution was replaced with a 0.03 mg/ml solution of biotin-HRP in PBS at room temperature. After 2 hours, the tissue was rinsed several times with PBS, reacted

with the luminal for 4 min and blotted. It was then placed in the imager for chemiluminescence detection (high sensitivity, 80 sec acquisition time, aperture fully open). Series of bright dots were observed on the tissue surface, indicating that the tissue was specifically reduced in the desired areas.

The experiment was repeated again except that the small molecule biotin-maleimide was used as the alkylating agent. The tissue was reduced as before and washed in PBS and was then placed in a Petri dish and incubated with a 12 μM solution of biotin-LC-maleimide in PBS (with 10% v/v DMSO to assist in solubilizing the reagent). The reaction was allowed to proceed at room temperature for 4hours. The tissue was then washed in PBS and reacted with 0.2 mg/ml solution of avidin-HRP conjugate in PBS conjugate for 2 hrs at room temperature. After this time, the tissue was rinsed several times with PBS, reacted with luminal for 4 min and blotted. The tissue sample was then placed in the imager, which was set for chemiluminescence detection (high sensitivity, 80sec acquisition time, aperture fully open). A series of bright dots were observed, indicating that the tissue was specifically reduced in the desired areas.

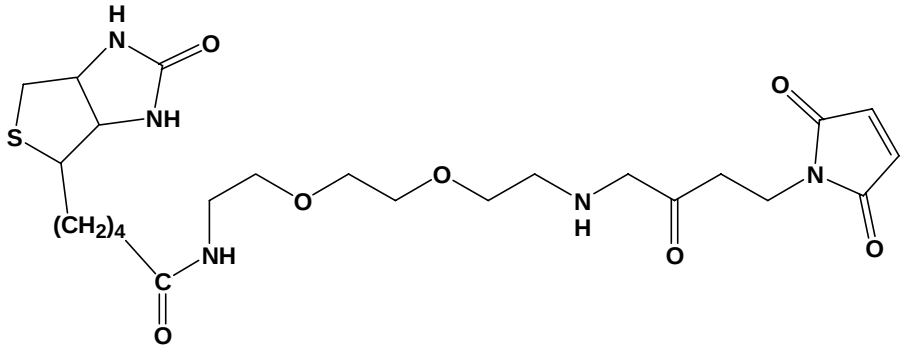

Figure 79. Structure of biotin-LC-maleimide, biotinyl-3-maleimidopropionamidyl-3, 6-dioxaoctanediamine

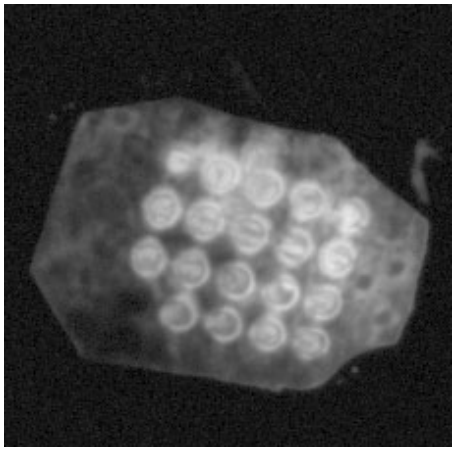

Figure 80. Chemiluminescent image of pericardium after site specific modification (avidin maleimide, biotin-HRP, luminal). The positive signal appears as series of bright dots.

Experiment 8: Site Specific Modification using a 96 well plate and Chemiluminescent Detection

A sample of fresh pericardium was cut to fit on the plastic 96 well plate using a scalpel blade. 150 μL of a 0.1 M solution of TCEP in PBS was added to wells to be reduced, the remaining wells served as controls and received 100 μL of PBS. The tissue sample was placed over the plate and a solid glass cover was clamped into place using spring clamps. The apparatus was turned upside down so that the reagents contact the tissue surface and the reaction was allowed to proceed for 45 min at room temperature. After this time, the apparatus was dissembled and the pericardium was rinsed in PBS and placed in a Petri dish. It was then placed in avidin-maleimide solution (0.2 mg/ml in PBS) and was incubated at 4^0C for 15 hrs. The tissue was then washed in PBS (3x2 min) and reacted with biotin-HRP in a Petri dish (0.03 mg/ml in PBS) for a further 2hrs. After this time, the tissue was rinsed several times with PBS, reacted with luminal for 4min and blotted to remove excess buffer. It was then placed in the imager for chemiluminescence

detection (ultrasensitivity, 100 sec acquisition time, aperture fully open). Bright dots were observed on the tissue surface, indicating that the tissue was specifically reduced and alkylated in the desired areas. Similar results are also obtain with a 384 and 1536 well plates (see experiments 12 and 13 and figure 78 and 79)

Figure 81. Bovine pericardium being mounted on a 384 well plate for site specific reduction of the tissue

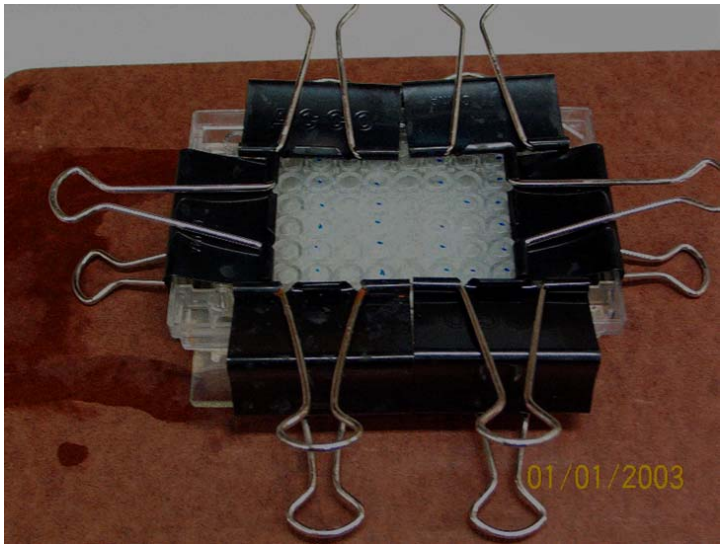

Figure 82. Bovine pericardium placed over a plate containing TCEP, covered with a glass plate, clamped with spring clamps, and the apparatus turned upside down.

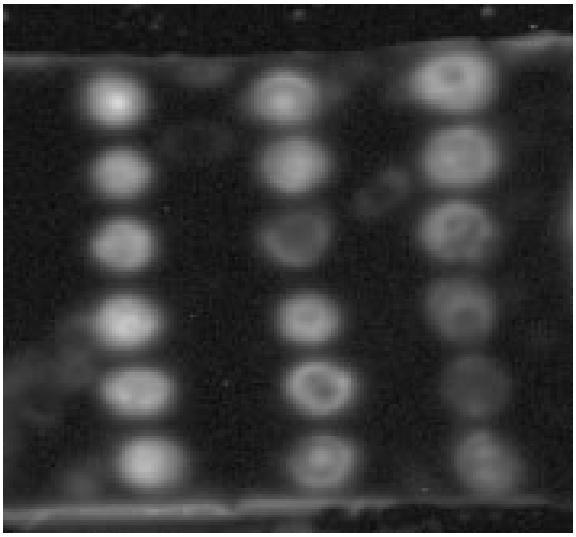

Figure 83. Chemiluminescent image from site specific reduction using a 96 well plate. The positive signal appears as series of bright dots, while the controls do not give off any signal.

The experiment was also repeated for the biotin-maleimide/avidin-HRP combination of reagents. This gave identical essentially results, as expected, to the previous experiment.

Experiment 9: Reduction of Bovine Pericardium with DTT and BME with Chemiluminescence Detection

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Two set of experiments were carried out to investigate the use of DTT and BME as reducing agents for the pericardium. The reactions were identical to that of the TCEP experiment (including tissue washes, alkykation with avidin-maleimide, further tissue washes, reaction with biotin-HRP, washes, incubation with luminal and imagining) with the exception that 100mM solutions of DTT or BME (in PBS) was used to reduce the disulfide bonds on the surface of the pericardium. The resultant images indicated that BME was a better reducing agent than the DTT. When reductive ability of BME was compared to that of TCEP, TCEP reduced slices emitted a stronger chemiluminescent signal when compared with BME reduced slices.

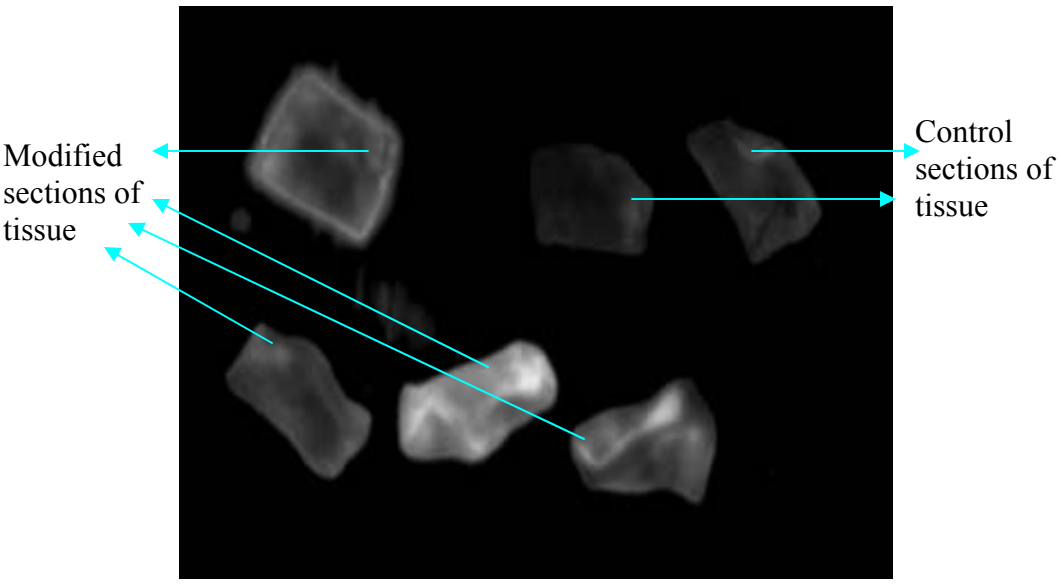


Figure 84. Chemiluminescent image with BME reduction. The modified sections of tissue appear brighter when compared to the control sections.

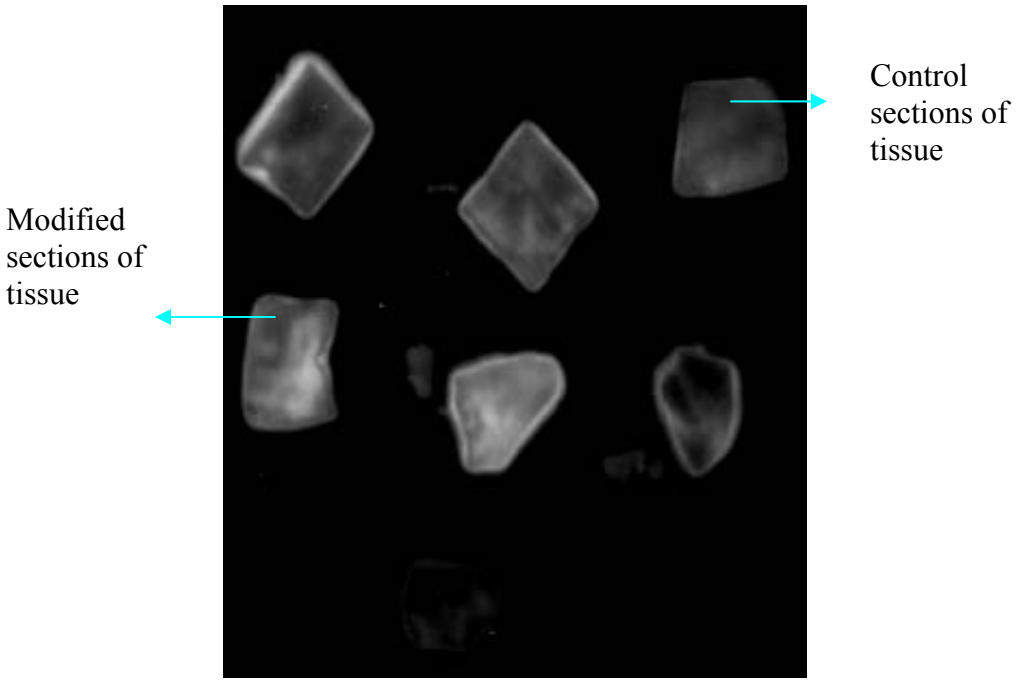


Figure 85. Chemiluminescent image with DTT reduction. The control sections (top row) appear identical to modified sections (bottom row) under fluorescence imaging.

Experiment 10: Quantification of the Amount of Protein Immobilized on the Pericardium with Chemiluminescent Detection

Several strips of pericardium were reduced with TCEP and alkylated with avidin-maleimide as in previous reduction procedures with pericardial tissue as described in experiment 1. At the same time, a slot blot was prepared with serial dilutions of avidin of (20, 10, 5, 2.5, 1.25, and 0.625 μg/mL) blotted on the nitrocellulose membrane, corresponding to 4, 2, 1, 0.5, 0.25, and 0.125 μg total protein applied to each slot (7 mm x 0.75 mm). The membrane was then placed into the Petri dish and processed for chemiluminescence with the tissue sample. The tissue and the nitrocellulose membrane were immersed in a PBS solution of biotin-horseradish peroxidase (0.03 mg/ml in PBS) for 2 hours at room temperature, then washed with PBS three times for ten minutes with a change of solution between each wash, and finally immersed in the chemiluminescence substrate (Supersignal® West Pico, luminal (Pierce)) at room temperature for 4.5 minutes. The tissue and membrane were then removed from the luminal solution, blotted with paper towels to remove excess solution, placed on a sheet of plastic film, and placed into the imager for detection of the chemiluminescence using ultrasensitive chemiluminescence settings with the aperture completely open for a 100 second acquisition. The positive signal from the modified regions of the tissue appeared as a series bright signal. From the slot blot, only the four highest concentrations of avidin were evident under these conditions. The strongest signal came from the application of 10 μg of avidin to the slot, which corresponds to about 2 μ/mm^2 if quantitative immobilization is assumed. Since the signal intensity on the tissue was approximately the same as the strongest signal from the slot blot (determined using freely available NIH

Image J 1.32j imaging software)[225], it was determined that the amount of protein immobilized on the tissue was approximately in the range of 1-4 μg/mm^2.

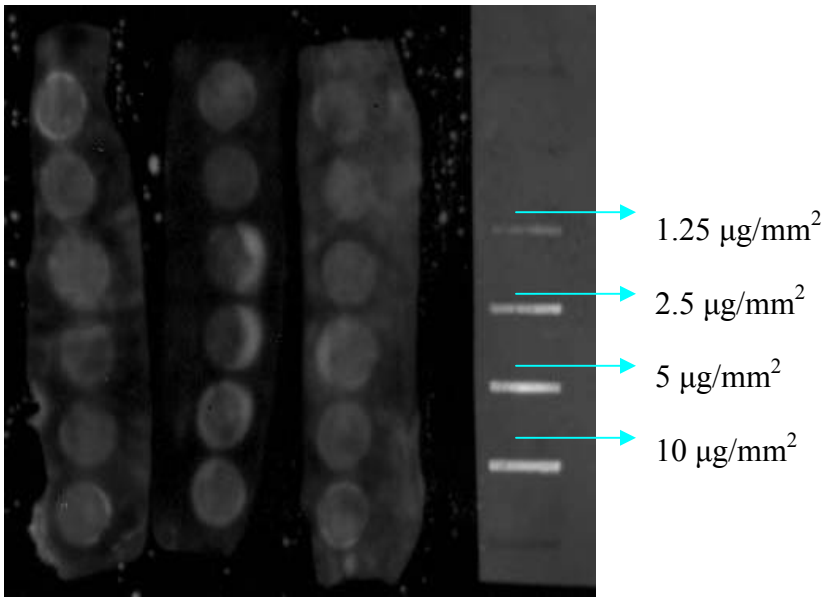


Figure 86. Reduced bovine pericardium strips with slot blot membrane for quantification of protein immobilized on tissue.

Experiment 11: Site specific Modification to produce a 'BU' Pattern on Pericardium Using a 96 well Plate and Chemiluminescent Detection.

A sample of fresh pericardium was cut to fit on the plastic 96 well plate using a scalpel blade. 150 μL of a 0.1M solution of TCEP in PBS was added in a 'BU' pattern to wells to be reduced; the remaining wells served as controls and received 150 μL of PBS. The tissue was reduced for 45min at room temperature. The remainder of the experiment (including the tissue washes, alkylation with avidin-maleimide, further tissue washes, incubation with biotin-HRP, further tissue washes, and incubation with luminal) was identical to experiment 10. The chemiluminescence was detected using ultrasensitive

chemiluminescence settings with the aperture completely open for a 100 second acquisition. The positive signal gave a bright 'BU' pattern against a dark background.

Experiment 12: Site Specific Modification to produce a BU Pattern on Pericardium Using a 384 well Plate and Chemiluminescent Detection

A sample of fresh pericardium was cut to fit on the plastic 384 well plate using a scalpel blade. 30 μL of a 0.1M solution of TCEP in PBS was added in a 'BU' pattern to wells to be reduced, the remaining wells served as controls and received 30 μL of PBS. The remainder of the experiment was identical to previous procedures (including reduction, washes, alkylation with avidin-maleimide, washes, incubation with biotin HRP, further tissue washes, incubation with luminal and imaging) as described in experiment 11.

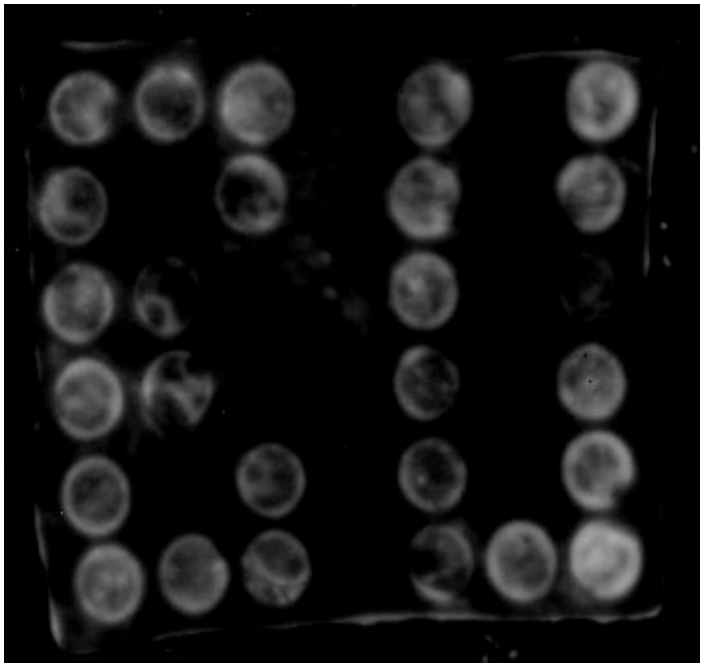


Figure 87. Chemiluminescent image of pericardium showing 'BU' after site specific protein immobilization using a 96 well plate. The tissue was modified in a way to have

the modified ‘pixels’ light up in a ‘BU’ pattern for Baylor University. The pixel resolution is ~4 mm in diameter.

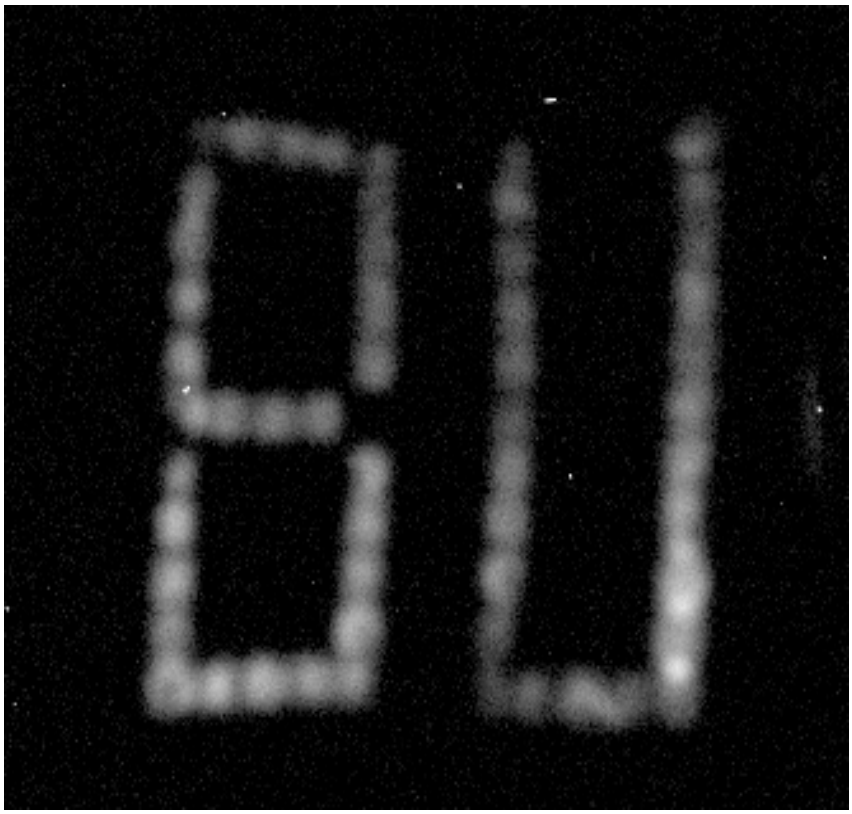


Figure 88. Chemiluminescent image of pericardium showing ‘BU’ after site specific protein immobilization using a 384 well plate. The ‘pixel’ resolution on the ‘BU’ is ~2 mm in diameter.

Experiment 13: Site Specific Modification to Produce a ‘BU’ pattern on Pericardium Using a 1536 well plate and Chemiluminescent Detection

A sample of fresh pericardium was cut to fit on the plastic 1536 well plate using a scalpel blade. 7 μL of a 0.1 M solution of TCEP in PBS was added to wells (in a BU pattern) to be reduced, the remaining wells served as controls and received 7 μL of PBS. The remainder of the experiment was identical to previous procedures including washes, alkylation with avidin-maleimide, washes, incubation with biotin-HRP, further tissue washes, incubation with luminal and imaging (see figures 86 and 87).

Figure 89. Bovine pericardium after being reduced with TCEP on 1536 well plate. A translucent BU pattern is only visible on the tissue surface in the areas where the reagent was contacted with the tissue.

Figure 90. Chemiluminescent image of pericardium displaying 'BU' after site specific protein immobilization using a 1536 well plate. The pixel resolution on the 'BU' is ~ 1 mm in diameter.

Experiment 14: Dose Response Investigation of TCEP on Pericardium and Chemiluminescent Detection

A sample of fresh bovine pericardium was cleared of fat and cut to fit on a well plate. Various concentrations of TCEP in PBS were added to columns 1, 3, 5, 7, 9, and 11 of the plate at 100 µL solution per well (0.1, 0.05, 0.025, 0.015, 0.01, and 0.005 M TCEP, respectively).

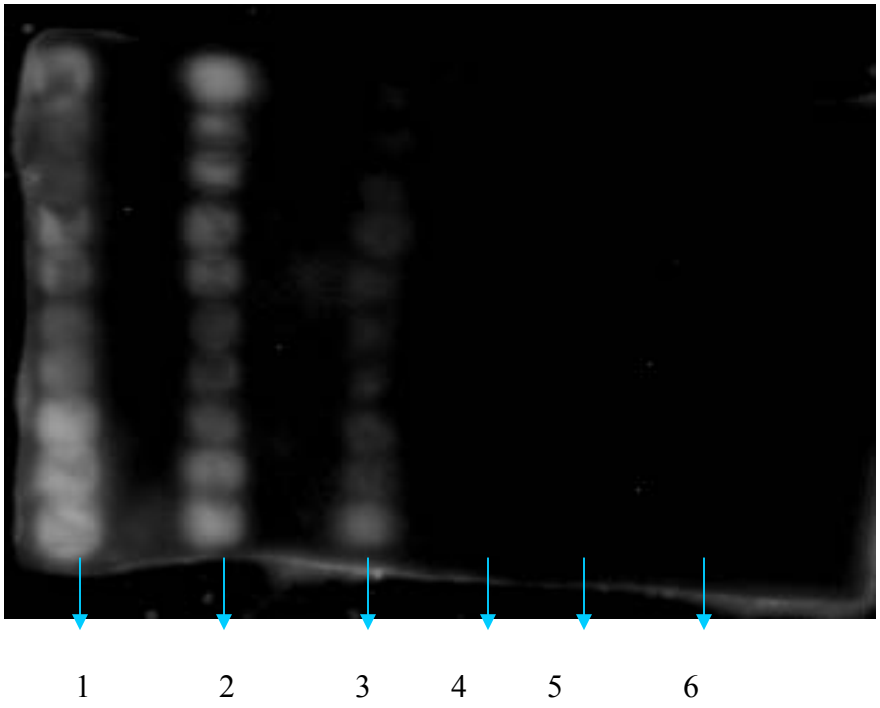


Figure 91. Chemiluminescent image from dose response experiment with TCEP. Concentrations of TCEP are as follows: Row 1= 100mM; Row 2=75mM; Row 3=50mM; Row 4=25mM; Row 5=12. 5mM and Row 6=control (PBS). The brightness of the dots is proportional to the concentration of TCEP. The greater the concentration of TCEP, the brighter the dots appear.

The wells in the control columns (2, 4, 6, and 8) contained PBS. The tissue sample was placed over the plate, and the remainder of the experiment proceeded as described in Example 3, including the tissue washes, alkylation with avidin-maleimide, further tissue washes, incubation with biotin-HRP, further tissue washes, and incubation

with luminol. The positive signal appeared as a series of bright dots with the brightest dots appearing in the columns having the greatest concentration of TCEP. The relationship of signal to the concentration of reducing agent is clearly shown in Figure 88. As the concentration of TCEP was increased, the chemiluminescent signal emitted also got stronger from the surface of the tissue.

Experiment 15: Time Exposure Effect of TCEP on Bovine Pericardium and Chemiluminescent Detection

Bovine pericardium was cleaned and cut to fit over the 200 μL VWR pipette tip holder. The tissue slice was then placed over the smooth side of the tip holder, covered with a solid glass plate and clamped into place using spring clamps. 100 μL of a 0.1M solution of TCEP in PBS was added to each well in column 1. This is repeated at 10 minute time intervals for columns 2, 3, 4, 5, and 6 for a total reaction time of 50min. The tissue was then removed from the apparatus, washed in PBS and placed in a new Petri dish. At the same time a slot blot was prepared with serial dilution of avidin as in experiment 10. The remainder of the experiment proceeded as in previous experiments (including the tissue washes, alkylation with avidin-maleimide, further tissue washes, incubation with biotin-HRP, further tissue washes, and incubation with luminal). As seen in Figure 89, the minimum reaction time for the reduction with TCEP to emit chemiluminescent signal detectable under these conditions was observed to be 20 min. There seems to be no change in the amount of protein immobilized between 40-50 min reaction time, based on the intensity of the signal of the slot blot strip and reacted rows on the pericardium.

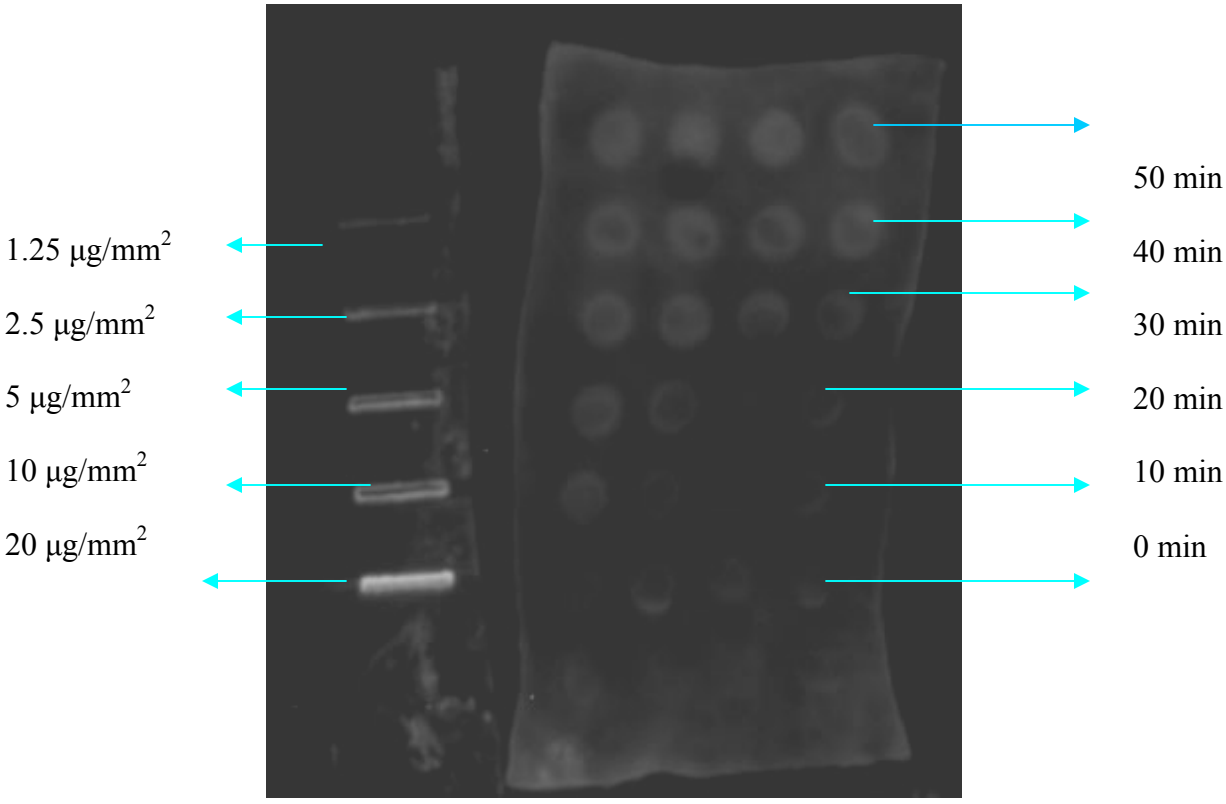


Figure 92. Chemiluminescent image from variable exposures of TCEP with pericardium and slot blot strip with serial dilutions of avidin for quantification. The brightness of the dots is proportional to the length of exposure of the tissue to TCEP. Greater the length of exposure to TCEP, the brighter the dots appear.

Experiment 16: Site Specific Immobilization of Avidin-maleimide with 96 well plate on a Entirely Reduced Section of Pericardium and Chemiluminescent Detection

A sample of fresh bovine pericardium was cleared of fat and cut to fit on a 96 well plate. It was placed in Petri dish and the entire tissue was reduced with 4ml of 0.1 M solution of TCEP in PBS for 45 min at room temperature. 100 µL of avidin-maleimide (0.02 mg/ml in PBS) was added to wells to be modified on a 96 well plate, while the remaining wells served as controls and received 100 µL of PBS. The reduced tissue was placed over the plate, covered with a glass slide and clamped into place using spring clamps. The apparatus was flipped over so that the reagents made contact with the

tissue surface and the reaction was run for 15 hrs at 4^0C. The tissue was then removed and washed several times in PBS and incubated with 4ml of the biotin-HRP solution (0.03 mg/ml in PBS) for 2 hrs at ambient temperature. The remainder of the experiment (including the tissue washes and incubation with luminal) proceeded as in previous experiments. The positive results were also similar to that of experiment 1.

This experiment was also repeated for the biotin-maleimide and avidin-HRP combination and results were essentially identical to the above experiment.

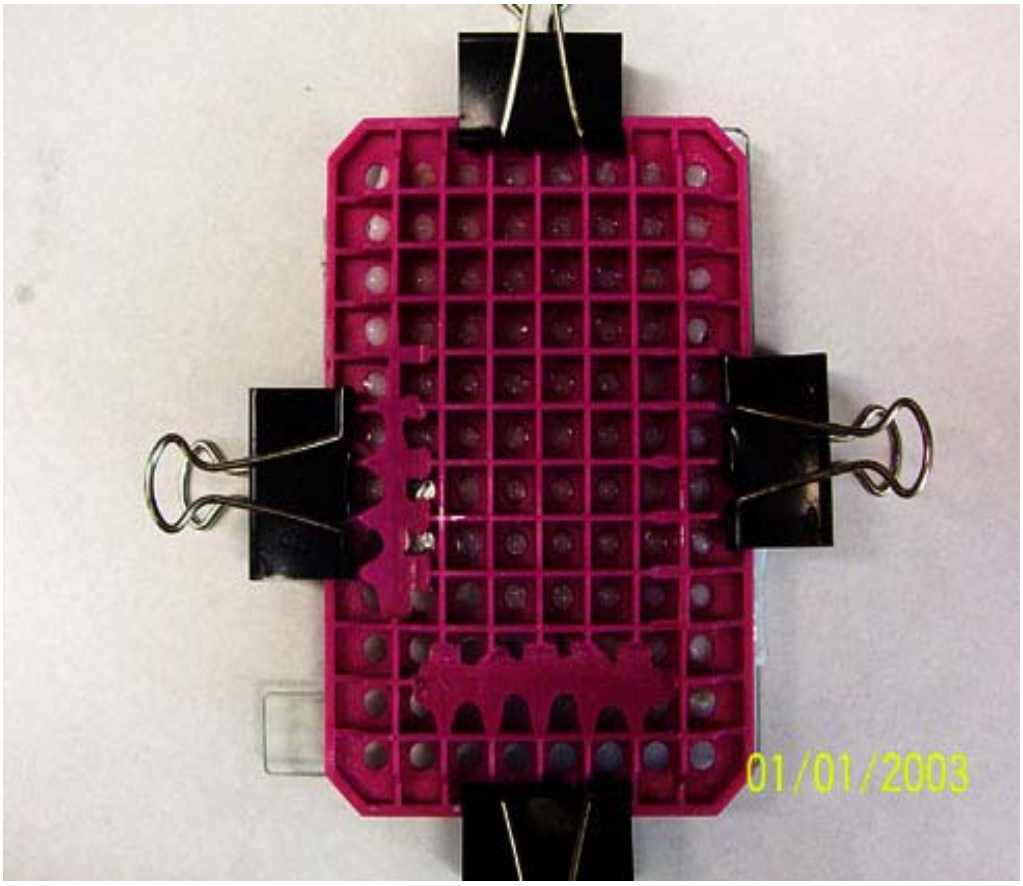


Figure 93. VWR tip holder as seen from above, with pericardium sandwiched in between the plastic plate and glass slide, and clamped with spring clamps. This set up was used to conduct all the blocking studies (see experiment 17 and 18). The reagents were added to open wells using a micro pipetter.

Experiment 17: Blocking Reduced sites with N-ethyl maleimide, Before Reaction with Avidin- maleimide and Chemiluminescent Detection

The pericardium was cleaned and cut to fit over the 200 µL VWR pipette tip holder (see Figure 90). The tissue slice was then placed over the pipette holder, covered with a solid glass plate and clamped into place using spring clamps. 100 µL of a 0.1 M solution of TCEP in PBS was added to wells in columns 1-5, and the remaining wells in column 6 served as controls and received 100 µL of PBS.

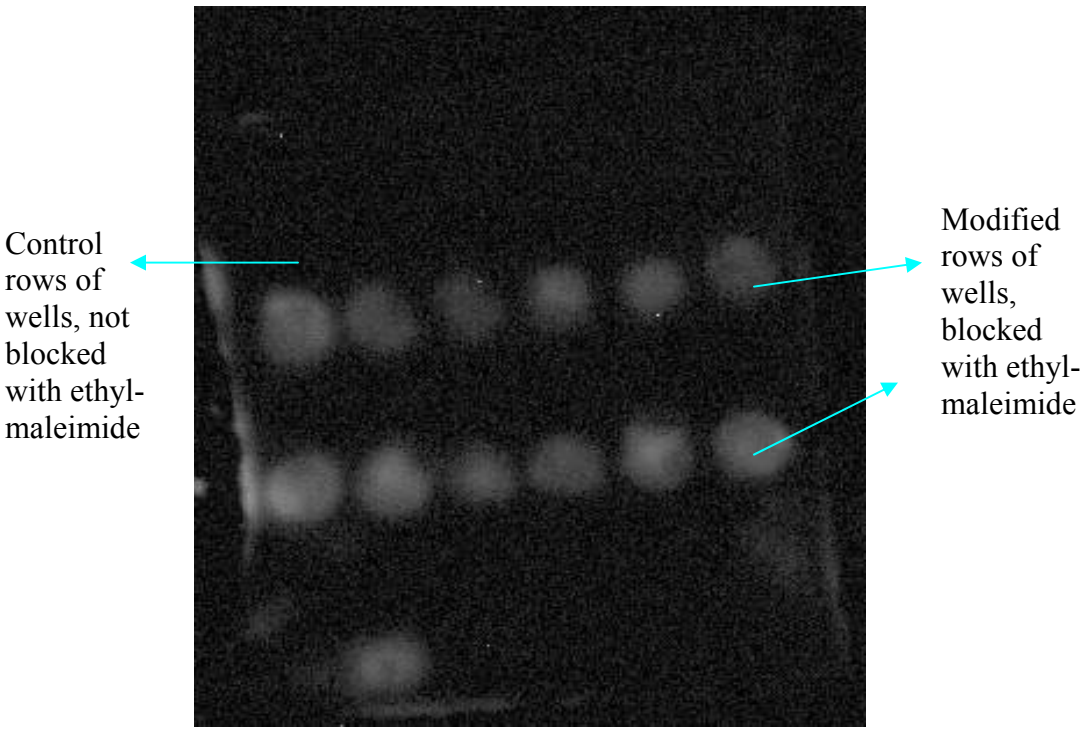


Figure 94. Chemiluminescent image from blocking experiment with N-ethyl maleimide. N-ethyl maleimide was used to block the wells reacted with TCEP. The wells blocked by the ethyl-maleimide are not visible on the image, while the unblocked control (which were reacted with avidin-maleimide) wells are seen as bright dots.

The tissue was then reduced for 45 mim at room temperature. After this time, the wells were emptied out by inverting the entire the apparatus, refilled with PBS and inverted again to wash out the wells of any excess reagent. 100 µL of N-ethyl maleimide

(100 mM in PBS with 10% DMSO v/v to solubilize the reagent) was added to columns 1, 3,5 and 6, while the remaining wells in column 7 received 100 μL of PBS (also with 10% DMSO). The reaction was then run for 2 hrs at room temperature with gentle agitation.

After this time, the wells were emptied and washed several times with PBS as before by inverting the apparatus. The entire apparatus was then dissembled and the tissue was washed several more times in PBS. The remainder of the experiment proceeded as in previous experiments (including the tissue washes, alkylation with avidin-maleimide, further tissue washes, incubation with biotin-HRP, further tissue washes, and incubation with luminal). As expected, the resultant imaged indicated that the wells which were reduced and then blocked with the ethyl maleimide before reacting with avidin maleimide did not emit any chemiluminescent signal.

Experiment 18: Reduction with TCEP, Biotinylated, Blocked with Avidin, Reacted with Avidin-HRP and Chemiluminescence Detection

The pericardium was cleaned and cut to fit over the 200 μL VWR pipette tip holder (see Figure 90). The tissue slice was then placed over the pipette holder, covered with a solid glass plate and clamped into place using spring clamps. 100 μL of a 0.1M solution of TCEP in PBS was added to columns 1, 3, 5 and 7, and the remaining wells served as controls and received 100 μL of PBS. The tissue was then reduced for 45mim at room temperature. After this time, the wells were emptied out by inverting the apparatus, refilled with PBS and inverted once again to wash out the wells of any excess reagent. 100 μL of biotin-maleimide (12 mM in PBS with 10% DMSO) was added to columns 1, 2, 3 and 5, while the remaining wells received 100 μL of PBS (also with 10% DMSO). The reaction was run for 2hrs at room temperature with gentle agitation. After

this time, the wells were emptied and washed several times with PBS as before by inverting the apparatus. The wells in columns 1 and 2 were then reacted with a 0.2 mg/ml solution of avidin in PBS to block the biotin immobilized in these columns. After two hours, the wells were emptied and washed several times with PBS as before by inverting the apparatus. The entire apparatus was then dissembled and the tissue was washed several more times in PBS. The remainder of the experiment proceeded as in previous experiments (including the tissue washes, incubation with avidin-HRP, further tissue washes, and incubation with luminal). As expected, the resultant image indicated that the wells which were reduced reacted with biotin-maleimide and then blocked with the avidin before reacting with avidin–HRP did not emit a chemiluminescent signal. This experiment was repeated for various conencentrations of HRP, but failed to improve the results.

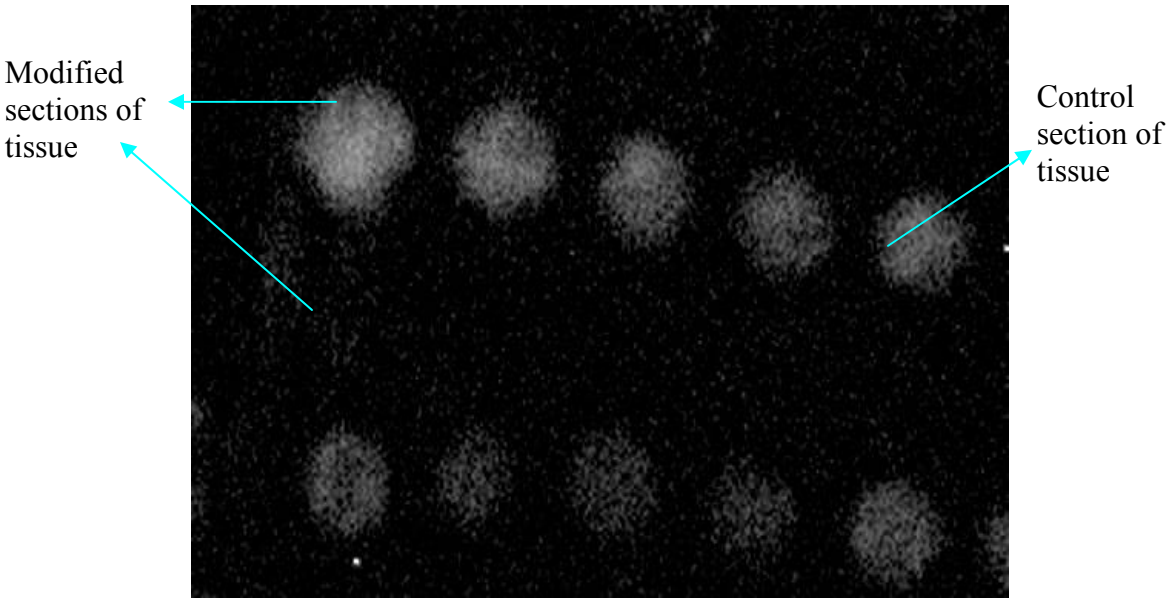


Figure 95. Chemiluminescent image from wells blocked and unblocked with avidin

Experiment 19: Site Specific Modification of Pericardium by Painting TCEP on the Surface of Pericardium with Chemiluminescent Detection

The pericardium was cut into a square, about 10cm/side, and placed on wet paper towels. 100 μL of a 0.1M of TCEP in PBS was added by hand to the pericardium using a micropipette in the pattern of an 'X'. This was repeated three more times for a total TCEP volume of 400 μL. The tissue was left undisturbed for 45min covered with a plastic lid to prevent drying. After this time, it was washed with PBS and the remainder of the experiment (including the tissue washes, reaction with avidin-maleimide, tissue washes, incubation with biotin-HRP, further tissue washes, and incubation with luminal) was similar to previous procedures. The chemiluminescence was detected using ultrasensitive chemiluminescence settings with the aperture completely open for a 100 second acquisition. An 'x' pattern was visible against a dark background.

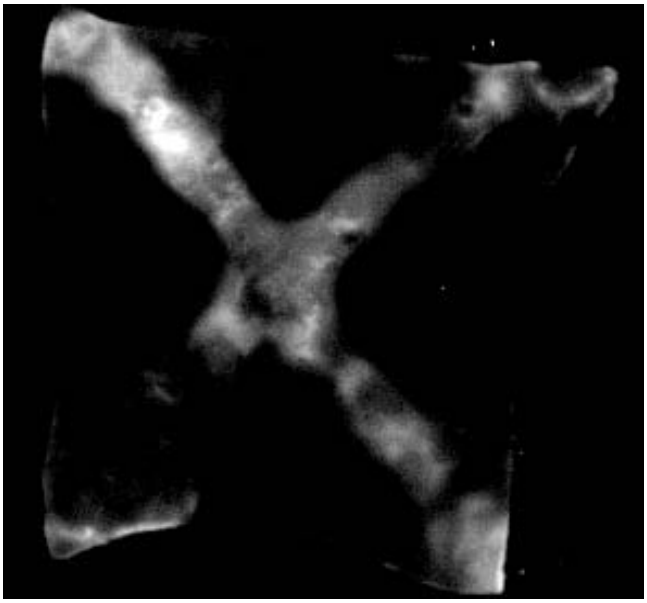


Figure 96. Chemiluminescent image from painted X. The area of tissue on which the TCEP was 'painted on' appears as the bright 'X' shape.

Experiment 20: Water-based Modification and Chemiluminescence Detection

The bovine pericardium was cut to fit a 384 well plate and soaked in DI water for 2 hours. 75 µl of a 0.1M solution of TCEP in DI water was added in 'BU' pattern to the wells to be reduced and the remaining wells received 75 µl of DI water. The reaction was carried out for 45min at room temperature with gentle agitation. After this time, the tissue was washed in DI water and incubated in a 0.02 mg/ml avidin-maleimide solution in DI water for 15 hrs at 4^0C. The tissue was then washed several times and incubated with a solution of 0.03 mg/ml biotin-maleimide solution in DI water for a further 2 hrs. After this time, the tissue was once again washed in water, reacted with the chemiluminescence substrate luminal for 4 min, blotted to remove excess reagent and imaged. The chemiluminescence was detected using ultrasensitive chemiluminescence settings with the aperture completely open for a 100 second acquisition. The resultant image had far less background chemiluminescence when compared to identical reactions carried out in PBS.

Figure 97. Chemiluminescent image of a "BU' from water base reaction

Experiment 21: Reductive Modification of Fetal pig Skin with Chemiluminescence Detection

A sample of fetal pig skin (second trimester) was cleared of fat and connective tissue and cut to fit on a well plate. 10 μL of a 0.1 M solution of TCEP in PBS was added to wells to be reduced, the tissue sample was placed over the plate, a solid glass cover was clamped into place using spring clamps, and the entire apparatus was flipped over so that the reagents contacted the tissue surface. After the tissue had been exposed for 50 minutes at room temperature, the apparatus was dissembled and the tissue was transferred to a small Petri dish and thoroughly washed with PBS (3x2 min with a change of solution between each wash). The PBS wash solution was then replaced by a solution of avidin-maleimide (0.25 mg/ml in PBS) and the tissue incubated overnight (approximately 15 hours) at 4ºC. After this time, the tissue was then once gain washed with PBS three times for ten minutes with a change of solution between each wash to remove any unreacted avidin. At the same time a slot blot was also prepared with serial dilution of avidin as in experiment 10. Both the modified tissue and slot blot membrane were placed in Petri dish, and the remainder of the experiment (including the tissue washes, incubation with biotin-HRP, further tissue washes, and incubation with luminal) was similar to previous procedure. The chemiluminescence was detected using ultrasensitive chemiluminescence settings with the aperture completely open for a 100 second acquisition.

Only the two highest concentrations of avidin on the slot blot were evident under these conditions. The strongest signal came from the application of 4μg of avidin to the slot, which corresponds to about 1 μg /mm^2 if quantitative immobilization is assumed. Since the signal intensity on the tissue was approximately the same as the strongest signal

from the slot blot (determined using freely available NIH Image J 1.32j imaging software), it was determined that the amount of protein immobilized on the tissue was at approximately 1 μg /mm^2.

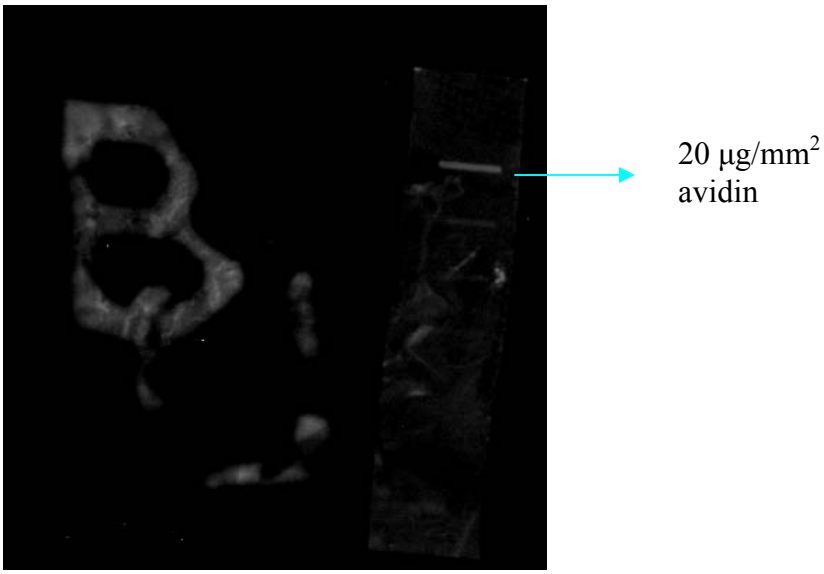


Figure 98. Chemiluminescent BU image on fetal pig skin with slot blot avidin strip for quantification of proteins

Experiment 22: Reductive Modification of Bovine Meniscus with Chemiluminescent Detection and Chromogenic Detection with HRP Substrate TMB

The bovine meniscus was cleaned of fat and connective tissue and longitudinal sections were made approximately 5cm in thickness. The slices were placed in compartments of a 12 well plate and 500 μL of a 0.1 M solution of TCEP in PBS was added to wells to be reduced, the remaining wells served as controls and received 500 μL of PBS. The slices were reduced for 45min at room temperature. They were then washed, placed in new wells and alkylated with 0.02 mg/ml solution of avidin-maleimide in PBS for 15 hours at 37^{0}C. After this time, the slices were washed several times in PBS and incubated with 0.03 mg/ml solution of a biotin-HRP in PBS for two hours. The

tissue slices were then washed, blotted, reacted with the luminal for 4min, blotted once again and placed into the imager for chemiluminescence detection. The chemiluminescence was detected using ultrasensitive chemiluminescence settings with the aperture completely open for a 100 second acquisition. The modified slices gave a much stronger signal when compared to controls, as seen in Figure 96**.**

After the tissue sections were imaged for chemiluminescence, they were washed thoroughly in PBS and placed in a solution of TMB, a chromogenic substrate for HRP. The slices were gently agitated in a TMB solution at room temperature and after about 1hr, a light blue precipitate was formed on the modified sections. The blue precipitate was visible to the naked eye.

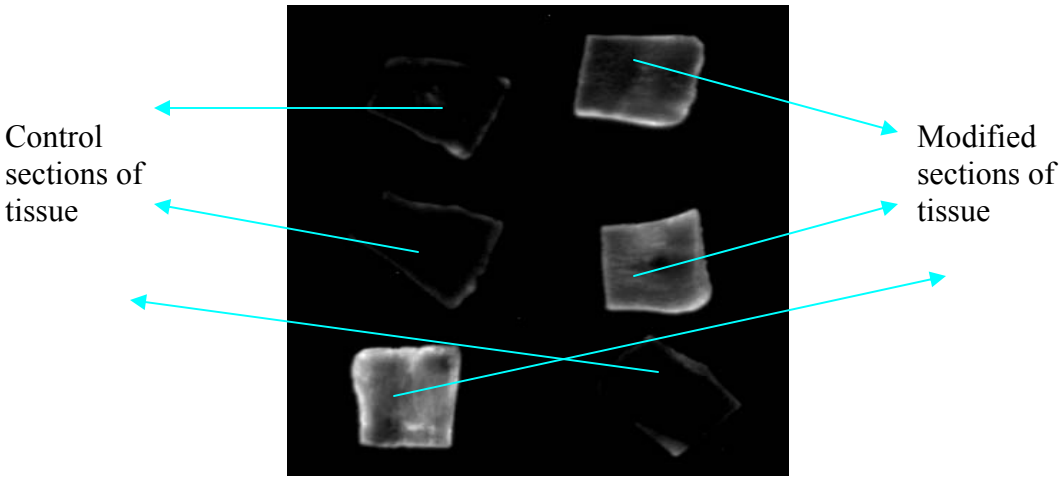


Figure 99. Chemiluminescence image of reduced meniscal sections

Experiment 23: Reductive Modification Cornea with Chemiluminescent Detection

The bovine cornea was removed from whole bovine eyes and thoroughly washed in PBS for 30 min. They were then horizontally sectioned with a scalpel blade to give slices approximately 1 cm in thickness and placed in compartments of a 12 well plate.

300 µL of a 0.1 M solution of TCEP in PBS was added to wells to be reduced, the remaining wells served as controls and received 300ul of PBS, and the reaction was carried out for 45 min at room temperature. The remainder of the experiment (including the tissue washes, alkylation with avidin-maleimide, washed, incubation with biotin-HRP, further tissue washes, and incubation with luminal) was similar to previous procedure. The resultant imaged indicated that the modified corneal section had a slightly stronger chemiluminescent signal when compared to the control.

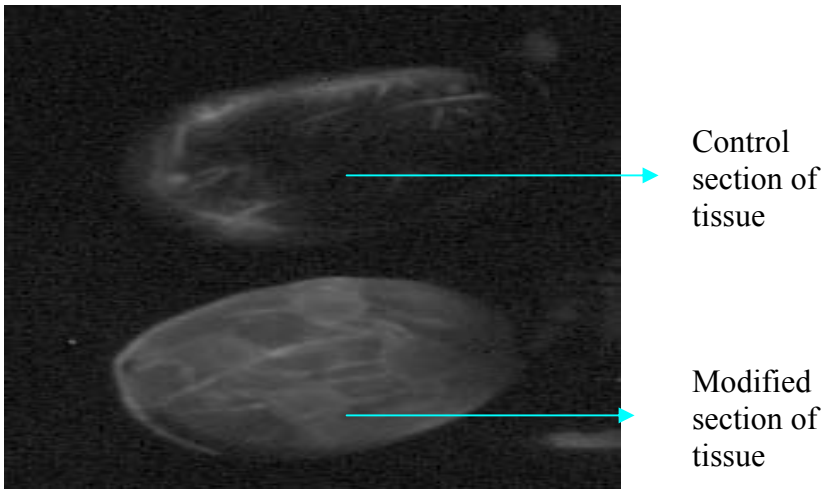


Figure 100. Chemiluminescence image of reduced corneal sections

Experiment 24: Reductive Modification of a Collagen Coated Plate with Chemiluminescent Detection

100 µL of a 0.1M solution of TCEP in PBS was added to each well in columns 1 and 3 of 96 well collagen coated plate, while well in columns 2 and 4 served as controls and received 100 µL of PBS. The wells were reduced for 45 min at room temperature. The wells were then emptied and rinsed several times with PBS. 100 µL of a 0.02 mg/ml solution of avidin-maleimide was added to each well and the reaction was allowed to

proceed at 4°C for 15 hrs. After this time, the plate was washed and the wells were filled with 100ul of 0.03 mg/ml biotin-HRP solution in PBS and incubated for 2 hrs at room temperature. The wells were once again rinsed in PBS, incubated with the chemiluminescence substrate for 4min, emptied out, and the entire plate was placed into the imager. The modified wells all gave a much stronger signal when compared to the controls.

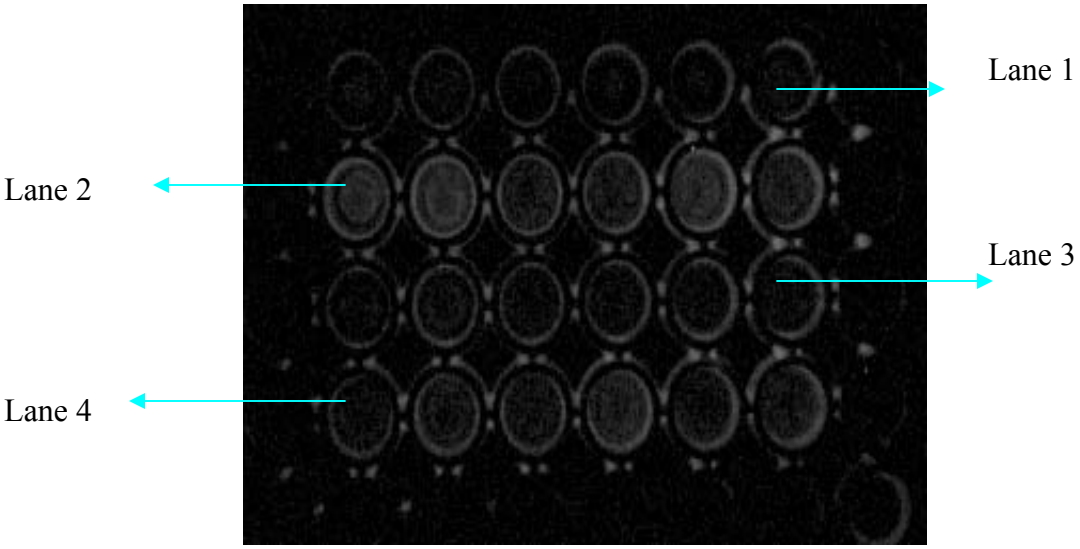


Figure 101. Chemiluminescence image of the collagen plate. The control wells in lanes 1 and 3 are relatively dimmer when compared to modified wells in lanes 2 and 4.

Experiment 25: Reductive Modification of Collagen Sheet with Chemiluminescent Detection

The collagen sheets (Bio-glide, Medtronic, Inc.) were cut into sections and hydrated in PBS for 30min. They were then placed into compartments of a 12 well plate and reduced with 100 µL of 0.1M TCEP solution of PBS per square, while the control squares received 100 µL of PBS. The squares were reduced for 45min at room temperature. They were then washed, placed in new wells and alkylated with 0.02 mg/ml

solution of avidin-maleimide in PBS for 15 hours at 4^0C. The remainder of the experiment (including the tissue washes, incubation with biotin-HRP, further tissue washes, and incubation with luminal) was similar to previous procedure. The modified sections gave a much stronger signal when compared to controls, as seen in Figure 99

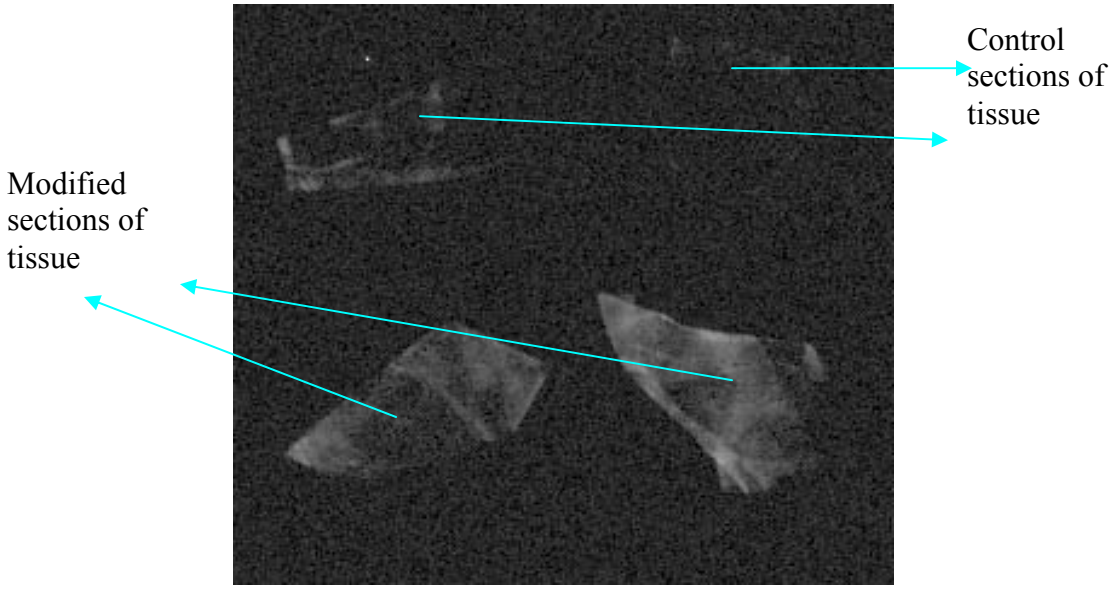


Figure 102. Chemiluminescence image of reduced collagen sheet sections.

Experiment 25: Enzymatic Digestion of Biotinylated Pericardium with Chemiluminescent Detection

Three different assays were performed to determine if the biotin immobilized on the reduced tissue was digested by the enzymatic activity in fresh serum or by the common protease enzymes, trypsin and pepsin. If the biotin was being digested, the chemiluminescent signal should decrease progressively with time since the quantity of biotin present on the tissue surface would decline. The chemiluminescence for each experiment was detected using ultrasensitive chemiluminescence settings with the aperture completely open for an 80 second acquisition.

Incubation with bovine serum. The pericardium was reduced with TCEP, cut into strips and alkylated with biotin-maleimide as in previous procedures. The strips were then incubated at 37^0C with fetal calf serum for time intervals of 2, 4, 6, 8, and 15hrs, while the control strips were incubated in PBS. After the desired reaction time, each strip, along with a control strip, was reacted with 0.03 mg/ml avidin-HRP, washed, incubated with luminal for 4min, blotted and imaged. We did not see any dramatic decrease in the chemiluminescent signal for any of the time intervals when compared with control strips. This indicated that the biotin was immobilized on the surface via the maleimide link was not prone to digestion by any enzymes in calf serum.

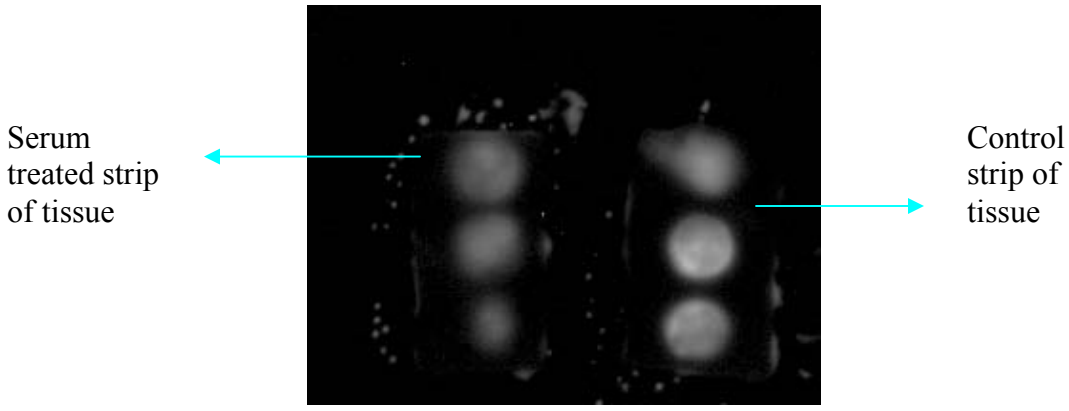


Figure 103. Chemiluminescent image of biotinylated bovine pericardial strips after 15 hrs of incubation in serum.

Incubation with trypsin. The pericardium was reduced with TCEP, cut into strips and alkylated with biotin-maleimide as in previous procedures. The strips were then incubated with 10mM concentration of trypsin in PBS for time intervals of 2, 4, 6, 8, and 16hrs, while the control strips were incubated in PBS. After the desired reaction time, each strip, along with a control strip, was reacted with 0.03 mg/ml avidin-HRP, washed,

incubated with luminal for 4 min, blotted and imaged. We did not see any dramatic decrease in chemiluminescent signal for any of the time intervals when compared with control strips. This indicated that the trypsin was also not able fully digest the biotin-maleimide bound onto the surface of the tissue.

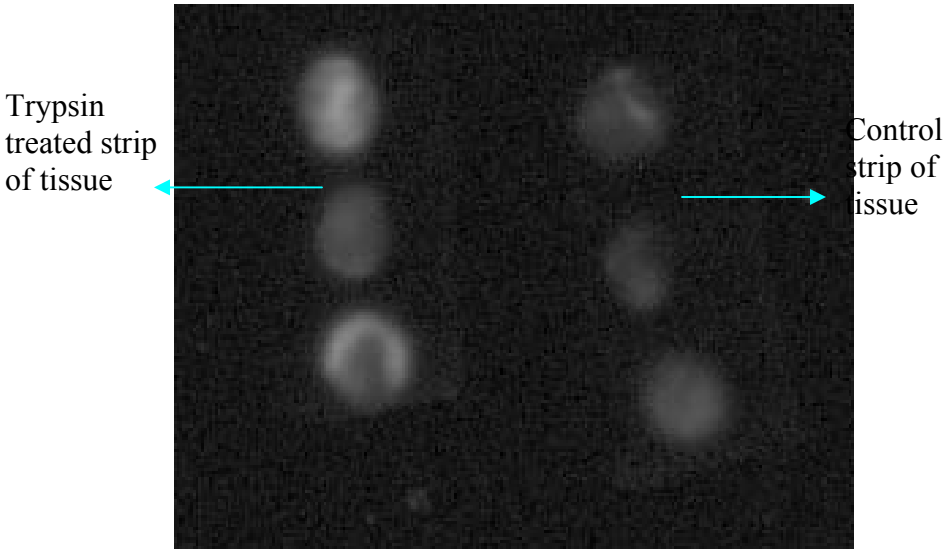


Figure 104. Chemiluminescent image of biotinylated bovine pericardial strips after 16hrs of incubation in trypsin.

Incubation with pepsin. The pericardium was reduced with TCEP, cut into strips and alkylated with biotin-maleimide. Several strips were alkylated with avidin-maleimide to serve as a control. The avidin control strip was used to ensure that the enzyme was active. Pepsin is a common protease and should digest the avidin, but be ineffective against biotin.

All the strips were placed in 100 mM solution of pepsin in PBS and incubated for time intervals of 4, 8, 12 and 16 hrs. At each time interval one biotin strip, along with a control avidin strip was removed and washed in PBS. The biotin strip was reacted with

biotin-HPR and the avidin control strip was reacted with biotin-HRP as in previous procedures (see experiment 1). After the two hours, both strips were washed in PBS, incubated with the luminal and imaged as before. The control strip appears bright, but no site specific signals are present on it, while the on biotin strip, the 'dots' are still visible, for up to 6hrs of incubation with the enzyme. However, over time (8 hrs and longer), the signal on the biotin strip does degrade and both control and reaction strips appear to be similar, see Figure 102. We hypothesized that this may not necessarily be due to biotin being digested but rather the underlying tissue being degraded by pepsin and hence the immobilized biotin is released from the tissue surface thereby reducing its availability.

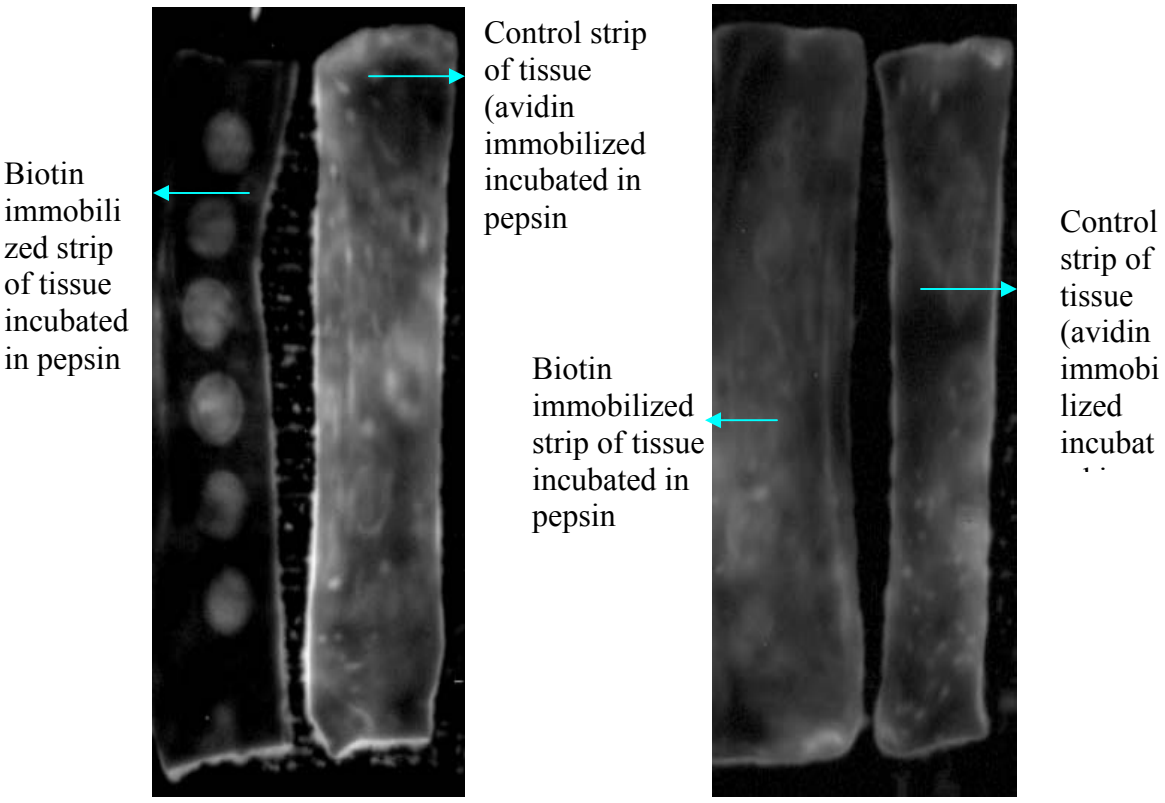


Figure 105. Chemiluminescent images after 6 hr incubation with pepsin (left) and image after 16 hr incubation with pepsin (right). Control strip is on the right for each image.

Experiment 26: Modification of neutravidin and avidin using a bifunctional crosslinker, immobilization on to reduced tissue surface and chemiluminescence detection.

10 mg of the neutravidin or avidin were dissolved in 500 μL of PBS buffer and 1 mg of LC-SMCC in 50 μL of DMSO was added to the protein. The reaction mixture was incubated for 1hr at room temperature. After this time, the avidin-SMCC and neutravidin-SMCC conjugate was purified using gel filtration chromatography (see experiment 4). The purified protein fractions were pooled to give a total volume of 0.5ml per protein sample, and the absorbance was measured at 280 nm using a UV spectrophotometer. The concentration of each protein was determined using the Beer-Lambert law, $A=\varepsilon bc$, where ε=is the molar absorbitivity with units of L mol^{-1} cm^{-1}, b= the path length of the sample – that is, the path length of the cuvette in which the sample is contained in cm, and c= concentration of the compound in solution, expressed in mol L^{-1}. The concentration of the avidin-maleimide was calculated to be 4 mg/ml, while the neutravidin-maleimide was calculated to be 5 mg/ml.

The proteins were then immobilized on the surface of pericardium using the reductive strategy by the procedures used in experiment 6 and 0.2 mg/ml concentration of each protein. For comparison, 0.2 mg/ml concentration of avidin-maleimide manufactured by Pierce was also immobilized on the tissue surface. All the tissues were then imaged together for chemiluminescence to measure the quantity of each type of protein immobilized on the tissue surface. The chemiluminescence for each experiment was detected using ultrasensitive chemiluminescence settings with the aperture completely open for an 80 second acquisition.

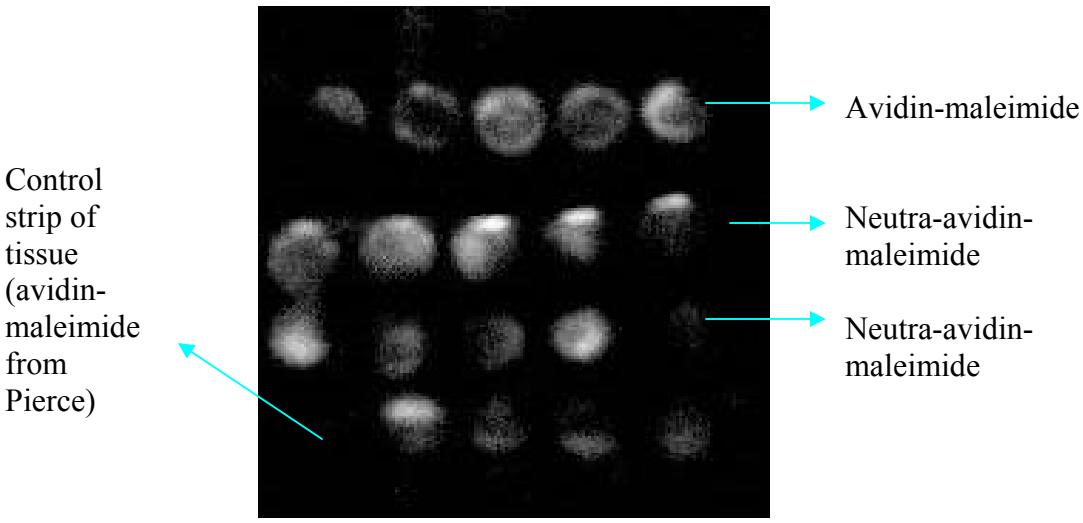


Figure 106. Chemiluminescent image from immobilized avidin-maleimide, neutral-avidin-maleimide and control (avidin-maleimide) from Pierce

Experiment 26: Reductive Modification of Bovine Aorta Reduction with Chemiluminescent Detection

The bovine aorta was cleaned of excess connective tissue and sectioned into squares, about 2 cm/side. They were placed in compartments of a 12 well plate, and reduced with a 0.1 M solution of TCEP. The control slices received only PBS and the reaction was carried out for 45min at room temperature. The remainder of the experiment (including the tissue washes, alkylation with avidin-maleimide, washes, incubation with biotin-HRP, further tissue washes, and incubation with luminal) was similar to previous procedures. The chemiluminescence for each experiment was detected using ultrasensitive chemiluminescence settings with the aperture completely open for time intervals of 100, 200 and 300 second acquisitions.

Unfortunately, we were unable to detect any signal that represents modification on any of the aorta sections.

Experiment 27: Reductive Modification of Skeletal Muscle with Chemiluminescent Detection

The skeletal muscle was cleaned of any connective tissue and longitudinal sections were made using a microtome blade. The chunks were placed in compartments of a 12 well plate and reduced in 0.1 M solution of TCEP in PBS, while the controls were left in PBS. After 45 min, the chunks were washed and placed in new wells and the remainder of the experiments (including the tissue washes, alkylation with avidin-maleimide, further tissue washes, incubation with biotin-HRP, further tissue washes, and incubation with luminal) was identical to experiment 1. The resultant image displayed no noticeable difference between the controls and modified tissue. The chemiluminescence for each experiment was detected using ultrasensitive chemiluminescence settings with the aperture completely open for a 300 second acquisition.

Section Five: Photo-Immobilization of Proteins on Fresh Tissue Surfaces

Background

The ease and convenience of using light as a switch to control the temporal and spatial features of a chemical process makes photochemical approaches attractive for the use in biomolecule (protein) immobilization. The most commonly used compounds for biomolecule photoimmobilization chemistry include aryl azide, diazirine and benzophenones. The active intermediates derived from these photosensitive moieties can react with the surrounding chemicals by non-specific insertion into chemical bonds.[226]

Aromatic azides are efficiently photolyzed by illumination with an ultraviolet light at 300-350 nm. The reactive molecule produced by this photolysis is a nitrene, which reacts rapidly and nonspecifically with either solvent molecules or with functional

groups on biomolecules. Almost any functional group or amino acid can be modified, since the nitrene is very reactive. Recent improvements in azide-based protein modification reagents have resulted in perfluorinated azides that generate nitrene intermediates with greater stability, thus giving reagents with higher efficiency of reaction (up to 40%) of reaction with the protein.[227] One of the primary uses of these highly reactive reagents is to carry out photoaffinity labeling experiments. In these experiments, the aromatic azide is attached to a drug or other molecule which binds specifically to a protein binding site (an example is an enzyme inhibitor or a nucleotide analogue) and then photolyzed. The location and type of bond formed in this process provides information about the environment near the binding site. In addition to their role as photoaffinity labels, aryl azides are useful as heterobifunctional cross-linkers. Succinimidyl azidobenzoate (SAB), 4-maleimido-benzophenone and p-azido-phenylacyl-bromide have been employed to couple proteins through dark reaction with amines or thiols followed by light activation.[228, 229]

Benzophenones are like azides in that they are photoactivatable by ultraviolet light, but once they have been activated, they can either react with functional groups or return to the ground state. Thus, these molecules can sometimes be reactivated if they do not react on the first activation. These reagents are also used as photoaffinity labels in a manner similar to that of the aromatic azides.[230]

The immobilization of biomolecules to hydrogels is an important tool to control both chemical and physical properties of the gel while at the same time providing bioactive materials for a variety of biotechnology applications including biomimetic materials for cell adhesion, affinity chromatography absorbents and solid phase

biocatalysts.[231] Photo-immobilization has also been used as a technique for immobilizing biomoleules on surfaces devoid of functional groups especially in the area of tissue engineering. It has also been used for protein microarray production on various surfaces.[232]

We hypothesized that the methods used to photo-immobilize biomolecules on hydrogels and various other solid surfaces using photoreactive molecules such as aryl azides could be adapted for use on tissue surfaces.

There are many examples where photo-immobilization has been used to immobilize a biomolecule on a polymer surface. Luo et al synthesized an agrose hydrogel material bearing immobilized 2-nitrobenzyl-protected biomolecules that are amenable to further peptide and protein immobilization. In another example, Cao et al were able to photo-immobolize ovalbumin (OVA) onto agarose gel. The OVA was modified with benzophenone, by reacting the primary amine groups of OVA with 4-benzoylbenzoic acid succinimide ester. The modified OVA were then dispersed into the agarose gel and photo-immobilized in situ by UV-irradiation.[233] In a similar example by the Ito group, insulin was modified with a photoreactive group and immobilized on polystyrene plates for cell culture assays. 4-azidobenzoic acid was reacted with N-hydroxysuccinimide to give N-(4-azidobenzoyloxy) succinimide. This was then reacted with insulin to give the azidophenyl derivatized insulin. The photo reactive insulin was added into the wells of a polystyrene plate and dried. The wells were then irradiated using a UV lamp which resulted in the insulin being photo-immobilized onto the wells of the plate. The immobilized insulin was shown to enhance cell growth when compared

with unbound insulin.[234] These experiments serve to prove that immobilization does not reduce the effectiveness of a biomolecule.

4-substituted 1, 8 naphthalamides

Our group has extensively studied the use of 4-substituted 1, 8 naphthalamides as protein crosslinkers and as photochemical tissue bonding agents. Photochemical tissue bonding (PTB) is a technique for sealing tissue with potential applications in many surgical specialties. In this method, the photochemically active dye is applied to the surface of the tissue to be rejoined, the tissues are intimately approximated and visible light applied to the interface. It has been proposed that the dye forms a reactive species, which react with potential electron donors and acceptors such as amino acid (e. g. tryptophan, tyrosine, and cystine) of proteins at both tissue surfaces. Strong covalent bonds are formed between the approximated surfaces, forming instantaneous protein crosslinks or nanosutures.[235] This mechanism contrasts with laser tissue welding, where the laser energy is used to increase temperature in the tissue and form non-covalent "welds" through protein denaturation.

Although the exact mechanism of PTB is not fully understood, formation of crosslinks in collagen type I molecules by photochemical methods have been reported. PTB has also been used to repair corneal lesions and skin graft adhesions. 1,8 naphthalamides have also been used successfully to crosslink type I collagen from bovine Achilles tendon.[236] Ex vivo and in vivo experiments have both show the ability of the naphthalamides to photochemically bond tissue. In one such study conducted with sheep, and artificial lesion was created in the knee meniscus. Several of the animals were treated by photochemical tissue bonding of the tear with the naphthalimides. These

animals returned to normal activity shortly after surgery, although the untreated animals showed a substantial limp.[237] Ex vivo experiments have also demonstrated the photochemical tissue bonding of human meniscal and articular cartilage.[238]

Research Strategies with Photochemical Methods

While working on the photochemical tissue bonding of meniscus, we were also inspired to use the naphthalimides to crosslink biomolecules to tissue surfaces. Before the onset of the covalent methods, we explored the ability of 1,8 naphthalimides analogs to photochemically crosslink various proteins to tissue surfaces. We especially liked the idea of photochemical crosslinking, since it would be a more convenient way, in terms of speed, for immobilizing any molecule on a tissue surface. For these experiments, we synthesized two naphthalamide analogs, JZ-11 and JZ-101(which were two of the best crosslinking dyes)[239] and a several biotinylated proteins. We then attempted to immobilize the biotinylated proteins on to tissue surfaces using the dye and an argon light source. The proteins were detected via a chromogenic assay much like the ones used in the direct alkylation methods (avidin-β-galactosidase and x-gal). We also immobilized several biotinylated amino acids (tyrosine, hystidine and tryptophan) using the naphthalamide dyes to investigate the role of these amino acids in photochemical crosslinking. Unfortunately, we were not able to detect any proteins or amino acid on the tissue surface with the chromogenic assays. We carried out similar experiments on collagen coated plates with several of the dyes and the biotinylated proteins and amino acids. We thought the use of collagen coated plates might provide a more uniform source of collagen as opposed to the meniscus, and in this case were able to barely detect the proteins, but not the amino acids (via chromogenic assays). Our subsequent attempts to

improve on these methods were to no avail and therefore decided to abandon the photochemical route.

After the success of chemiluminescence to detect proteins modifications on tissue surfaces, we decided to take another look at the photochemical approach to tissue bonding. We reasoned that the initial failure of the experiments might have been due to poor sensitivity of the chromogenic assays, and by using the more sensitive chemiluminescence assay it could be possible to detect the immobilized biomolecules. To test our reasoning, we used a photoactivatible biotin-azide and immobilized it onto the surfaces of pericardium and skin using a visible light source (halogen lamp). The immobilized biotin was then detected using an avidin-HRP and luminal based chemiluminescence assay. In these cases, there was clear evidence from the chemiluminescence images that the biotin was immobilized on the tissue surfaces. We also used an argon lamp as an alternative light source and also had similar results on pericardium.

Materials and Methods

Chemicals and Reagents

Except where otherwise noted, reagents used for the synthesis were obtained from Aldrich Chemical CO., Milwaukee, WI, and Fisher Scientific, Pittsburgh, PA (including Across reagents), Pierce and Molecular Probes, and used directly as purchased. Solvents such as dichloromethane, hexane, ethanol, ethyl acetate and methanol were obtained from commercial sources (via Baylor University Chemistry Department stockroom) and were

distilled prior to use. Deionized water (DI) was obtained via a US Filter system provided by Baylor Dept. of Chemistry.

Bovine pericardium was obtained from Animal Technologies INC in Tyler, Texas. The pericardium was cleaned of all excess fat using a microtome blade. The connective tissue was carefully removed by scraping with the blade and care was taken not to damage any of the actual pericardium. The cleaned pericardium was placed in a PBS solution at room temperature for up to 2hrs before use. Unused pericardium was stored at -20^{0}C and defrosted as needed.

The fetal calf skin was obtained from Animal Technologies and the rat skin was obtained from department of neuroscience at Baylor University. The fetal pig skin was washed in PBS and trimmed of any excess fat using a microtome blade. It was then allowed to soak in PBS until use. Unused skin was stored at -20^{0}C and defrosted and hydrated in PBS before use. The rat skin was obtained fresh, and the hair was removed by shaving or with the use of Nair. It was then soaked in PBS for up to 2 hrs before use or stored at -20^{0}C.

Instrumentation

Proton (1H) and carbon (13C) were obtained on a Bruker AVANCE 300 NMR running XWIN-NMR 3.1 software, at 300MHz for proton and 75MHz for carbon. Chemical shifts are expressed in ppm (∂), peaks are listed as singlets (s), doublets (d), triplet (t) or multiplet (m), with the coupling constant (J) expressed in Hz. HPLC was carried out with a Beckman System Gold, composed of a model 168 detector and a model 126 solvent module using a Alltech Alltima C-18 5M 33mm*7mm “rocket” column. For sample analysis, eluant A consisted for 0.1% TFA in water and elutnt B, 0.1% TFA

in 9:1 acetonitrile-water with a flow rate of 2.5 ml/mim. GC-Mass spec was obtained on a Hwelett Packard instrument. The fluorescence and chemiluminescence was detected using the Bio-Rad Laboratories Inc Fluor-S Multi-Imager and analyzed using Bio-Rad software. The chromogenic images were recorded using a Kodak 2 mega pixel digital camera. The lamp source for sample irradiation was an EFSO Ultracure 100ss spot curing lamp with 100 W Xenon Short-Arc Reflector bulb used at a power output of 500 mW and 2.45 W/cm^2

Experimental Methods for Photoactive Protein Immobilization and Crosslinking

Experiment 1: Synthesis of Naphthalamide Compounds

Synthesis of JZ-11. 4-amino-1,8 naphthalic anhydride (105 mg, 0.5 mmol) was added to 25 mL of N-ethyl-ethane-1,2-diamine (51 mg, 0.5 mmol) in 5 ml of ethanol. The resulting suspension was heated to 80^0 C for 1 hr. The reaction was then concentrated under vacuum and applied to preparative TLC (1:5 methanol:dichloromethane, Rf =0.31), and obtained 128 mg (0.43 mmol) as a yellow powder in 86% yield.

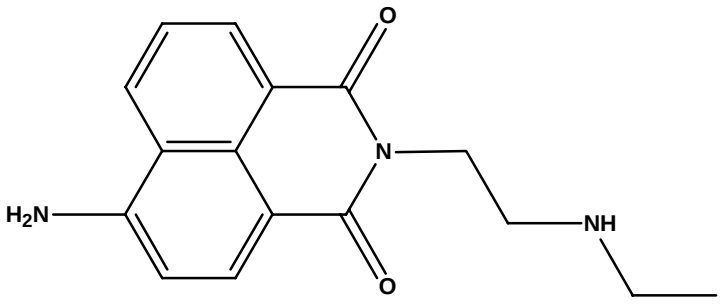


Figure 107. Structure of JZ-11 (**1**)

^{1}H ($CDCl_3$, 300MHz)

8.55 (1H, d, J = 8.3Hz), 8.45 (1H, m), 8.30 (1H, d, J = 8.3Hz), 8.27 (1H, d, J = 8.4), 6.83 (1H, d, J = 8.4Hz), 4.33 (2H, t, J = 7.2), 3.68 (2H, bs, NH), 3.45 (1H, bs, NH), 3.01(2H, t, J = 7.2), 2.47 (2H, q, J = 7.1), 1.12 (3H, t, J = 7.1)

^{13}C-NMR ($CDCl_3$, 75MHz)

165.4; 165.0; 150.28; 134.7; 131.34; 127.2; 124.8; 122.2; 120.5; 104.6; 59.8; 48.6; 45.7; 40.2; 20.2, 13.3

Synthesis of JZ-101. JZ-101 was synthesized in a similar manner to JZ 11. The amine used was 2,2'-(ethylenedioxy)bis(ethylamine). Preparative TLC was applied (1:3 methanol:dichloromethane, Rf=0.33) to give 115 mg, 67% yield.

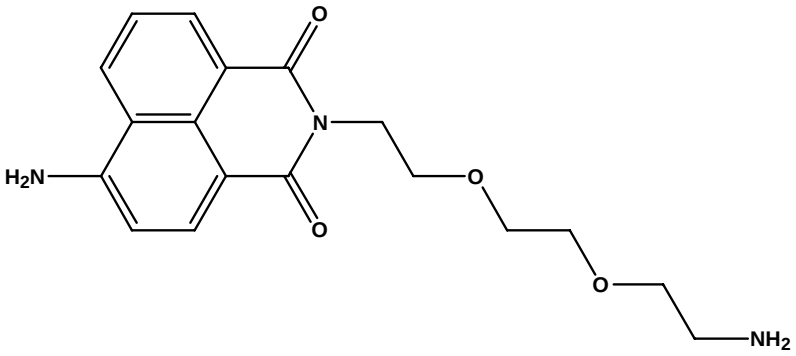


Figure 108. Structure of JZ-101 (**2**)

^{1}H ($CDCl_3$, 300MHz)

8.56 (1H, d, J = 8.4), 8.36 (1H, d, J = 8.4), 8.20 (1H, d, J = 7.3), 8.06 (1H, d, J = 8.4), 4.50 (2H, m, NH), 4.40 (2H, t, J = 6.2), 4.23 (2H, t, J = 6.2), 4.00 (2H, m, NH), 3.25 (2H, t, J = 5.2), 3.32 (2H, t, J = 5.2), 2.76 (2H, m), 2.64 (2H, m)

^{13}C-NMR ($CDCl_3$, 75MHz)

165.2, 164.4, 149.9, 134.2, 131.7, 130.1, 127.43, 125.2, 123.0, 120.7, 111.9, 109.6, 74.2, 71.2, 70.2, 68.7, 42.1, 39.3

Experiment 2: Biotinylation of Bovine Serum Albumin (BSA)

20 mg of BSA was dissolved in 1ml of 0.1M sodium bicarbonate buffer. 200 μL of a 20 mg/ml solution 6-((6-((biotinoyl)amino)hexanoyl)aimine)hexanoic acid(biotin-xx-se) in 1 ml DMSO was then added to the BSA. The mixture was incubated at room temperature for 2 hr with gentle shaking.

Purification of the biotinylated BSA was carried out by chromatography using Bio-Spin 6 columns. The exclusion limits for the Bio-Spin-6 was 6000 Kda. The columns were inverted several times to resuspend the gel and to remove any air bubbles. The tips were then snapped off the columns and then placed in 2 ml microcentrifuge tubes. The caps were removed and the columns were allowed to drain by gravity to the top of the gel bed. The collected buffer was discarded, the columns placed in the centrifuge tubes and the tubes were centrifuged for 2 min at 1,000 x g to remove excess buffer. The dry columns were placed back into clean microcentrifuge columns and the 75 μL of the biotinylated BSA was added to each column. The columns were centrifuged for 4min at 1,000 x g and the collected fractions were pooled to give a total sample volume of about 750 μL.

Degree of Biotinylation

To determine the degree of biotinylation of the BSA, we used the FluoReporter Biotin-xx Protein Labeling Kit. 1 ml of the HABA (4'-hydroxyazobenzene-2-carboxylic

acid) was added to a series of microcentrifuge tubes. 100 μL of the biotinylated-BSA was added to one tube and vortexed. For a positive control, 50 μL of 2mg/ml biotinylated IgG standard with a know degree of biotinylated and 50 μL of PBS was added to the second tube. For a negative control, 100 μL of PBS was added to the third tube. The samples were vortexed and incubated at room temperature. After 10 min, the samples were then centrifuged for a further 5 min to remove any precipitate. A PBS blank was run on the spectrophotometer at the absorbance recorded at 500nm. The absorbance of the sample containing the 100 μL PBS and the 1ml HABA complex was also recorded at 500nm, followed by he absorbance of the positive control at 500nm. Finally, the absorbance of the sample comprising the biotinylated BSA and the avidin-HABA complex was read and recorded at 500 nm.

The degree of biotinylation (moles of biotin per mole of protein) was calculated using the equation-

$(\Delta A_{500} \times BSF \times MW \times 10^3) / (mg/ml\ protein \times 0.1mL)$

Where ΔA_{500} is the absorbance of the biotinylated sample

BSF is the biotin sensitivity factor is the absorbance of the standard biotinylated sample (value printed ob bottle)

The equation was first used to compare the positive control value calculated to the value listed on the bottle, which were identical, thus the assay was shown to be functioning properly. The degree of biotinylation for the BSA sample calculated using the above equation was 3 moles of biotin per mole of BSA.

Colorimetric Dot Blot Assay

A colorimetric dot blot assay was conducted to ensure biotinylation of the BSA The nitrocellulose membrane was cut into 2 cm x 2 cm squares and placed in PBS buffer for 5 mim. The wet membrane was transferred onto a piece of blotting paper and 3 μL of the biotinylated-BSA was added to the center of the square. The protein was allowed to dry on the membrane (about 5 min). The protein bound membrane was then placed in a blocking buffer (PBS with 5%BSA) for 45 min. After this time, the membrane was washed in PBS (2x5 min, with a change of buffer between washes) in a glass dish. The washed membrane was transferred to a dry plastic dish and incubated with avidin-β-galactosidase (0.5 mg/ml) for 2 hrs. The nitrocellulose membrane was then washed 3 times in PBS with 5 min per wash. The nitrocellulose membrane was then placed in a dry plastic dish and incubated with the x-gal (1 mg/ml in PBS) solution. After about 1hr, blue precipitate formed at the center the each square where the biotinylated BSA was added.

Experiment 3: Biotinylation of Rnase and Lysozyme

The Rnase and lysozyme were each dissolved in bicarbonate at a concentration of 15 mg/ml. They were then reacted with 200 μL of a 20 mg/ml solution of biotin-NHS in DMSO for 2 hrs. The proteins were purified using gel exclusion chromatography and their degree of biotinylation calculated using the Flout kit.

The biotinlylated Rnase and lysozyme were also dot blotted on nitrocellusose membrane and a western blot was used to test for biotinylation. The degree of biotinylation was 3 moles of biotin/1mole of lysozyme and 4moles of biotin/mole of Rnase.

Experiment 4: Protein Immobilization of Biotinylated Proteins on Bovine Meniscus and Chromogenic Detection

The bovine meniscus was cleaned, washed and sectioned into squares about 2 cm/side. The sections were placed in bicarbonate buffer (pH 8. 5) for two hours prior to use. Each of the section was then placed on a glass slide and the slide was secured to the clamp stand. Equal molar amounts of JZ-11 (24 mmol) and biotinylated BSA were mixed (40 μL total volume) and added to the surface of the tissue using a micropipette. The argon lamp light guide was positioned 20 mm from the surface of the tissue and the whole experimental set up was covered in aluminum foil to prevent leakage of light. The tissue then was irradiated for 5 min with the light source.

Figure 109. Chromogenic image from photoimmobilization with biotinylated proteins. Both controls (top 2 section) and modified sections (bottom 3 section) look identical.

For a control, we used equal volume of dye and PBS on the tissue with 5 min of irradiation. All the slices were washed in PBS (3x5 min) with a change of solution between washes and placed in individual wells on a 12 well plate. 1ml of avidin-β-galactosidase (0.5 mg/ml in PBS) was added to each well and the reaction was run for 2

hr at room temperature. After this time, the tissue slices were washed in PBS (3x5 min) with a change of buffer between washes and then incubated at room temperature with 1ml solution of x-gal (1 mg/ml in PBS) in each well. Unfortunately, we were unable to see any blue precipitate on any tissue surface. The experiment was repeated with biotinylated Rnase and biotinylated lysozyme. These proteins also gave negative results.

Experiment 5: Protein crosslinking Using Collagen plates and Chromogenic Detection.

100 μL of the biotinylated protein (1 mg/ml in PBS) and 100 μL of the JZ-11 dye (24 mmol) were added to rows 1, 3 and 5 of the collagen coated 96 well plate. For a control, 100 μL of biotinylated protein (1 mg/ml in PBS) mixed with 100μL of PBS was added to rows 2, 4 and 6 of the same plate. The plate was placed on stand and positioned 5mm from the light guide attached to the argon light source. The wells were each radiated with the light for 5 min. After all the wells were treated, they were then emptied out and washed thoroughly in PBS (3x5 min). 200 μL of the blocking buffer (PBS with 5%BSA) was placed in each of the wells and the plate gently agitated at room temperature for 1hr. The wells were washed in PBS (3x5 min) and 200 μL of the avidin-β-galactosidase (0.1mg/ml in PBS) was added to each well and incubated at room temperature. After three hours, the wells were once again washed in PBS (2x5min) with change of buffer between washes. Finally, 200 μL of the x-gal (1 mg/ml) solution was added to each well and the plate was incubated at room temperature for about 2 hrs. The wells that were reacted with protein and light all gave a more intense blue color when compared to the controls. The experiment was then repeated with a stronger blocking buffer (PBS with 10% BSA) to reduce the non-specific binding but there was no change in the result. We also repeated the experiment for 7 min and 10 min of irradiation with

the light, but even with the longer light exposure, there was no change in the results. The experiment was also carried out with different biotinylated proteins including Rnase, and lysozyme with 10 min exposure to the light. These proteins also gave similar results.

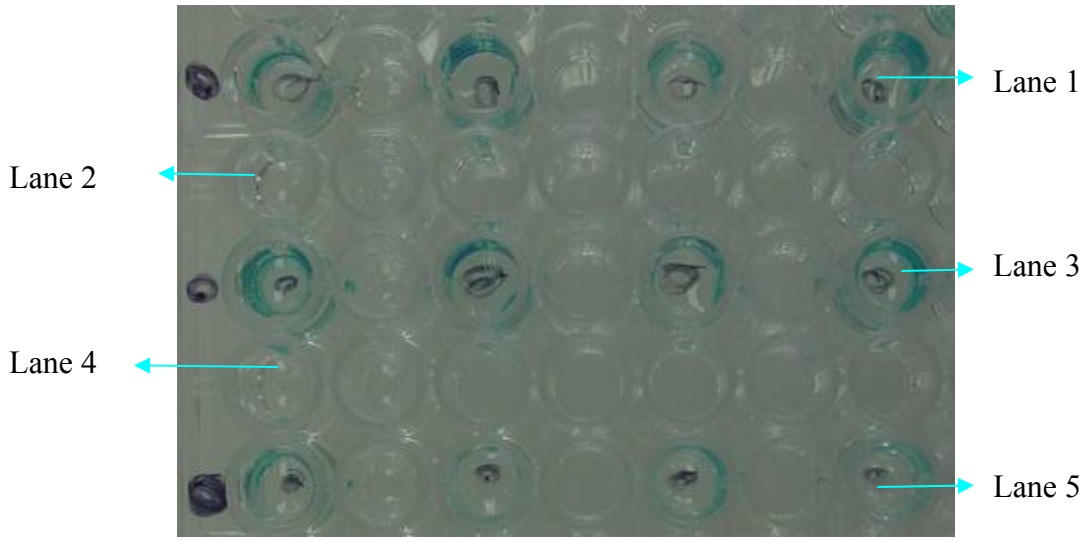


Figure 110. 96 well collagen plate after reacting with biotinylated BSA. The blue color has developed in the modified wells (lanes 1,3 and 5), while the controls (lanes 2 and 4) remain fairly clear.

Experiment 6: Crosslinking Using Collagen coated Plates and Biotinylated Amino Acids and Chromogenic Detection

The collagen plates were prepared for use as in the previous experiment. The biotinylated amino acids used were tryptophan, histidine and tyrosine, each at a concentration of 20 mg/ml in PBS. For controls, we used equal amounts of biotinylated protein and PBS without any dye. Rows 1, 3 and 5 of the plate contained the each of the different biotinylated proteins, while wells of rows 2, 4 and 6 contained the controls. The wells were positioned and irradiated individually with the light for 5 min as before. The wells were then washed in PBS and the colorimetric assay was carried out as described

for the pervious experiment with the biotinylated proteins. Unfortunately, we did not see blue precipitate in any of wells.

Biotinylation of Tryptophan

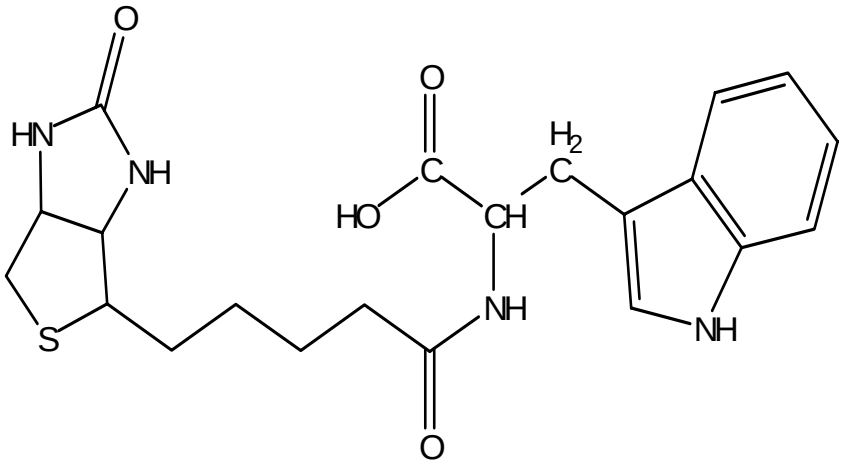


Figure 111. Structure of biotinylated-tryptophan (**3**)

20 mg of tryptophan was dissolved in 1ml of bicarbonate buffer and to it was added 200 μL of biotin-xx-se solution (20 mg/ml in DMSO). The reaction was then carried out for 2 hrs at room temperature. The biotinylated tryptophan was purified using HLPC using a C-18 column HPLC fractions containing product were pooled and concentrated under a strong vacuum. The pure biotinylated tryptophan was dissolved in bicarbonate buffer to give a stock solution of 20 mg/ml. The analytical method consisted of starting at 5% B and then increasing it to 90% B over 7 min. Then it was taken to 100% B over another 1min, and then back to 5% B in another 1min). The preparative HLPC method for purifying the product consisted of 5-60% B over 22 min, hold for 1 min, 100% B for 2 more min, and back down to 5% B over 2 min.

^{1}H (D_2O, 300MHz)

δ 7.18 (2H, m), 6.80 (1H, m), 5.61 (1H, m), 4.60 (1H, m), 3.51 (2H, m), 3.36 (1H, t, J = 7.2), 3.16 (1H, m), 2.90 (2H, d, J=7.2), 2.18 (2H, m), 1.62 (2H, m), 1.41 (2H, m), 1.30 (2H, m), 1.29 (2H, m)

^{13}C-NMR (D_2O, 75MHz)

δ 177.0, 173.5, 163.2, 136.5, 122.8, 119.5, 114.6, 113.1, 112.2, 110.5, 110.0, 60.7, 53.2, 48.4, 38.4, 36.4, 34.3, 30.4, 30.2, 26.0 25.6

Biotinylation of Tyrosine

20 mg of tyrosine was dissolved in 1 ml PBS and reacted with 200 μL of biotin-xx-se (20 mg/ml in DMSO). The reaction was run for 2 hr at room temperature with gentle shaking. After this time, the reaction mixture was concentrated under vacuum and purified using HPLC. Chromatography was performed using a C-18 column. The analytical method consisted of starting at 5% B and then increasing it to 90% B over 7 min. Then it was taken to 100% B over another 1 min, and then back to 5% B in another 1 min). The preparative HLPC method for purifying the product consisted of 5-55% B over 24 min, hold for 1 min, 100% B for 2 more min, and back down to 5% B over 2 min (see figure 109).

^{1}H (D_20, 300MHz)

6.96 (2H, d, J=8.5). δ 1.29 (2H, m), 6.68 (2H, d, J=8.5), 4.85 (1H, t, J=7.4), 4.75 (1H, m), 4.60 (1H, m), 3.36 (1H, m), 3.04 (2H, d, J = 7.4), 2.97 (2H, d, J = 7.1), 2.18 (2H, t, J= 7.1), 1.57 (2H, m), 1.62 (2H, m)

^{13}C-NMR (D_2O, 75MHz)

δ 179.0, 174.5, 163.5, 154.5, 136.6, 132.8, 130.2, 129.3, 58.5, 48.9, 48.4, 37.1, 36.4, 34.3, 30.2, 26.0, 25.6

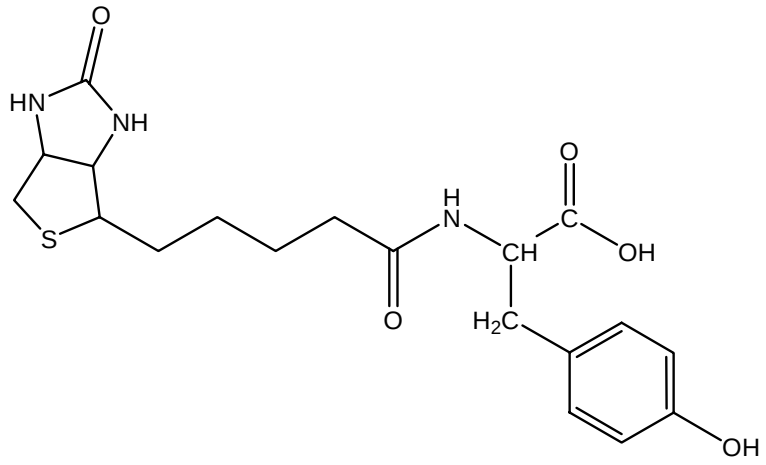


Figure 112. Structure of biotinylated tyrosine (**4**)

Biotinylation of Histidine

10 mg of histidine-(MTT)-OH was dissolved in methanol with 2% TEA (to assist in slolubilizing the reagent). 500 μL of the biotin-xx-se (20 mg/ml in DMSO) was added to the histidine-mtt and the reaction was run at room temperature for 6 hrs. The reaction mixture was concentrated under vacuum and NMR was taken to confirm the presence of the product. The product was purified using HPLC to give 8 mg of the pure His-MTT-biotin (see figure 110).

Chromatography was performed using a C-18 column. The analytical method consisted of starting at 5 % B and then increasing it to 90 % B over 7 min. Then it was taken to 100 % B over another 1min, and then back to 5 % B in another 1 min. The preparative HLPC method for purifying the product consisted of 5-60 % B over 22 min, hold for 1 min, 100 % B for 2 more min, and back down to 5 % B over 2 min

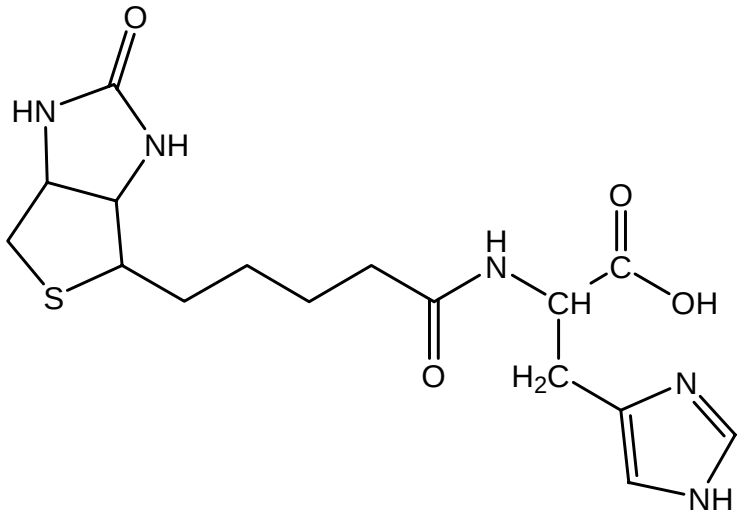


Figure 113. Structure of biotinylated histidine (**5**)

The His-MTT-biotin was then deprotected to remove the MTT group by dissolving the powder in 5 ml of TFA for 1 hr at room temperature. The final His-biotin was then purified with HPLC to obtain 5 mg of the final product. Chromatography was performed using a C-18 column. The analytical method consisted of starting at 5 %B and then increasing it to 90 %B over 7 min. Then it was taken to 100 %B over another 1min, and then back to 5 %B in another 1 min. The preparative HLPC method for purifying the product consisted of 5-60 % B over 22 min, hold for 1 min, 100 % B for 2 more min, and back down to 5 % B over 2 min.

^{1}H (D_2O, 300MHz)

δ 8.73 (1H, s), 1.29 (2H, m), 7.66 (1H, s), 4.85 (1H, t, J=7.5), 4.75 (1H, m) 4.60 (1H, m), 3.36 (1H, m), 3.04 (2H, d, J = 7.5), 2.97 (2H, d, J = 7.1), 2.18 (2H, t, J= 7.1), 1.62 (2H, m), 1.57 (2H, m)

^{13}C-NMR (D_2O, 75MHz)

δ 178.2. 174.9, 163.5, 135.9, 134.8, 123.0, 59.2, 53.3, 48.5, 38.4, 36.9, 34.5, 30.9, 30.2, 25.2, 24.0

Experiment 7: Photo modification of bovine pericardium using a visible light source and chemiluminescence detection.

The pericardium was cleaned of fat, cut of fit a pipette tip holder plate and incubated in PBS for 2 hrs prior to use.

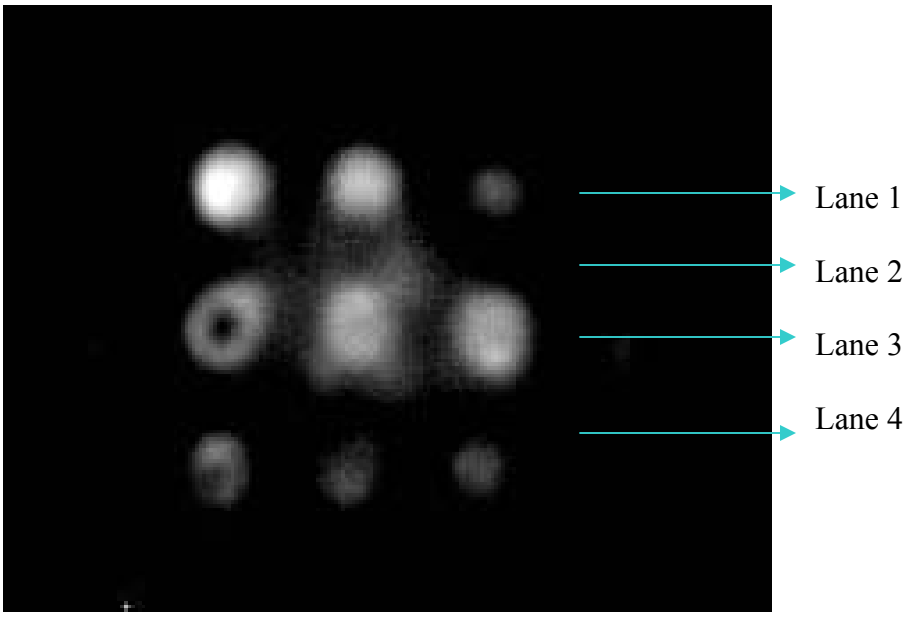


Figure 114. Chemiluminescent image from photoimmobilization of biotin-azide. The regions of tissue modified with the biotin-azide appear as a series of bright dots. Lanes 1-3 contain wells modified with the reagent and light, while lane 4 contains the control wells.

It was then placed over the smooth side of a 200 µL VWR pipette tip holder and solid glass plate was clamped into place using spring clamps. 30 µL of biotin-azide (0.5 mg/ml in PBS) was added to the wells to be modified, while the controls received 30 µL

of PBS. Another control was also set up where the tissue surface received 30 μL of reagent but no light. The whole apparatus was placed on an ice bed, and placed under a 300 W halogen lamp for 10 min.

After this time, the entire apparatus was disassembled and tissue was washed for 10 min in PBS with several changes of buffer. It was than placed in a 0.03 mg/ml solution of avidin-HRP in PBS for 3hrs at room temperature. The tissue was then washed thoroughly in PBS, incubated with luminal for 4 min, blotted to remove excess reagent and imaged (chemiluminescence detection-high sensitivity, 80 sec acquisition time, aperture fully open). The positive signal appeared as bright dots on the surface of the tissue, while the controls remained dark.

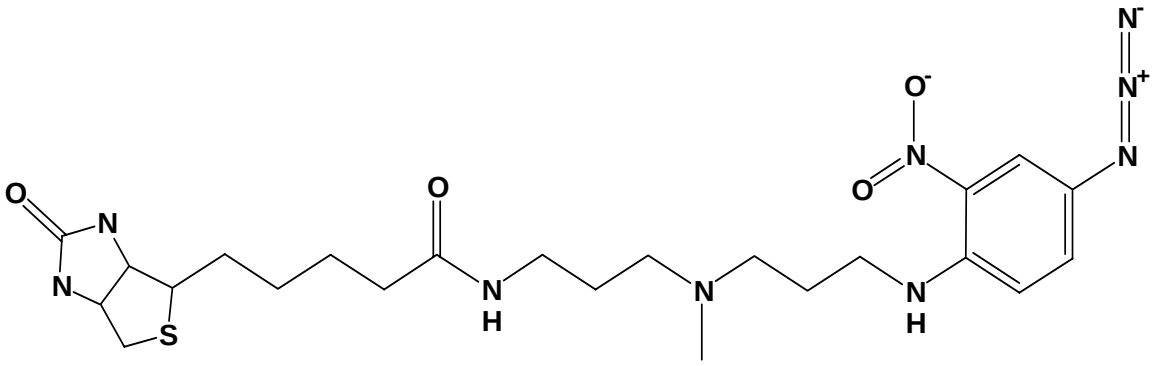


Figure 115. Structure of photoactivatable biotin

Experiment 8: Photo Modification of Fetal pig skin and Chemiluminescence Detection

The fetal pig skin was cleaned of fat, cut of fit a pipette tip holder plate and incubated in PBS for 2 hrs prior to use. It was then placed over the smooth side of a 200 μL VWR pipette tip holder and solid glass plate was clamped into place using spring clamps. 30 μL of biotin-azide (0.5 mg/ml in PBS) (see figure 112) was added to the wells to be modified, while the controls received 30 μL of PBS. The whole apparatus

was placed in an ice bed, and placed under a 300W halogen lamp for 10 min. After this time, the entire apparatus was disassembled and tissue was washed for 10 min in PBS. It was then placed in a 0.03 mg/ml solution of avidin-HRP in PBS for 3 hrs at room temperature. The tissue was then washed thoroughly in PBS, incubated with luminal for 4min, blotted and imaged (chemiluminescence detection-ultrasensitivity, 100 sec acquisition time, aperture fully open). The positive signal appeared as bright dots on the surface of the tissue, while the controls remained dark.

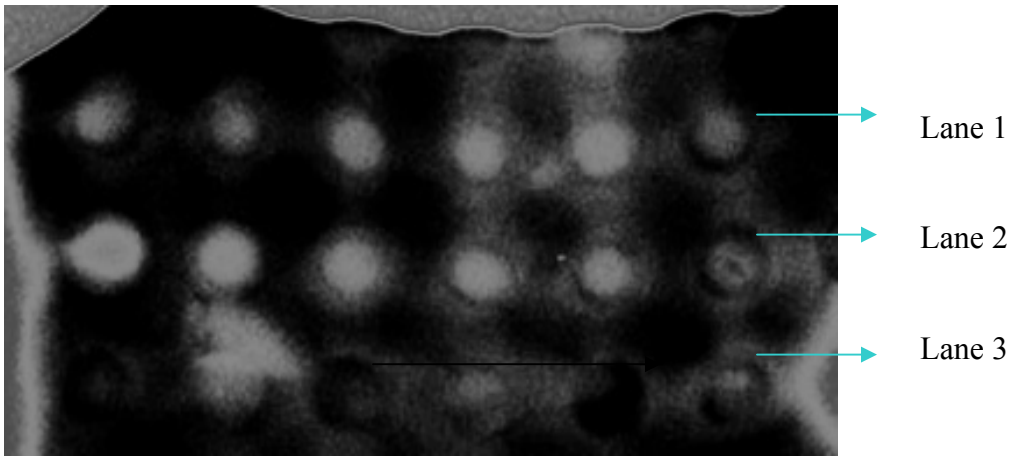


Figure 116. Chemiluminescence image of biotin immobilized on fetal pig skin. The positive signal is seen as a series of bright dots. Lanes 1 and 2 contain the wells modified with reagent and light, while lane 3 contains the control wells.

Experiment 9: Time Trial and Chemiluminescence Detection

The fetal pig skin was cleaned to fat, cut to fit a pipette tip holder plate and incubated in PBS for 2 hrs prior to use. It was then placed over the smooth side of a 200 µL VWR pipette tip holder and solid glass plate was clamped into place using spring clamps. The entire apparatus was immersed in an ice bed and placed under the 300 W halogen lamp. 30 µL of the biotin-azide (0.5 mg/ml in PBS) was added to three wells

every 2 min for a total reaction time of 10min, while the control wells for each time interval received 30 μL of PBS. The remainder of the experiment including, tissue washes, reaction with avidin-HRP, further tissue washes, incubation with luminal and imaging was identical to experiment 1. The resultant image indicated that the minimum reaction time of the biotin-azide with the light to detect a chemiluminescent signal (chemiluminescence detection-ultrasensitivity, 100 sec acquisition time, aperture fully open) from the tissue surface was 5 min.

Experiment 10: Photo Modification of Bovine Pericardium with Argon lamp and Chemiluminescence Detection

The pericardium was cleaned of fat, cut to fit a pipette tip holder plate and incubated in PBS for 2 hrs prior to use. It was then placed over the smooth side of a 200 μL VWR pipette tip holder and solid glass plate was clamped into place using spring clamps. 30ul of biotin-azide (0.5 mg/ml in PBS) was added to the wells to be modified, while the controls received 30 μL of PBS. The whole apparatus was placed in an ice bed, and placed under an argon lamp. The light guide was positioned 5mm above each well and was irradiated with the argon lamp for 300 sec per well. After this time, the entire apparatus was disassembled and tissue was washed for 10 min in PBS. It was than placed in a 0.03 mg/ml solution of avidin-HRP for 3 hrs at room temperature. The tissue was then washed thoroughly in PBS, incubated with luminal for 4 min, blotted and imaged (chemiluminescence detection-ultrasensitivity, 100 sec acquisition time, aperture fully open). The positive signal appeared as bright dots on the surface of the tissue, while the controls remained dark.

Time exposure. Time exposure experiment was also carried out, where the biotin-azide was irradiated with the argon light source for 200, 300, 600, 800 and 1000 sec on the pericardium. Control experiments received reagent without light or light and no reagent for each of the time intervals. The remainder of the experiment including incubation with avidin-HRP, tissue washes, incubation with luminal and chemiluminescence detection was identical to the previous procedure. The positive result appears as a series of bright dots, while the controls remained dark.

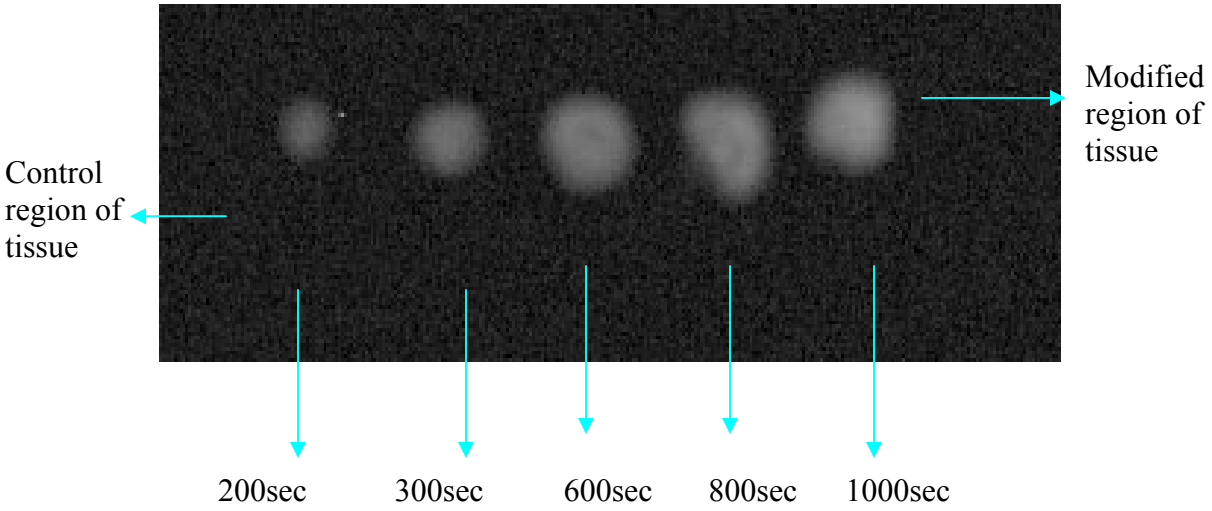


Figure 117. Chemiluminescent image from photo-immobilization of biotin using argon light source and various time exposures. The positive signal appears as a series of bright dots.

Experiment 11: Adsorption of Reagent onto Tissue, Photo-immobilization and Chemiluminescence Detection

The pericardium and fetal pig skin was cleaned of fat and cut to fit a 96 well plate. 30 µL of the biotin-azide (0.5 mg/ml in PBS) was added to each well to be modified, and the remaining wells (controls) received 30 µL PBS. The skin and pericardium were placed over their respective plates, covered with a solid glass slide and clamped into place using spring clamps. The apparatus was turned upside down and both the tissues

were incubated at 4°C for 15 hrs. After this time, the tissue were removed from the apparatus and thoroughly washed in PBS. Each tissue was then placed in a Petri dish containing cold PBS and irradiated with the halogen lamp for 10 min. The remainder of the procedure, including tissue washes, reaction with avidin-HRP, further tissue washes, incubation with luminal and chemiluminescence, were similar to that of experiment 5. We only detected a positive signal from the skin (bright dots) where the reagent was absorbed onto the surface.

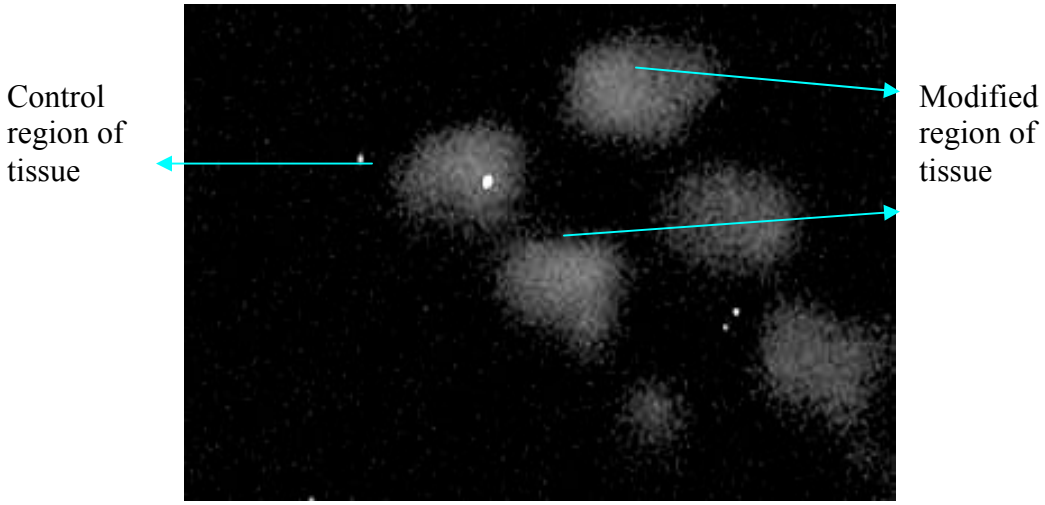


Figure 118. Chemiluminescent image from absorption of photoactive biotin unto the surface of skin, followed by immobilization with light. The regions of tissue where the reagent was absorbed appear as a series of bright dots, while the controls emit no signal.

Conclusion to Tissue Modification Strategies

The direct alkylation was the first strategy we investigated for the modification of fresh tissue surfaces. The initial sets of experiments with the strategy were primarily aimed at modifying meniscal tissue. Since then we have expanded the scope of this method to other tissues such as skin and pericardium. The modification of meniscal

tissue with the direct alkylation strategy with chromogenic detection was successful, but due to the variability of the tissue, we were not able to obtain consistent results. The fluorescence assays were successful at detecting the modification on the tissue surface, but there was always interference from the natural fluorescence of the tissue. When we attempted an indirect fluorescent assay (i. e. , when the fluorescent probe was indirectly conjugated on to the tissue surface using biotin and avidin conjugated fluorescent probe), we were unable to visualize any modification on the tissue due to the natural fluorescence of the tissue. We could have avoided the natural fluorescence of the tissue by using a fluorophore that has its emission in the UV or IR regions, but our imager was not set up to detect fluorescence in these ranges. The chemiluminescence assays were the most successful at visualization the medication on tissue, since the natural fluorescence of the tissue was not a factor in this type of assay. Some of the experiments with chemiluminescence detection did have some non-specific signal since we were using the avidin-biotin system.

Out of the tissue types investigated, the fetal pig skin was most successfully modified by the direct alkylation method. We were able to achieve the best resolutions, and the highest amount of protein immobilized on the tissue surface when compared to other tissue types. Since our discovery that chemiluminescence is a better visualization technique for our tissue modification experimentation, some of the early experiments with chromogenic and fluorescent based detections need to revisited with chemiluminescence. Since one of our main goals is to use these methods for aiding meniscal repair, further studies need to be conducted to refine these methods for use on the meniscus.

An oxidative strategy was explored since it was another common method of performing protein modifications. This strategy was successful at modify several tissue types including fetal pig skin, bovine pericardium and bovine meniscus. We used chemiluminescence to visualize any modification on the tissue surfaces since it has previously been shown to be the most effective method. Our attempts to use fluorescence based detection was once again unsuccessful due to the interference of the natural fluorescence of the tissue. Though we were successful at conducting gross modification of the various tissue types, we were unable to site-specifically modify any of the tissues using this method. The images from the experiments conducted to investigate spatial resolution displayed a good deal of non-specific signal making it very difficult to visualize the modification. We attempted to reduce background signal with the use of strong detergents, or blocking buffers, but to no avail. Our attempts to modify other tissue types such as bovine skeletal muscle and bovine aorta were unsuccessful. The exact reason for this needs further investigation.

In the future, the oxidative strategy should be explored with the goal of affording more spatial resolution and better signal-to-noise ratio. Various experimental conditions must be explored to expand the strategy into other tissue types such as bovine aorta or bovine skeletal muscle or to investigate the resistance of these tissues to be oxidatively modified. As with the other methods, fluorescence could be potentially used to detect modification provided we use a probe above or below the natural fluorescence of the tissue (~520 nm). The chromogenic based detection is attractive since the modifications could potentially be visualized with the naked eye. The chromogenic assay was not investigated for this method and therefore deserves further investigation. Once we are

able to have more spatial control over the oxidative modification, quantification experiments need to be conducted to better compare the three different strategies on various tissue types.

The reductive strategy was used successfully to modify a variety of tissue types including, bovine pericardium, fetal pig skin, bovine cornea and bovine meniscus. This was the most thoroughly investigated strategy due to the remarkable success we have had in modifying a variety of tissue types. Out of these tissue types, bovine pericardium was the most susceptible to reductive modification. We also obtained good results with the fetal pig skin. The bovine pericardium also displayed remarkable spatial resolution, up to about 1 mm when using the 1536 well plates. Most of the experiments had some amount of background chemiluminescence, presumably because we were using an avidin-biotin assay for the modification. Avidin and biotin generally tend bind non-specifically, and our attempts to reduce the non-specific binding by using blocking buffer such as BSA and detergents were unsuccessful. One surprising result was that when the modifications were carried out with using water as opposed to PBS buffer, the resultant images appeared to give enhanced signal-to-noise ratio. We are not exactly sure as to why this is the case and further experimentation is needed to determine the effect of water on tissue modification. Out of the three detection methods we used to visualize modification on the tissue surface (chromogenic, fluorescence, and chemiluminescence), the chemiluminescence was best able to detect the modifications on tissue surface. The fluorescence assay was successful at times, but always had interference from the natural fluorescence of the tissue.

We investigated several reducing agents for reducing the tissue surfaces including TCEP, DTT and BME. Out of these, TCEP was the best at reducing tissue surfaces. TCEP was also the most convenient since it does not have to be removed before reacting the tissue with maleimide probes. TCEP can also be used in fairly low concentration (50 mm) and with about 30 min reaction time on the tissue surface.

Out of the different tissue modification strategies, the photo-immobilization is the most convenient due to the fact that it can be done in about 200sec. We think that our initial attempts to immobilize proteins appeared to be unsuccessful due to the poor detection limits of the chromogenic assay. Now that chemiluminescent has proved to be a superior method of detection, the all the previous experiments need to be repeated with this type to detection. The immobilization of the various biotinylated amino acids such as tyrosine will also shed some light to understand the mechanism of photochemical tissue bonding.

We have only modified two tissue types, bovine pericardium and fetal pig skin, with this strategy. Further studies need to be carried out to expand this methodology to other tissue types such as meniscus, aorta, and cornea. As with the other strategies, we also need to conduct more quantification experiments to better compare this strategy between the different tissue and other methodologies. In our work, we have only used an aryl azide conjugated molecule for the photo-immobilization. However there are other photo-reactive molecules such as benzophones that might prove to be more efficient for this strategy that have yet to be utilized. We also investigated only two naphthalamides for immobilization of biotinylated amino acids, but there are varieties of these compounds that also need to be investigated for this method.

In general, the bovine pericardium was the most successfully modified tissue in with all these strategies. By using serial dilutions of avidin on a slot blot membrane, we were able to quantify the amount of protein being immobilized on the various tissue surfaces using the different modification methods. It was evident from the quantification experiments that there was a considerable heterogeneity on the tissue surfaces. In general amount of protein immobilized on the tissue surfaces using the reductive and direct alkylation method was in the order of 1-5 $\mu g/mm^2$. Currently we are conducting similar experiments with the oxidative and photo-immobilization strategies for quantification data.

Understanding tissue modification in terms of histology will also help in have a better understanding of the phenomena. Therefore, we are presently exploring the use of techniques like fluorescence microscopy to give a better understanding of the modifications at a microscopic level. Eventually, several *in vivo* studies will be carried out in order to investigate the toxicity of tissues modified using these types of methodology. Other tissue types such as cornea and aorta also need further investigation with this type of modification. In general most of these experiments also need to be repeated with quantification standards in order to have a better understand of our ability to modify tissue types. We are also currently investigating new methods to improve our detection of tissue modifications.

We successfully developed four different strategies to modify fresh tissues surfaces. A variety of tissue types such as bovine cornea, pericardium, aorta, meniscus and fetal pig were investigated. Since most of this work was inspired with the hope of repairing injured meniscus, currently we are focusing our efforts to better effect meniscal

modification using all the different strategies. Eventually the hope is to move away from our model assay into testing these methods for immobilization of growth factors and other healing agents of interest. These strategies could also prove useful in the field of tissue bonding and this is another area that will hopefully be investigated in the near future.

CHAPTER TWO

Synthesis of Labeled 3-O-methyl dopa and 4-O-methyl dopa for Use in Mass Spectrometry

Background

Mass Spectrometry and Isotopically Labeled Internal Standards

The use of internal standards in quantitative bioanalysis is a common and accepted procedure. An internal standard is used as a means to correct for variability in dilutions, evaporation, recovery, adsorption, degradation and instrumental parameters such as injection volume.[240] The internal standards used in quantitative bioanalysis LC/MS assays are either structural analogs or stable isotopically labeled (SIL) analogs of the analyte. SIL internal are common compounds in which several atoms of the analyte are replaced by their stable isotopes, such as ^{2}H (D, deuterium), ^{13}C, ^{15}N or ^{17}O. Labeling with three to eight ^{2}H or ^{13}C atoms or a combination of both is most common. Since a compound and its SIL analog will theoretically co-elute, it is important that the mass difference between the two atoms is at least three mass units in order to be able to separate them in the mass analyzer and to prevent "cross-talk". When the difference is less than three mass units, the isotope peaks of the analyte may interfere with the signal of the internal standard. Furthermore, the SIL internal standard should be pure enough to prevent any contributions to the analyte response.[241]

Methods for Synthesis of Labeled Compounds

During the last several decades, development of new synthetic methods for the stable isotope labeling of organic compounds have been extensively studied for the investigation of biosynthetic pathways, chemical reaction mechanisms and kinetics, the catalytic mechanism of enzymes, analysis for secondary and tertiary structures of biomolecules, analysis of drug metabolism and residual particles and so on.[242, 243] For the preparation of deuterium-labeling of organic compounds, there are two main methods- the multistep synthetic method starting from deuterium labeled small synthons and the post synthetic H-D exchange displacement of the hydrogen bound to the carbon of an unlabeled compound by deuterium using a catalyst.[244]

The synthetic method, in which the deuterium is directly and specifically inserted, often yields high deuterium abundance, but is limited by the chemistry required. In addition, the molecule labeled might be changed due to the severity of the required reaction conditions. The exchange method yields lower deuterium abundance, often with the label being distributed over many sites on the molecule, but has the advantage that it does not require separate synthetic steps and are less likely to disrupt the structure of the molecule being labeled.[245]

Aromatic compounds can exchange hydrogens when treated with acid by electrophilic aromatic substitution reactions. These acid-catalyzed exchange reactions are obviously a convenient tool for the labeling of intact target molecules. Originally applied to the synthesis of d_6-benzene, it has also been demonstrated with phenol, where the usual directive effects apply. This orientation effect and the general acid catalyzed reaction point toward an ordinary arenium ion mechanism. Besides strong mineral acids,

BF_3. D_20 was also used to prepare a series of deuterated simple aromatic compounds in a convenient fashion.[246]

Base-catalyzed reactions may be used to gain desired labeling when the starting compounds are acid sensitive. Strong bases such as CH_3OD and NaOD are often used for these types of exchange reactions. For example, d_3 prostaglandin (PE) E2 was prepared by equiliberating PGE_2 methyl ester in CH_3CH_2OD in the presence of anhydrous potassium acetate at room temperature.[247]

Transition metals have also been investigated for possible catalytic properties for H/D exchange reactions. Nickel has been used to prepare d_6-benzene and D_2O under tortuous conditions (450^0C). Better results were obtained with platinum black after reduction with Adams catalyst. Rhodium(III) chloride has also been employed as a homogenous catalyst for isotopic hydrogen exchange in the deuteration of aromatic compounds and alkanes. The use of ethylaluminium chloride and a range of other halides, such as $NbCl_5$, WCl_6 and $SbCl_5$ as catalysts for the rapid deuteration of simple organic compounds, were described utilizing d_6-beneze as the isotope source. [248]

Transition-metalcatalyzed C-H bond activation combined with the deuteration of the activated substrate has been a topic of current interest and is an underdeveloped methodology. Many approaches, which invoke Ir[3], Rh[4], Co[5], Pt[6], Ru[7] and Mn[8], are uniformly conducted under homogeneous conditions.[249]

While homogeneous catalysts are used in many cases, heterogeneous catalysts are crucial for application of the methodology to industry because they are easily removed from the reaction mixture using only simple filtration. Existing techniques utilize Pd/C-D_2, Pd/C-DCO_2K, Pd/ C D_2O, PtO_2-D_2-D_2O, Rh/SiO_2-D_2, Raney Co-Al- D_2O and K10

clay-D_2O.[250] However, such conventional procedures for the incorporation of deuterium into the C-H bonds of organic compounds are often limited to activated positions of the molecules, leading to low levels of deuterium incorporation, and usually require a vast amount of the catalyst, addition of acidic or basic additives, and/or deuterium atmosphere. Most recently, Matsubara et al., reported a remarkable H-D exchange transformation with 10% Pd/C in subcritical D_2O (250°C) in the absence of hydrogen.[251]

Another synthetic method for the incorporation of deuterium into a compound is the use of reducing agents which contain deuterium. $LiAlD_4$ or $NaBD_4$ assisted reduction is the most general protocol for introducing deuterium into molecules with reducible sites.[252] Lithium trialkylbohorohydride (superhydride) is another highly reactive nucleophilic reducing agent that is being used for deuterium labeling. This reagent is capable of reducing ester, hindered alkyl halides and toluene-p-sulphonates, in addition to exhibiting great sterioselectivity and steriospecificity, as in the reduction of expoxides. Catalytic deuteration of double bonds provides a rapid route to the desired labeled compounds. The homogenous catalyst tris-9-triphenly-phosphine rhodium(I) chloride (Wilkinson catalyst) and deuterium gas were used for the specific deuteration of a series of n-mono-olefins.[253]

Biological generation of standards using labeled precursors is another method providing rapid access to desired compounds, when only minute amounts are required. The possibility to readily obtain even complex biomolecules in a suitable labeled form has found considerable attention. Thus, deuterated 11- dehydro-thromboxane can be biologically synthesized by incubation of d4-TXB2 with high-speed supernate from the centrifugation of guinea pig liver homogenate in the presence of NAD.[254]

Metabolic Disorders

Disorders of monoamine neurotransmitter metabolism have been increasingly recognized. Mono-amines, also called biogenic amines, include serotonin and the two catecholamines, dopamine and norepinephrine. These compounds have numerous roles including modulation of psychomotor functions, hormone secretion, cardiovascular, respiratory and gastrointestinal control, sleep mechanisms, body temperature, and pain.[255] Aromatic L-amino acid dercarboxylase (AADC) is an enzyme that converts 3, 4-dihydroxyphenylalanine (L-dopa) to dopamine and 5-hydroxytryptophan to serotonin. Inherited deficiency of this enzyme leads to decreased levels of these two neurotransmitters, resulting in severe early onset neurological disorders.[256]

In 1990 Hyland and Clayton identified the first human patients with AADC deficiency by screening cerebrospinal fluid samples from children with unidentified neurological disorders for abnormalities of neurotransmitter metabolites. They reported male monozygotic twins born to first-cousin parents who presented at the age of 2 months with severe hypotonia and paroxysmal movements consisting of crying followed by extension of the arms and legs, oculogyric crises and cyanosis. They also showed occasional choreoathetoid movements of the extremities. Later, defects in temperature regulation and postural hypotension were observed.

Laboratory analyses showed a greatly decreased concentration of homovanillic acid (HVA) and 5-hydroxyindoleacetic acid (5-HIAA) in the CSF, as well as decreased whole blood serotonin and plasma catecholamines. There was a significant elevation in the urinary excretion of L-DOPA, 5-hydroxytryptophan (5HTP) and 3-methoxytyrosine, all of which precede the AADC step in the biochemical pathway. The findings

demonstrated that serotonin and dopamine synthesis were affected in both the central and peripheral nervous systems, consistent with a deficiency of AADC. AADC enzyme activity was severely reduced in plasma and in liver tissue (1% of control). Treatment with a monoamine oxidase inhibitor, a dopamine agonist and pyridoxine resulted in a striking improvement in tone and movement. The parents were asymptomatic, but had biochemical profiles consistent with their being heterozygous for AADC deficiency.[257]

In the absence of AADC activity, L-dopa is methylted to 3-0-methyl dopa which is then accumulated in blood, urine and cerebrospinal fluid in infants and children with a deficiency of this enzyme. 3-O-methyl dopa therefore, provides a biochemical marker that can be used to screen for this disease.[258] Thus, the goals of this research were to synthesis a labeled analog of 3-O-methyldopa for the use in mass spectrometry for the screening of AADC deficiency (see Figure 116 for strucutes of 3-O and 4-O methyl dopa).[259]

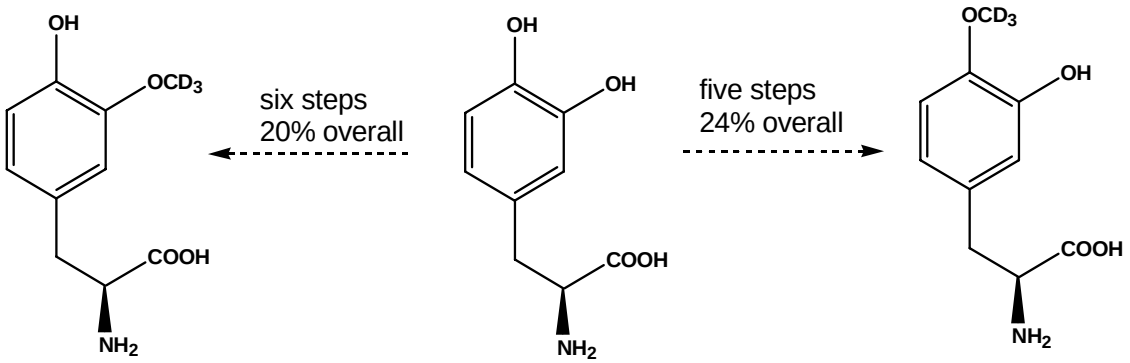


Figure 119. Structures of (left to right) 3-O-methyl dopa, L-Dopa, 4-O-methyl dopa

Synthetic Strategy

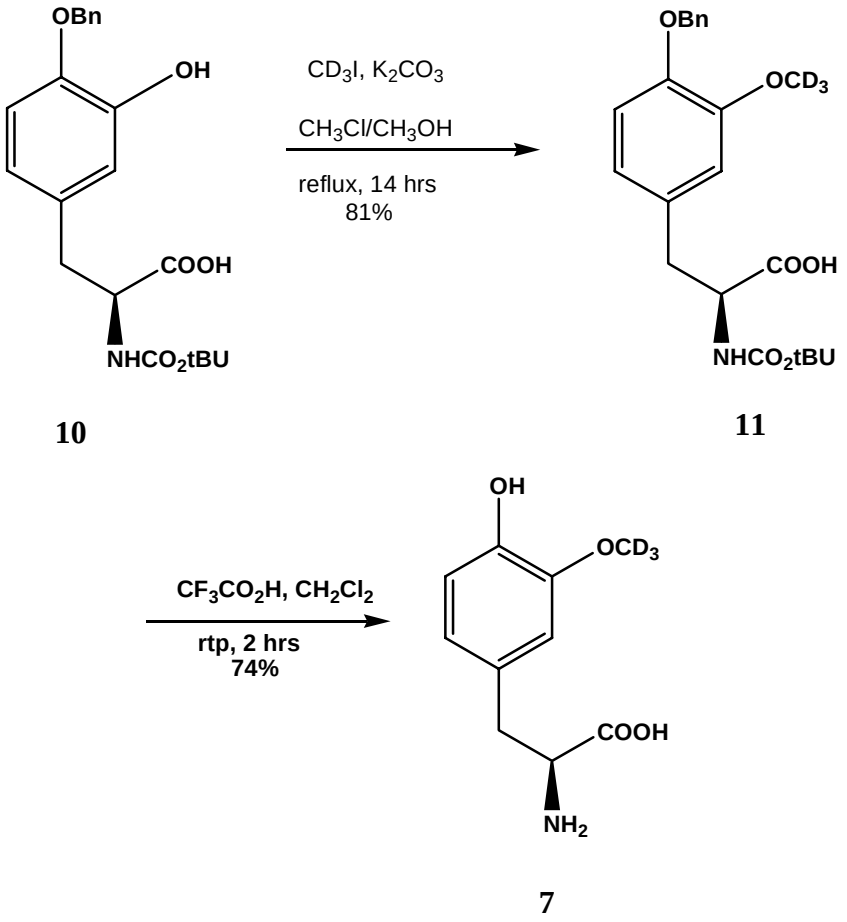
Our initial idea was to synthesize a cyclic mexthoxy acetal, followed by a selective ring opening with a reducing agent to afford the methylated dopa. We started with the fairly simple approach of reacting L-dopa with cesium fluoride and dichloromethane in DMF. After exploring several routes, we were able to synthesize the methoxy acetal using cesium flouoride, bromo, chloro- methane in DMF. The next step was to selectively open the acetal using a reducing agent, but we were unable to find a suitable set of conditions to carry out the reaction.

The next strategy we studied was the convenient direct methylation of L-Dopa using a strong base and methyl iodide. We tried several bases along with deuterated methyl iodie but this "simple" approach proved to be problematic as were unable to get a selective methylation.

Next, we turned our attention to selectively protected dopa precursors synthesized by Jung and Lazarova. These compounds are readily available from tyrosine by a sequence of reactions involving the Reimer-Tiemann formylation and a Dakin oxidation.[260] Similar strategies for the synthesis of selectively protected dopa have been reported by the Boger group[261] and the Chen group.[262] This synthetic strategy, starting with tyrosine, allows for the selective methylation at the 3 or 4 position on the benzene ring of dopa at good yields and allows for easy purification. Accordingly we used this strategy for the synthesis of our target compounds 3-O-methyl and 4-0-methy dopa.

The straight forward synthesis of labeled 3-O-methyl dopa began with the selectively protected dopa analogue **10** which is available from tyrosine in 4 steps with 33% yield. The deuterium label was incorporated by alkylating **10** with excess CD_3I,

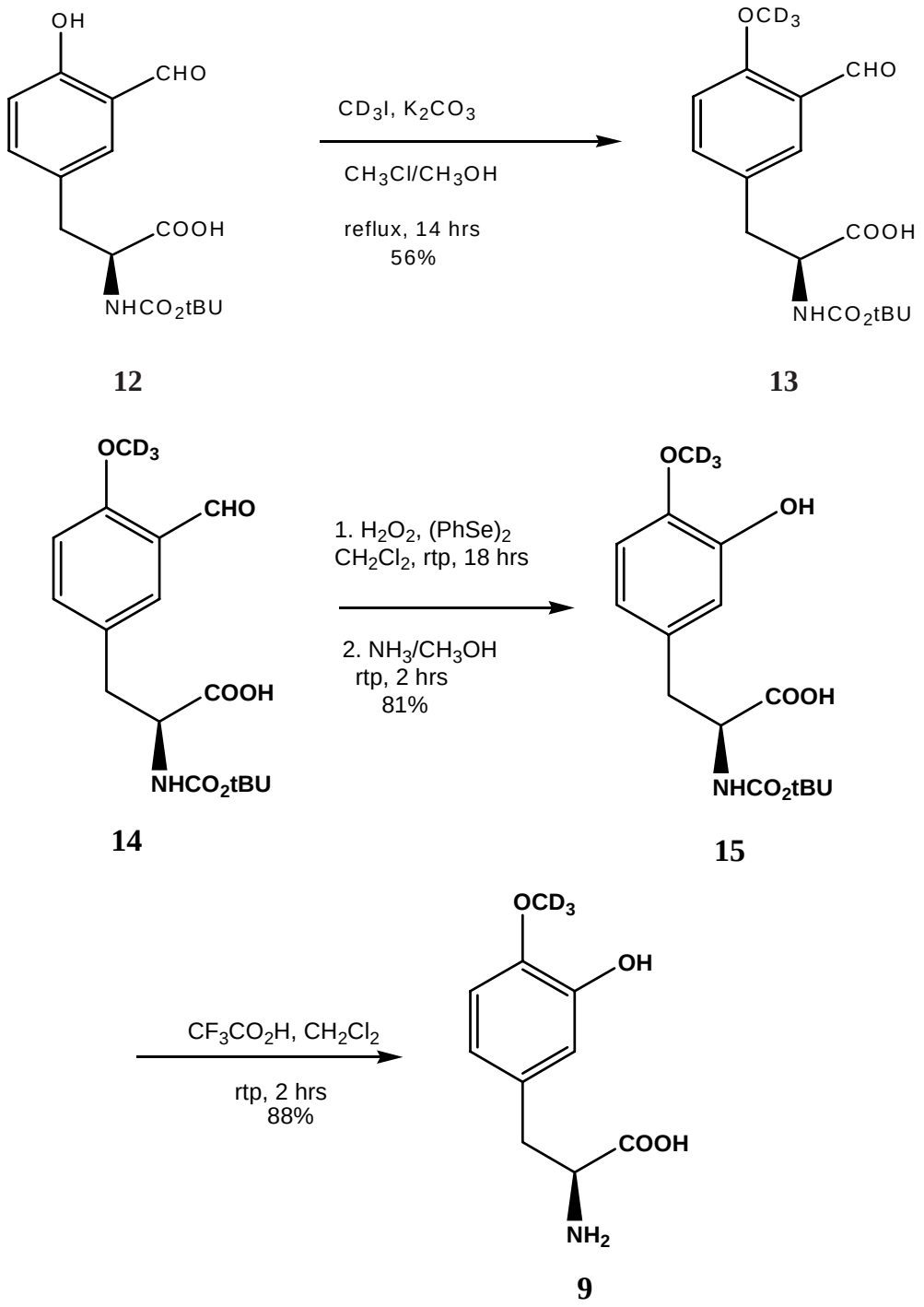
which was followed by the removal of both protecting groups (N-tBOC and O-benzyl) by treatment with trifluoroacetic acid (TFA) in dichloromethane. Crude **7** was purified by preparative reverse phase TCL (water/methanol/sodium octylsulfonate 9:1:1 mM), and the ion paring reagent was subsequently removed by filtration through H^+ -form Dowex-50 X 4 (NH_4OH elutant).



Scheme1. Synthesis of 3-O-methyl dopa

Preparation of 4-O-methyl dopa began with hydroxyl benzaldehyde (**12**), which was synthesized in two steps from tyrosine (59% overall). Introduction of the label was again accomplished by alkylation with excess CD_3I. Diphenyl diselenide-mediated

oxidation of the alkylated benzaldehyde **14**, followed by the aminolysis of the resulting formate ester, afforded the protected dopa derivative **15**, which was deprotected and purified in analogy with the 4-O-methyldopa derivative.



Scheme 2. Synthesis of 4-O-methyl dopa

Conclusion

One of the major technical advances in newborn screening is the use of an analytical instrument known as a tandem mass spectrometer. Research and development in the newborn screening applications of tandem mass spectrometry were started in the early 1990's and they continue today. More than 30 disorders of body chemistry can be detected in a single analysis of a dried blood spot collected from neonates using this technique. The tandem MS analysis includes amino acid, and 3-0-methyldopa can be incorporated into the screening process to help identify patients with AADC deficiency. Accurate quantification with tandem MS, however, requires incorporation of a stable isotope internal standard for each compound analyzed. Therefore, we have developed syntheses of stably labeled 3-0-dopa and its isomer 4-0-methyl dopa for the use as SIL internal standard in mass spec analysis.

As noted in the introduction, aromatic compounds can exchange hydrogens when treated with acid by electrophilic aromatic substitution reactions. These acid catalyzed exchange reactions are obviously a convenient tool for the labeling of intact target molecules. Recently, we have also investigated the used of deuterated acids to carry out exchange reactions with the phenyl protons of dopa. We realized that this could be more convenient route to preparing labeled dopa analogs. Unfortunately, out of the two deuterated acids (deuterated hydrochloric and sulfuric) investigated for this method, neither one was able to cleanly carry out deuterium exchange with the phenyl ring of dopa.

Materials and Methods

Chemicals and Reagents

Except where otherwise noted, reagents used for the synthesis were obtained from Aldrich Chemical CO., Milwaukee, WI, and Fisher Scientific, Pittsburgh, PA (including Acros reagents) and used directly as purchased. Solvent such as dichloromethane, hexane, ethanol, ethyl acetate and methanol were obtained from commercial sources (via Baylor University Chemistry Department stockroom) and were distilled prior to use. Tetrahydrofuran (THF) was dried and distilled over potassium metal and benzophenone according to standard procedure prior to use. Deionized water (DI) was obtained via a US Filter system provided by Baylor Dept. of Chemistry.

Instrumentation

Proton (^{1}H) and carbon (^{13}C) were obtained on a Bruker AVANCE 300 NMR running XWIN-NMR 3.1 software at 300MHz. Chemical shifts are expressed in ppm (∂), peaks are listed as singlets (s), doublets (d), triplet (t) or multiplet (m), with the coupling constant (J) expressed in Hz. HPLC was carried out with a Beckman System Gold, composed of a model 168 detector and a model 126 solvent module using an Alltech Alltima C-18 5M 33mm*7mm "rocket" column. GC-Mass spec was obtained on a Hwelett Packard.

Synthesis of Compounds

Synthesis of 3-O-methyl Dopa

N-[(1,1-Dimethylethoxy) carbonyl]-L-tyrosine

Triethyl amine (5. 81ml, 41. 4mmol) was added to a solution of L-tyrosine (5g, 27.6 mmol) in a 1/1 mixture of dioxane/water (100 ml). The reaction mixture was cooled to 0^0 c with an ice water bath, and di-tert-butyl dicarbonate (6.6 g, 30.4 mmol) was added on one batch. After 30min, the cold bath was removed and the reaction was stirred under nitrogen at room temperature for 18 hrs. The reaction mixture was concentrated by rotovapp, and the residue was diluted with water and ethyl acetate. The water layer was washed with ethyl acetate, acidified to pH 1 with 1M HCl and back extracted with three 100 ml portions of ethyl acetate. The organic extracts were washed with brine, dried over $MgSO_4$, and evaporated to give the Boc protected amine as white foam (7. 12g, 92%). The product was used in subsequent steps without further purification.

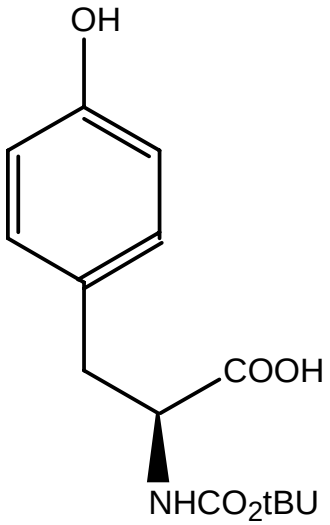


Figure 120. Structure of N-[(1,1-dimethylethoxy) carbonyl]-L-tyrosine (**16**)

^{1}H NMR ($CDCl_3$)

δ 12.52 (1H, bs), 7.0 (2H, d, *J* = 7.8 Hz), 6.74 (2H, d, *J* =7.8 Hz), 5.92 (1H, bs, OH), 5.06 (1H, bs, NH), 4.58 (unresolved m, 1H), 3.02 (2H, unresolved m,), 1.42 (9H, s, Boc)

^{13}C NMR (CD_3OD, 75 MHz)

δ 174.5, 156.7, 157.1, 131.2, 129.1, 116.1, 80.2, 57.8, 37.8, 28.5

N-[(1,1-dimethylethoxy)carbonyl]-3-(3-formyl-4-hydroxyphenyl)- L-alanine

Powdered sodium hydroxide (1.71 g, 42.72 mmol) was added to a suspension of the N-Boc tyrosine (2 g, 7.12 mmol), water (0.25 ml, 14.13 mmol) and 30 ml of chloroform.

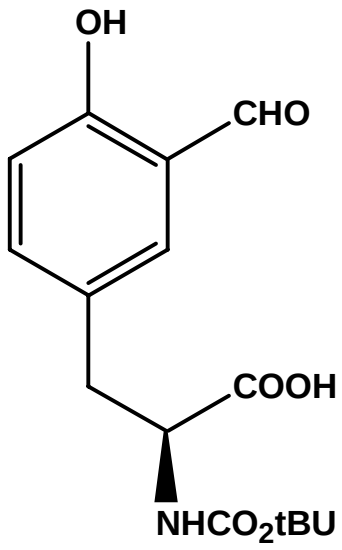


Figure 121. Structure of N-[(1,1-dimethylethoxy)carbonyl]-3-(3-formyl-4-hydroxyphenyl)- L-alanine (**12**)

The mixture was refluxed at 40^0 C. Additional sodium hydroxide was added (0.42 g, 10.68 mmol) after 1 and 1.5 hr. The reaction was then diluted with water and

ethyl acetate and the aqueous layer was acidified to pH 1 using 1 M HCl and back extracted with three 100 ml portions of ethyl acetate.

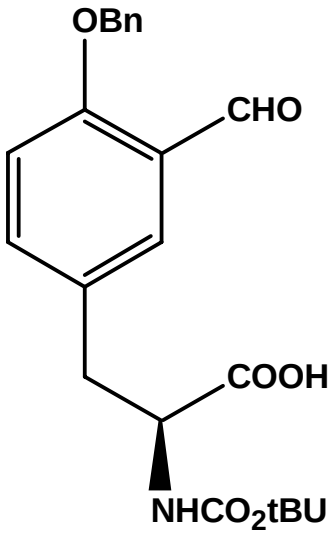


Figure 122. Structure of N-[(1,1-dimethylethoxy)carbonyl]-3-[3-formyl-4-(phenylmethoxy)phenyl]-L alanine **(11)**

The organic extracts were washed with brine, dried over $MgSO_4$ and concentrated to give a brown solid. The solid was dissolved in 10ml of chloroform and 5 g of silica was added. The solvent was then evaporated leaving behind a crude product absorbed onto the silica gel. The product containing the silica gel was packed into a Biotage FLASH sample injection module (SIM) cartridge which was then eluted though a silica FLASH 40S cartridge using 12:1 ratio if chloroform:methanol with 1% acetic acid as the eluent. The fractions containing the product were concentrated leaving behind the desired product as brown oil (0.72 g, 33%).

^{1}H NMR ($CDCl_3$)

δ 12.5 (1H, bs), 10.90 (1H, s, OH), 9.84 (1H, s, CHO), 7.36 (1H, s), 7.33 (1H, d, J = 8.3 Hz,), 6.93 (1H, d, J =8.3 Hz), 5.28 (1H, bs, NH), 4.96 (1H, unresolved m), 3.17 (1H, unresolved m), 3.04 (1H, unresolved m), 1.40 (9H, s, Boc);

^{13}C NMR ($CDCl_3$, 75 MHz)

δ 196.6, 175.6, 160.5, 155.3, 138.1, 134.2, 127.6, 120.4, 117.7, 80.4, 54.2, 36.8, 28.2

N-[(1,1-dimethylethoxy)carbonyl]-3-[3-formyl-4- (phenylmethoxy)phenyl]-L alanine

A solution of anhydrous potassium carbonate (0.506 g, 3.66 mmol) in 2/1 chloroform/ methanol (6 mL) was refluxed for 15 min. The 3-formyl-*N*-Boc- L-tyrosine **12** (0.257 g, 0.832 mmol) and benzyl bromide (0.148 mL, 1.25 mmol) were added, and the mixture was refluxed for 4 hours. The reaction was then diluted with water and ethyl acetate. The aqueous layer was acidified to pH 1 with 1 N HCl and backextracted with ethyl acetate. The organic extracts were washed with brine, dried over $MgSO_4$, and concentrated to give the desired product **17** as yellow oil (0.237g, 71%), which was used in the next step without further purification:

^{1}H NMR ($CDCl_3$)

δ 12.5 (1H, bs), 10.52 (1H, s, CHO), 7.67 (1H, s), 7.41 (6H, m); (1H, d, J =8.6 Hz), 5.18 (2H, s), 4.98 (1H, bs, NH), 4.58 (1H, unresolved m), 3.18 (1H, unresolved m), 3.10 (1H, unresolved m), 1.42 (9H, s, Boc)

^{13}C NMR ($CDCl_3$, 75 MHz)

δ 189.9, 175.3, 160.2, 155.3, 144.5, 137.0, 136.0, 129.2, 128.7, 128.2, 127.3, 124.8, 113.3, 80.3, 70.5, 54.1, 36.9, 28.2

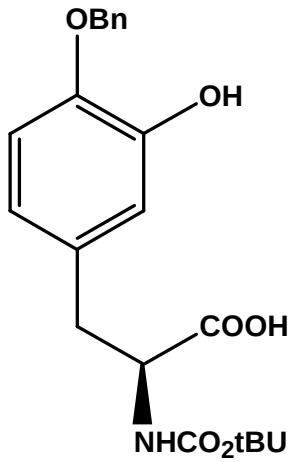


Figure 123. Structure of N-[(1,1-dimethylethoxy)carbonyl] 3-hydroxy-4-benzyloxy-phenylalanine **(10)**

N-[(1,1-dimethylethoxy)carbonyl] 3-hydroxy-4-benzyloxy-phenylalanine

Diphenyl diselenide (0.003 g, 0.01 mmol, 0.03 eq) and aqueous hydrogen peroxide (0.5 ml of a 35% aqueous solution, 4.6 mmol, 15 eq) was added to a solution of compound **17** (0.1 g, 0.31 mmol, 1 eq) in dichloromethane (3 ml). The reaction mixture was stirred under N_2 at room temperature for 24 hr and then diluted with water (5 ml) and ethyl acetate (20 ml). The layers were separated and the organic layer washed with brine, dried over $MgSO_4$, and concentrated under vacuum. The crude residue was redissolved in 1 ml methanol, 3 ml of 2 M ammonia in methanol was added, and the reaction stirred at room temperature for 2 hr. The mixture was then concentrated and diluted with water (5 ml) and ethyl acetate (10 ml). The layers were separated and then the aqueous layer was acidified to pH 1 with 1N HCl and back extracted with ethyl acetate (3 x 25 ml). The combined organic extracts were washed with brine, dried over $MgSO_4$, and concentrated to afford 0.08 g (0.25 mmol, 81%) of reasonably pure **10** as a yellow oil. **10** was used in the next step without further purification.

^{1}H NMR ($CDCl_3$)

δ 12.5 (1H, bs), 7.39 (5H, m), 6.85 (1H, d, J = 8.2 Hz), 6.76 (1H, s), 6.64 (1H, d, J = 8.2 Hz), 5.60 (1H, bs, OH), 5.28 (2H, s), 5.06 (1H, bs, NH), 4.53 (1H, unresolved m), 3.04 (2H, unresolved m), 1.40 (9H, s, Boc)

^{13}C NMR ($CDCl_3$, 75 MHz)

δ 176.2, 153.6, 142.7, 142.5, 136.4, 129.6, 128.6, 128.2, 126.7, 121.1, 115.9, 111.6, 81.1, 71.0, 55.2, 37.1, 28.9

N-[(1,1-dimethylethoxy)carbonyl] 4-benzyloxy-3-[$^{2}H_3$]-methoxyphenylalanine

A suspension of anhydrous potassium carbonate (0.5 g, 3.6 mmol, 4.4 eq) in chloroform/methanol (2/1; 6 ml) was refluxed under N_2 for 15 min and then cooled. Compound **12** (0.25 g, 0.81 mmol, 1 eq) and CD_3I (1 ml, 4.5 mmol, 5.5 eq) were added and the mixture was refluxed under N_2 for an additional 14 hrs.

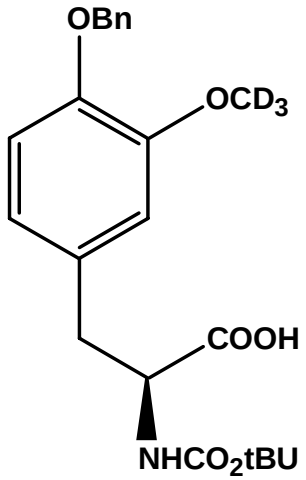


Figure 124. Structure of N-[(1,1-dimethylethoxy)carbonyl] 4-benzyloxy-3-[$^{2}H_3$]-methoxyphenylalanine **(11)**

The reaction mixture was diluted with water and ethyl acetate, and the aqueous layer was acidified to pH 1 with 1N HCl, the layers separated, and the aqueous layer back extracted with ethyl acetate (3 x 50 ml). The combined organic extracts were washed with brine and water, dried over MgSO4, and concentrated under vacuum. Flash column chromatography was carried out using a Flash 40S column and the Biotage Flash system (75 g silica gel, 1% acetic acid in 22:1 ratio of CH_2Cl_2/MeOH). This afforded the desired product (0.17 g, 0.45 mmol, 56%) as brown oil.

^{1}H NMR ($CDCl_3$, 300MHz)

δ 12.5 (1H, bs), 7.39 (5H, m), 6.85 (1H, d, J = 8.2 Hz), 6.76 (1H, s), 6.64 (1H, d, J =8.2 Hz), 5.60 (1H, bs, OH), 5.28 (2H, s), 5.06 (1H, bs, NH), 4.53 (1H, unresolved m), 3.04 (1H, unresolved m), 1.40 (9H, s, Boc)

^{13}C NMR ($CDCl_3$, 75 MHz)

δ 176.9, 155.6, 145.7, 145.1, 136.4, 129.7, 128.6, 128.2, 127.7, 121.0, 115.9, 112.6, 80.1, 71.1, 56.3, 55.0 (C-D, sept, J = 36), 37.1, 28.4

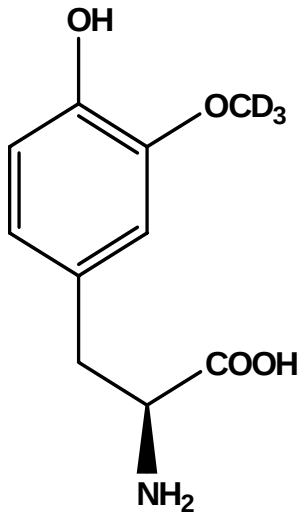


Figure 125. Structure of 4-hydroxy-3-[$^2H_3$]-methoxyphenylalanine (**7**)

4-hydroxy-3-[$^2H_3$]-methoxyphenylalanine

Compound **11** (0.2 g, 0.50 mmol, 1 eq) was dissolved in 2ml of dichloromethane and 2 ml of triflouroacetic acid was carefully added. The reaction was stirred under N_2 for 4 hr at room temperature. The reaction mixture was then concentrated under vacuum and the product dissolved in 1ml water. Preparative reverse phase TLC (1mM 1-octanesulfonic acid [sodium salt] in water/methanol [9/1], pH 2) afforded an oil that was dissolved in 10% acetic acid (3 ml), loaded on an ion exchange column (Dowex 50, 4-400, acid form, 5 g) that was then washed extensively with water. Elution of the resin with concentrated ammonium hydroxide, followed by concentration under vacuum, afforded the desired product (0.08 g, 0.37 mmol, 74%) as a light brown powder.

^{1}H NMR ($CDCl_3$)

δ 12.35 (1H, bs), 6.82 (1H, d, J= 7.8 Hz), 6.75 (1H, d, J = 7.8), 6.54 (1H, m), 5.92 (1H, bs, OH), 5.06 (2H, bs, NH), 4.58 (1H, m, unresolved), 3.02 (2H, unresolved m)

^{13}C NMR (CD_3OD, 75 MHz)

δ 175.5, 157.7, 157.1, 131.2, 129.1, 116.1, 70.5, 53.8 (C-D, sept, J = 35), 37.8, 28.1

Synthesis of 4-0 methyl dopa

N-[(1,1-dimethylethoxy) carbonyl] 3-formyl-4-[$^2H_3$]-methoxyphenylalanine

A suspension of anhydrous potassium carbonate (0.5 g, 3. 6 mmol, 4. 4 eq) in chloroform/methanol (2/1; 6 ml) was refluxed under N_2 for 15 min and then cooled. Compound **12** (0.25 g, 0.81 mmol, 1 eq) and CD_3I (1 ml, 4.5 mmol, 5.5 eq) were added and the mixture was refluxed under N_2 for an additional 14 hrs. The reaction mixture

was diluted with water and ethyl acetate, and the aqueous layer was acidified to pH 1 with 1N HCl, the layers separated, and the aqueous layer back extracted with ethyl acetate (3 x 50ml). The combined organic extracts were washed with brine and water, dried over $MgSO_4$, and concentrated under vacuum. Flash column chromatography was carried out using a Flash 40S column and the Biotage Flash system (75 g silica gel, 1% acetic acid in 22:1 CH_2Cl_2/MeOH). This afforded the desired product (0.17 g, 0.45 mmol, 56%) as brown oil.

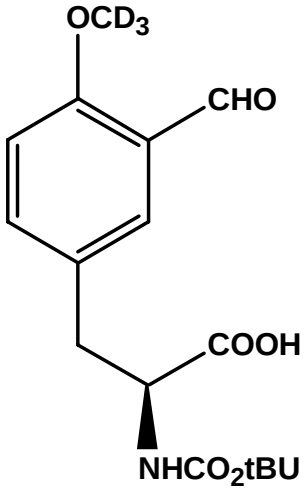


Figure 126. Structure of N-[(1,1-dimethylethoxy) carbonyl] 3-formyl-4-[$^2H_3$]-methoxyphenylalanine **(14)**

^{1}H NMR ($CDCl_3$)

δ10.90 (1H, s, OH), 9.84 (1H, s, CHO), 7.36 (1H, s), 7.33 (1H, d, J = 8.3 Hz,), 6.93 (1H, d, J =8.3 Hz), 5.28 (1H, bs, NH), 4.96 (1H, unresolved m), 3.17 (1H, unresolved m), 3.04 (1H, unresolved m), 1.40 (9H, s, Boc);

^{13}C NMR ($CDCl_3$, 75 MHz)

δ 196.6, 175.6, 161.5, 154.3, 144.2, 138.1, 134.2, 127.6, 120.4, 117.7, 80.4, 54.2 (unresolved m), 36.8, 28.8

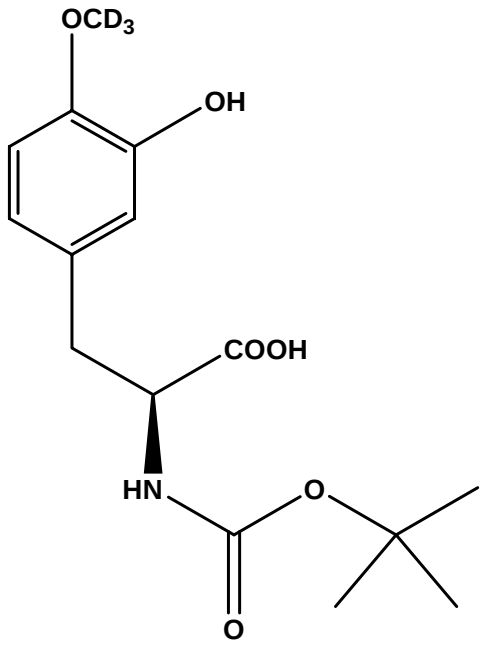


Figure 127. Structure of N-[(1,1-dimethylethoxy) carbonyl] 3-hydroxy-4-[$^{2}H_3$]-methoxyphenylalanine **(15)**

N-[(1,1-dimethylethoxy) carbonyl] 3-hydroxy-4-[$^{2}H_3$]-methoxyphenylalanine

Diphenyl diselenide (0.003 g, 0.01 mmol, 0.03 eq) and aqueous hydrogen peroxide (0.5 ml of a 35% aqueous solution, 4. 6 mmol, 15 eq) was added to a solution of compound **14** (0.1 g, 0.31 mmol, 1 eq) in dichloromethane (3 ml). The reaction mixture was stirred under N_2 at room temperature for 24 hr and then diluted with water (5 ml) and ethyl acetate (20 ml). The layers were separated and the organic layer washed with brine, dried over $MgSO_4$, and concentrated under vacuum. The crude residue was redissolved in 1 ml methanol, 3 ml of 2M ammonia in methanol was added, and the reaction stirred at room temperature for 2 hr. The mixture was then concentrated and diluted with water (5 ml) and ethyl acetate (10 ml). The layers were separated and then the aqueous layer was acidified to pH 1 with 1N HCl and back extracted with ethyl acetate (3 x 25 ml). The combined organic extracts were washed with brine, dried over MgSO4, and concentrated

to afford 0.08 g (0.25 mmol, 81%) of reasonably pure **15** as a yellow oil. **15** was used in the next step without further purification.

^{1}H NMR ($CDCl_3$)

δ 12.35 (1H, bs), *7.0* (1H, d, *J*= 7.8 Hz), 6.74 (1H, d, *J* =7.8 Hz), 6.48 (1H, m), 5.92 (1H, bs, OH), 5.06 (1H, bs, NH), 4.58 (unresolved m, 1H), 3.02 (2H, unresolved m,), 1.42 (9H, s, Boc)

^{13}C NMR (CD_3OD, 75 MHz)

δ 171.6, 161.5, 154.3, 144.2, 138.1, 134.2, 125.6, 121.4, 117.7, 80.4, 54.2 (C-D, spet. J = 35), 36.8, 28.2

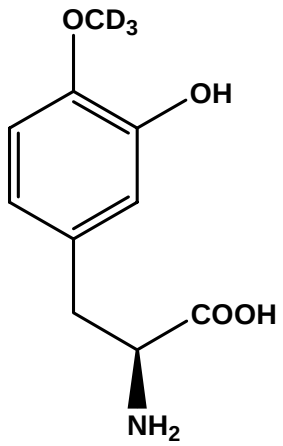


Figure 128. Structure of3-hydroxy-4-[$^{2}H_3$]-methoxyphenylalanine (**9**)

3-hydroxy-4-[$^{2}H_3$]-methoxyphenylalanine

Compound **15** (0.1 g, 0.32 mmol, 1 eq) was dissolved in 2ml of dichloromethane and then 2ml of triflouroacetic acid was carefully added. The reaction was stirred for 2 hr at room temperature and then concentrated under vacuum. Preparative reverse phase TLC (water/ methanol [9/1] with 1mM 1-octanesulfonic acid sodium salt, pH 2) afforded

an oil that was dissolved in 10% acetic acid (3 ml), loaded on an ion exchange column (Dowex 50, 4-400, acid form, 5 g) that was then washed extensively with water. Elution of the resin with concentrated ammonium hydroxide, followed by concentration under vacuum, afforded the desired product (0.06 g, 0.28 mmol, 88%) as a light brown powder.

^{1}H NMR ($CDCl_3$)

δ 12.30 (1H, bs), 10.0(1H, bs, OH), 6.8 (2H, d, *J*= 7.8 Hz), 6.54 (1H, m), 5.92 (1H, bs, OH), 5.06 (1H, bs, NH), 4.58 (1H, m, unresolved), 3.02 (2H, unresolved m)

^{13}C NMR (CD_3OD, 75 MHz)

δ 173.5, 153.7, 157.1, 131.2, 129.1, 115.1, 71.5, 52.8 (C-D, spet, J = 35), 37.8, 25.2

CHAPTER THREE

Synthesis of Metoclopramide Derivatives

Introduction

The benzamides represent a class of drugs, which have experienced an unusually broad application for the clinical development. So far these agents are being, or have already been, developed as clinically relevant antipsychotics (sulpiride), antiarrhythimics (procainamide), diabetics (nicotinamide), local anesthetics (lidocaine), and antiemetics (metoclopramide).[263, 264] The diverse clinical application of these drugs is paralleled by an equally diverse mode of action which can involve effects on tumor microcirculation, inhibition of DNA repair and PARP (poly ADP ribose polymerase), and binding to dopamine (D_2) and hydroxytryptamine (5-HT3) receptors.[265]

Metoclopramide (4-amino-5chloro-N-2 methoxybenzamide, MCA) is a multiply substituted N-(tertiary amino alkyl) benzamide. MCA is most commonly used as an antiemetic, especially in the treatment of chemotherapy-induced nausea and vomiting.[266] In a series of clinical studies it was established that MCA was able to sensitize the radiation therapy and chemotherapy of certain cancers. Although the mechanism of MCA as a radio and chemosensitizer is not completely known, two potential radiosensitizing properties have been proposed; increase the level of DNA damage and an inhibition of DNA repair in the presence of radiation. MCA was also shown to inhibit NF-κB (a transcription factor that protects cells from cytotoxic exposure to DNA damage) and hence facilitate apoptosis.[267]

Apoptosis, or programmed cell death (or "cell suicide"), is a form of cell death in which a controlled sequence of events (or program) leads to the elimination of cells without releasing harmful substances into the surrounding area. Many types of cell damage can trigger apoptosis, and it also occurs normally during development of the nervous system and other parts of the body. Imbalance of apoptosis can lead to several diseases and can also lead to tumor development, since the rate of cell death and cell proliferation are the determining factors for tumor growth.[268] Therefore, MCA can play a key role in anti-tumor therapy as a facilitator of apoptosis.[269]

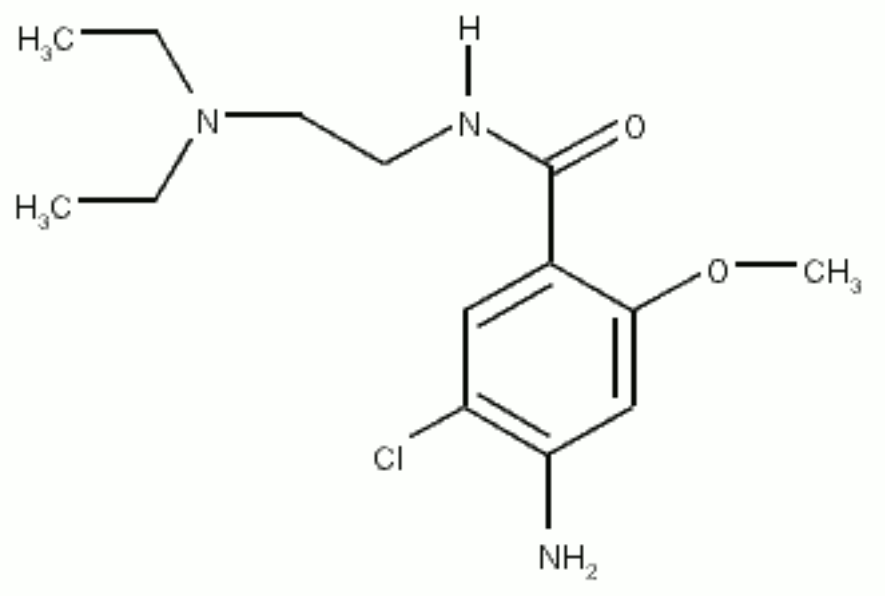


Figure 126. Structure of Metoclopramide

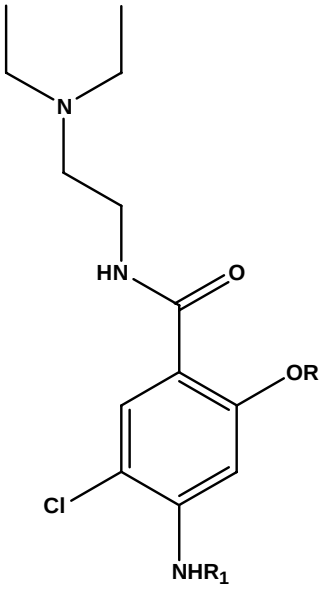
The clinical usefulness of high dose MCA as a radio-and chemosensitizer is limited by its central nervous system (CNS) side effects. Primary side effects include drowsiness, acute extrapyramidal reactions, akathisia (generalized motor restlessness) and drug induced Parkinson's. MCA has also been shown to increase irritability and anxiety. These CNS side effects are chemically related to the ortho-methoxy group in

MCA, which planarizes the molecule and allows it to have a high affinity for dopamine D2 receptors.[270]

Research Goals

MCA is a potentially valuable drug that has had its usefulness limited due to its CNS side effects. Since the ortho-methoxy group was suggested to be the cause of the CNS side effects, we hypothesized that modifications at the ortho-methoxy group could decrease the CNS side effects and increase cytotoxicity. Several derivatives of the O-substituted MCA were synthesized and their structure activity relationship (SAR) was studied.

The straightforward synthesis of the MCA analogs began with the synthesis of the MCA-phenol from metoclopramide. This was accomplished with the base mediated removal of the orthomethoxy group from metaclopramide to give the MCA-phenol. To synthesize the various analogs, MCA-phenol was deprotonated with NaOH, and reacted with a variety of alkylide halides. To obtain the acylaylated products, once the MCA was reacted with the desired alkylide halides, the compound was then reacted with acetic anhydride. The compound were then purified and tested for cytotoxicity using an MTT assay. The MCA-analogs were screened against three different cell lines, and MCA was used as the control drug. The cell lines used were the two H2982, HL-60 and PMBC (non-cancerous, control), with does concentrations for each drug at 5, 10, 50, 100, 250 and 500 μM. Cell viability was reported as an IC_{50} value for each drug, as determined by the dose that resulted in 50% inhibition of cell growth compared to the controls.



Compound

18 R=CH_3, R_1=H

19 R=benzyl, R_1=H

20 R=benzyl, R_1=CH_3CO

21 R=$(CH_2)_{10}I$, R_1=H

22 R=$(CH_2)_{10}$MCA, R_1=H

23 R=$(CH_2)_{10}I$, R_1=CH_3CO

24 R=$(CH_2)_{10}$MCA, R_1=CH_3CO

Figure 129. Structures of synthesized MCA analogs

Conclusion

Six Metoclopramide analogs were synthesized and their structure activity relationship was evaluated by measuring the cytotoxity of the analogs by MTT assays. The MTT assay is widely used to measure cell proliferation and to screen for anticancer drugs. The six MCA analogs were tested on three different cell lines. H2982 cells are human lung adenocarcinoma cell lines and are known to be selectively resistant to apoptosis. The HL-60 cells are human B cell leukemia cells and are prone to spontaneous apoptosis, therefore they are very sensitive to drugs which induce apoptosis. PBMC are peripheral blood mononuclear cells and were used as control cells (non-cancer cells). The mean IC_{50} values were obtained from a minimum of five repeat experiments and represent the doses that resulted in 50% inhibition of cell growth compared to the untreated control cells. The results showed that the MCA analogs were more toxic to cancer cell lines when compared to the control MCA. However, these MCA analogs

were also just as toxic to the PBMC (non-cancer cells, see Figure 126). This indicated that the drugs did not distinguish between cancerous and non cancerous cell types, and if used clinically, they would have detrimental side effects. Based on these results, as well as results from other analogs synthesized by our group, we decided not to continue with further MCA analog synthesis.

Table 1. IC_{50} values for the MCA-analogs in three different cell lines

Drug	H2982 IC_{50} (μM)	S. D.	HL-60 IC_{50} (μM)	S. D.	PBMC IC_{50} (μM)	S. D.
MCA	530	170	357	150	410	85
MCA-benzyl	115	13	87	14	72	12
MCA-benzyl-acetylated	130	34	118	45	88	10
MCA-monomer	123	16. 5	104	25. 2	118	12
MCA-dimer	117	15	86	15	102	19
MCA-monomer-acetylated	85	12	63	15	66	40
MCA-dimer-acetylated	42	22	64	11	80	13

Materials and Methods

Chemicals and Reagents

Except where otherwise noted, reagents used for the synthesis were obtained from Aldrich Chemical CO., Milwaukee, WI, and Fisher Scientific, Pittsburgh, PA (including Across reagents) and used directly as purchased. Solvents such as dichloromethane, hexane, ethanol, ethyl acetate and methanol were obtained from commercial sources (via

Baylor University Chemistry Department stockroom) and were distilled prior to use. Deionized water (DI) was obtained via a US Filter system provided by Baylor Dept. of Chemistry.

Instrumentation

Proton (^{1}H) and carbon (^{13}C) NMR were obtained on a Bruker AVANCE 300 NMR running XWIN-NMR 3.1 software at 300MHz. Chemical shifts are expressed in ppm(∂), peaks are listed as singlets (s), doublets (d), triplet (t) or multiplet (m), with the coupling constant (J) expressed in Hz. HPLC was carried out with a Beckman System Gold, composed of a model 168 detector and a model 126 solvent module using a Alltech Alltima C-18 5mm 33mm*7mm "rocket" column.

Synthesis of Compounds

Before any analogs were to be synthesized, MCA had to be converted into the MCA-phenol. This compound was then used as the starting material for subsequent analog synthesis. MCA-phenol was synthesized as outlined in Figure 127 via the procedure outlined by Kato S. et al.[271]

4-amino-5-chloro-N-(2-diethylamino-ethyl)-2-hydroxy-benzaldehyde (MCA phenol)

10.004 g (30 mmol) of metoclopramide (MCA) HCl salt was added to 100 ml of DMF in a round bottom flask with a dry argon feed. 1.80 g (74 mmol) of NaH was then added to the reaction and the reaction was stirred over ice for about 5 min. 3.4 ml of ethanethiol was added and the reaction was stirred at room temperature for 30 min, followed by refluxing for 4 hr. The reaction mixture was concentrated using a rotovap,

leaving a dark brown liquid. The brown liquid was dissolved in water followed by 3 washes with 100 ml portions of chloroform.

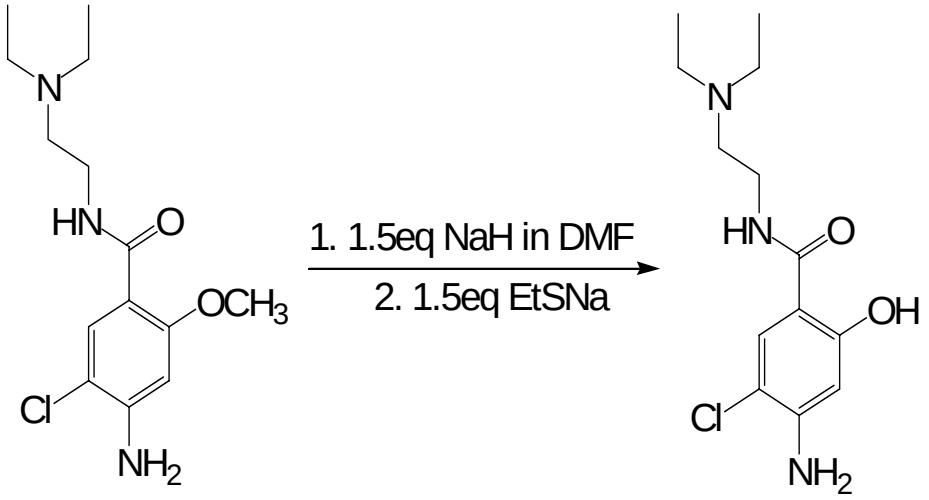


Figure 130. Synthesis of MCA phenol (18)

The water layer was neutralized with 10% HCl and then washed with 3 portions of 100 ml ethyl acetate. The ethyl acetate layers were pooled and washed with water and brine. The ethyl acetate extract was then dried over $MgSO_4$ and rotovapped, leaving a hard yellow solid (7.5 g, 88% yeild). The purity and structure of the product was verified by TLC and proton NMR.

^{1}H-NMR ($CDCl_3$, 300MHz)

δ 8.56 (1H, s), 7.02 (1H, s), 6.27 (1H, s); 5.85 (2H, s), 5.29 (1H, s), 3.42 (2H, t, J = 6.7), 2.61 (2H, t, J = 6.7); 2.04 (4H, q, J = 2.4), 1.05 (6H, t, J = 2.4)

^{13}C NMR ($CDCl_3$, 75 MHz)

δ167.9, 159.2, 145.6, 116.8, 111.2, 113.3, 107.5, 55.0, 46.4, 42.4, 13.3

MCA-benzyl Ether

4-amino-2-benzyloxy-5-chloro-N-(2-diethylamino-ethyl)-benzamide

To 1 g (3.5 mmol) of MCA-phenol in 15 ml of NaOH was added 3ml of benzyl chloride and 3.4 g (11 mmol) of tetrabutyl ammonium bromide in 5 ml of dichloromethane. The reaction was then vigorously stirred at room temperature, and was complete at the end of 4 hr as observed by TLC. The light yellow solution was then added to a separatory funnel containing 50 ml of 1N NaOH. The organic product was extracted with 3 portions of 100 ml dichloromethane. The organic extract was then washed with DI water and brine and dried over MgSO4.

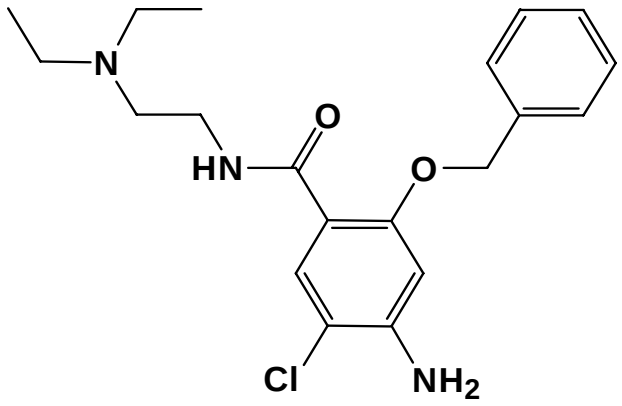


Figure 131. Structure of 4-amino-2-benzyloxy-5-chloro-N-(2-diethylamino-ethyl)-benzamide **(19)**

The washed dichloromethane layer was filtered and rotovapped down to dryness to yield a yellow solid. The crude product was purified using flash column chromatography (Biotage-Flash system) on silica using dichloromethane:hexane:triehylamine (1:2:1) as solvent to yield 0.85 g, 65% yield, of the final product. The purity of the product was verified using TLC and NMR.

^{1}H NMR ($CDCl_3$, 300MHz)

δ 8.56 (1H, s), 7.36 (1H, s), 7.18 (2H, m), 7.11 (2H, m), 7.01 (1H, m), 6.09 (1H, s), 5.85 (2H, m), 5.02 (2H, s), 3.30 (2H, t, J = 7.2Hz), 2.66(2H, t, 7.2Hz), 2.40 (4H, q, J = 7.14), 1.20 (6H, t, J = 7.14)

^{13}C NMR ($CDCl_3$, 75 MHz)

δ 164.9, 159.8, 151.5, 141.0, 129.5, 126.7, 126.1, 125.1, 112.7, 110.5, 102.5, 77.4, 55.0, 46.4, 43.3, 13.8

Acylation of the MCA-benzyl Ether

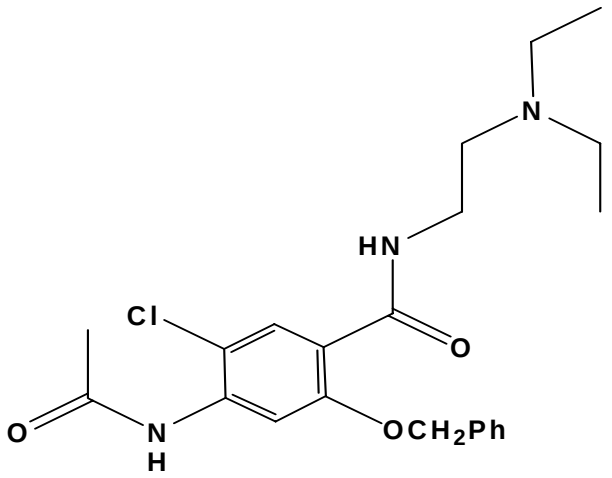


Figure 132. Structure of 4-acetylamino-2-benzyloxy-5-chloro-N-(2-diethylamino-ethyl)-benzamide ***(*20)**

4-acetylamino-2-benzyloxy-5-chloro-N-(2-diethylamino-ethyl)-benzamide

Acetylation at the para amino position was performed by mixing 0.5 g (~1. 5mmol) of MCA-benzyl ether with 5 ml of acetic anhydride and heating to 100^0C in an oil bath for 1hr. The reaction completion was confirmed by TLC. The reaction was cooled to room temperature and added to a seperatory funnel containing 50 ml of 1M

NaOH. This mixture was extracted with 3 portions of 50 ml dichloromethane, the organic extracts dried over $MgSO_4$, and rotovapped leaving a brown oily product. The product was dissolved in dichloromethane to which 5 g of silica was added, and the solvent was evaporated leaving behind a crude product absorbed onto the silica gel. The silica gel containing the crude product was packed into a Biotage FLASH sample injection module (SIM) cartridge which was then eluted though a silica FLASH 40S cartridge using dichloromethane:hexane:triethly amine (1:2:1) using Biotage FLASH 40 chromatography system. The fractions containing the product were pooled and rotovapped down to yield 0.2 g, 33% yield, of the final product. The purity of the product was verified by TLC and NMR.

^{1}H-NMR ($CDCl_3$, 300MHz)

δ 8.56 (1H, s), 7.26 (1H, s), 7.19 (2H, m), 7.1 (2H, m), 7.01 (1H, m), 6.09 (1H, s), 5.02 (2H, s), 3.30 (2H, t, J = 7.2Hz), 2.66 (2H, t, 7.2Hz), 2.40 (4H, q, J = 7.14), 2.02(3H, s), 1.20 (6H, t, J = 7.14)

^{13}C-NMR ($CDCl_3$, 75 MHz)

168.2, 167.9, 157.2, 151.5, 140.0, 129.5, 127.7, 127.2, 127.1, 112.8, 111.5, 102.5, 77.4, 55.0, 46.4, 43.3, 17.6, 13.7

MCA Momoner

4-amino-5-chloro-N-(2-diethylamino-ethyl)-2-(10-iodo-decyloxy)-benzamide

1 g of the MCA phenol was dissolved in 5ml of 1M NaOH and 3. 6 g of tetrabutyl ammonium bromide was added. The mixture was allowed to stir for five minutes and then 4.5 g of diiodiodecane dissolved in 7 ml of dichloromethane was added. The

reaction mixture was stirred under argon for 15 hrs at room temperature, at which time it was confirmed to be complete by TLC.

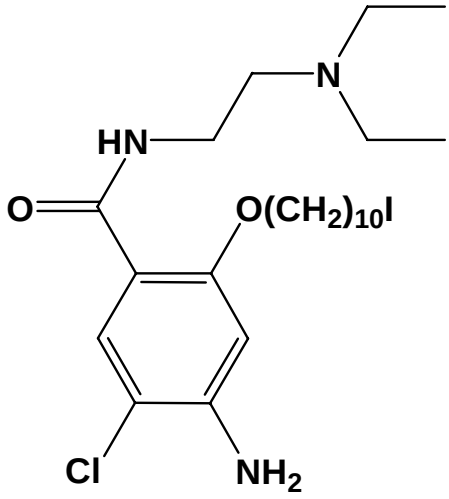


Figure 131. Structure of 4-amino-5-chloro-N-(2-diethylamino-ethyl)-2-(10-iodo-decyloxy)-benzamide ***(21)***

The product was extracted by washing the reaction mixture with three 100 ml portions of dichloromethane. The organic layer was washed with water and brine, dried over $MgSO_4$ and rotovapped to dryness leaving behind 0.5 g of a brown oily liquid. The liquid was redissolved in ethanol, 5 g of silica was added to the mixture and dried to a powder. The product containing silica was packed into a SIM cartridge and run through a FLASH 40S column using 1:1:1 ratio of dichloromethane, hexane and triethyl amine respectively. The fractions containing the pure product were pooled and rotovapped leaving 0.3 g, 16% yield, of the MCA-monomer. The purity of the product was confirmed by TLC and NMR.

^{1}H-NMR ($CDCl_3$, 300MHz)

δ 8.56 (1H, s), 7.60 (1H, s), 6.09 (1H, s), 5.00(2H, bs, NH_2), 3.94 (2H, t, J = 8.0), 3.33 (2H, t, J = 7.02), 3.00 (2H, s), 2.66 (2H, t, J = 8.0), 2.40 (4H, t, J = 7.3), 1.71 (2H, tt, J = 7.02, 7.3), 1.29-1.12 (14H, m) ,1.06 (6H, q, J = 7.4),

^{13}C-NMR ($CDCl_3$, 75 MHz)

167.9, 155.5, 154.2, 129.9, 112.0, 110.6, 102.5, 101.4, 71.9, 55.0, 46.4, 43.3, 34.3, 32.5, 30.3, 29.9 29.6, 25.7, 23.1, 13.7, 6.8

MCA Dimer

1g of the MCA phenol was dissolved in 5 ml of 1 M NaOH and 3.6 g (5 mmol) of tetrabutyl ammonium bromide was added.

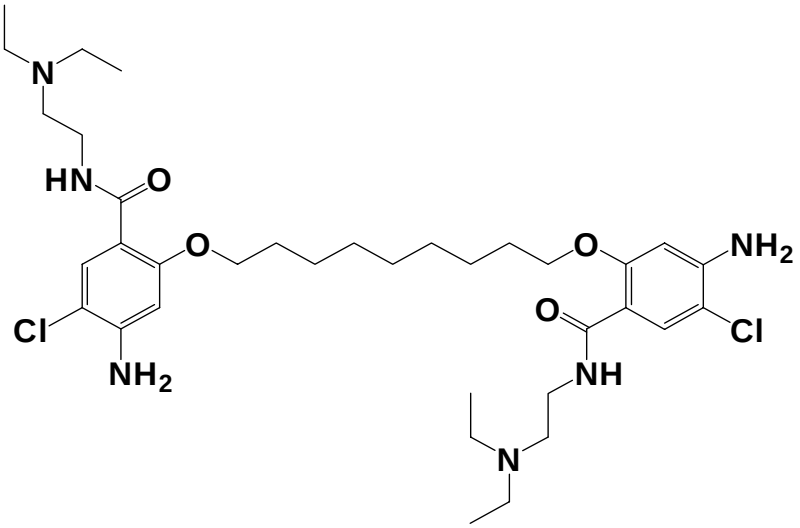


Figure 132. Structure of MCA dimer (**22**)

The mixture was allowed to stir for five minutes and then 4.5 g of diiodiodecane dissolved in 7 ml of dichloromethane was added. The reaction was stirred under argon for 15 hrs at room temperature, at which time the reaction was confirmed to be complete

by TLC. The product was extracted by washing the reaction mixture with three 100 ml portions of dichloromethane. The organic layer was washed with water and brine, dried over $MgSO_4$ and rotovapped to dryness leaving behind 0.5 g of a brown oily liquid. The liquid was redissolved in ethanol and 5 g of silica was added to the mixture. The ethanol was evaporated using reduced pressure leaving the product absorbed onto the silica. The product containing silica was packed into a SIM cartridge and run through a FLASH 40S column using 1:2:1 ratio of dichloromethane, hexane and triethyl amine respectively. The fractions containing the pure product were pooled and rotovapped leaving 0.4 g, 17% yield, of the MCA-dimer. The purity of the product was confirmed by TLC and NMR.

^{1}H-NMR ($CDCl_3$, 300MHz)

δ 8.58 (2H, s), 7.60 (2H, s), 6.09 (2H, s), 5.00 (4H, bs, NH_2), 3.94 (4H, t, J = 8.0), 3.33 (4H, t, J = 7.02), 2.66 (4H, t, J = 7.02), 2.40(8H, t, J = 7.3), 1.86(12H, q, J = 7.4), 1.71 (4H, tt, J = 8.0, 7.3), 1.29-1.12 (10H, m)

^{13}C-NMR ($CDCl_3$, 75 MHz)

165.7, 156.2, 155.2, 129.1, 110.6, 102.5, 71.9, 55.0, 46.4, 43.3, 34.3, 32.5, 29.6, 23.2, 13.5, 6.8

MCA monomer Acetylated

4-acetylamino-5-chloro-N-(2-diethylamino-ethyl)-2-(10-iodo-decyloxy)-benzamide

1 g (2mmol) of the MCA-monomer was dissolved in 5 ml of acetic anhydride. The reaction was stirred under argon for 4 hr at 100^0C, at which time TLC revealed the reaction to be complete. The mixture was cooled to room temperature and added to a

separatory funnel containing 1M NaOH, which was then extracted with three 50 ml portions of dichloromethane.

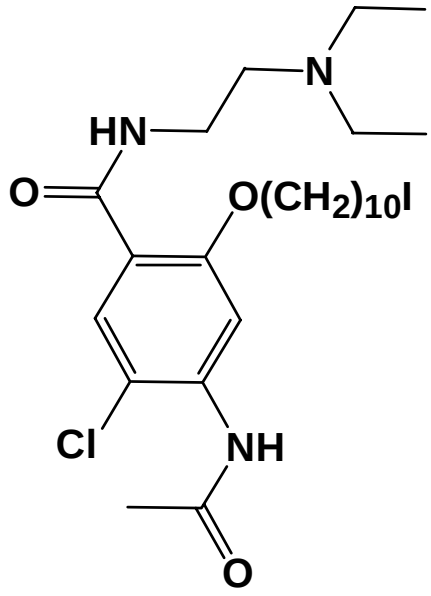


Figure 133. Structure of 4-acetylamino-5-chloro-N-(2-diethylamino-ethyl)-2-(10-iodo-decyloxy)-benzamide (**23**)

4g of silica gel were added to the combined dichloromethane layers which were concentrated using a rotovapp, leaving the product absorbed onto the silica. The silica was packed into a SIM cartridge and eluted though a FLASH 40s silica column using the Biotage Flash chromatography system1:3:1 ratio of dichloromethane, hexane and triethyl amine respectively. The factions containing the product were pooled and rotovapped leaving behind 0.42 g, 40% yield, of MCA-monomer.

^{1}H-NMR ($CDCl_3$, 300MHz)

δ 8.56 (1H, s), 7.60 (1H, s), 6.19 (1H, s), 5.53 (2H, bs, NH_2), 3.87 (2H, t, J = 8.1), 3.33 (2H, t, J = 7.02), 3.02 (2H, s), 2.66 (2H, t, J = 7.02), 2.40(4H, t, J = 7.3), 2.02 (2H, s), 1.86 (6H, q, J = 7.4), 1.71 (2H, tt, J = 8.0, 7.3), 1.29-1.12 (14H, m)

^{13}C-NMR ($CDCl_3$, 75 MHz)

167.1, 156.5, 153.2, 129.7, 112.0, 110.6, 105.2, 102.5, 101.5, 71.9, 55.0, 54.8, 46.4, 43.3, 34.3, 32.5, 30.1, 29.9 29.4, 25.6, 23.2, 13.7, 6.7

MCA-dimer-Acetylated

1g of the MCA-dimer-acetylated was synthesized as above with a reaction time of 5 hr. The yield of the pure MCA-dimer acetylated was 0.4 g, 36% yield.

The purity of the product was verified by TLC and NMR.

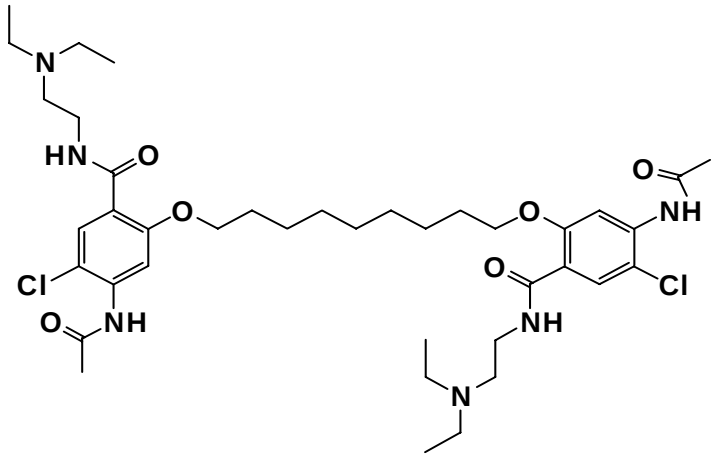


Figure 134. Structure of MCA-dimer acetylated ***(*24**)

^{1}H-NMR ($CDCl_3$, 300MHz)

δ 8.50 (2H, s), 7.64 (2H, s), 6.19 (2H, s), 5.50 (4H, bs, NH_2), 3.92 (4H, t, J = 8.0), 3.33 (4H, t, J = 7.0), 2.66 (4H, t, J = 7.02), 2.45(8H, t, J = 7.3), 2.12 (6H, s), 1.86(12H, q, J = 7.4), 1.71 (4H, tt, J = 8.0, 7.3), 1.29-1.12 (10H, m)

^{13}C-NMR ($CDCl_3$, 75 MHz)

165.7, 156.2, 155.2, 129.1, 110.6, 102.5, 71.9, 55.0, 54.0, 46.4, 43.3, 34.3, 32.5, 29.6, 23.2, 15.6, 13.5, 6.8

Cytotoxicity Assays

The analogs synthesized were tested for cytotoxicity by determining their IC_{50} upon exposure to normal and cancer cell lines. The tests were carried out with the help of Elizabeth Keschman (MS, Institute of Biomedical Studies, Baylor University, 1999)

Cell Culture

Human B cell leukemia HL-60 cells were supplied by Oxigene Europe (Lund, Sweden). Cells were maintained in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS), 2nM L-glutamine, 10nM HEPES buffer, 2mM MEM sodium pyruvate (all obtained from Gibco-BRL, Grand Island, NY) and antibiotics (Sigma, St Louis MO). Peripheral blood mononuclear cells (PBMC) were obtained from various normal human donors. They were also maintained in RPMI-1640 medium supplemented with 10% FCS, 2nM L-glutamine, 10% Concanavalin A (Con A, Sigma, St Louis, MO) and antibiotics. Both cell types were maintained at 37^0C in a 5% CO_2 atmosphere and passaged regularly when confluent. Cell density and viability were assayed by trypan blue excusion.

MTT Assay

The MTT Cell Proliferation Assay is a colorimetric assay system which measures the reduction of a tetrazolium component (MTT) into an insoluble formazan product by the mitochondria of viable cells. After incubation of the cells with the MTT reagent for approximately 2 to 4 hours, a detergent solution is added to lyse the cells and solubilize the colored crystals. The samples are read using an ELISA plate reader at a wavelength of 570 nm. The amount of color produced is directly proportional to the number of

viable cells. The MTT system is a quantitative, more sensitive test. Because there is a linear relationship between cell activity and absorbance, the growth or death rate of cells can be measured; the trypan blue test is qualitative and indicates only if a cell is alive. The MTT assay can also be adapted to high-throughput screening, whereas trypan blue tests must be read individually.

To test for cytotoxicity, cancer cells were seeded at 2000 cells per well and PBMC were seeded at 10,000 cells per well in 90ul RPMI-1640 with 10%FCS in a 96 well plate. The cells were incubated for 24 hrs at 37^0 C before treatment. Serial dilutions of the drugs were added to the cells and the final culture volume was brought to 100 μL per well. For each concentration, triplicate wells were run and each experiment was performed at least five times. The cells were incubated for 5-6 days at 37^0 C. The PBMCs required stimulation with ConA every 2-3 days during incubation. After 5-6 days, cell viability was analyzed by the MTT (3-(4,5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide) assay. Optical densities were measured at 429nm using a Labsystem 96 well multiscanner (Fisher Scientific, Dallas, TX) and the data were analyzed using Microsoft Excel. Cell viability was reported as an IC_{50} value for each drug, as determined by the dose that resulted in 50% inhibition of cell growth compared to control cells.

Table 2. IC_{50} values for MCA-analogs

Drug	H2982 IC_{50} (μM)	S. D.	HL-60 IC_{50} (μM)	S. D.	PBMC IC_{50} (μM)	S. D.
MCA	530	170	357	150	410	85
MCA-benzyl	115	13	87	14	72	12
Drug	H2982 IC_{50} (μM)	S. D.	HL-60 IC_{50} (μM)	S. D.	PBMC IC_{50} (μM)	S. D.
MCA-benzyl-acetylated	130	34	118	45	88	10
MCA-monomer	123	16. 5	104	25. 2	118	12
MCA-dimer	117	15	86	15	102	19
MCA-monomer-acetylated	85	12	63	15	66	40
MCA-dimer-acetylated	42	22	64	11	80	13

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