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Insights into cellular adhesion: reducing agent and thiol blocker treatments

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Thesis

**INSIGHTS INTO CELLULAR ADHESION:
REDUCING AGENT AND THIOL BLOCKER TREATMENTS**

by

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ABSTRACT

Expected trace or low-level amounts of deoxyribonucleic acid (DNA) swabbed or cut from evidence prove crucial in the removal of as much of that DNA as possible from the substrate in order to obtain a probative profile. While cotton substrates contain polar side groups aiding in the retrieval of biological matter, purifying DNA further from that fabric proves difficult. Failure to remove the cells from the substrate before amplification could result in the presence of unwanted inhibitors from the substrate, inhibiting the polymerase chain reaction (PCR).

Cellular adhesion molecules (CAMs), specifically integrin, are known to play a role in cellular adhesion to a variety of matrices. Through reduction and oxidation (redox) reactions, integrin is able to shuffle its allosteric disulfide bonds, exposing free thiols that may be necessary in promoting adhesion.

Through the preparation of epithelial cells placed onto a cotton fabric swatch or a cotton swab, different variables were able to be tested including the use of disaccharides to reduce cell adhesion, the use of reducing agents to strengthen cell adhesion, and the use of thiol blockers to inhibit the effects of the reducing agents. Through this experimentation, 0.75 M D-(+)- Trehalose Dihydrate showed to improve cellular recovery from the cotton fabric, while reducing agents such as Dithiothreitol

(DTT) and Tris(2-carboxyethyl)phosphine Hydrochloride (TCEP) impeded cellular recovery. However, unexpected results occurred with the use of N-ethylmaleimide (NEM) thiol blocker, leading to ambiguous results. Through these experiments, insights into the cause of cellular adhesion has been revealed.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
CAM	Cell Adhesion Molecules
CODIS	The Combined DNA Index System
CRF	Cellular Release Fraction
°C	Degrees Celsius
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
ECP	Epithelial Cell Preparation
FBI	Federal Bureau of Investigations
T _g	Glass Transition Temperature
g	Grams
In	Inch
Kd	Kilodalton
MF	Material Fraction
qPCR	Quantitative Polymerase Chain Reaction
T _m	Melting Temperature
μL	Microliters
mL	Milliliters
mM	Millimolar
M	Molar
NEM	N-ethylmaleimide

ng	Nanograms
PBS	1X Phosphate-buffered Saline
PCR	Polymerase Chain Reaction
PDI	Protein Disulfide Isomerase
redox	Reductive/Oxidative Reactions
RCF	Relative Centrifugal Force
RPM	Rotations per Minute
STR	Short Tandem Repeats
SDS	Sodium Dodecyl Sulfate Lysis Buffer
TE	Tris-EDTA Buffer
TCEP	Tris(2-carboxyethyl)phosphine Hydrochloride

1. INTRODUCTION

1.1 DNA Recovery in Forensic Casework

Forensic deoxyribonucleic acid (DNA) analysis utilizes the Polymerase Chain Reaction (PCR) to amplify short tandem repeats (STR) from the human genome (1). STR loci typically range in repeat unit length from four to five base pairs, and many in the number of repeats, and the complexity of those repeats. These polymorphic qualities of STR analysis make them useful to individualize and create probative linkages between humans. Most commercial forensic STR kits used in forensic laboratories amplify twenty-four of the most commonly used autosomal loci in a multiplex fashion (1)(2). In the future, more STR loci may be included in common commercialized kits to further provide DNA discrimination especially in complex cases involving trace or low level amounts of DNA (1).

Significant quantities of DNA have been shown to be retained on common substrates such as cotton fabrics or swatches (3). Substantial amounts, 20-76% of DNA collected from a cotton cloth or swab is lost during the extraction process due to the type of extraction performed, type of substrate the DNA was collected onto, the amount of DNA present, and the condition of the DNA (3)(4). Collecting and releasing a maximum amount of DNA is crucial to generate the clearest, most informative profile (3).

1.2 Common Collection Methods for DNA Evidence

Collecting the sample is the first step in generating a DNA profile. Subsequent to collection of the cells, the cells must be released from the substrate, then lysed in order to analyze the DNA. The term cellular recovery, used throughout this paper, will refer to the process of cellular removal from the substrate followed by cell lysis in order to purify and quantify the DNA. Collection, however, is crucial, as collecting a maximum amount of DNA is beneficial to generate an informative profile. Common collection methods include swabbing, cutting, and tape lifting. Swabbing and tape lifting are useful when cutting is not practical. Swabbing works best on smooth, non-porous surfaces, while tape lifting works better on porous, rough surfaces (5). The double swab technique to collect DNA has been a favored technique. This technique involves swabbing an area with a moistened swab then following up with a dry swab (6). While swabbing is very effective to minimize destruction and collect DNA from non-porous surfaces, cutting is preferential when low levels of DNA are suspected on fabric substrates. While cutting will help to minimize the loss of DNA, it may introduce chemicals or dyes from the fabrics that can have inhibitory effects on the PCR reaction (5).

Each collection method has its benefits and drawbacks, therefore, the analyst working on the case must be able to look at the sample and determine what collection method will maximize the lift and release of enough cells to yield an informative DNA profile.

1.3 DNA Extraction Methods

DNA is packaged, consolidated and stored within the membrane of the cell nucleus. The DNA is coupled to positively charged proteins called histones that link to form the basic unit of DNA packaging known as the nucleosome. The nucleosome includes approximately 146 base pairs of DNA wound around eight histone proteins. Together all the nucleosome units coil up and form the chromatin structure, or the final packaging of the DNA. In order to obtain a probative profile, an analyst must disrupt these membranes and remove the proteins surrounding the DNA molecules, via DNA extraction. Ideally, the extraction process should remove not only these protective membranes and proteins complexed to the DNA, but also should remove any other cellular proteins or substrate chemicals that could elicit inhibitory effects on PCR. In theory, the process of DNA extraction is simple; lyse the cell membranes to release the DNA, digest the proteins, and purify the DNA. However, ensuring the removal of all the inhibitors and cellular contents, as well as minimizing the loss of DNA may prove difficult (7).

1.3.1 Cell Lysis Followed by DNA Purification

Indirect lysis is a two-step extraction method that involves breaking the cellular membranes and digesting the proteins, followed by purifying the DNA. Cell lysis is typically performed using Sodium Dodecyl Sulfate Lysis Buffer (SDS) and Proteinase K, while DNA isolation utilizes a series of washes and filtering. Organic extractions, such as phenol/chloroform use an organic phenol/chloroform mixture to separate the DNA in

solution from the organic components of the cell. This method “cleans up” the DNA by removing a majority of the other cellular material, however, phenol and chloroform are hazardous chemicals to work with (7).

Silica based extractions separate the DNA from the cell material through the use of chaotropic salts and silica filters or beads to which the DNA adsorbs. Similar to the phenol/chloroform method, silica based extractions are able to efficiently separate out the DNA from the other cellular material while eliminating the use of harmful organic solvents. However, silica based extractions may lose DNA over the silica filters (7)(8).

1.3.2 Direct Cell Lysis

Direct lysis breaks the cellular membranes and digests the proteins from the DNA all in one step. Thus, the DNA, along with the cellular material remain in the solution together. Direct lysis procedures are fast and use minimal tube transfers that allow for higher DNA recovery, but fail to isolate the DNA away from possible PCR inhibitors. Chelex® 100 resin is one example of a direct lysis method. Chelex® 100 utilizes the principles of ion exchange to bind to metal ions and other cellular components. The mixture is heated to break open the cells and denature the proteins, leaving the DNA unbound and PCR ready (7)(8).

1.3.3 EAI Proteinase

Proteins and enzymes discovered and isolated from thermophilic organisms have proved to be extremely useful in the biotechnology industry due to their unique abilities

to withstand extreme environmental conditions. These thermophilic organisms have been shown to have similar proteins to well-known mesophilic organisms with only slight differences in their amino acid sequence (9). A gene from a neutral thermostable proteinase isolated from the thermophilic organism, *Bacillus* sp. EA1 strain, the proteinase was found to be extremely useful in DNA extraction methods (10). This proteinase is active around 70 degrees Celsius (°C), but inactive at higher temperatures of 85°C to 90°C. It has also been found to work compatibly with buffers commonly utilized in DNA extractions and is hydrolytic, eliminating the need for detergents, thus causing inhibitors to remain insoluble (9).

MicroGEM Bio Corporation has marketed ForensicGEM™ (ZyGEM Corporation Limited, Hamilton, New Zealand), a recombinant version of this proteinase that has been used for direct lysis extractions in DNA and RNA extractions. The lack of tube transfers and elimination of detergents allow for maximum extraction of DNA, making ZyGEM a desirable option for extracting low level samples (9).

1.4 Structure of the Eukaryotic Epithelial Cellular Membrane

The cell membrane is a complex intertwinement of carbohydrates, lipids, proteins, and phospholipids forming a bilayer structure. The phospholipid groups are made up of a polar head attached to two non-polar fatty acid chains. The head group faces outward into either the interior or the exterior of the cell. In between these head groups are the tails, facing inwards towards each other forming the interior of the membrane as shown in Figure 1. This arrangement of the phospholipids maximizes hydrophilic interactions on

the borders of the membrane while simultaneously maximizing hydrophobic interactions within the membrane creating a desirable low free energy state (11).

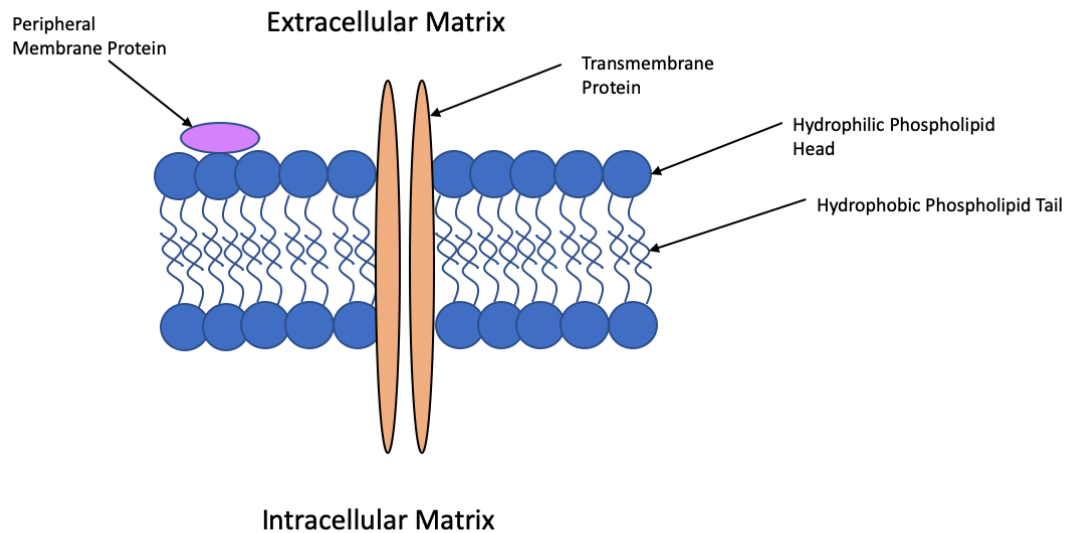


Figure 1. General depiction of the structure of a cell membrane.

The membrane is described as dynamic and fluid with the ability to change based on variations in its environment. The lipids in the membrane, such as the phospholipids, move rotationally and laterally throughout the surface of the membrane. The degree of saturation, length of the fatty acid tails, the amount of cholesterol, and the addition of heat or cold can all make a membrane more or less fluid. Increasing the fluidity of the membrane will increase the disorder and permeability of the structure (12)(13).

A variety of different protein types have been found within the membrane. These proteins can either be integral transmembrane proteins, spanning the width of the entire membrane or peripheral proteins attached to either the internal or external surface of the

membrane. Peripheral proteins are held onto the membrane by weak noncovalent interactions, therefore, they are not strongly correlated with the membrane (11). A majority of the proteins found on or within the cell membrane may not be individual components. Instead they can assemble in groups, moving throughout the membrane to work together to initiate and maintain cell signaling, cell adhesion, ion transport and a variety of other essential functions (13). Changes within the exterior or interior cellular environment will ultimately lead to downstream changes in the structure and function of the cell membrane (12).

1.4.1 Dehydration of the Cell Membrane and Corresponding Proteins

Water is an essential molecule needed for proper membrane assembly, stability, and function. Water acts as a spacer within the membrane, separating the phospholipid head groups to maintain an optimal level of fluidity. Not only does water help to organize the phospholipids, but water also helps to organize other lipids and proteins found within the membrane structure. When the cell membrane dehydrates, which commonly occurs in many forensic case samples before examination, the structure of the membrane will change via changes in the hydrophilic interactions between the phospholipid heads (14). When drying occurs energy is transferred from the source to the water within the cell causing a phase transformation of the cell from liquid to solid, removing vapors away from the cell (15). This will cause a decrease in the space in between the phospholipids and proteins, packing them together, thus increasing the membrane melting temperature

(T_m) and causing the membrane to become leaky (16)(17). This change can be damaging to the cell and prevent optimal functioning of the cell and its membrane components (18).

1.5 D-(+)- Trehalose Dihydrate

D-(+)- Trehalose Dihydrate is a naturally occurring disaccharide composed of two glucose units linked via an α - α 1,1 glycosidic linkage. Trehalose performs crucial functions within several anhydrobiotic organisms, serving as a source of energy and carbon, and providing protection from stress involving heat and dehydration (14). The ability of these anhydrobiotic organisms to survive for decades under extreme dehydration and stress is correlated closely to their high concentrations of trehalose, and trehalose's natural ability to act as a stabilizer for cell membrane and proteins encouraging restoration of the membrane back to a similar structure as in the presence of water (14)(18).

1.5.1 Glass Transition Temperature and Vitrification

The glass transition temperature (T_g), is described as “the melting point of amorphous materials” (19). It is the temperature it takes for a substance to transition from a solid state to a more fluid or rubbery state via the addition of heat or water. The glass transition process is complex and is effected by several factors such as morphology and molecular weight, however, full elucidation of why and how the glass transition works is not well understood (17)(19).

Vitrification is defined as the transformation of an organized crystalline structure into an amorphous glass structure. With respect to T_g , vitrification requires that the substance have a glass transition temperature (20). Vitrification has been shown to be efficient at stabilizing cellular membrane components such as proteins, and has been used previously as a bio-preservation and cryoprotective agent (20)(21).

Disaccharides such as sucrose and trehalose are unique as they have the ability to vitrify and form a glass-like matrix in a dry or dehydrated state. However, the T_g of trehalose is the highest of any disaccharide at 115°C, 43°C higher than that of sucrose. The high T_g of trehalose may be responsible for its protective qualities (20)(21).

The ability for trehalose to form a glass-like matrix under dehydrated conditions can help to slow chemical reactions and limit protein movement. This in turn will help to maintain protein structure and function, and prevent unwanted protein aggregation and protein-protein interaction. Furthermore, denatured proteins have a higher free energy state, and therefore have the need to bind to a ligand to lower their energy. In the presence of trehalose, proteins will preferentially bind to the trehalose helping to return them to a lower energy state (17).

While trehalose is regarded as a universal protein stabilizer, especially because it shows no negative effects towards membrane proteins, observations indicate that it does not stabilize all proteins equally well. The nature of the protein's structure, including its primary sequence, length, and side chains can impact the ability of trehalose to stabilize. However, it is not fully known what exact primary structures are more inclined to be stabilized by the introduction of trehalose (22).

The removal of water from a cell will also decrease the free space between the lipid heads of the cell membrane thus increasing the Van der Waal forces between them. This will increase the T_m or the temperature it takes to melt the chains to form a liquid phase. The vitrification of trehalose may help to inhibit the increase in the T_m , thus protecting the cell membrane. However, this will only be beneficial if the T_g of trehalose exceeds the T_m (17).

1.5.2 The Water Replacement Hypothesis

The water replacement hypothesis suggests that trehalose can replace the lost water molecules within the membrane by forming stable hydrogen bonds with the polar heads of the phospholipids. This will mimic the structure and spacing of the membrane as in the presence of water therefore restoring both the membrane's structure and function (14). Furthermore, trehalose can protect the proteins in the same way, by binding to the polar groups of the proteins maintaining their structure, and preventing denaturation during drying (17).

Currently there is not enough evidence to claim that vitrification and the glass transition temperature play a larger role in trehalose's protective qualities than the water replacement hypothesis. It is instead thought that these two theories work in conjunction and one is not mutually exclusive of the other (14).

1.6 Disulfide Bonds

Disulfide bonds are essential in the structure and function of a wide variety of proteins found within eukaryotic cells, including membrane proteins. These covalent bonds formed between the sulfur atoms of two cysteine amino acid residues occur within and between proteins or protein subunits, thus impacting the structure and the function of a protein (23). Disulfide bonds can be classified as structural or functional bonds. Structural disulfide bonds are typically located within the interior of the protein. They assist in protein folding and stabilization and will remain relatively constant throughout the lifespan of the protein, exhibiting few structural changes.

Functional disulfide bonds can change their formations, through reduction and oxidation (redox) reactions thus initiating a change of function. Disulfide bonds are within allosteric control, meaning that a change in the bond structure will cause a change elsewhere in the protein. This change can be as simple as the breakage or formation of a single disulfide bond or as complex as the breakage and formation of several bonds initiating a process known as disulfide bond shuffling. Any rearrangement of disulfide bonds, whether large or small, will result in structural and functional protein changes (23)(24).

1.7 Thiol Blockers

Maleimides such as N-ethylmaleimide (NEM) are a common thiol reactive reagents that will bind readily to free thiols. NEM will react with a free thiol creating a thioether, blocking any future reactions with that thiol. However, before the addition of

the NEM blocker, free thiols must be exposed via the breakage of disulfide bonds by a reducing agent. Dithiothreitol (DTT) and tris(2-carboxyethyl)phosphine hydrochloride (TCEP) are two common reducing agents used to break disulfide bonds. However, DTT unlike TCEP contains two free thiol groups in its structure, as shown in Figure 2, thus competitively binding to NEM blocker. Due to this, it is recommended to use TCEP in conjunction with the NEM blocker in order to prevent unwanted blocker binding to the DTT thiols.

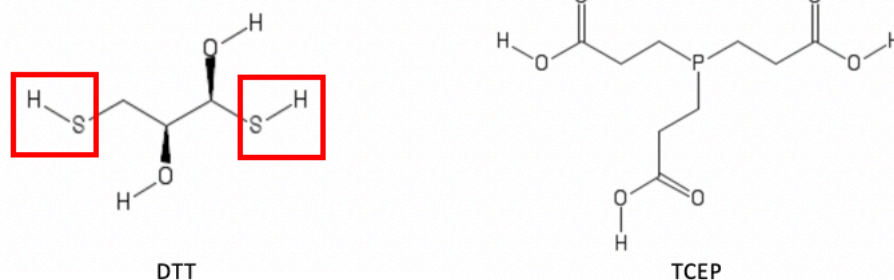


Figure 2. Depiction of the chemical structure of DTT (left) and TCEP (right). DTT has two thiol groups, highlighted in red, which are not ideal for use with thiol blockers.

TCEP has also been shown to be more stable in a neutral and a higher pH as well as perform as a stronger and faster reducing agent than DTT, thus making it a desirable reducing agent to use (25)(26).

1.8 Cell Adhesion Molecules

Cell adhesion molecules (CAMs) are a broad category of signaling molecules that are organized into four distinct groups; integrins, cadherins, selectins, and the immunoglobulin superfamily. These molecules play a crucial role in initiating and mediating cell signaling and interactions involved in embryonic development, inflammation, the immune response, vascular and epithelial cell maintenance, cell organization, cell aggregation, and cell-cell cell-matrix adhesions. These cellular interactions evoked by the CAMs are varied and can be homotypic, heterotypic, can occur between the cell and the extracellular matrix (ECM), or can be a combination of all three (27).

1.8.1 Integrin

Integrin is a heterodimeric transmembrane protein that is involved in mediating cellular adhesion. Its overall structure is made up of two main subunits, the alpha and beta units. Each subunit contains a large extracellular domain, transmembrane domain, and a small cytoplasmic tail that anchors to the cell's cytoskeleton facilitating communication within the cell and its environment. The alpha subunit has a molecular weight of 120 to 180 kilodaltons (Kd), while the beta subunit is slightly smaller with a molecular weight between 90-110 Kd. Integrins are a broad category of CAMs, that can be further divided up into more than twenty specific types of integrin depending upon the beta subunit and its ligand binding specificity. However, even with these varied

categories of integrin, cysteine residues within the beta subunit have been shown to be highly conserved and crucial in structural and functional disulfide bonding (23)(28).

Integrin is mainly found in its low affinity binding state also known as its inactive state. In this state, integrin takes on a bent conformation (29). Once a specific signaling protein binds to its ligand binding site, integrin is activated and undergoes a conformational change into a high affinity extended state, as shown in Figure 3.

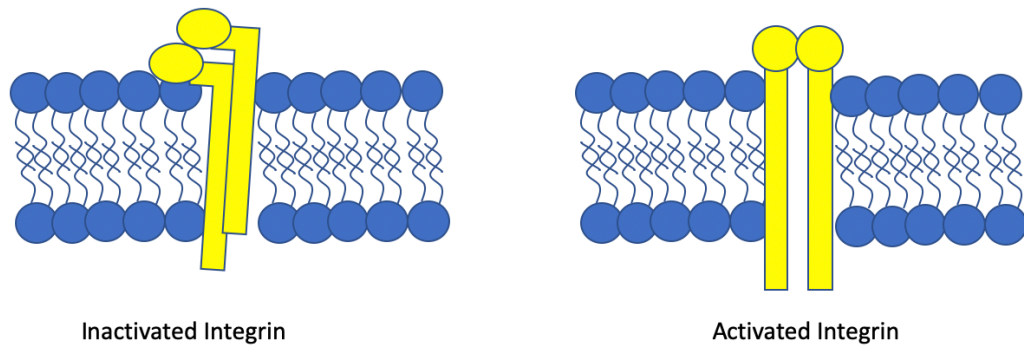


Figure 3. General depiction of integrin in its bent inactive state followed by integrin in its straightened active state. The full mechanism of why and how integrin changes conformation and activates is not fully elucidated.

While the mechanism of activation is not fully elucidated, the highly conserved disulfide bonds within integrin's core structure have been shown to stabilize integrin in its bent conformation, while also acting as a redox switch to trigger integrin activation (23)(29). Levin et al. demonstrated through a series of cysteine knock out mutations of the $\beta 3$ subunit of $\alpha IIb\beta 3$ integrin, that the activation of integrin is much more complex


and can involve the reduction and oxidation of more than one disulfide bond, thus making it difficult to understand the specifics of integrin activation (28).

1.8.2 Integrin and Reducing Agents

Integrins have a cysteine rich domain that can be changed by the introduction of redox chemicals or enzymes. Changing the structure via reduction will ultimately promote integrin activation (30). Within certain eukaryotic and prokaryotic cells, enzymes such as protein disulfide isomerase (PDI) whether endogenous or exogenous will mediate initial integrin activation through reduction of the disulfide bonds. Once the disulfide bonds break, free thiols are formed and integrin is able to readily promote cell adhesion and aggregation (31)(32). Rosenberg et al demonstrated that PDI targets a variety of different integrin types, but will not have an effect on non-integrin receptors, thus highlighting a unique feature of the integrin molecule and its role in adhesion (31).

Furthermore, Yan et al. demonstrated that exogenous DTT treatments of cells has been shown to activate integrin and to increase the levels of free thiols from 2.5 in its bent conformation to 6.6 after the addition of DTT (23)(30). Furthermore, it was found that breaking the disulfide bonds, followed by blocking the free thiols formed inhibited the activation that DTT initially caused. This demonstrates that not only must disulfide bonds be broken, but free thiols must be readily available in order to maintain activation of integrin, and must be present at the time of adhesion (23)(31)(33). These results from various references are summarized below in Table 1.

Table 1. Summary of experimental results obtained from a variety of different peer-reviewed references.

	REDUCING AGENT	BLOCKER	CELL TYPE & MATRIX TYPE	RESULTS
NI ET AL. 1998	0.1 – 100 mM DTT 0.001 – 1 mM Mn ²⁺	N/A	IM9 Cells Fibronectin	<ul style="list-style-type: none"> • Increase in adherence in presence of both DTT and Mn²⁺ • DTT and Mn²⁺ seem to induce conformational changes leading to activation
YAN & SMITH. 2001	3 mM DTT	N/A	AS-1 Cell Fibrinogen	<ul style="list-style-type: none"> • Application of DTT increased cell adhesion • Conformation change of integrin following the application DTT seen via peptide mapping • DTT induces a disulfide bond rearrangement within integrin • Number of free cysteines within the integrin structure increased approximately by 2X within the presence of DTT • Correlation of reduction of disulfide bonds in integrin's redox site to a transition into integrin's active state
MARGARITIS ET AL. 2011	3mM & 10 mM DTT	10 – 500 μM NEM	Platelet cells Collagen	<ul style="list-style-type: none"> • DTT amplified platelet adhesion • DTT treatment followed by NEM treatment inhibited platelet adhesion in dose dependent manner
METCALFE ET AL. 2011	2.5 mM TCEP Various enzymatic treatments	N/A	2B4 Mouse cells	<ul style="list-style-type: none"> • Observed allosteric membrane proteins with disulfide bonds • Enzymatic treatments gave an increase in free sulfhydryl groups than TCEP treatment • Redox-mechanism on these allosteric surface proteins
LEVIN ET AL. 2013	N/A	N/A	β3 Subunit of platelet αIIbβ3 integrin	<ul style="list-style-type: none"> • Mutants with disulfide bond disruption will prohibit disulfide exchange thus being unable to activate • Suggest disulfide bond rearrangement is essential for integrin activation
POPIELARSKI ET AL. 2018	200 μM TCEP PDI	20 mM NEM (10 mins) PDI Blockers	Human skin fibroblast Collagen	<ul style="list-style-type: none"> • Thiol blockers and PDI blockers reduced adhesion of human skin fibroblasts to collagen matrix • PDI and TCEP increase attachment of cells to matrix, however, exact mechanism of PDI is unclear
ROSENBERG ET AL. 2019	Exogenous PDI	0-200 μM pCMPS 0-1 mM DTNB	Human skin fibroblast Fibronectin Collagen 	<ul style="list-style-type: none"> • Percent attachment of HSF cells to collagen and fibronectin increased when exposed to PDI • Percent attachment of HSF cells to collagen and fibronectin decreased when exposed to PDI followed by blocker. • Exposure of free thiols is needed for integrin activation • Disulfide exchange is needed for integrin activation and cell adhesion

1.9 Fabric Textiles and Swabs

Both fabric swatches and swabs are composed of either natural, semi-synthetic, or synthetic fibers that are intertwined together to complete the finished textile. These fibers are constructed in different ways to produce textiles or swabs with various thread counts, lengths, widths, and other properties that will ultimately affect their ability to pick up and

release biological material. Swatches and swabs constructed with a lower thread count will create larger spaces in between the threads to allow the cells to penetrate deep within the fibers. The opposite is true for textiles with a higher thread count (34).

1.9.1 Cotton

Cotton is one of the most abundant natural fibers used in textile manufacturing. Pure cotton is composed of 95% sugar, specifically polymers of cellulose. These cellulose polymers are composed of repeating glucose units linked in a $\beta 1$ to $\beta 4$ manner. Due to this composition cotton has many hydroxyl and amine groups found throughout its structure (35). These hydroxyl and amines groups have a high hydrogen binding potential thus allowing the cotton material to be very effective at picking up and holding onto the cells via a strong dipole-dipole bond, unlike many synthetic fibers (34). This strong bond is beneficial for maximizing cell pick up, but can be problematic when the time comes to release the cells from the cotton fibers.

1.10 Aim

The purpose of this study was twofold. First, to further establish the use of trehalose dihydrate to improve cellular recovery from cotton fabrics and swabs. Second, to study the effects that common reducing agents have on cellular adhesion. Through extensive literature reviews we have found that CAMs such as integrin activate via a series of redox reactions, see Table 1. Given that there are reports in the literature that support integrin activation when exposed to a reducing agent, it is reasonable to ask the

question of whether the use of DTT prior to cell deposition onto fabric would have a similar effect. With an understanding of why the cells adhere to cotton so strongly, we may be able to develop a method to prevent or reverse this adhesion for more efficient cellular recovery.

2. MATERIALS AND METHODS

2.1 Epithelial Cell Preparation (ECP)

Approximately 2 milliliters (mL) of saliva was collected and divided equally into two separate 2 mL collection tubes labeled tube 1 and tube 2. 1X Phosphate-buffered saline (PBS) (Thermofisher, Waltham, MA) was added to each tube up to the 2 mL mark. Each tube was then vortexed for 10 seconds and centrifuged for 3 minutes at 800 relative centrifugal force (rcf). The supernatant for each tube was drawn off and discarded, leaving behind an epithelial cell pellet. This process was repeated two more times beginning with the addition of the 1X PBS to wash the cells. The pellet in tube 2 was then combined with the pellet in tube 1. 1X PBS was added to the now empty tube 2, vortexed, and then combined with tube 1. Tube 1 was vortexed and centrifuged for 3 minutes at 800 rcf. The supernatant was drawn off and discarded. Approximately, 50 microliters (μL) of Tris-EDTA (TE) Buffer (10 millimolar (mM) Tris, 0.1 mM EDTA) was added to tube 1, bringing the total volume of the tube up to approximately 200 μL . Each liquid epithelial cell preparation was quantified, using the Quantifiler® Trio Kit (Applied Biosystems, Foster City, CA) prior to its addition onto a fabric swatch or a cotton swab.

2.1.1 Addition of ECP to Fabric Cotton Swatches

105-F02-ISO adjacent cotton fabric swatches were obtained from Testfabrics, Inc (West Pittston, PA). The cotton fabric swatches were cut into ½ inch (in) x ½ in squares then placed on a glass slide (Thermofisher, Waltham, MA). Either 50 ng, 10 ng, or 2 ng of epithelial cells was pipetted onto the center of the swatch. The glass slide with the swatch on top was placed in a biohazard hood covered with a Kimwipes™ Sheet (Kimberly-Clark™, Irving, TX) to prevent contamination of the swatches. The swatches were dried overnight, then packaged separately in paper envelopes until needed.

2.1.2 Addition of ECP to Cotton Flocked Swabs

Puritan® DNA-Free PurFlock® Ultra Tipped Applicator (Puritan Medical Products Company LLC, Guilford, ME), swabs were used to test for similarities and differences in cell adhesion compared to the Testfabrics, Inc cotton swatches. A scalpel (FEATHER®, Osaka, Japan) was used to make a vertical cut along the length of the cotton swab. The swab was then unwrapped from its wood post and laid flat on a glass slide. In all the experiments utilizing swabs, 50 nanograms (ng) of DNA was pipetted onto the center of the outer surface of the swab. The swabs were allowed to dry overnight in the same manner as described in section 2.1.1.

2.2 Baseline Extraction

A cotton fabric swatch or a cotton flocked swab were placed in a 0.5 mL tube, 178 μ L of TE Buffer was then added to the tube. The tube was placed in a Thermal Mixer (Thermofisher, Waltham, MA) for 1 hour at 56 °C and 400 rotations per minute (rpm).

The swatches or swabs were removed from the tube, placed in a spin basket, and centrifuged at 14,000 rpm for 3 minutes. The swatch or swab was then placed in a new 0.5 mL tube. The tube containing the swatch or swab was labeled as the material fraction (MF). The tube containing the liquid flow through was labeled as the cellular release fraction (CRF).

Eighty nine microliters of CRF liquid was pipetted into a 0.2 mL tube. Ten microliters of 10X Blue Buffer (ZyGEM Corporation Limited, Hamilton, New Zealand) and 1 μ L of ForensicGEM™ (ZyGEM Corporation Limited, Hamilton, New Zealand) were added bringing the total volume up to 100 μ L. This process is depicted below in Figure 4.

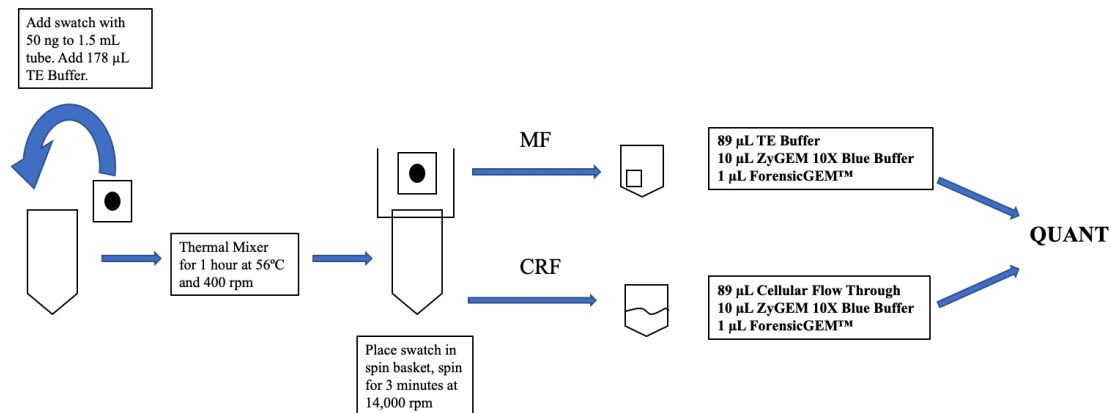


Figure 4. Diagram depicting the general baseline extraction procedure using cotton swatches .

2.3 Experimental Variations to the Baseline Extraction Protocol

Each experiment outlined in this section (section 2.3) follows the general baseline extraction protocol as outlined in section 2.2, Baseline Extraction. A control baseline extraction sample consisting of 50 ng of DNA dried onto a cotton swatch was run alongside each baseline variable to ensure consistency.

2.3.1 Varying Mass of DNA

An ECP was made according to section 2.1, Epithelial Cell Preparation, and was quantified. Based upon the quantification result, the ECP was diluted via a 1:10 dilution and a 1:100 dilution using TE Buffer. ECPs having DNA concentrations of 50 ng, 10 ng, and 2 ng were pipetted directly onto the center of separate fabric swatches, and allowed to dry overnight as outlined in section 2.1.1.

2.3.2 Varying Concentrations of Trehalose Dihydrate

D-(+)- Trehalose Dihydrate (Sigma-Aldrich, St. Louis, MO) solution in TE Buffer was created at three separate concentrations, 0.1 molar (M), 0.75 M, and 2 M.

A 0.1 M Trehalose solution was created using 0.38 grams (g) of Trehalose added to 10 mL of TE Buffer.

A 0.75 M Trehalose solution was created using 2.84 g of Trehalose added to 10 mL of TE Buffer.

A 2 M Trehalose solution was created using 3.78 g of Trehalose added to 5 mL of TE Buffer.

Cotton fabric swatches were then soaked in either 0.1 M, 0.75 M, or 2 M Trehalose solutions for 10 minutes. The swatches were removed from the Trehalose solution and allowed to dry overnight in a biohazard hood, covered with a Kimwipes™ Sheet.

This was followed by the addition of 50 ng of DNA onto the center of the swatch and once more allowed to dry overnight.

2.3.3 Trehalose Dihydrate on Swabs

Puritan® DNA-Free PurFlock® Ultra Tipped Applicators were prepared as outlined in section 2.1.2, Addition of ECP to Cotton Flocked Swabs. The swabs were laid flat in a weigh boat. 0.75 M Trehalose was added for 10 minutes, ensuring to saturate the

entire swab. The swabs were then removed from the Trehalose solution and allowed to dry overnight.

Epithelial cells having 50 ng of DNA were then added to the dried Trehalose swabs and again allowed to dry overnight. Swabs with cells added but without the addition of Trehalose were also prepared, as outlined in section 2.1.2, and run alongside the Trehalose soaked swabs

2.3.4 Dithiothreitol (DTT)

A DTT/ECP solution was created at two different concentrations; 3 mM and 30 mM.

The addition 0.6 μ L of 1 M DTT (Sigma-Aldrich, St. Louis, MO) was added to 200 μ L of epithelial cells, creating a final concentration of 3 mM.

Similarly, 3 μ L of 1 M DTT was added to 100 μ L of epithelial cells, creating a final concentration of 30 mM.

The DTT/ECP solution, containing 50 ng of DNA, was added to the center of the cotton swatches and was allowed to dry overnight.

Cotton swatches soaked in 0.75 M trehalose were prepared and dried overnight as outlined in section 2.3.2. A 3 M DTT/ECP solution, containing 50 ng of DNA, was pipetted to the center of the swatch, which were then dried overnight.

2.3.5 *Tris(2-carboxyethyl)phosphine Hydrochloride (TCEP)*

A TCEP/ECP solution was created at three different concentrations; 1 mM, 10 mM and 100 mM.

To create a final concentration of 1 mM, 0.4 μ L of 0.5 M TCEP (Sigma-Aldrich, St. Louis, MO) was added to 200 μ L of epithelial cells.

To create a final concentration of 10 mM, 1 μ L of 0.5 M TCEP was added to 50 μ L of epithelial cells.

To create a final concentration of 100 mM, 10 μ L of 0.5 M TCEP was added to 50 μ L of epithelial cells.

A total of 50 ng of the TCEP/ECP solution was added to the center of the cotton swatches and was allowed to dry overnight.

2.4 NEM Thiol Blocker

Seven conditions were run, including a standard baseline extraction swatch, as outlined in section 2.2, and a 10 mM TCEP swatch as outlined in section 2.3.5. The remaining four conditions included a 20 mM NEM only swatch, a 10 mM TCEP treatment followed by 20 mM NEM blocker treatment, with the blocker remaining in the solution for either 10 minutes or for 1 hour; and the reverse, a 20 mM NEM blocker treatment, with the blocker remaining in the solution for either 10 minutes or for 1 hour, followed by a 10 mM TCEP treatment. After each of these conditions were created 50 ng of DNA was pipetted onto a cotton fabric swatch, and allowed to dry overnight. The

following day a baseline extraction protocol was run on each condition, as outlined in section 2.2.

2.4.1 NEM Blocker Only Treatment

A 200 mM NEM blocker stock solution was created using 0.1 g of NEM (Sigma-Aldrich, St. Louis, MO) dissolved in 4 mL of deionized water. Ten microliters of 200 mM NEM stock solution was added to 100 μ L of pre-quantified epithelial cells to create a 20 mM blocker solution.

2.4.2 10 mM TCEP Treatment Followed by 20 mM NEM Blocker Treatment

A 10 mM TCEP solution was created as outlined in section 2.3.5. The cells were allowed to remain in the presence of the TCEP for 10 minutes. Following the 10 minutes the cells were centrifuged for 3 minutes at 800 rcf. The TCEP supernatant was then drawn off and discarded. The cells were brought back up to a final volume of 100 μ L using $\frac{1}{2}$ X PBS Buffer.

Next, NEM blocker solution was added to the cells to create a final concentration of 20 mM. The cells were allowed to remain in this solution for either 10 minutes or 1 hour before being applied to the cotton swatches.

This procedure is outlined below in Figure 5.

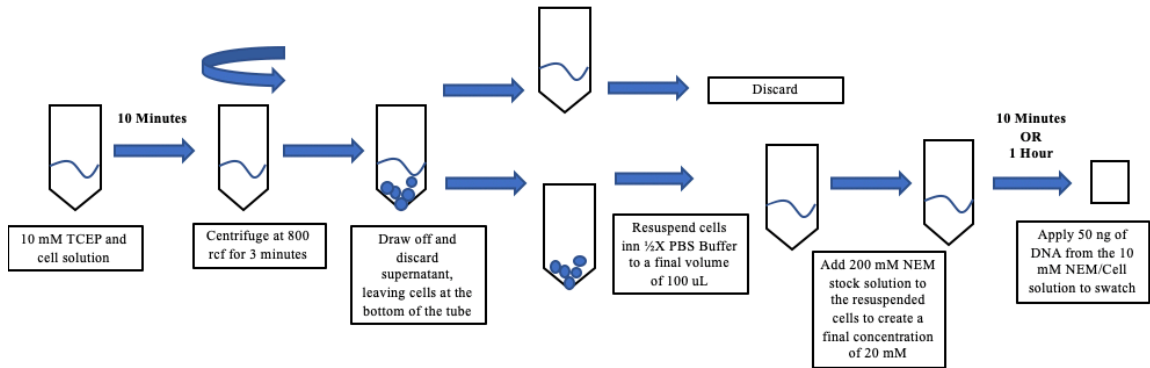


Figure 5. Schematic diagram of a 10 mM TCEP treatment followed by a 20 mM NEM treatment as described in section 2.4.2.

2.4.3 20 mM NEM Blocker Treatment Followed by 10 mM TCEP Treatment

A 20 mM NEM and cell solution was created as outlined in section 2.4.1. The cells were allowed to remain in the presence of the NEM blocker for either 10 minutes or 1 hour. Following this, the solution was centrifuged for 3 minutes at 800 rcf. The NEM blocker supernatant was drawn off and discarded. The remaining solution of cells was brought back up to a final volume of 100 μ L using $\frac{1}{2}$ X PBS buffer.

Next, TCEP was added to the 100 μ L cell solution to create a final concentration of 10 mM. The cells were allowed to remain in the presence of the TCEP for 10 minutes, before being applied to the cotton swatches.

This procedure is outlined below in Figure 6.

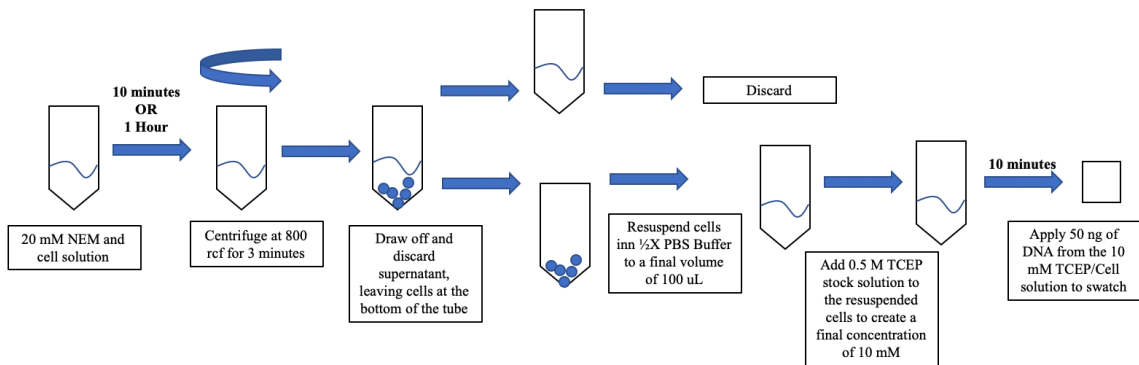


Figure 6. Schematic diagram of a 20 mM NEM treatment followed by a 10 mM TCEP treatment as described in section 2.4.3.

2.5 ZyGEM Extraction

The samples in 0.5 mL tubes were incubated in Veriti Thermalcycler (AppliedBiosystems, Foster City, CA). Samples in the 0.2 mL tubes were incubated in the SimpliAmp Thermalcycler (AppliedBiosystems, Foster City, CA). The samples in both the 0.2 mL and 0.5 mL tubes were incubated at 75°C for 15 minutes followed by 95°C for 5 minutes, then 4°C until removed from the Thermal Cyclers. No following purifications were performed.

2.6 DNA Quantification

DNA quantifications were done using the Quantifiler® Trio Kit (Applied Biosystems, Foster City, CA), with a standard curve run for each sample set. The samples were run on the 7500 Real-Time PCR Instrument (Applied Biosystems, Foster City, CA)

following manufacturers' instructions. Internal positive control values were normal unless otherwise noted.

2.7 Percent Recovery Calculations

These calculations were based on the quantification value in nanograms obtained from section 2.6, DNA Quantification.

The mass was first calculated for both the MF and the CRF. For the CRF the quantification value was multiplied by 100, which was the total volume in the ZyGEM Digest. Due to the fact that only half of the CRF was used in the digest, the above value was multiplied by two to account for the total volume of the CRF.

The mass was calculated for the MF by multiplying the quantification value by 100, which was the total volume in the ZyGEM Digest.

After calculating the mass for the CRF and for the MF the percent recovery was calculated using the following formula, $\frac{\text{Mass of CRF}}{\text{Mass of CRF} + \text{Mass of MF}} * 100$.

3. RESULTS

A baseline extraction was performed alongside all experiments. Each baseline run for the duration of this thesis was averaged together and recorded as the total baseline. The total baseline is the value that was used for comparison and results from each experimental variable. The run baseline, as observed in each of the graphs below, shows the average baseline obtained from only the baseline extraction run alongside the experimental condition. A one tailed student's T test was used to determine the

significance between the total baseline and each experimental variable, unless otherwise noted.

3.1 Baseline Extraction

The baseline extraction procedure served as a key point of comparison between each experimental variable, to determine the impact on cellular recovery. The procedure involved using a consistent 50 ng of DNA pipetted onto a cotton fabric swatch, followed by an incubation of 1 hour at 50°C and 400 rpm. This baseline procedure was developed by former student, Sylvia Speidel, through modifications to the recommendations outlined in the QIAmp DNA Investigator extraction kit (36).

The average baseline cellular recovery value obtained from a total of 19 runs was 24%. This means that 24% of cells are being removed from the fabric, while 76% of the cells remain adhered to the fabric substrate. These results reiterate the issue of strong cellular adhesion to cotton fibers, proving problematic especially in cases involving low level DNA samples or the presence of inhibitors.

3.2 Varying Initial DNA Mass

Different amounts of DNA were pipetted and dried onto a cotton swatch to determine if a comparable percentage of DNA is recovered off of the cotton fabric at low (2 ng), medium (10 ng), and high (50 ng) amounts. An analysis of variance (ANOVA) test determined that a comparable percentage of DNA was released from the swatches at

each amount. The extent of cellular recovery between 2 ng, 10 ng, and 50 ng was not significant, represented with a p-value of 0.99. The results are summarized in Figure 7.

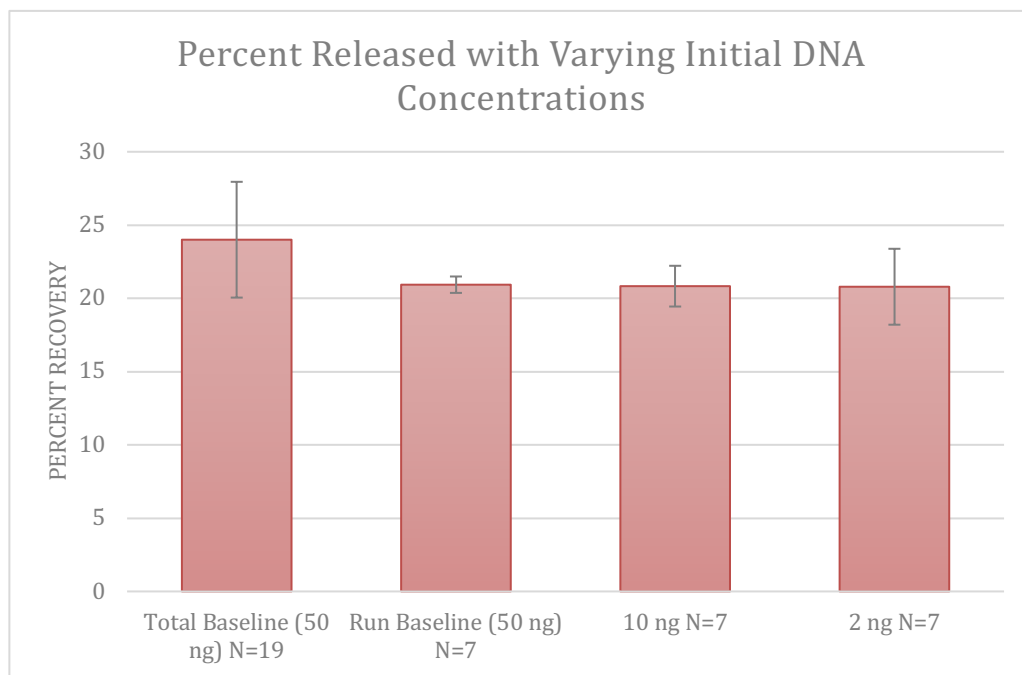


Figure 7. Percentage of cells recovered at 50 ng, 10 ng, and 2 ng of DNA.

These results demonstrate that regardless of the initial amount of DNA pipetted onto the swatch the cell recovery remains similar, averaging results between 20% - 24% cellular recovery.

3.3 Varying Trehalose Dihydrate Concentrations

It was previously demonstrated that 0.75 M trehalose yielded statistically significant increase in cell recovery compared to baseline recovery (36). Based on this information we decided to use a range of different trehalose concentrations to see what would yield the most promising results.

It was noticed that 2 M and 0.75 M trehalose yielded a significant increase in cellular recovery, yielding 53.81% and 70.5% recovery respectively. These results yielded significant p-values of 0.00016 and 0.00001. However, 0.1 M trehalose, only recovered 33.79% of cells compared to 24% recovery of baseline, determined by a p-value of 0.73. The results are summarized in Figure 8.

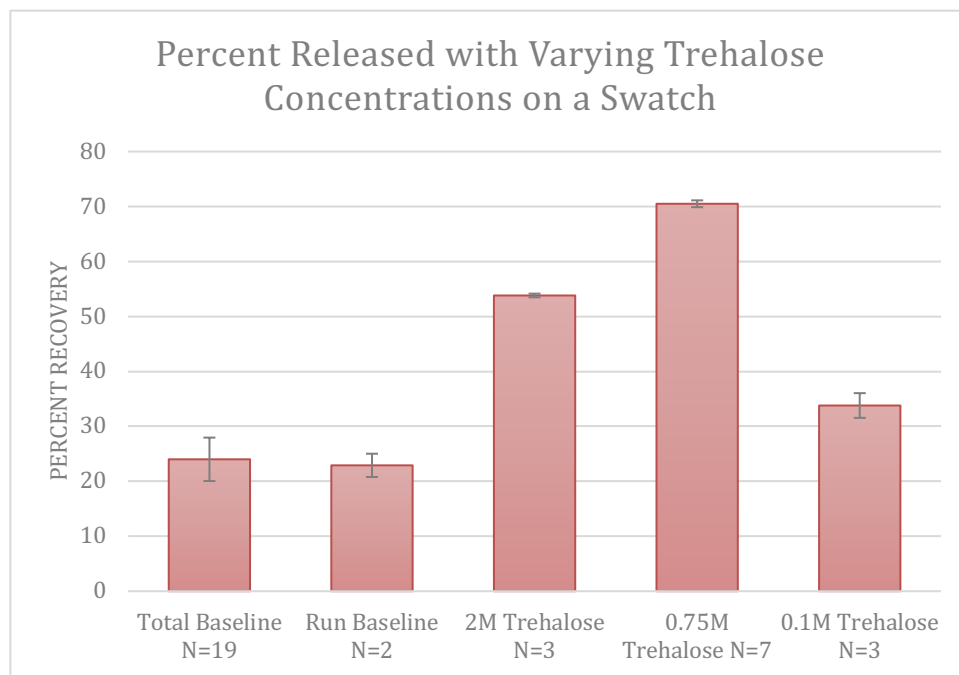


Figure 8. Baseline results compared to the percent cellular recovery under three different trehalose conditions.

Cellular recovery with the addition of 2 M trehalose compared to 0.75 M trehalose were similar thus the results were not statistically significant (p-value of 0.063). This may be due to the small sample size. However, considering the data obtained by Speidel, and the slightly higher percent recovery seen in Figure 8 using 0.75 M trehalose, we decided to continue to use 0.75 M trehalose for future experiments (36).

3.4 Cotton Flocked Swabs

Cellular recovery from cotton flocked swabs was compared to recovery using the baseline procedure. Epithelial cell recovery from swabs averaged 14.78% and did not yield a significant difference (p-value of 0.050) when compared to the baseline results of 24% from the swatches. The addition of 0.75 M trehalose recovered an average of 26% thus did not significantly increase cellular recovery from the swabs (p-value of 0.16), in contrast to the results using cotton swatches. The results are summarized in Figure 9.

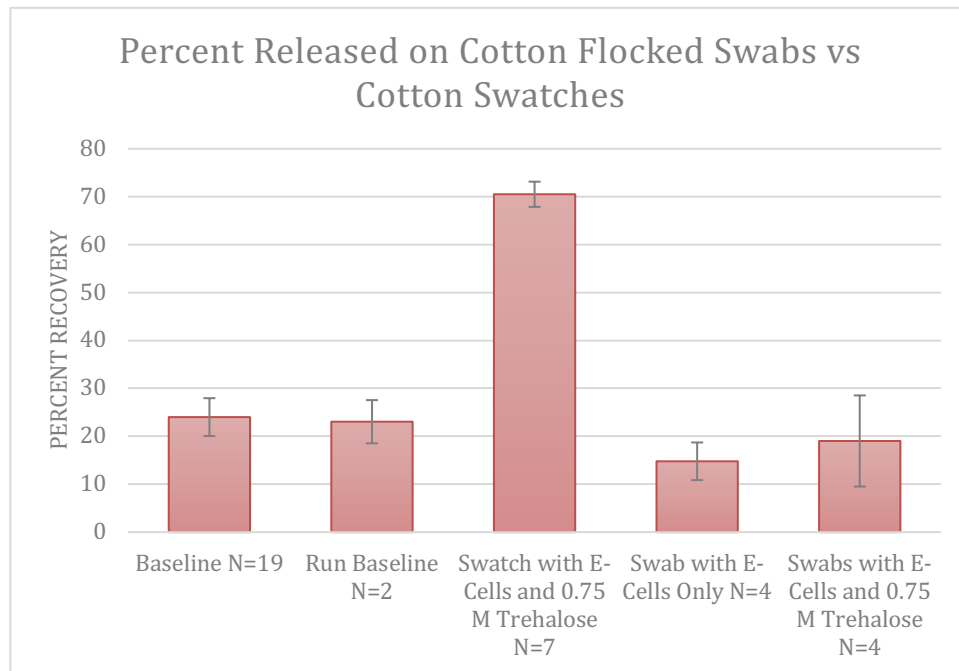


Figure 9. Percent of cells recovered from cotton swabs compared to cotton swatches.

It is unclear why trehalose increased cellular recovery significantly on the cotton swatches, but not on the cotton swabs, especially considering that baseline cellular recovery between the swatches and swabs without trehalose yielded similar results. One possible explanation for these unexpected results may be due to differences in the

thickness or the construction of the fibers for each substrate, thus changing how trehalose is able to interact with and aid in increasing cellular recovery. However, more research and experimentation must be done in order to try to understand this difference.

3.5 Reducing Agents

3.5.1 Effects of DTT on Cellular Recovery

To determine the effects of reducing agents on cellular adhesion we added 3 mM and 30 mM DTT to an ECP prior to cell deposition and drying. It was noticed that 3 mM DTT significantly increased cellular adhesion to the cotton swatch yielding a cellular recovery of 11.09%. Similarly, 30 mM of DTT also strengthened cellular adhesion lowering recovery to 10.78%. Compared to the baseline cellular recovery of 24%, both 3 mM and 30 mM DTT yielded significantly lower cellular recovery percentages, with p-values of 0.00074 and 0.02 respectively.

The addition of 0.75 M trehalose to the cotton swatch was then tested to see if trehalose would help improve cellular recovery within the presence of DTT. Cellular recovery was seen to significantly increase to 47.71% compared to the initial 11.09% of 3 mM DTT without the addition of trehalose (p-value of 0.00001). While the addition of trehalose under reducing conditions improved cellular recovery, the result was significantly lower than the cellular recovery obtained without the presence of DTT (p-value of 0.0018). These results are summarized in Figure 10.

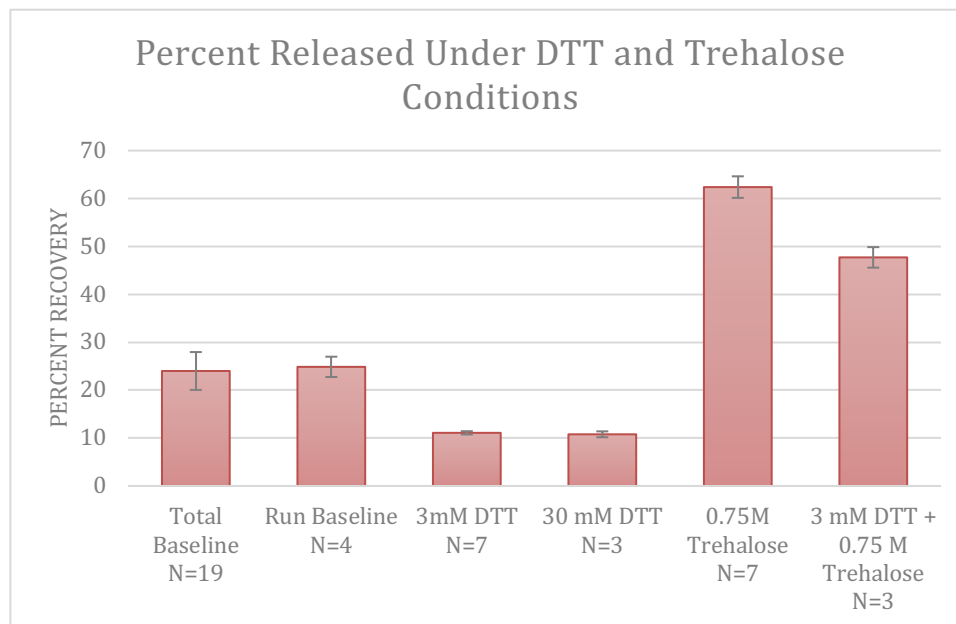


Figure 10. Percent recovery of epithelial cells with 3 mM DTT, 30 mM DTT, and 3 mM DTT with 0.75 M trehalose added to the swatch.

We theorize that the DTT may be acting upon CAMs, specifically integrin, initiating a redox reaction. This redox reaction will activate integrin by breaking the disulfide bonds, and exposing free thiols, resulting in increased cell adhesion.

DTT treated cells placed on a trehalose treated swatch showed an increase in cellular recovery compared to a swatch without trehalose. It is difficult to theorize what properties of the cotton swatch, trehalose, and the cell surface may result in improved cellular recovery.

3.5.2 Effects of TCEP on Cellular Recovery

To attempt to confirm that it is the reducing action of DTT that increased cellular adhesion, the experiment was repeated using TCEP at 1 mM, 10 mM, and 100 mM

concentrations. 1 mM TCEP did not show a significant decrease in cellular recovery, compared to baseline, at an average of 14.62% (p-value of 0.067). However, the use of 10 mM TCEP decreased cellular recovery to 12.23%. This result was significantly lower than baseline recovery (p-value of 0.0003). The addition of 100 mM TCEP created unexpected results with what seems to yield a very high percent recovery, however, quantitative polymerase chain reaction (qPCR) results showed significant DNA degradation within the qPCR results were observed, thus no further work was done with 100 mM TCEP. The results are summarized in Figure 11.

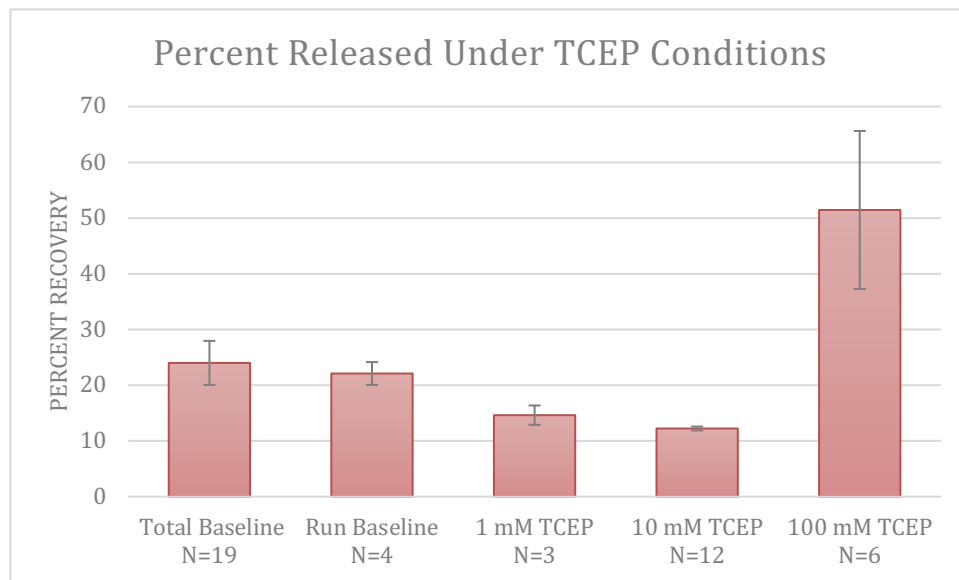


Figure 11. Percent recovery of cells with 1 mM TCEP, 10 mM TCEP, and 100mM TCEP. *These unusual results are explained below.

The effects of 10 mM TCEP on cellular recovery were very similar to the effects of both 3 mM and 30 mM DTT on cellular recovery. Therefore, we can further theorize

that it is the reduction potential of the reducing agent causing these strong effects on cell adhesion.

It is unclear why 100 mM TCEP had such unusual results coupled with a large average degradation index of 57. However, due to these unexpected results 10 mM TCEP was used for future experiments.

3.6 Thiol Blockers

It has been shown by researchers that free thiols, made available by the breakage of disulfide bonds within integrin, are one of the many components necessary for cell adhesion. Our experiments examining cell adhesion within the presence of reducing agents correlated to the results obtained by several of these researchers as outlined previously in Table 1. With this information, it is predicted that by reducing the disulfide bonds in integrin via 10 mM TCEP, followed by blocking the free thiols with 20 mM NEM, cellular recovery percentages should be similar to baseline recovery. However, a 20 mM NEM blocker treatment directly followed by a 10 mM TCEP treatment, should yield cellular recovery percentages similar to that of a TCEP treatment alone.

The addition of cells treated with 20 mM NEM with an incubation of 10 minutes onto a cotton swatch recovered 30% of cells. As we predicted, this result was not statistically different than our average baseline percent recovery of 24% (p-value of 0.24). We expected the NEM to block any free thiols that were not directly involved in integrin activation, thus the NEM alone should not change cellular recovery compared to baseline. Our results supported this, but does not confirm this theory.

Adding 20 mM NEM to an ECP for 10 minutes followed by 10 mM TCEP yielded 16% cellular recovery. Similarly, leaving the NEM on the swatch for 1 hour yielded 15% cellular recovery. As expected, the NEM treatment followed by the TCEP treatment yielded cellular recoveries that were similar to cellular recoveries obtained from TCEP treatments alone (p-value of 0.051). However, this same experimental treatment was not significantly different from baseline (p-value of 0.078), thus the TCEP did not promote cell adhesion as strongly within the presence of NEM, as it does alone. However, this could also be due to a small sample size.

The addition 10 mM TCEP to an ECP followed by 20 mM NEM with an incubation time of 10 minutes yielded an average of 12% cellular recovery. This cellular recovery is much lower than we expected. We theorize this could have been due to a short NEM incubation period, thus not allowing enough time for the NEM to block all the available free thiols. However, it should be noted that a large standard deviation within the data was observed, possibly due to a low sample number.

Increasing the incubation time to 1 hour yielded a noticeably large average degradation index of 84.5, coupled with a high standard deviation. While the results indicated that cellular recovery increased to 72%, they cannot be deemed reliable due to variability within the data. The results are summarized in Figure 12.

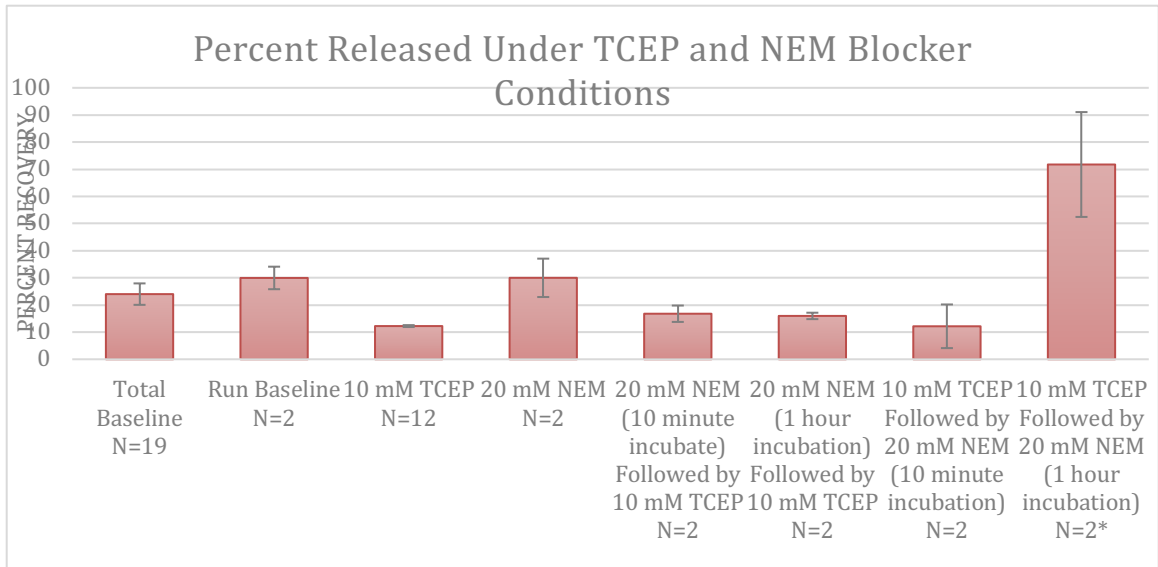


Figure 12. Percent recovery of cells under various conditions involving 10 mM TCEP and 20 mM NEM thiol blocker.

While the outcome of this data was not what we expected, it does not indicate that our theory regarding integrin activation and cell adhesion is wrong, instead more in-depth research must be done to study the specifics of epithelial cell adhesion.

4. CONCLUSIONS

4.1 Summary of Results

Low (2 ng), medium (10 ng), and high (50 ng) initial concentrations of DNA dried onto a cotton swatch yielded similar cellular recoveries, ranging between 20%-24%. The addition of trehalose at medium (0.75 M) and high (2 M) concentrations significantly increased cellular recovery to 70.5% and 53.8%, respectively. However, a lower trehalose concentration of 0.1 M, did not yield a significant increase in cellular recovery.

Samples dried onto cotton swabs yielded statistically similar cellular recoveries as samples dried onto cotton fabric swatches. The introduction of 0.75 M trehalose significantly increased cellular recovery from the cotton swatch from an average of 24% to 70.5%, however, this increase in cellular recovery was not seen when 0.75 M trehalose was added to the cotton swab.

The addition of reducing agents, such as DTT and TCEP, significantly decreased cellular recovery, from a baseline average of 24% to 11.09% for 3 mM DTT and 13.07% for 10 mM TCEP. Increasing the concentration of the DTT solution to 30 mM, continued to yield significantly lower cellular recoveries compared to baseline. One millimolar TCEP also yielded lower cellular recoveries, but was not significantly lower than baseline recoveries. One hundred millimolar TCEP yielded unexpected and ambiguous

results that were unable to be analyzed completely, due to a large degradation index and a high standard of deviation.

The application of 0.75 M trehalose dried onto a swatch followed by the addition of 3 mM ECP and DTT solution increased cellular recovery to 47.01%. This was seen to be a larger recovery than seen within the absence of trehalose, but not as large as the recoveries obtained without the presence of DTT.

The addition of the NEM thiol blocker to the ECP did not seem to impact cell adhesion, yielding a similar cellular recovery to baseline. Conversely, treatment of cells with NEM, incubated for either 10 minutes or 1 hour, directly followed by treatment with TCEP yielded percent recoveries of 16% and 15% respectively. These results were not significantly different from TCEP only treatment or baseline.

The addition of TCEP followed by NEM with either a 10 minute or one hour incubation yielded unexpected and ambiguous results. A 10 minute incubation of the NEM following the TCEP, yielded only 12% cellular recovery. A higher cellular recovery was expected however, we theorize that the 10 minute incubation time for the NEM with the cells may not have been long enough. A one hour incubation of the NEM following the TCEP treatment, yielded 72% cellular recovery. These results had an

unusually high degradation index and a wide standard deviation, making them ambiguous and unreliable for any interpretation.

4.2 Future Considerations

The use of 0.75 M trehalose continued to yield a higher cellular recovery percentage compared to baseline on cotton fabric swatches, as seen previously by Speidel (36). However, the application of trehalose onto a swab did not significantly increase cellular recovery, as it had on the swatch. We only used one brand of swabs thus another brand could be used to see if similar results are observed. Furthermore, we deposited cells directly onto the swab, however, it would be useful to see the recovery results of using swabs to pick up cells deposited onto a surface.

We have observed lower cellular recoveries with the addition of a reducing agent, thus it would be important to explore if we would continue to see this pattern of decreased cell recoveries if swabs were used instead of fabric swatches. Furthermore, it would be important to see if a thiol blocker would also produce similar results on swabs, as it did on the swatches. However, future reducing agent experiments should be run alongside a reducing agent only treated swatch to serve as a control. This control will ensure that it is not the reducing agent interacting with the fabric causing the increase in cell adhesion.

Lastly, this thesis focused on the use of epithelial cells to attempt to understand cellular adhesion. However, forensic laboratories do not only deal with epithelial cells, thus the use of other cell types, such as sperm and blood cells would be important to test

to determine how similar they adhere to a substrate and what effect trehalose or a reducing agent would have on their adhesion.

LIST OF JOURNAL ABBREVIATIONS

Am J Med	The American Journal of Medicine
Annu Rev Physiol	Annual Review of Physiology
Antioxid Redox Signal.	Antioxidants and Redox Signaling
Asian Pac J Reprod	Asian Pacific Journal of Reproduction
BBA	Biochimica et Biophysica Acta
Biochem Biophys Res Commun	Biochemical and Biophysical Research Communications
Exp Cell Res	Experimental Cell Research
Forensic Sci Int Genet	Forensic Science International: Genetics
Int Congr	International Congress Series
Int J Food Sci Technol	International Journal of Food Science & Technology
J Biol Chem	Journal of Biological Chemistry
J Forensic Sci	Journal of Forensic Sciences
J Therm Anal Calorim	Journal of Thermal Analysis and Calorimetry
Mol Membr Biol	Molecular Membrane Biology
Nat Rev Genet	Nature Reviews Genetics
Open Biol	Open Biology
Pharmacol Res	Pharmacological Research
PLoS One San Franc	Public Library of Science San Francisco

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