MECHANICAL INVESTIGATIONS OF THE PRIMARY CELL WALL AND
ATTEMPTS AT MODIFICATION OF NOVEL CORE ADHESIVES FOR USE IN IRON
FOUNDRIES

A Thesis in
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by
Paul J. Munson

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The thesis of Paul J. Munson was reviewed and approved* by the following:

Nicole R. Brown  
Associate Professor of Wood Chemistry  
Thesis Co-Advisor

Dan G. Sykes  
Senior Lecturer in Chemistry  
Thesis Co-Advisor

Karl T. Mueller  
Professor of Chemistry

Daniel J. Cosgrove  
Professor of Biology  
Eberly Chair of Biology

Barbara J. Garrison  
Shapiro Professor of Chemistry  
Head of the Department of Chemistry

*Signatures are on file in the Graduate School
ABSTRACT

1. The primary cell wall of plants display interesting mechanical properties such as stiffness to resist the turgor forces generated by the living cell and extensibility to allow it to grow. The interactions occurring between the polysaccharides cellulose, xyloglucan (XG), and pectin were examined thermo-mechanically. Heat treatment of plant tissues for brief periods (<15 min) resulted in an increase in the stiffness of the tissues in the linear viscoelastic region of up to 46% compared to controls. Heat treatment of tissues containing little or no xyloglucan (celery parenchyma and xxt1/xxt2 Arabidopsis mutants) result in higher stiffness after heating of up to 60% which leads to the thought that XG rearrangement may not be the major cause of the stiffness. Rearrangement of the pectin / cellulose matrix is the major factor. This agrees with sum frequency generation (SFG) data that show a re-dispersement of cellulose during heating in tests involving onion epidermal cell walls. X-ray spectroscopy also revealed no aggregation of cellulose microfibrils occurring during heating.

Microelectromechanical System (MEMS) testing was accomplished on heat treated onion epidermal cell wall to distinguish if the stiffness was primarily caused at the tissue level or at the single wall level. Results indicated that the wall section became less stiff after heat treatment, which implicates the tissues and possible the middle lamella in the stiffening. The cell wall sections however show greater strength at break of 46% than controls and have a higher strain of 67%. This indicates that the cell wall structure is undergoing rearrangement during heating, and that no one factor is decisive in the observed stiffening.
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PREFACE

The graduate school career I have encountered has been a tedious one; however, the experiences encountered have served to make me a more well rounded chemist and scientist in general. I have had the pleasure of working in four different groups; each of which has expanded my knowledge and skill in areas ranging from biological evaluation of compounds and natural products chemistry, to analytical chemistry and plant biology. While there was no one strict focus in my 8 year stay, it can be said that in today's multi-disciplinary workforce, the training and experience I have acquired has led to a more useful individual that is able to adapt to whatever the scientific need may be. While my stay in any of these labs has been relatively short (~2 years), I believe that I have retained much of the knowledge gained from each of these experiences, making me a well rounded individual able to see the broader picture in many situations.

My initial work focused on medicinal and bio-organic chemistry, and the interaction of compounds at the cell surface. While generally not as synthetically demanding as a natural products lab, the practical application of the products that were made and the biological evaluation of the compounds led to a deep appreciation for the methodology that goes into compound evaluation in medicinal chemistry. This experience also gave an appreciation for the amount of work that goes into the development of useful compounds, as many of the compounds made in the lab were based on those that came previously.

The second lab experience allowed me to hone my synthetic methodology skills. I decided when looking for a new group that getting more experience in a straight synthetic lab would expand my horizons and allow me to be of greater value in the future. As such, I joined a natural products lab, where I worked on two major projects; the first of which focused on investigating the methodology that was developed in the Feldman lab: the allenyl azide cyclization pathway. This was a great way to learn new chemistry and reinforce bench practices. This initial work did lead to a publication in the Journal of Organic Chemistry. After finishing the project, a project involving the synthesis of a natural product using the developed methodology was attempted, expanding my appreciation for the amount of work that goes into multi-step syntheses. While only an
11-step synthesis, the route challenged my synthetic skill and helped me to learn more about what goes into a full synthesis both on paper and at the bench. This project also taught me about failure, disappointment, and how to overcome difficulty when faced with both. This opportunity allowed me to broaden my abilities in retro-synthetic analysis and how to manage a forward synthesis.

All in all, despite the tenure I have had during graduate school, I have had the pleasure of getting to know a great many people and learn about attitudes, politics, academia, and most importantly I've learned my limits, wants, and needs. I do not regret this time of my life. As strenuous as it may have been, I have learned and grown more than I imagined I would have when I began. I believe my training here will be of great value in my future.
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I would also like to give thanks to Dr. Nicole Brown for giving me a chance in my moment of need. I have learned much in your lab and have been grateful for your insight and scientific curiosity and I have appreciated the opportunities I have had in your lab for learning new things.

Thank you to my committee members Dr. Dan Cosgrove and Dr. Karl Mueller for taking the time to interact with me and be a part of my intellectual journey. Dr. Cosgrove thank you also for giving me a chance when I was in need, you have expanded my scientific horizons more than I could have imagined when I started at Penn State.

Next I would like to thank all of the countless people who have helped me finish this work, specifically Dr. Brett Diehl who has been one of the best lab mates I could have asked for. Who all at once has been the source of much consternation and light heartedness in the lab. Without our constant banter and intellectual discussion, I would not have made it through with my sanity. Drs. Yong Bum Park, Sarah Kiemle and everyone else in the Cosgrove lab for your help in learning plant biology and the support in growing and sectioning plants. I would be remiss to not thank my prior advisors, as while things did not work out in your labs, I was still able to learn and gain valuable insight into how chemistry works.

I would like to thank the DOE EFRC Center for Lignocellulose Structure and Formation for funding and being an enormous spring board for scientific inquiry. I would also like to thank Penn State's NSF GK-12 CarbonEarth Program for funding in my first two years in the Brown Lab. The experiences I gained helping teach science to 8th-graders has given me a greater perspective of how people perceive science as a whole.

Finally, I would like to thank my family most of all. For all the support over the last eight tumultuous years. You have been there for me every step of the way with words of encouragement and have believed in me even when I did not. Without your love and support I never would have made it out of Indiana, much less graduate school. There is not enough thanks I could give, except to continue on into my Air Force career and make you proud.

Thank you to all who have helped me on this journey!
Chapter 1. Thermo-mechanical Investigations of the Primary Cell Wall

Preparing for publication

1.1 Introduction:

The primary cell wall of plants serves several important functions, such as protection from pathogen and abiotic stresses,\(^1\) is able to withstand turgor pressures and tensile stresses (>2.5 atm),\(^2\) and allows for cell expansion during growth.\(^3\) These characteristics arise from the interactions of cellulose, hemicelluloses, and pectins within the cell wall.\(^4\) While the physical characteristics and compositions of the primary cell wall have been well evaluated, the full nature of the chemical and physical interactions between cellulose microfibrils and the surrounding matrix polymers remains unclear. A better understanding of the molecular interactions that give ligno-cellulosic materials their strength and pliability is not only of fundamental interest, but may provide insight into how recalcitrant composites from natural materials could be designed ex-situ, or alternately may guide deconstruction of the cell wall for use in bio-fuels.

Of interest here is the primary cell wall (Fig 1.1), where there are little to no covalent linkages between polymers and strength is primarily governed by hydrogen bonding.\(^1,5,6\) It has been widely accepted that the main load bearing super molecular structure in the primary wall in dicotyledonous plants arises from H-bonding interactions between cellulose microfibrils and the xyloglucan (XG) network.\(^7,8\) XG has the ability to tightly bind to cellulose,\(^9\) conceptually producing an ordered framework that permits the transfer of stresses while still allowing for wall extensibility. The tethered cell wall model assumes a loose association of XGs \textit{with} each other to impart extensibility to the wall, while cellulose resists the force of turgor pressure being exerted perpendicular to the direction of growth.\(^10\) It was first thought that XGs completely cover cellulose, however recent studies utilizing ssNMR have shown have XG has less cellulose contact than previously thought, indicating that the pectin matrix also contributes to the observed stiffness.\(^11-13\) These studies, along with new results utilizing substrate specific endoglucanases,
have brought the traditional tethered model of the cell wall into question (Fig 1.2).\textsuperscript{14}

While xyloglucan does have an impact on wall mechanics, it seems that certain domains include more structurally significant tethers than others. Several models have been proposed regarding the interactions occurring between cellulose and XG domains.\textsuperscript{15} Key parameters underlying such models, such as the number of interacting sugars per unit length and the strength or “adhesion” of these domains to each other, remain unclear. The interactions of hemicelluloses within the primary cell wall must allow for some movement of cellulose microfibrils in relation to each other, and has been observed previously during growth, which induces some separation between microfibrils.\textsuperscript{10,16,17} Digestion utilizing cellulose and xyloglucan specific endoglucanases resulted in no decrease in the creep of the associated samples; however, when utilizing an endoglucanase that had hydrolytic activity for both cellulose and the xyloglucan showed a significant loss of cell wall cohesion under stress.\textsuperscript{14} These results indicate a much closer association of cellulose microfibrils that does not allow access to portions of the xyloglucan matrix. These tight interactions are hypothesized to impart most of the connective strength in the primary cell wall.

Here, thermo-mechanical investigations of the primary cell wall were attempted to determine the temperature dependent nature of the XG-cellulose binding and quantify the amount of hydrogen bonding present between. Specifically, we hypothesize that with increasing temperature, hydrogen bond “tethers” between XG and cellulose would break. Depending on the nature of the connections, this should happen at finite temperature ranges dependent on the degree of hydration, the tightness of the interactions, and the amount of XG-cellulose bonding regions present. If the system were truly composed primarily of tethered networks of load bearing XGs and cellulose, without major pectin interaction, tissue stiffness would be highly affected by temperature increases. Distinct hydrogen bonding patterns should become evident as Brownian motion increases, as observed by stiffness decrease. The temperature at which this occurs will relate to the number of bonds present between XG and cellulose. The disruption of
hydrogen bonding should cause an increase in the mobility of the microfibrils with respect to each other, resulting in observable changes in mechanical characteristics. The research herein also describes the processes taken to help elucidate the role pectin interactions play in cell wall stiffness, as pectins are thought to regulate elastic stretching in the cell wall, inhibit XG stretching during expansion, and control the porosity of the cell. While it has been shown that pectins have only a small binding affinity for cellulose, the results here implicate pectin matrix rearrangement as a possible mechanism that occurs during heating to increase stiffness within the cell wall.

1.2 General Experimental:
1.2.1 Plant tissues:
Cucumber (Cucumis sativus) hypocotyls from 4 day old etiolated seedlings were collected on ice and frozen at -80 °C as previously described. Tissues were brought to RT and pressed under weight (500 g) between glass slides for 5 min to remove excess water and then heat inactivated still between glass slides via boiling for 15 s. All samples were then stored at -80 °C until used.

Celery (Apium graveolens) was obtained from commercial sources and parenchyma tissue was isolated from the vascular tissue. Parenchyma was then sectioned into thin slices (30 x 3 x 0.5 mm), pressed, and heat inactivated as described previously. All samples were then stored at -80 °C until used.

Maize (Zea mays) coleoptiles were harvested from 4 day old etiolated seedlings were collected on ice, bisected, and frozen at -80 °C. Samples were then pressed under weight for 5 min and heat inactivated for 15 s via boiling water. All samples were then stored at -80 °C until used.

Jumbo white onion (Allium cepa) was obtained commercially and strips of epidermis (30 x 1.5 mm) were isolated from the abaxial side of the 5th scale. Samples were placed on a glass slide, frozen at -80 °C and then heat inactivated between glass slides in boiling water for 15 s. All samples were then stored at -80 °C until used.

Arabidopsis (Arabidopsis thaliana) petioles from Col-0 and xxt1/xxt2, mutants were harvested from 3 wk old plants grown under 24 hr light and prepared as above. PMEI, tsd2, and qua2 mutant petioles were harvested at 3 wks and 4 wks to obtain petioles large enough to fit
into the DMA instrument (15 mm minimum length length). All samples were stored at -80°C until used.

1.3.2 Thermo-mechanical Analysis (DMA):

**Temperature Ramps:** DMA analysis was performed as follows: the apical 15 mm of cucumber hypocotyls were allowed to warm to RT and then clamped in a DMA submersible tension clamp (TA Instruments, Q800 DMA) and placed in 20 mM HEPES buffer (pH = 6.9) at room temperature (RT) and equilibrated for 5 min. A pretension force of 0.05 N was applied to the samples for 5 min prior to the temperature ramp. Temperature ramps were performed from 23°C to 80°C at a rate of 3°C min⁻¹ with a constant force of 0.05 N applied to the sample.

**Bath heating & Time dependence:** Tissue samples were prepared as stated above. Samples were then heated in an 80°C water bath pressed between glass slides for 0, 1, 5, 15, 40, and 60 min and subsequently quenched in ice water for 1 min. After warming to RT, samples were clamped in a Q800 DMA (TA instruments) submersible clamp, placed in 0.02 M HEPES buffer (pH = 6.9) at RT and extended via force ramp from 0.01 N to break, and the resulting stiffness (Nm⁻¹) was obtained from the initial 200 µm of displacement.

**XEG Digestion:** The apical 3 cm of thawed cucumber hypocotyls were excised and abraded with a carbarundum slurry (300-mesh) to facilitate buffer and enzyme penetration. Heat inactivated hypocotyls were incubated with xyloglucan specific endoglucanse (XEG, Novozymes), 100 µg/ml in 100 mM ammonium acetate buffer, pH 5, with 0.02 % NaN₃) for 24 hrs at 27 °C for complete digestion of available XEG susceptible XG domains. After digestion, the supernatant was collected for sugar analysis and the hypocotyls were washed in ddH₂O (3 x 5 min), collected, and then frozen at -80 °C. Samples were kept at -80 °C until utilized for DMA analysis.

1.3.3 Sum Frequency Generation (SFG):

Dried onion epidermal peels after heat treatment and extension were taped on a standard microscope slide (VWR International) and SFG spectra were collected in reflection mode as described previously. Experiments were performed using a SFG spectrometer (EKSPLA,
Vilnius, Lithuania), pumped by a picosecond Nd:YAG 1,064 nm laser operated at a 10 Hz repetition rate. A 532 nm visible laser pulse was generated through frequency doubling of the Nd:YAG output. A tunable 2.3–10 μm infrared laser pulse was generated through optical parameter generation and amplification processes. The p-polarized infrared beam and s-polarized visible beam were temporally and spatially overlapped at the epidermal slice surface at incidence angles of 56° and 60° with respect to the surface normal, respectively. The p- and s-polarizations are defined as the electric field of the light within and out of the laser incidence plane, respectively. The SFG signal was detected in the reflection geometry with a beam collimator to increase the signal collection efficiency. The SFG signal was directed into a monochromator with s-polarizer and recorded with a photomultiplier tube and spectra were taken at 4 cm\(^{-1}\)/steps in the C-H stretch vibration region (2,700–3,050 cm\(^{-1}\)) and 8 cm\(^{-1}\)/steps in the O-H stretch vibration region (3,100–3,800 cm\(^{-1}\)). The SFG signal was averaged over 50 laser pulses and normalized with the IR and visible input laser intensities at each point. The probe volume was approximately 200 μm × 150 μm wide over the sample and approximately 20 μm deep from the external surface of the sample.\(^{22}\)

To study the orientation dependence of the SFG signal from onion epidermal peels, the spectra were recorded with the laser incidence plane along the longitudinal and transverse directions of onion cell major axis. The SFG peaks and tentative assignments from cellulose in onion epidermal peels has been discussed previously.\(^{22-24}\)

**X-Ray Spectroscopy**

Small angle X-ray scattering (SAXS) measurements were performed using a Molecular Metrology pinhole camera system. The source was operated at 45kV and 0.67mA while the sample to detector distance was 1.5m and 0.5m for Chamber 1 and Chamber 2, respectively. After correcting for the background, the data was fit using a simple Lorentzian function in order to estimate the peak position and determine the d-spacing. Wide angle X-ray scattering (WAXS) patterns were collected at 45 kV, 40 mA with fixed divergence and receiving slits (1/4 deg. divergence, ½ deg. anti-scatter, ¼ deg receiving) on a PANalytical Empyrean x-ray diffractometer using Cu K-alpha radiation from 5–40 deg. 2-theta. The system was equipped with a PIXcel silicon strip detector operated in 1D mode (step~0.026 deg/count 300s).
1.4 Results and Discussion:

1.4.1 Bath heating and time dependence:

Stiffness values were obtained within the linear viscoelastic region of the stress strain curve from 1-2% extension (100 - 300 nm). Cucumber hypocotyls exhibited a 46% increase in stiffness compared to controls (p = 0.001) after heat treatment via incubation at 80 °C for 15 min and subsequent quenching in ice water (Fig 1.3a). The stiffening was initially thought to involve the re-patterning of hydrogen bonding within the cellulose/polysaccharide matrix; because of this, the amount of time the tissues spend at 80°C should have an impact on the amount of stiffness increase observed. The stiffness of the tissue increased linearly until approximately 15 minutes of heating, where maximum stiffness was observed (Fig 1.3b). Many of the hypocotyls isolated after 60 min were too soft to clamp and fell apart during manipulation. This is possibly due to softening of the middle lamella during the heating, causing loss of intercellular adhesion.

Table 1.1 Average stiffness values for bath time dependance with associated p-values utilizing an unpaired, 2-tailed t-test.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Control</th>
<th>No Inactivation</th>
<th>5 Min</th>
<th>10 Min</th>
<th>15 Min</th>
<th>40 Min</th>
<th>60 Min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ave. Stiffness (N/m)</td>
<td>75.3</td>
<td>78.3</td>
<td>87.2</td>
<td>99.1</td>
<td>105.00</td>
<td>92.11</td>
<td>101.2</td>
</tr>
<tr>
<td>p-value vs. Control</td>
<td>0.276</td>
<td>0.100</td>
<td>0.004</td>
<td>0.001</td>
<td>0.063</td>
<td>0.074</td>
<td></td>
</tr>
</tbody>
</table>

Figure 1.3 Stiffness measurements of 4 day etiolated cucumber hypocotyls. a) Stiffness measurements via DMA before and after 80 °C heat bath for 15 min. and subsequent quenching in ice water while tissues were pressed between glass slides. Error bars denote standard error. n = 12 b) Stiffness measurements made at various exposures to the 80 °C heat bath. Error bars signify standard error. All sample sets have n ≥ 13, except for 40 and 60 min, where the associated samples were less amenable to manipulation, n= 6 and n=3 respectively.
4.2 Temperature Effects in Different Plant Tissues:

To investigate the mechanism of stiffening, we compared the results from cucumber hypocotyls with other tissues containing different ratios of matrix polysaccharides: celery parenchyma contains little (~3%) XG, maize coleoptiles as a general monocot system, and onion epidermis is a single cell wall layer. All four tissues showed an increase in stiffness with heating (Fig 1.4). Cucumber that was not heat treated prior to testing was also examined to determine if the 15 second boiling for heat inactivation of enzymes would affect the mechanical properties of the samples (Fig 1.3b). No statistical difference between the stiffness of the non-heat treated and the heat inactivated control was observed (p = 0.276), which confirmed that initial boiling of the samples was not affecting the observed mechanical properties of the tissues.

All samples display a linear increase in stiffness according to incubation time up to 15 min, except for the single tissue layer of onion epidermis (Fig 1.4c). After 10 min of heating, onion epidermal peals were no longer as stiff and breakage happened across the ML of the tissue as opposed to across cell membranes as observed via microscopy. Maize and cucumber tissues showed total stiffness increases (controls vs. 15 min. heated) of 31% (p < 0.001) and 46% (p =

Figure 1.4 Stiffness of tissues after heat treatment between glass slides at 80 °C for incubation times of 5, 10, and 15 min for a) cucumber hypocotyls, b) celery parenchyma, c) fifth layer onion epidermis and d) maize coleoptiles. All samples except onion showed maximum stiffness was achieved after 15 minutes incubation. Error bars denote standard error (n=12 - 18).
respectively (Figs 1.4a&d), while the XG deficient celery parenchyma had a stiffness increase of 62% (p = 0.0001) (Table 1.2). The increase observed in the XG deficient celery tissue suggests larger order rearrangement of pectins and cellulose are occurring to cause the observed increase in stiffness in this tissue.

In order to evaluate the observed heat-related stiffening, cucumber hypocotyls were digested with XG-specific endoglucanase (XEG). If XG is causing the stiffening, then heat treatment allows the remaining network to undergo increased molecular motion, allowing the matrix to reform more interactions upon cooling. This would presumably lead to more hydrogen

![Figure 1.5 a) Stiffness of 24 hr XEG digested Cucumber hypocotyls before and after heat treatment at 80 °C for 15 minutes. The buffered control shows less stiffness than non-buffered hypocotyls, but XEG digestion does not show a significant loss of stiffness (p=0.114). Error bars denote standard error. n = 6 b) Stiffness differences between heated and control WT and xxt1/xxt2 arabidopsis mutant petioles. There is no significant difference observed in the WT petioles. A stiffness increase of 46% compared to controls was observed in xxt1/xxt2 petioles (p < 0.0001). Error bars signify standard error n= 10.]

<table>
<thead>
<tr>
<th>Sample</th>
<th>Control Stiffness (N/m)</th>
<th>Heated Stiffness (N/m)</th>
<th>Increase (%)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cucumber hypocotyls</td>
<td>75.3 ± 5.7</td>
<td>105.0 ± 6.1</td>
<td>46.3</td>
<td>0.001</td>
</tr>
<tr>
<td>Maize coleoptiles</td>
<td>39.1 ± 2.4</td>
<td>55.1 ± 2.9</td>
<td>30.7</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Onion epidermis</td>
<td>20.1 ± 1.1</td>
<td>30.6 ± 2.6</td>
<td>30.7</td>
<td>0.001</td>
</tr>
<tr>
<td>Celery parenchyma</td>
<td>58.1 ± 2.5</td>
<td>94.3 ± 3.2</td>
<td>62.2</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Arabidopsis petioles</td>
<td>57.3 ± 4.6</td>
<td>71.0 ± 10.1</td>
<td>25.0</td>
<td>0.249</td>
</tr>
<tr>
<td>xxt1/xxt2</td>
<td>17.1 ± 1.1</td>
<td>25.1 ± 1.6</td>
<td>46.7</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

*a Onion epidermis was heated for 10 Min.

*b All values have p ≤ 0.05 according to an unpaired, 2 tailed t-test except WT Arabidopsis petioles, which show no significant difference between controls and heated samples.
bonding between the matrix polysacharrides to give an observed increase in the overall stiffness of the walls, removal of XG should result in a drastic loss of stiffness. XEG digested samples displayed very similar results to prior experiments (Fig 1.5a). Control XEG digested samples did not display a significant difference in stiffness (24.8 ± 3.5 N/m) as compared to buffered controls (29.0 ± 3.91 N/m, p = 0.444), which was expected as XEG does not digest load bearing XG domains. After heating, it was thought that the hypocotyls would retain little stiffness, as there should be little free XG left to increase tethers between microfibrils; however, a 42.9% (p = 0.1233) increase in the stiffness was observed, a standard t-test of the sample set however shows no significant difference within the sample set. It is possible that 80°C is enough to detach structurally significant XG domains from cellulose, allowing them to form more / stronger tethers to cellulose. This observed structure change that occurs from a short interval of heating suggests that the pectin matrix also plays a role in cell wall mechanics, as pectins are much more mobile that XG or cellulose microfibrils in the cell wall. This was also evidenced in the increase of stiffness in cell walls shown to have little or no XG. It is also possible that at temperature, it is possible that pectins are rearranging in the ML to form tighter interactions, and allow for tissue stiffening. This may be due to pectin de-esterification and increased interactions with other pectin chains, mobile portions of XG, or Ca²⁺ ions.

To further examine the role that XG impacts the sample stiffness, xxt1/xxt2 arabidopsis petioles, that have been shown to lack XG, were tested. These samples also show a significant increase of stiffness after heating (46.7%, p = 0.0001) compared to WT arabidopsis petioles (Fig 5b). These findings further indicate that the pectin matrix, more than XG, is the main portion of wall that is contributing to the observed increase in stiffness.

1.4.3 Examination of Arabidopsis Mutants

As a means of further examining how the pectin matrix influences the stiffness of the cell wall, three Arabidopsis mutants with differing pectin alterations were tested:
PMEI, which expresses a pectin methyl esterase inhibitor,\textsuperscript{30} and methyl transferase deficient mutants, \textit{tsd2} and \textit{qua2} that have been shown to influence homogalacturonan synthesis and cell to cell adhesion.\textsuperscript{31,32} Each of these mutants should result in different cell wall mechanics based on the branching and esterification of their pectin side chains. Demethylesterification of pectins has been implicated in increased cell wall elasticity during organ formation and cell to cell adhesion via the Ca\textsuperscript{2+} chelation of demethylated pectins.\textsuperscript{33,34} Although the phenotypes of the petioles were visually different, DMA analysis of 3 wk petiole controls showed differing stiffness between only WT arabidopsis and \textit{tsd2} mutants (p =0.02) (Fig 1.6). There were also no significant differences observed between control samples and heat treated pairs, except \textit{tsd2} which showed a stiffness increase of 36\% (p = 0.05). These results correspond with earlier attempts to examine WT arabidopsis petioles, as such the results of these experiments were inconclusive. This was not attempted with other plant tissues as the availability of such mutants is very limited.

1.4.4 SFG Analysis

Figure 1.7 shows the SFG spectra collected from onion epidermal peels. The SFG spectra show a broad peak in the alkyl stretching region centered around 2920 cm\textsuperscript{-1}, consistent with a previous SFG study of intact epidermal peels from the fifth scale of an onion bulb.\textsuperscript{35} The broad alkyl stretch could be due to the assembly of cellulose microfibrils typically observed in the primary cell wall cellulose samples compared to distinct features observed in secondary cell wall samples.\textsuperscript{24,36} The hydroxyl stretching regions centered at 3320 cm\textsuperscript{-1} is assigned to intra and inter chain hydrogen bonding in cellulose microfibrils.\textsuperscript{37}
In order to investigate the changes in cellulose orientation and assembly in onion intact peels due to extension and temperature, the dependence of the SFG signal on the azimuthal angle between the polarization of incident IR beam and the alignment of cellulose microfibrils in the onion cell walls was examined. Figure 1.7a shows the SFG spectra of intact epidermis without any treatment collected in longitudinal and transverse direction to the cell major axis. The difference in alkyl/hydroxyl intensity ratio between two spectra and the enhancement of hydroxyl intensity in SFG spectra collected along the transverse cell axis indicates the cellulose microfibrils are primarily aligned transversely to the cell major axis, and is consistent with the surface and net orientation observed previously for fifth scale of onion bulb.22 When epidermal peels were extended along the cell major axis, the alkyl/hydroxyl intensity ratio did not change drastically (Fig 1.7b). This shows that full extension did not change the net cellulose microfibril orientation in the epidermal peel. The total SFG intensity slightly decreased, which could be due to the decrease in thickness of the sample that is linearly related to the total SFG intensity. The application of heat without physical extension showed some changes in the SFG spectra (Fig 1.7c). The alkyl stretch peak was still broad but the peak center slightly shifted towards higher
wavenumber. The alkyl/hydroxyl stretch ratio between longitudinal and transverse direction did not change with the ratio close to one for both sample orientation. The organization of the cellulose microfibrils may have been effected due to the higher temperature bath, possibly due to pectin motion. The possibility of pectin removal was examined colorimetrically and no pectin was observed in the heating bath after treatment, indicating the stiffness increase is not due to pectin removal from the cell wall. The more aligned cellulose microfibrils in the intact scale has become more dispersed after heat treatment. After extension and heat treatment, the SFG spectra show typical spectral features observed previously for highly dispersed cellulose microfibrils of the innermost scale of onion bulb. The SFG peaks in the alkyl stretch region have sharper features at 2850 cm$^{-1}$ and 2944 cm$^{-1}$ and the intensity of the hydroxyl stretch does not change substantially between the two sample orientations. It could be envisaged that the use of the temperature bath disturbed the original alignment of cellulose microfibrils in the epidermal cell wall; this dispersed orientation of cellulose microfibrils can be manifested in the peak intensity ratio which do not vary based on the azimuthal angle.

1.4.5 XRay Analysis

WAXS was utilized to observe any changes in the crystal spacing of cellulose. Both onion and celery tissues display scattering at a 2θ angle of 22.2° which corresponds to d spacing of 4 Å, and comes from the (002) peak of cellulose I (fig 1.8). It is thought that the peak at 14.4° in

![Figure 1.8 WAXS data for a) onion epidermal peals, and b) celery parenchyma. No difference was observed between heated samples and controls, indicating there were no quantifiable changes to cellulose crystallinity during heating.](image_url)
celery parenchyma comes from the overlay of (101, 100) peaks in cellulose I (fig 1.8b). There is no change between the controls and heated samples, therefore it is unlikely a change from cellulose I to cellulose II occurred during heat treatment. The lack of a change in crystallinity size also suggests there is no change in the aggregation of the cellulose microfibrils in the samples, as there is no observable change in the d spacing of the samples. Analysis using SAXS data also indicated there was no associated change in the crystalline structure of cellulose between heated and unheated samples.

1.5 Conclusions

Plant tissues stiffen following brief (<15 min.) heat treatments at 80 °C. Using DMA, a heat dependant stiffening of cell tissues was observed and characterized. Upon heating tissues at 80 °C for 15 min, tissues displayed increases in stiffness of up to 60% compared to controls. Most tissues such as cucumber hypocotyls and onion epidermis show initial stiffness increases of approximately 42% while tissues lacking in XG, such as celery parenchyma (62%) and xxt1/xxt2 arabidopsis (46%) showed higher increases after heating. This leads to the thought that pectins play a major role in the observed stiffness, pectin influences were not able to be confirmed utilizing pectin mutant data.

Spectroscopic analysis utilizing X-Ray scattering indicates that there is no aggregation of cellulose microfibrils, while SFG analysis suggests that there is a major rearrangement of the overall cell wall matrix after heating. This realignment of the glucan network causes an increase in the stiffness of the cell walls and therefore the tissues. These results have given a look into the connectivity of the primary cell wall. By examining deformations in the elastic region of the cell wall, it eliminates influence of slippage of microfibrils influencing the results. The stiffening of cell walls at temperature reinforces the thought that pectins play a major role in withstanding the forces that are present within the cell by inhibiting movement of cellulose within the wall. The fact that no change in the physical properties of cellulose is observed within the cell wall after heat treatment indicates the change in stiffness must come from a change in the overall superstructure of the wall.
1.6 References


Chapter 2. Investigation of the Effect of Heat Treatment on Onion Epidermal Cell Wall Mechanical Properties Using Microelectromechanical System (MEMS) Analysis

- Preparing for publication

2.1 Introduction

The primary cell wall of plants has the unique ability to both withstand the turgor pressure generated within living cells,\(^1,2\) and still be extensible to allow for plant growth.\(^3,4\) These mechanical properties come from the interactions of cellulose, hemicelluloses, and pectins within the cell wall which form a complex superstructure that gives the cell wall its properties.\(^5,6\) The way these polymers interact to give the cell wall its functionality is of primary interest, as the connectivity of the polymers has not been fully elucidated.

Much work has been done to examine mechanical properties of plant cell walls at the tissue level (macro-scale) using extension measurements.\(^3,7-9\) However, while useful, these studies do not give a full picture of the mechanics happening within a cell wall. Tissues contain a higher order, three dimensional geometry composed of multiple cell walls adhered together via the middle lamella (ML). Mechanical tests of plant tissues reveal the cumulative influence of forces distributed among different cell types through cell to cell interaction, the ML, and even intercellular space.\(^10-12\) Hence, it is desirable to examine the mechanical properties of the cell wall at the subcellular level. Recent work by Zamil and Puri demonstrates an efficient method for examining cell wall mechanical properties at the sub cellular level utilizing microelectromechanical systems (MEMS).\(^13,14\) To date, these systems have been utilized in vacuo in concert with SEM imaging, yet the native cell wall is hydrated, and dehydration has been shown to affect wall mechanics.\(^3\) A MEMS system that allows fully hydrated walls to be tested is of great interest.

Much of the stiffness and elasticity of the cell wall under tensile stresses is thought to arise from the interactions of cellulose and xyloglucans (XG) within the cell wall. Recent work has shown however that pectins may play a larger role in resisting extension at small forces, and inhibits the movement of cellulose and XG in the wall.\(^9,15\) We have recently observed that plant tissues that have been incubated at 80°C for short periods of time exhibit a stiffening effect in the elastic region of extension. In effort to distinguish wall stiffening from tissue stiffening, we utilized a MEMS extensometer that allows for the walls to be hydrated to examine the effect heat treatment has on the cell wall of onion epidermal cells.
2.2 Experimental

Fresh white onion (Allium cepa) bulb was purchased locally. Considering the oldest or outermost fleshy layer as scale #1, the fifth scale was removed and excised into 15x5 mm pieces. The outer epidermal skin was then peeled off, which produced a cell wall profile composed of an open array of epidermal cells resembling a shoebox. This is a well documented technique to expose the cell wall, and has recently been used to image and characterize cell wall mechanical properties at the sub-cellular scale.\textsuperscript{13,16,17} The cell wall profiles were immediately stored in freezer at -85 °C, and were used as a consistent source of cell wall material for subsequent cryotoming to keep samples from the same onion and same scale.

2.2.1 MEMS Device

The test setup consists of a piezoelectric actuator (Model: AG LS25; Manufacturer: Newport, Bozeman, MT, USA) and a 3D printed force sensor beam (Figure 2.1a). Together, the arrangement works as a microextensometer. The displacement resolution of the actuator, was 440 nm. The force sensor beam stiffness was 30 N/m, and based on the displacement resolution provided a 13.2 µN force resolution. The force sensor beam was designed considering the low modulus value of the primary cell wall and was fabricated using an industrial type 3D printer (Proto3000, Vaughan, Ontario,Canada). Due to the complicated shape (folded flexure beam) of the force sensor, rather than using an analytical beam formulation, the sensor was calibrated.
empirically (Figure 2.1b). A unique feature of this MEMS setup is that the force sensor is attached with an adjustable height micromanipulator (Figure 2.1a). The very small size of the sub-cellular scale samples required that the sample be sitting in the same plane on both fixed and moving ends (Figure 2.1a). An image analysis based technique was used to measure stretching and the applied force exerted on the samples. The microextensometer used in this study differs from the prior MEMS based tensile devices in force sensor beam design and assembly. A folded type sensor (Figure 2.1a) was adapted to make the sensor more flexible, which aids in the testing of soft biological samples. In addition, the sensor was fabricated separately and assembled with the actuator later, allowing more space to manipulate small biological samples. This part of the design feature is crucial and facilitates the ability to pick-and-place, as well as grip cryotomed samples under an optical microscope. By adapting a separate force sensor technique, we were able to manipulate and test hydrated plant cell wall samples at subcellular scale.

2.2.2 Subcellular scale sample preparation

The freshly peeled cell wall profiles were immediately put into cryomatrix (Richard-Allan Scientific, Kalamazoo, MI) and stored at -85 °C. The peels were then cryotomed into 35 µm pieces. Specific orientation of the slices were maintained so that the slicing was made along the growth axis of the cells. After cryosectioning, the samples were washed and preserved in DI water and all the experiments were performed within 36 hours of sample preparation. The sample thus prepared is an array of single cells connected by intercellular boundaries or middle lamella (Fig 2.2a).

2.2.3 Heat treatment of samples

For heat treated sample preparation, the cell wall profile was peeled off and set on the bottom, neat of centrifuge tubes 1 (mL). The tubes were then placed in an 80°C water bath for 10 minutes and subsequently quenched in ice water for 1 minute. After cooling, the intact onion samples were stored in their tubes at -80 °C until cryotomed and utilized in testing.

2.2.4 Pick-and-place sample placement and gripping
The cryotomed onion epidermal cell wall section preserved in DI water (Fig 2.2a) was picked using a precision tweezers and carefully placed between the fixed and movable end of the microextensometer. Afterwards, the sample was manipulated very precisely to keep one cell (bounded by two edges of ML) between the edges of fixed and movable end (Fig 2.2b). Then the grip was established using cyanoacrylate adhesive in such a way that the effective sample become a fragment of the cell wall within the boundary of a single cell (Fig 2.2c). The grip was cured overnight at room temperature and fixed humidity conditions to attain sufficient strength to undergo testing in the hydrated state.

2.2.5 Test under optical microscope with continuous hydration

The samples were soaked in water for 10 minutes to re-hydrate before the experiments began. Due to the very small size, the wall fragment samples were very prone to dehydration, for which an arrangement was made for continuous supply of water to the samples in the form of nano droplets. We used a nano mist spray atomizer (Anzikang Nano Handy Mist Spray Atomization Facial Humectant Steamer Moisturize Beauty Instrument) to spray DI water on the sample in five second intervals. The continuous supply of water ensured that the samples were completely wet and at 100% relative humidity throughout the stretching stage. The samples were
stretched from slightly taut to fracture at a rate of 1.9 μm per second. Every two seconds a high resolution image was taken, which provides necessary information to calculate stretching and exerted force on the samples. Fracture stress and strain were collected from the stress-strain plot. The T/L ratios were calculated as follows:

\[ Transverse \ strain \ (T) = \frac{Width_{\text{max}} - Width_{\text{initial}}}{Width_{\text{initial}}} \]

\[ Longitudinal \ strain \ (L) = \frac{Length_{\text{max}} - Length_{\text{initial}}}{Length_{\text{initial}}} \]

\[ \frac{Transverse \ to \ Longitudinal \ strain \ ratio \ (T/L)}{Transverse \ strain \ (T)} = \frac{Transverse \ strain \ (T)}{Longitudinal \ strain \ (L)} \]

2.3 Results and Discussion

Five samples were utilized for both control and heat treated experiments and were tested from slightly taut to fracture. Figures 2.3a and b show the stress-strain plots of control and heat treated experiments respectively. As the samples exhibited a great deal of non-linearity past 5% strain in their mechanical responses, we divided the whole strain range into four groups. In

![Graphs](image)

Figure 2.3: (a) Stress-strain diagram of control samples (n=5). The numerical number 1,2,3 and 4 indicates 4 ranges of strain of 0-5, 5-10, 10-15, and 15-Fracture respectively. (b) stress-strain diagram of heat treated samples (n=5). Same four ranges of strain were defined as control experiments. (c) The modulus of elasticity (E) values of control experiments corresponding to each four strain ranges. The values at top of column plot show the average value of E for a specific range. (d) The modulus of elasticity values for heat treated samples corresponding to four strain ranges.
Figures 2.3a and 2.3b, the numbers 1,2,3, and 4 mark the four range groups corresponding to strain ranges from 0-5%, 5-10%, 10-15%, and 15% to break. The ranges were selected such that in each range the modulus of elasticity (E) can be calculated considering linear regression of the points. Figures 2.3c and 2.3d indicate the corresponding modulus of elasticity (E) values to be 297 MPa and 310 MPa for the control sample and heat treated samples respectively. There were no significant differences observed between the values for E calculated and two inferences can be made from these results: either the cell wall itself is not changing during heating and the stiffness increases observed in tissues after heat treatment come exclusively from interactions of the intercellular spaces and ML, or the cell wall section is not as supported as it would be in to withstand transverse force due to lack of the reinforcement of a bordering cell wall (Fig 2.4). The latter is evidenced by changes in the transverse to longitudinal strain (T/L ratio) for the samples at wall fracture (Table 2.1). The fracture stress, fracture strain, and T/L ratios for all onion samples are shown in table 2.1. The T/L ratio for heated sample is almost 40% (p = 0.002) lower than that of the controls, which can be attributed to the increase in fracture strain of the heated samples. The heated samples break at higher strains (67%) than controls, which indicates some changes in the wall structure is occurring during heat treatment that makes the wall more plastic. The stress at fracture for the heated cell wall samples is also significantly higher, being 48% (p = 0.02) greater than controls. This could occur from
realignement of cellulose within the pectin matrix during the heat treatments, as we have previously observed in tissue level experiments using sum frequency generation (SFG). The lack of a change in E suggests that the major changes occurring happen between load bearing xyloglucan and cellulose more so than the pectin matrix, as they are the main contributors to overall tensile strength of the wall.\textsuperscript{9,18} The main problem with this assumption, is that there is no hindrance to necking and strain hardening in these samples that would not occur with a reinforcing, neighboring cell wall. This illustrates the differences between tissue level and subcellular mechanics. The ML in tissues allows for stresses to transfer to other cell walls under tensile loading, where in these experiments, no such transfer can occur and the wall fragment bears all of the force. This allows for movement of the polymers in both the longitudinal and transverse directions and can be seen in the increased T/L ratio in the heat treated samples.

2.4 Conclusion

This study utilized a new MEMS approach that allows for the testing of fully hydrated cell wall samples. Using this methodology, samples from the fifth epidermal peal of onion were tested after heat treatment at 80°C for 10 min. No major change in the elastic modulus (E) were observed after the heat treatment, however major changes in the fracture stress and strain of the cell walls were observed. Heated cell walls showed increased plastic response with higher average fracture strain (67%) and stress (77.7 MPa) compared to controls (45% and 52.5 MPa). This indicates a rearrangement of the wall superstructure is occurring during heat treatment. These results also indicate that the transfer of stresses between cell walls in tissues and the local versus global alignment of cell wall polymers may make a difference in tissue level mechanics.
2.5 References


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Chapter 3. Characterization and Attempted Modifications of Novel Gelatin / Alkali Silicate Core Binders for Use in Metal Foundries

3.1 Introduction.

Iron foundries constitute a major base of the US industrial sector, accounting for nearly 7.4 million metric tons of metal castings every year. Forming a significant portion of the American manufacturing sector, US foundries keep metal manufacturing more affordable, as shipping time and cost inhibit bulk imports of metal cast products. This in turn helps keep US manufacturing competitive in the world market. The major limitation faced by foundries today comes from increased EPA regulations on carbon and organic pollutant emissions. Current regulations define the maximum amount of Volatile Organic Carbon (VOC) emissions allowed by industrial plants; which limits the amount of product a foundry can produce. Using current methodologies, the burning of the core adhesives utilized in the foundry process accounts for approximately 70% of a foundry's emissions. By lowering the amount of VOC's generated by the casting process, it should be possible to increase the amount of product generated per year by a foundry. This would be of benefit not only financially to foundries, but also for the environment. Foundry workers would also benefit by the lowered the amount of pollutants that would be generated within the foundries themselves. The main effort in trying to limit emissions during metal casting has come from the development of novel core binders; which have been shown to exhibit desirable characteristics similar to conventional binders, but with ~95% lowered emissions during the casting process.

The research herein describes the characterization of a newly developed gelatin/alkali silicate hybrid binder system and attempts to improve upon the physical properties of the binder system. A number of the interactions between silica sand and gelatin are described, as well as silica and gelatin surface modifications that were made to increase the thermal stability of the binder system.

3.2 Iron Foundry Cores and the Development of a Novel Binder System

The metal casting process involves taking molten metal and pouring it into molds that define the shape of the part to be made. The typical casting process uses green sand; which is a mixture of clay, sand, and coal for the outer mold that defines the outside of the part to be cast.
For more complex molds containing hollow portions, such as the impellors used in motors and vacuum pumps, something to take up the hollow spaces in a part during the casting process is required (Fig 1). For these intricate inner portions, cores are used. Green sand, however, is not conducive to making these intricate hollow portions of parts.

Cores are typically made up of high purity silica sand and a small amount of an adhesive (1-3%), or binder, that holds the sand grains together prior to metal casting. Binders must have the thermal stability needed to withstand molten metal temperatures at the molten metal/core interface (1230-1510 °C) to retain its shape during casting, yet conversely be able to be easily removed after the metal has cooled. These two aspects tend to be mutually exclusive, making a paradoxical situation for those attempting to make improved binder systems. The major adhesives utilized in foundries today are petroleum based, as they have good thermal stability and storability. The main concern with the use of these adhesives is once exposed to molten metal temperatures, they burn and release much of a foundry's VOC and hazardous air pollutant (HAPs) emissions. The reason conventional polyurethane binders are still used today is they demonstrate the desired characteristics needed in a core binder. While the conventional systems provide both of these attributes, the large amount of HAPs produced by their pyrolysis leads to the desirability of new systems to be developed; both in the interest of environmental sustainability but also for worker safety, as many of the gasses produced are known toxins (Table 3.1). Other binder systems, specifically that of gelatin, have been marginally utilized, as the byproducts of burning the organic protein are generally harmless and lead to a largely reduced amount of HAP emissions. Gelatin alone, however, has little thermal stability, decomposing at 550 °C. This is well under the temperatures encountered in the metal casting process which range from 1510 °C at the core surface to 50-300 °C just inches into the core. This leads to defects, such as erosion, veining or pitting, in the castings and causes a loss of both time and money for foundries. Another system
utilizes inorganic binders such as alkali silicates that have tremendous thermal stabilities; their shakeout efficiencies, however, tend to be extremely poor.\textsuperscript{4–7} Meaning that after forming the cores, it is extremely difficult to remove the cores, which also presents an increased cost in manpower and lowered efficiency.

The amount of VOCs and HAPs produced by foundry cores makes the development of new binder systems paramount to the environmental sustainability of foundries. This fact prompted the use of collagen as a binder system, and has been shown to produce almost 70-95\% less VOCs than traditional phenolic urethane based binders.\textsuperscript{8} Something was needed to increase the thermal stability of the system to make it more amenable to the casting process. With this in mind, alkali silicates were chosen as possible additives for the binder. This was due to fact that silicates are already utilized in making foundry cores, and they have been shown to cross-link with polyamine containing peptides.\textsuperscript{9,10} As an inorganic additive, there would be no contribution to VOC emissions. Dr. John Fox was able to develop this novel binder system and showed that the mechanical characteristics of the alkali silicate/ gelatin binder system were better than those using gelatin or alkali silicate alone.\textsuperscript{2} While this system does produce less VOC emissions, there have been problems with casting defects in foundry level testing. These problems prompted the need to design a system that modifies collagen, silica, or both in attempt to impart better characteristics to the binder system.

### Table 3.1 Emissions from typical core binder resins vs collagen based binders.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Emissions</th>
<th>Phenolic Urethane Resin (Sigma Cure 7121 and 7156)</th>
<th>Biodiesel Resin (Sigma Cure 705 and 305)</th>
<th>Collagen-based Binder</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>C1–C4, methane</td>
<td>42.0</td>
<td>12.2</td>
<td>72.6</td>
</tr>
<tr>
<td>2</td>
<td>Toluene</td>
<td>107.8</td>
<td>11.6</td>
<td>78.6</td>
</tr>
<tr>
<td>3</td>
<td>Aniline</td>
<td>32.1</td>
<td>1.7</td>
<td>4.1</td>
</tr>
<tr>
<td>4</td>
<td>Benzene</td>
<td>35.4</td>
<td>3.4</td>
<td>3.1</td>
</tr>
<tr>
<td>5</td>
<td>Ethylbenzene</td>
<td>28.4</td>
<td>17.6</td>
<td>1.79</td>
</tr>
<tr>
<td>6, 7</td>
<td>o-, m-, p-Xylene</td>
<td>0.18</td>
<td>0.05</td>
<td>0.33</td>
</tr>
<tr>
<td>8</td>
<td>Nitrobenzene</td>
<td>0.77</td>
<td>0.12</td>
<td>0.94</td>
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<tr>
<td>9</td>
<td>Phenol</td>
<td>0.78</td>
<td>0.08</td>
<td>0.78</td>
</tr>
<tr>
<td>10</td>
<td>Tert-butylbenzene</td>
<td>11.92</td>
<td>14.7</td>
<td>13.88</td>
</tr>
<tr>
<td>11</td>
<td>Cresol</td>
<td>11.0</td>
<td>0.30</td>
<td>1.53</td>
</tr>
<tr>
<td>12</td>
<td>cresol</td>
<td>1.10</td>
<td>0.30</td>
<td>1.53</td>
</tr>
<tr>
<td>13</td>
<td>2,6-Dimethylphenol</td>
<td>2.27</td>
<td>0.33</td>
<td>2.47</td>
</tr>
<tr>
<td>14</td>
<td>naphthalene</td>
<td>4.11</td>
<td>0.65</td>
<td>16.27</td>
</tr>
<tr>
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<td>Dimethylphenol</td>
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<td>0.11</td>
<td>0.58</td>
</tr>
<tr>
<td>16</td>
<td>3,5-Dimethylphenol</td>
<td>ID</td>
<td>ID</td>
<td>ID</td>
</tr>
<tr>
<td>17</td>
<td>Naphthalene</td>
<td>0.81</td>
<td>0.24</td>
<td>0.47</td>
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<tr>
<td>18</td>
<td>1-Methyl-naphthalene</td>
<td>9.20</td>
<td>0.81</td>
<td>0.74</td>
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<tr>
<td>19</td>
<td>1-Methyl-naphthalene</td>
<td>4.16</td>
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<tr>
<td>20</td>
<td>Dimethylphenyl (isomers)</td>
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<td>1.12</td>
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<td>21</td>
<td>Methyl oleate</td>
<td>14.27</td>
<td>1.23</td>
<td>39.92</td>
</tr>
</tbody>
</table>

1 Hazardous air pollutants listed in Title III of the 1990 CAAA are indicated by the numbers with italic font. Abbreviation key: ND, not detected; ID, intermittently detected. 2 C6H5 isomers included penta-methylbenzene isomers, 1-methyl/2-(1-methylthyl)-benzene, 1-methyl/3-(1-methylthyl)-benzene, and 1-methyl/4-(1-methylthyl)-benzene etc. GC-FID results calibrated with sec-butylbenzene (C8H11)
3.3 Characterization of the Novel Binder Systems

Initial efforts were made to define the chemical interactions occurring between the sands and binder systems to determine what chemical modifications to increase the thermal stability of the molds while keeping the shakeout efficiency constant would be possible. To do this, an understanding of gelatin and silica re-activities were needed. Utilizing the methods developed by Dr. Fox, a number of molds with differing amounts of binder were made and examined via SEM. The images obtained indicated that relatively little surface area of the sand grains was involved in the binding of the systems (Fig 3.2). The maximum distance the binder was seen to bridge was 6.2 µm. Both NMR and IR studies of the resulting powders obtained from crushing the samples were inconclusive, as the mixes were primarily made up of silica, with relatively small amounts of the binder actually present; therefore, much of the initial characterizations were macroscale measurements of the mechanical properties obtained by Dr. Fox in the initial development of the binder system. Analyses of the starting materials was attempted in effort to determine the chemical interactions occurring within the binder system alone. Knowing the interactions occurring within the binder system should allow for a more informed pathway for modification of the binder system and improve its properties.

3.3.1 Characterization of Gelatin

Collagen is a triple-helical based protein found ambiguously throughout animal tissue. As a structural protein, collagen displays a tensile strength equal to that of steel. Collagen is instrumental in the development of bone and is of major interest in the medicinal field for use in

Figure 3.2 SEM images of core mold cross-sections. a) Core made using 3% w/w binder showing total surface. b) Close up of a single junction where binder is holding grains together.
bone and skin grafts and is the main component in connective tissues. The primary structure of collagen is formed mainly of Gly (26%) and follows a Gly-X-Y pattern, where X and Y constitute any other amino acid, but are generally Pro (16%) and Hyp (14%) which allows for the protein's tight, left-handed twist. The amide backbone of the single strands then associate to form right handed triple helices, forming a relatively strong adhesion via hydrogen bonding (Fig 3.3). These bonds give collagen its reversible adhesive properties. Collagen denatures at 54 °C in the presence of water and then is able to recombine upon cooling into less ordered, random helices. Other amino acids included in the side chains, such as Lys, point outwards and upon oxidation, can undergo cross-linking to other strands forming largely cross-linked fibrils. It is these fibrils which impart the total strength of the protein and allow for large, branching hydrogels to form. It is this functionality that make it useful as an adhesive. Once the hydrogels are dehydrated via continued heat, while the strength of the resulting collagen is less than that of associated coils, the protein does retain much of its strength.

Initial investigations and characterization attempts utilizing known methods of collagen characterization and purification failed. This led to an investigation as to whether it was inexperience in the procedures being performed, or the obtained collagen samples were simply so impure that traditional methods did not apply. A purified sample of collagen was purchased and characterized first to determine what should be found. IR of the protein looked as it should, with amide peaks at 1626 and 1537 cm\(^{-1}\) which is indicative of the strained Gly-Hyp-Pro dominated α-helical structure of the molecule\(^{12,13}\). Comparison of this sample with a sample of the collagen being used in the binder system led to an interesting discovery; that it was not collagen that was being utilized, but gelatin, a more hydrolyzed form of collagen. Gelatin, as opposed to collagen, contains much less Lys, is much less cross-linked, and is composed of a more heterogeneous mix of oligomers, from 80-300 KDa. To confirm this, a commercial grade sample of gelatin was obtained and spectra were compared to that of the material used in the
developed binder. It was then determined that gelatin, not collagen, was being used for the core binders, Fig 3.4. This did not change the desired route of modification however, as gelatin contains much of the same functionality as collagen, just with less amine crosslinks.\textsuperscript{11}

While much research has been done to physically characterize gelatin and its bulk properties for use in foods and as a hydrogel,\textsuperscript{11–13} not much has been done to chemically characterize the heteropolymer for further chemical modification. Upon further examination of literature, it was discovered that many polymer gels are better analyzed via NMR in their gelled states due to locking of the protein into a more confined state. Figure 3.5 shows the $^1$H NMR of gelatin in D$_2$O. It should be noted that in D$_2$O, the protons typically associated with amide bond
backbones is not observed, possibly due to coil formation and fast proton exchange. There is still some variability within side chain environment, as well as increased T₂ relaxations which account for peak broadening. Peak assignments were made utilizing the known amino acid shifts as well as comparison to earlier attempts to characterize the heteropolymer.

Once it was determined that individual amino acid residues could be identified within the ¹H NMR spectra, an initial study to determine if the inclusion of silicate in the gels would be observable was performed. By adding increasing amounts of sodium silicate, a shift in the ε-proton of Lysine can be observed (Fig 3.6). This is thought to be due to removal of the ammonium and amine protons during silicate addition, as the pKₐ of the Lys side chain is ~10 and the pH of the added alkali silicates is in excess of 11, well above that needed to remove the

Figure 3.6 ¹H NMR spectra of gelatin with added amounts of Na₂SiO₂. A downfield shift in the ε-proton of Lys can be observed with an increase in the silicate concentration from 3.01 to 2.62 ppm can be observed as the concentration of Na₂SiO₂ increases from 1% (blue) to 5% (red) to 10% w/w (green).

Figure 3.7 Overlay of IRs with differing amounts of silicate added, from 0% (red) to 50% w/w (green). The most notable differences come from increasing absorbances at 2400 and 1100 cm⁻¹, which come from the absorbance of silica particles. All spectra are normalized the amide I band of the gelatin backbone at 1630 cm⁻¹.
amine protons. The shielding affect of which is observed in the $^1$H NMR spectra. The changes associated with gelatin can also be observed via FTIR, Fig 3.7. A significant decrease in the amide stretch at 1500 cm$^{-1}$ can be seen with an increase in silicon-oxygen bending region at 1100 cm$^{-1}$.

3.3.2 Characterization of Foundry Sands

The first part of this project involved the characterization of silica sand surfaces to determine the chemistry occurring in gelatin/ alkali silicate binder systems. While a number of characterizations utilizing IR have been attempted with sands, most have been done using reclamated green sands and not virgin sands. Also, many of these analyses involve physical methods, such as sieving and turbidity; a more chemical method was desired for this project and NMR and FTIR methods were utilized for characterization. Using virgin sands prior to modification allows for a more thorough evaluation of modified silicas, both in this project and for industry as a whole. The term silicas includes a wide range of materials including natural silicas, such as quartz and cristobalite, to synthetically made colloidal silicas and silica gels. The wide range of applications that silicas are used for makes them of valuable interest. The resilience and general lack of reactivity associated with silicas makes them ideal substrates for solid phase chemistry. Much work has been accomplished to define the surface and reactivity of differing types of silicas, the most useful of which belong to the silica gels.\textsuperscript{15,16} Silica gels are primarily used in chemistry as a means of analytical separations. HPLC utilizes modified silica gels in columns as stationary phases that can be modified to display desired functionality. This allows for efficient separation of desired compounds based on desired chemical interactions. The main benefit of silica gels is their porosity. Some silica gels display surface areas of more than 500 m$^2$/g via nitrogen adsorption, giving a large interaction surface for molecules.

Silicas have a high heat resilience, which makes the use of silica sands useful for iron foundry work. Quartz sand, the main component of foundry cores, has two major temperature transitions prior to melting: a transition from $\alpha$-quartz to $\beta$-quartz at 573 °C and $\beta$-quartz to $\beta$-cristobalite at 1050 °C. Cristobalite does not melt until reaching 1705 °C (Fig 3.8). Issues arise however from the initial change in the crystal lattice of the material from $\alpha$ to $\beta$, as the density of the silica decreases corresponding to expansion from trigonal to a hexagonal structure. Sometimes, this can cause the sand cores to expand, causing pitting and veining when exposed to molten iron temperatures. This prompts the need to utilize adhesives that allow for thermal
expansion of the sand.

Mesoporous silica gels were used as model systems for modifications of silica sands as they have more surface area for modification. This allows for easier analysis of the resulting materials as the surface area to mass ratio for quartz silicas is extremely low. Foundry sand with an average diameter of 300 µm has a surface area of approximately 10 m$^2$/g while silica gels can average over 200 m$^2$/g for particles of a similar diameter. As such, it was much easier to start with silicas for the characterization of the materials. Generally, silica gels are treated with different alkoxy silanes in effort to introduced desired chemistry to their surfaces, allowing for the variability found in analytical separatory columns.

### 3.4 Proposed Modifications to the Binder Systems

Due to the veining and pitting problems occurring within a number of the molds made with the novel binder system, a number of possibilities for improving the thermal stability of the system were conceived. Organic modifications of the substrates to allow them to undergo covalent bonding reactions during curing would dramatically increase the thermal stability of the molds, as the energy put into the system via molten metal would no longer only have to overpower hydrogen bonding, but now actual physical bonds between the binder system, silica sand, and silicate. Some evidence to help with this has been accomplished using known cross-linking agents such as formaldehyde and gluteraldehyde.$^{11,17-19}$ These tend to be toxic and are not ideal for use in an industrial setting.

The first thoughts involved increasing the affinity that gelatin has for silicates in solution. It has been shown that a number of plants, sponges, and diatoms are able to fix silicic acid out of their environments and utilize it to form biosilica composites.$^{20,21}$ The forms in which the silicas are utilized is very specific to the organism and the proteins used to fix the silica. Diatoms, for
example, produce a specific class of proteins called silafins, which are specifically utilized for the ordered structuring of poly-salicic acid. Silafins are known to contain a large number of amine containing side chains and allow for specific condensation of silica within its secondary and tertiary structures (fig 3.9).\textsuperscript{20,22} Generally, biomineralization of silicas occurs in proteins containing high amounts of proline and acidic side chains such as Asp and Glu, as well as polymers having high amine content. Gelatin and collagen, being largely of this generic make up should be able to follow this ability, and this biomineralization pathway is similar to that of the formation of hydroxyapatite, the main component in bone.\textsuperscript{23} This information led to the thought that the addition of more Lys and Arg residues onto gelatin may aid in the association of alkali silicates in the binder system and increase thermal stability. Aminosilanes have been shown to influence the mechanical properties of cements as well,\textsuperscript{24} therefore adding amine functionality to the utilized silica sands should also help to increase the interaction of sand with both silicate and gelatin.

### 3.5 Experimentals

SEM images were obtained using a FEI Nova NanoSEM 630 FESEM. All FTIR spectra were obtained utilizing a Thermo-Nicolet 6700 FTIR with globar source, DTGS detector with Diamond ATR attachment and KBr beam splitter. Data analysis was performed utilizing EZ OMNIC software. TGA was performed using Q500 TGA (TA Instruments) using 50 mg of sample per run. The temperature ramp was ran from RT to 1000°C at a constant rate of 50°C min\textsuperscript{-1} and the data was worked up using TA software analysis. \textsuperscript{1}H NMR spectra were obtained using a Bruker 400 MHz NMR. CP and SP MAS solid state NMR spectra were submitted and obtained from the Penn State NMR facility. All reactions were performed under argon atmosphere.
3.5.1 General Silica Modification:

Silica was etched in boiling Piranha solution (3:1 H$_2$SO$_4$ : 30 % H$_2$O$_2$) for 1 hr to remove any impurities. The silica was then filtered, washed with distilled H$_2$O and dried en vacuo. The samples silica (1 g) were then placed in a flask containing anhydrous toluene (20 mL) and the specified alkoxy silanes added according to table 1.2. After stirring for the specified time, the silica samples were filtered and washed with methanol and cured in an oven (150 ºC) overnight. The silica samples were then washed with DI water and acetone to remove any physically adsorbed silane and dried in vacuo. All samples were then examined via TGA to determine binding.

Azido Silica:

Triflic azide (5 mmol) in CH$_2$Cl$_2$ (15 mL) was added dropwise to a flask containing APTES modified silica (1 g), CuSO$_4$ pentahydrate (5 mg, 1 mol %) and K$_2$CO$_3$ (0.45 g, 3 mmol) in CH$_2$Cl$_2$ (25 mL). The mixture was then stirred overnight, filtered and the solid washed with H$_2$O, CH$_2$Cl$_2$, and MeOH. The silica was then dried under N$_2$ and analyzed via DSC and TGA.

Alkynyl Silica:

Propargyllic acid (122 µL, 2.4 mmol) was added to a solution of diisopropyl carbodiimide (372 µL, 2.4 mmol) in CH$_2$Cl$_2$ (25 mL) under stirring. After 30 min, the solution was added to silica (1 g) in CH$_2$Cl$_2$ (25 mL) and stirred for 2 hrs. The mixture was then filtered and the solid washed with CH$_2$Cl$_2$, H$_2$O, and Acetone. The silica was then dried en vacuo and analyzed via TGA and DSC.

3.5.3 General Gelatin Modification Procedure:

Protected Amino acid (1.5 mmol) was added to a stirring solution of EDC (1.7 mmol) and NHS (2.3 mmol) in DMSO (10 ml) under argon. After stirring for 2 hrs at 37 ºC, gelatin (1.5 g, 10% in DMSO) was added slowly via cannula. The mixture was then allowed to stir overnight. The mixture was poured into cold ethanol. The resulting white precipitate was filtered, washed with ethanol and acetone, and then dried under vacuum to give a yellowish gum. After further drying, the solid was then analyzed via FTIR and $^1$H NMR spectroscopy.
3.6 Results and Discussion

3.6.1 Modification of Mesoporous Silicas.

Initial experimentation was attempted on foundry sands utilizing a silane bonding procedure used typically for the synthesis of reverse phase silicas for use in HPLC columns. As silica sands contain the same siloxyl functionality on their surface as do silica gels. Initial attempts to characterize the products, however, met with failure. This was due to the relatively low surface area present on the sands and was not conducive to typical means of analysis such as NMR or IR. This led to the use of TGA as a possible means of analysis, but this also was plagued with irreproducibility due to thermal expansion of the sands causing jumping in the pan, and the small amount of material that charred off the sample was miniscule in comparison to overall sample mass, which shows little percent mass lost (Fig 3.10). The silica sands are primarily made up of quartz, a piezoelectric material, so heating and expansion also cause charging of the sample. This led to an indicated increase in the mass of the sample over time as well.

Figure 3.10 Initial TGA experiments to determine binding of APTES on the surface of silica sand. Major problems came from the "jumping" encountered at approximately 575 °C that appears as a major mass lost. A subsequent increase in the masses is observed, which is thought to occur from electrostatics resulting from heating quartz sands.
Three organosilanes were chosen for the modifications: aminopropyl triethoxy silane (APTES) 3, as it is known to show good binding chemistry and its primary amine functionality lends itself well to further modifications; amino(ethyl amino)propyl dimethoxy methyl silane (AEAPDMS) 2, because of its diamino moiety that would add binding sites for silicates in the molds via biosilification type interactions. Mercaptopropyl trimethoxysilane (MPTMS) 1 was utilized as a possible means of introducing covalent disulfide bridges in the molds to increase thermal stability. All three substrates are known to bind to silicas in similar fashion and are shown in figure 3.11. Due to the difficulties involved with analysis of foundry sand, it was

Figure 3.11 Proposed modifications to the silica surface. 4 and 5 were synthesized from 3.

![Diagram of organosilanes]

Figure 3.12 TGA results for bondings of mesoporous silica gel with APTES at different concentrations of APTES. All results are normalized to 100 %, after dehydration of the silica.
determined that mesoporous silica gel would give similar bonding characteristics with much better efficiency due to increased surface area of the samples and therefore increased binding sites. First attempts at bondings worked well, as is evidenced by the percent mass lost via TGA (Fig 3.12). Amino silanes initially coordinate with the surface hydroxyls via hydrogen bonding, and subsequent curing at 150 °C allows for removal of the silane alkoxy groups and bonding to the silica surface itself. Any physisorbed silane is then washed off the surface via acetone and ethanol. APTES bonding has been shown previously to only need 30 min of cure time before all coordinated silanes are bonded to the silica surface. This is due to the nature of the silane itself, APTES has the ability to self aggregate during curing, to form larger networks. This leads to higher bonding efficiencies and is dependent on the stir time of the mixture. APTES bonding for the mesoporous silica used was saturated at 2 mmol/g APTES to silica. This corresponds to a coverage of 1.5 mmol/g silica based on the percent mass lost. The observed mass lost is proportional to the amount of organic material bound to the surfaces. The same binding assays were performed for AEAPDMS and MPTMS. AEAPDMS showed similar mass lost to APTES (Fig 3.13), but at a lower binding coverage of 1.5 mmol/g silica due to both its larger size and dimethoxy silyl groups.

Figure 3.13 TGA analysis of AEAPDMS (diamino) silica bondings at different concentrations of substrate. A maximum bonding efficiency was achieved at 1.5 mmol/g silane to silica.

Figure 3.14 Trimer formation of APTES compared to single bond formation of AEAPDMS.
which inhibit the formation of the trimeric structure of APTES (Fig 3.14). MPTMS, however, showed very little bonding efficiency upon first analysis. This was due to the thiol moiety having little interaction with the silica surface. Not only does the amine functionality allow for initial coordination with the silica surface, it also makes the surface silanol more nucleophilic. \(^\text{15}\) To induce better binding of the MPTMS, imidizole was added to act as an amine base which activates the silica surface, and this allowed for silica modification (Fig 3.15)

NMR characterization of the bonded sands were then accomplished utilizing \(^{29}\)Si CP MAS NMR and \(^{13}\)C CP MAS NMR. Fig 3.16 shows the comparisons of the \(^{29}\)Si spectra from the different silica bondings. Fig 3.16a shows non-bonded silica gel displays δ's of -92, -102, and -113 ppm corresponding to geminal siloxyls, single hydroxyls and bridging hydroxyls respectively. \(^\text{15}\) This indicates that most of the surface is composed of single reactive siloxyl moieties. Upon reaction with APTES (Fig 3.16a) the signals at -92 and -102 ppm are greatly diminished, while new signals at -69 and -59 ppm arise, due to reaction with geminal and single siloxyl groups to form the new alkoxy silane. Two peaks arise due to branching of the silane during bonding (Fig 3.13). The bridging siloxyl groups are non-reactive, so their intensity remains constant. Fig 3.16b illustrates the bonding of AEAPDMS and shows only a single new peak at -21 ppm. Only one signal is observed because of the silanes steric bulk, which does not allow for the formation of the larger aggregates formed with both APTES and MPTMS. Figure 3.17 shows the \(^{13}\)C CP TOSS spectra obtained from the samples. Fig 3.17a indicates that no carbon is present in the silica gels prior to bondings. After binding of APTES, peaks at 41, 23,
and 8 ppm can be observed, which correspond to the propyl chain in the silicate product. The trimeric nature of the surface chemistry cannot be seen as the environment surrounding the propyl chains is nearly identical. AEAPDMS bonding shows the predicted 5 chemical shifts at 50, 38, 20, 11, and -5 ppm. MPTMS bound silica shows peaks at 25 and 9 ppm, with a much

Figure 3.16 $^{29}$Si CP MAS NMR spectrum of modified silicas. a) un-modified silica b) AEAPTMS modified silica. Single addition to the surface is shown by the single new silane peak at -21 ppm. c) APTES modified silica. The trimeric structure of the added silane can be seen by the appearance of two peaks at -59 and -69 ppm, corresponding to the pendant and central silicon atoms respectively. (see fig 3.13) d) MPTMS bound silica. The presence of the two new peaks may signify a dendritic bonding similar that of APTES.

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Figure 3.17 $^{13}$C CP TOSS NMR spectra obtain for modified silica gels. a) Unmodified Silica. b) AEAPDMS modified silica gel. c) APTES modified silica gel. d) MPTMS modified silica gel.
smaller peak at 44 ppm, which corresponds to the thiol carbon.

With this evidence, it was clear that bondings were going as expected, and so modifications to the amino silicas was done to create the precursors for clickable silicas. Azido triflate was added to the APTES bonded silica following a literature based procedure that utilized a catalytic mixture of copper sulfate and potassium carbonate used as a means of placing azides on the end of amino terminated peptides.\textsuperscript{17} Utilizing a different batch of amino silica, propanoic acid was used in a carbodiimide coupling to form the alkynyl substrate 5. The results of both can be seen in Fig 3.18, which shows the TGA overlay of all the bonded silicas. All of these were bonded at the 2.0 mmol/g silicate to silica gel ratio, and all of the substrates show a bonding efficiency of 1.5 mmol/g as was shown previously. The alkynyl and azido silicas indicate an increased mass loss that is proportional to the added mass to the amino silica substrate. Also, the initial loss of nitrogen from the azido silica can be seen as the first slope in the results. After these sucessful results, a number of bonding to silica sands were attempted, but again, nothing was truly identifiable through spectroscopic means. A simple experiment utilizing molybdic acid stain (generally used in conjugation with TLC to identify unsaturated copounds) was tried, and a difference in color between unmodified sand and Alkynyl sand was noticed, lending credence (albeit not conclusive) to the bonding of substrates to the sands. As such, the sands were kept and later utilized without further characterization.
Figure 3.19 Desired gelatin side chain modifications. All were attempted using carbodiimide couplings to make the corresponding amides with Lys side chains.

### 3.6.2 Modification of Gelatin

It was originally hypothesized that the additions could occur by initiating the diimide coupling utilizing the acid side chains Asp and Glu, as they are in higher concentration after isolation; however, attempts to do this resulted in immediate crosslinking of the gels themselves. Therefore, number of amino acid substitutions to the remaining Lys side chains of the protein were attempted utilizing DCC, EDC, and DIC couplings (Fig 3.19). The first modifications attempted were done to make modifications 1-4. Acetylation of the gelatin was attempted by adding acetic anhydride to a solution of gelatin in CaCO_3 and water. FTIR analysis of the product did indicate something occurred, as new stretches at 1013 and 951 cm\(^{-1}\) were observed (Fig 3.20). The resulting NMR however did not show any differences in the ε-proton for Lys, (Figure 3.20 FTIR of isolated Acylated gelatin. An addition of two stretches at 1013 and 951 cm\(^{-1}\) can be seen. There is also a slight relaxation of the Amide I stretch indicative of the tight twist of the Pro dominated helices.)
Figure 3.21 $^1$H NMR spectrum of the acylated gelatin. There is almost no difference from reference gelatin spectra.

Fig 3.21), leading to the belief that the reaction did not work. A number of other coupling reagents were tried, but none showed success. It was then hypothesized that perhaps the chosen reagent was the problem, and a number of the other couplings involving the aforementioned amino acids were attempted.

Boc protected Ala was utilized first as a possible means to show that the amine functionality is necessary for the binder system to work. Also, as a simple amino acid, it may lend itself to a more facile reaction. After EDC coupling, it seemed like the gel had changed physically, as it was able to solubilize much faster, and the melting temperature was lower than unmodified gelatin. The FTIR spectrum also showed the same change in the stretches as did the acyl modification at 1013 and 951 cm$^{-1}$. There is also a slight increase in the peak at 1165 cm$^{-1}$. Subsequent deprotection, however, yeilded identical spectra to gelatin, both with FTIR and $^1$H NMR. This was also the case for the other modifications attempted. Some difference could be noted in the spectra, but none gave definitive results to show that modification was a success. Upon attempted deprotection with dilute TFA, all spectra were identical to the starting gelatin. Due to this, neither of the precursors to the click cyclization were attempted.

### 3.7 Conclusions

The starting materials for the gelatin alkali silicate binder system was described, and the interaction of alkali silicate with the Lys side chains gelatin were observed. This is thoguht to be
the mechanism of how the binder gains its thermal stability. Chemical modifications to gelatin, silica sand, and mesoporous silicas were attempted. All attempts to modify gelatin to impart desired physical characteristics failed due to the complexity of the heteropolymer. Chemical modifications to mesoporous silicates (as a model system for sand) were extremely successful and demonstrated coverages of 2 mmol/g silica. Further work should be done to combine these systems with current physical testing to determine if the chemical changes used would impact the thermal stability and strength of the core binders.
### 3.10 References


Appendix A. Prior Group Projects: Synthetic Work

A.1 Synthesis and Analysis of Cholesterolamine Dimers and Blockers of Lipid Transport

In my first two years, I studied bio-organic chemistry with Dr. Blake Peterson. Research in the Peterson lab focused on synthetic cell surface receptors, specifically cholesteroamine conjugates and their influence on cell cytology and receptor mediated endocytosis was studied using these compounds.²,³ My projects focused on the creation and testing of cholesterolamine dimers that were intended to function similarly to membrane spanning proteins (Fig A.1). The compound would enter a cell membrane through hydrophobic interactions and subsequently one end of the dimer should “flip” to the cell interior either through the random relocation that occurs in cell membranes, or via flipases, proteins that the cell uses to cycle phospholipids and cholesterol. The spanning cholesterol-amine dimer should then allow for influencing internal cellular function via interaction of selected compounds with a designed receptor on the extracellular portion of the dimer. A number of the desired dimers were synthesized, though none showed in vitro activity.

The second project accomplished during my tenure in the Peterson lab was the creation and evaluation of di-acyl ureas that were found to block lipid transport within cell membranes (Fig A.2). A number of novel compounds were synthesized and tested in cells via flow cell cytometry based on uptake of cholesterolamines synthesized in this lab coupled with fluorescent tags. Lipid transport into cells via high density lipoprotein (HDL) controls many important cellular functions. It is hypothesized that the compounds used by the Peterson lab are incorporated into

Figure A.1 Mode of action for controlling cellular signaling via use of artificial transmembrane receptors. a) The dimer inserts into the membrane in a “U” shape. b) The less polar end of the molecule flips to the inner leaflet of the membrane presenting biotin to the cytosol. c) Streptavidin binds biotin being projected into the cytosol. d) Proteins fused to streptavidin influence cell signaling and endocytosis (e).
plasma membranes via a specific HDL uptake pathway involving scavenger receptor class B type 1 (SR-B1). In effort to lend credence to this, a number of inhibitors were designed based on work by Nieland, et al. who designed a number of compounds shown to inhibit lipid uptake via this pathway. Two of these compounds were made initially, BLTs 1 and 4, and inhibition was tested in Jurkat cells using the 1-Glu Penn compound, a fluorescently tagged cholesterolamine. After 1 hour incubation with 100 μM BLT compound, the cells were washed and incubated another 5 minutes with 1-Glu Penn. The cells were then analyzed for total fluorescence via flow cytometry. It was determined that BLT-4 showed the best inhibition of 1-Glu Penn and a number of analogues of this were made to optimize inhibition and possibly determine the mechanism of inhibition. The first series of analogues indicated that the electron rich moiety was necessary on the phenyl ring, with the presence of a chlorine being the most effective at inhibiting uptake. A number of chloro analogues were also synthesized. PBJ-5 proved to be the most inhibitory of the compounds at 50 μM. This project allowed me to gain experience in biological methods and cell cultures as well. Dr. Peterson then accepted a job with the University of Kansas Medicinal Chemistry program. I elected to stay at Penn State and join another group. This time working in organic synthetic chemistry.
A.2 Methodology of Allenyl Azide Cyclizations and Attempted Synthesis of Nodulisporic Acid F. - Allenyl Azide Methodology published

Dr. Ken Feldman’s lab focuses on organic methodology and natural product synthesis. I felt that this lab would allow me to gain more bench experience and further my knowledge of organic synthetic methods. My first project involved further investigating copper mediated allenyl azide cycloadditions to form annelated indoles. This methodology initially took advantage of trimethylenemethine chemistry developed by Little and coworkers (Fig A.3). It has since been shown that it is possible to do similar chemistry utilizing triazolines, and it was this work that sparked interest in the possibility of allenyl azide cyclizations. While studying a number of aryl tethered allenyl azides, it was discovered that by addition of a small amount of copper iodide to the reaction mixture during photochemical initiation, regiochemical control could be introduced to the systems, providing a single regioisomer as (Fig A.4). The proposed explanation for this comes from the mechanism of the reaction. Transition of an indolidene...
intermediate during the reaction hinders bond rotation, giving rise to a single isomer as the product. I worked to further the scope of this methodology (Table A.1).¹

After finishing the methodology, I moved on to the synthesis of a model compound of the nodulisporic acid family of natural products. Being diterpene indoles, it was thought that the allenyl azide methodology would fit well with its synthesis. Initial attempts towards the synthesis of the model system met with moderate success (Fig A.5). The synthesis of starting

![Synthetic route to the model system of Nodulisporic Acid F.](image)

Figure A.5 Synthetic route to the model system of Nodulisporic Acid F. Much progress was made through the route; the final cyclized product was not able to be isolated.
material was plagued by an initial 27% yield and subsequent steps required major chromatography steps, limiting the subsequently obtained products and slowing the pace of the route. While it took some time to reach the key steps, the initial allenyl azide cyclization did work as proven by NMR; however, the final cationic cyclization did not meet with success.

A.4 References:


Appendix B: Development and Characterization of an Inexpensive Portable Cyclic Voltammeter

B.1 Abstract

The teaching of instrumental analysis for many small colleges and high schools continues to be stymied by the high cost, complicated maintenance, high power requirements, and often the bulk of the instrumentation. Such issues have led us to develop inexpensive small scale instruments, that have shown to significantly enhance the achievement and confidence of students in our technology based labs and courses. We have dubbed this lab-based pedagogy, primarily aimed at inspiring and engaging science and engineering students, as SMILE (small, mobile instruments for laboratory enhancement). One instrument that has recently been designed, constructed, and characterized by staff and students at Penn State is the cyclic voltammeter. CV is a versatile electroanalytical technique that is used to monitor the redox behavior of chemical and biochemical species in solution. The CV instrument and CV technique readily lends itself to miniaturization, and facilitates the practical application of CV analysis within standard undergraduate and advanced high school laboratory courses. The entire miniaturized and portable instrument was designed for less than $50, thus allowing for deployment of multiple apparatus in an undergraduate lab with a modest budget.

B.2 Introduction

Analytical instrumentation has changed significantly and dramatically over the past three decades, and equipment that was the mainstay of well funded research centers and institutes is now routinely found in undergraduate chemistry teaching labs. The sensitivity and selectivity of analytical techniques has increased many-fold over this period. Likewise the low cost and availability of such analysis has led to a broad increase in public awareness of health, safety, and environmental issues relating to pharmaceuticals, food, energy, and industrial processes. These enormous advances in technology demand schools and colleges to train their students in the necessary STEM skills required to analyze a wide variety of samples with an equally wide variety of instruments, interpret data with due care, and at the same time to continue to further develop these analytical techniques, thus enabling our students to succeed in the modern workplace. A competent analytical chemist must have a sound knowledge of the various
techniques that are available for a given chemical system, and a reasonable understanding of the fundamental science behind the instrument being utilized. Knowledge of the limitations, accuracy, time, cost, and the ability to determine the adequacy of the data obtained for the task in hand is necessary for today's chemists.

Unfortunately the teaching of analytical chemistry in many schools and colleges continues to be thwarted by the high cost, complex maintenance, and the sheer bulk of many commercial instruments. For institutions with larger enrollments, providing each individual student with access to an instrument requires either a highly sophisticated lab rotation program, or the deployment of multiple expensive instruments. These troubling issues have led our research efforts to focus on developing inexpensive, small scale instruments for undergraduate, high school, and middle school students, a program that we have dubbed SMILE (small, mobile instruments for laboratory enhancement).1-3 At Penn State University, students in the upper level instrumental analysis course design, construct, characterize, and troubleshoot small portable instruments. Underclassmen, including visiting school students, are then taught how to assemble the instruments from kits and how to use the instruments in their chemistry labs.

The instrument described herein facilitates the practical application of cyclic voltammetric (CV) analysis within standard undergraduate and advanced high school level laboratory courses. The entire instrument was designed for less than $50 (excluding Pt working electrode), clearly an inexpensive economical alternative to a pricey commercial CV instrument that can cost upwards of $15K. A recent report4 has highlighted the cost issue by making use of a MicroLab sensor and interface as a potentiostat to control a Pine electrochemical cell that uses disposable carbon electrodes for a cost of $1500 per station; however, the student-built instrument that we describe herein has been constructed by undergraduate students for less than one-tenth of the cost of the Pine-MicroLab system. The CV instrument remains under constant development by our students, and the latest model has allowed us to obtain some remarkable cyclic voltammograms on a number of chemical compounds, thus highlighting the usefulness of CV in studying biological, inorganic and organic systems.

CV is a versatile electroanalytical technique employed to give qualitative and quantitative information about redox reactions within a potential range. As such, CV is an important tool for the study of reactions in the field of inorganic chemistry, organic chemistry, and biochemistry. The CV experiment is carried out in an electrolytic cell with three electrodes: The reference
electrode keeps the potential between itself and the working electrode constant, and the current is measured between the working and counter electrodes. In a typical CV experiment, the potential of the working electrode is varied slowly and linearly from an initial value to a predetermined limit where the direction of the scan is then reversed. Often an initial reduction is undertaken to form a product of interest, and then the voltage scan is reversed to oxidize the product back to the starting material. A cyclic voltammogram is obtained by recording the current produced at the working electrode during the entire potential scan. Depending on the information sought, single or multiple cycles, of varying scan rate and potential limits can be used.

In order to encourage greater participation, and provide a more memorable and lasting lab experience we have also developed a series of experimental labs for the student-built CV instrument. These labs comprise varying levels of sophistication and complexity to help students realize the many useful applications of the CV technique. The lab protocols could easily be geared for high school as well as a variety of chemistry and biochemistry undergraduate courses, from the freshman to the senior level. The development of these labs also provided an opportunity to gauge the reliability, reproducibility, limitations, and accuracy of data obtained by the student-built CV in comparison to a commercial BASi Epsilon CV50W instrument.

**B.3 Experimental Section**

The circuit for the CV potentiostat, as shown in Figure 1, is discussed below. The circuit described uses a Programmable Interface Controller (PIC) which requires access to a PIC programmer and software. An alternative, breadboard-based version of the CV potentiostat, which does not use a PIC, is described in the SI. The simpler version uses a commercial function generator which is tied to the 100k resistor on the inverting input of the LMC6484 op amp, the output of which drives the counter electrode. Voltammograms obtained using either version are identical.

**Instrument Design:** The custom-built CV instrument consists of two parts as shown in Figure 1: A commercial miniLAB-1008 unit, and a ‘student-built’ CV potentiostat. The miniLAB unit is a data acquisition (DAQ) device that provides an interface for Analog to Digital conversion that allows for computer analysis and manipulation of the acquired data. The CV-potentiostat
employs a platinum disc or glassy carbon working electrode, a platinum counter electrode, and a simplified ‘student-built’ Ag/AgCl reference electrode.

A. B.

Figure B.1: (A) MiniLAB 1008 for data acquisition from the potentiostat to a computer. (B) View of the circuit board found inside the student-built potentiostat.

A general list of components and their costs are given in Table B1. A complete detailed list of all components, materials, and vendors is provided in the SI. In order to build the instrument as shown, some machine work on the housing is required which adds a small additional cost that is not reflected in the above total. It is also possible that alternative housings could be developed, or no housing could be used, for less cost. The compact nature of the potentiostat circuit board lends itself to multiple packaging possibilities.
Table B1: A general list of parts and costs needed to build the CV apparatus.

<table>
<thead>
<tr>
<th>CV Part</th>
<th>Cost ($)</th>
</tr>
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<td>PIC 16F684 Programmable Chip</td>
<td>2.00</td>
</tr>
<tr>
<td>LMC 6484 Operational Amplifier</td>
<td>3.00</td>
</tr>
<tr>
<td>Plastic Housing</td>
<td>5.00</td>
</tr>
<tr>
<td>Power Supply Unit</td>
<td>5.00</td>
</tr>
<tr>
<td>Various resistors and capacitors</td>
<td>4.00</td>
</tr>
<tr>
<td>Ag-wire</td>
<td>2.00</td>
</tr>
<tr>
<td>Pt-wire</td>
<td>25.00</td>
</tr>
<tr>
<td>Pt working electrode</td>
<td>237.00</td>
</tr>
</tbody>
</table>

**Electrical Circuit:** The circuit diagram for the CV instrument is shown in Figure 2. The circuit consists of two main sub-circuits, a wave generator and current amplifier. The power supply used was a Lucent 120 V 60 Hz to 12 V, 300 mA DC adapter (not shown). The first circuit is comprised of the PIC16F684, three variable resistors, and an output filter, is used to produce a triangle waveform. The amplitude, slope, and DC offset of the triangle waveform are controlled by three variable resistors. Although early prototypes generated this waveform using more traditional analog electronics, the PIC microcontroller design uses fewer parts and is less critical of individual part’s values and tolerances. The triangle output is in the +/- 2.5 V range. The triangle waveform is coupled to a second sub-circuit, a typical potentiostat, which is built using the LMC6484 op amp. A phase-compensated op amp drives the counter electrode. The loop is closed by the high input impedance voltage follower connected to the reference electrode which also provides a V applied signal. The current through the sample is detected by a transimpedance amplifier which has three selectable gains. The output of the transimpedance amplifier is low pass filtered, to limit any high frequency noise, and is then applied to the input of the A/D in the data acquisition unit. Data processing and interpretation was performed with MathWorks Matlab (version 7.14). For those who have access to, or prefer to use, a commercial waveform generator, the output of the commercial unit can be tied to the 100k resistor on the inverting input of the LMC6484 op amp, the output of which drives the counter electrode. The remaining circuit can then be built on a regular digital breadboard. Detailed procedures for the construction of the CV apparatus are provided in the SI.
Ag/AgCl Electrode: The Ag/AgCl reference electrode was readily constructed by our analytical chemistry students in a similar manner to that described in literature.\textsuperscript{5-8} One end of a silver wire was connected to the positive-terminal of a 9V battery and the other end immersed in a 1M solution of HCl. A copper wire was connected to the negative-terminal and immersed in the 1M HCl solution as well. The silver wire remained immersed in the solution for 60 s to ensure a good coat. The data obtained using this electrode was compared to that obtained via a commercial Ag/AgCl electrode (MF2052, BASi), and both electrodes gave comparable results.

The CV experiment is carried out in an electrolytic cell with three electrodes: A working electrode, a counter (auxiliary) electrode, and a reference electrode. The working electrode used was either a commercially available Pt-disc electrode (BASi), the counter electrode was simply a 2mm Pt wire, and the reference Ag/AgCl electrode was made by the students. The electrodes were housed in a 50 mL beaker or a shot-glass (electrochemical cell) with a customized rubber or drilled teflon tops (stoppers), as shown in Figure B3.
**Figure B3:** Set-up for the CV test cell, electrodes and drilled Teflon stopper.

**Chemicals & CAS numbers:** Potassium Chloride (7447-40-7), potassium ferricyanide (13746-66-2), potassium nitrate (7757-79-1), hydroquinone (123-31-9), sodium monobasic phosphate (7558-80-7), sodium dibasic phosphate (7558-79-4), phosphoric acid (7664-38-2), acetaminophen (103-90-2), argon or nitrogen supply, nanopure water, and Tylenol gelcaps (Tylenol Extra Strength Rapid Release).

**Safety and Disposal:** All solutions must be disposed of in designated and labeled waste containers. Do not dispose of solutions down the drain. Do not inhale or ingest any solutions intentionally. If skin or eye contact occurs, flush with water immediately. Never leave the working and auxiliary electrodes in buffer solution for long periods of time (i.e. longer than the time it takes to collect data). The reference Ag/AgCl electrode should never be allowed to run dry; when the experiment is completed, rinse with deionized water and return the electrode to the saturated NaCl storing solution. Do not expose the Ag/AgCl electrodes to direct sunlight or any other UV source, since UV light decomposes AgCl, giving the electrode a black appearance. Hydroquinone may be toxic by inhalation, skin absorption and ingestion. Students will be using phosphoric acid, so great care must be taken because it is extremely corrosive. Students should wear safety glasses, latex gloves, long pants, and no open-toed footwear. Acid spills should be
carefully neutralized with bicarbonate before disposal. Use absorbent paper to mop up small spills. Large spills can be treated using an absorbent such as sand or Vermiculite.

**General Procedures:** The CV potentiostat was connected to the miniLAB-1008 data acquisition device, which in turn was attached via USB to a computer. The CV acquisition program was written in Java and is programmed to plot voltage versus current data. There are four switches on the CV potentiostat which allow adjustments to be made to the receiver gain (low, medium, high), amplitude, frequency, and DC-offset. The amplitude and DC-offset were set at the start of each set of experiments and not adjusted thereafter in order to retain reproducibility. The gain utilized in each experiment was based on the concentration of the test solutions; however, it was kept at mid-range by default. The frequency was adjusted at the start of each experiment so as to set the scan rate. Once calibrated it was important not to disturb the adjustment knobs on the instrument, since the settings are still somewhat over-sensitive. Detailed procedures for the calibration are provided in the SI.

Before each solution was analyzed, a cleaned and dried electrochemical cell was filled to ¾ of its capacity (about 35 mls), and the three electrodes were carefully inserted through the topper, submerged about 3 cm into the solution and connected to the potentiostat. The solution was then purged (sparged) with argon or nitrogen for 5 minutes while stirring. Following calibration, the scan rate (frequency) was calculated and set using a stopwatch. After a preliminary run to determine the final settings for the reaction, the experimental run was saved, transferred to Excel, tabulated and plotted. Duplicate runs were performed for each test solution to ensure reproducibility.

A BASi Epsilon CV50W electrochemical analyzer with a C3 stand and Faraday cage was also employed for each experiment, so as to compare and characterize the student-built instrument. A 6 cm long 2 mm platinum wire was used as the auxiliary electrode, a student-built Ag/AgCl was used as the reference electrode, and a 6 cm long 2 mm platinum wire or a glassy carbon electrode (MF2012, BASi) was used as the working electrodes. Slightly less noise was observed in the CV data when using the commercial glassy carbon electrode.

Three experiments of varying complexity were developed for the student-built CV instrument:
(a) For the K$_3$Fe(CN)$_6$ experiment, six solutions of the ferricyanide with concentrations ranging between of 0.2 mM and 5.0 mM were prepared in 1 M KNO$_3$. These solutions were used to plot a calibration curve of $I_{pa}$ (mV) and $I_{pc}$ (mV) *versus* Concentration (mM). From this plot, the concentration of the unknown K$_3$Fe(CN)$_6$ sample was directly calculated. The $E^\circ$ (mV) value and the precision and accuracy for the Fe(II)/Fe(III) redox couple was compared to literature values. In a second series of experiments, the scan rate was varied from 100 mV/s to 300 mV/s, and the effect of varying scan rate on the $I_p$ (mA) and $E_p$ (mV) parameters was observed. A plot of $\Delta E_p$ (mV) *versus* square root of the scan rate (mV/s) allowed for discussion of the Randles-Sevcik equation.

(b) The hydroquinone/quinone (H$_2$Q) experiment focused on a redox system from the world of organic chemistry. At least four known hydroquinone-buffer solutions at pH between 1 and 6 were prepared and then analyzed by CV. The pH of the solutions was measured immediately prior to use with a Metrohm pH meter 744 and a combined glass electrode. A calibration curve of pH *versus* $E^\circ$ (mV) was generated, and from this linear relationship the pH of an unknown sample was directly calculated. In addition, the number of electrons transferred and the diffusion coefficient of the electrochemical system were calculated using the Nernst equation and the Randles-Sevcik equation.

(c) The third experiment examined the electrochemical mechanism of 4-acetaminophenol (APAP), a component of many cold medicines, as it is oxidized to benzoquinone. This pH dependent transformation was observed by the CV instrument by monitoring the shift in the redox potentials and the appearance/disappearance of reaction intermediates as the pH was varied. A calibration curve of $I_{pa}$ (mV) *versus* APAP Concentration (mM) at a constant pH of 1.7 was also prepared. Using this calibration, the concentration of APAP in an unknown sample of Tylenol was directly calculated and compared to the manufacturers claimed content on the label.
B.4 Results and Discussion

The SMILE program that we have developed for high school and undergraduate students provides an opportunity to reinforce the concepts of electrochemistry in a more engaging and realistic manner. The student-built CV instrument takes no more than three hours to construct from kit form. The potentiostat can employ either a commercial glassy carbon electrode or a student-built platinum working electrode, a platinum counter electrode, and a simple student-built Ag/AgCl reference electrode. The principle function of the potentiostat is to control potential and measure current. The inputs to the potentiostat are the connections to the electrodes in the cell, and the outputs are signal lines reflecting the current and potential of the working electrode. The potentiostat is designed to be interfaced with a data acquisition unit and a computer to allow signals to be read directly by the controlling software, to allow experimental data to be manipulated, displayed, and stored. The miniLab-1008 DAQ unit was obtained from commercial vendors. The Javascript program for data acquisition and plotting was written in-house and provides data in a format that can be readily transferred to Excel for advanced plotting and analysis.

The potential of the working electrode in voltammetry experiments is controlled with respect to the reference electrode. The Ag/AgCl reference electrode used with this apparatus was readily constructed by our undergraduate students within 3 minutes. The Ag/AgCl electrode was found to be inexpensive, reliable, and gave consistently reproducible data, even after a semester of use. In order to calibrate the electrode potential, the Fe$^{3+}$/Fe$^{2+}$ redox couple was examined by a three-electrode BASi Epsilon instrument. A 10 mM K$_3$Fe(CN)$_6$ solution in 100 mM KNO$_3$ was used as the test. The electrode gave identical data to that obtained from a commercially available electrode (MF2052, BASi). A minor correction factor was calculated from this data and used in all subsequent runs. The peak potential difference ($\Delta$Ep) and peak height (Ip) remained constant for several cycles, which indicated that the fabricated electrodes were stable and reliable.

For comparison purposes a BASi Epsilon CV50W electrochemical analyzer with a C3-stand and Faraday cage was also employed for each experiment and the data collected allowed us to gauge the precision, accuracy, and quality of the student-built instruments. All scans were generally made in three segments for the BASi instrument and two segments for the student-built instrument: In general, a negative-going potential (cathodic) scan was made first followed by a
reversed potential scan towards the anode, and then finally allowed to return to the initial potential in the cathodic region.

Each of the three hour lab experiments was intentionally designed and developed to be of varying technical complexity and sophistication, and can thus be geared towards the aptitude and experience of students, and indeed the instructor. These CV experiments have been successfully conducted with high school students, college freshman, and senior college chemistry students. All CV data was first collected on a commercial BASi Epsilon instrument, and then subsequently collected on the student-built instrument for comparison. The final results were found to be remarkably similar.

Detailed experimental protocols and instructor notes are provided as SI. The instructor notes provide some helpful hints on how to prepare students for the experiments, for preparation of equipment, reagents and solutions, and details on the actual execution of the experiments. Typical data as generated by our students are also included in the instructor notes.

**Experiment #1: Ferro/Ferricyanide**

The ferro/ferricyanide couple was used as an example of an electrochemically reversible inorganic redox system. It is a relatively well-behaved couple, and allows for the introduction of the very basic concepts of electrochemistry and cyclic voltammetry to our students as well as helping them become familiar with the use and operation of the potentiostat. This experiment can be used to illustrate how electrochemical studies are conducted, to work up and plot the data, and how to utilize the CV technique to obtain quantitative analyses. Table B2 provides data from the BASi Epsilon instrument, and Table B3 shows data from the student-built instrument. In both cases a glassy carbon electrode (MF2012, BASi) was used as the working electrode. Complete CV plots for both instruments are given in the SI.

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<th>Iₚₐ (mA)</th>
<th>Eₚₐ (mV)</th>
<th>Iₚₑ (mA)</th>
<th>Eₚₑ (mV)</th>
<th>ΔEₚ (mV)</th>
<th>E° (mV)</th>
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Table B3: CV data from student-built instrument. Potentials are versus Ag/AgCl. Scan rate is 95mV/s.

<table>
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<tr>
<th>[K₃Fe(CN)₆] (mM)</th>
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<th>Eₚa (mV)</th>
<th>Iₚc (mA)</th>
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<td>133</td>
<td>98</td>
<td>182</td>
</tr>
<tr>
<td>0.75</td>
<td>-0.0649</td>
<td>246</td>
<td>0.0547</td>
<td>148</td>
<td>98</td>
<td>197</td>
</tr>
<tr>
<td>1.50</td>
<td>-0.1410</td>
<td>275</td>
<td>0.1202</td>
<td>171</td>
<td>104</td>
<td>223</td>
</tr>
<tr>
<td>3.00</td>
<td>-0.2575</td>
<td>287</td>
<td>0.2241</td>
<td>191</td>
<td>96</td>
<td>239</td>
</tr>
<tr>
<td>Mean Average</td>
<td></td>
<td></td>
<td></td>
<td>99</td>
<td>210</td>
<td></td>
</tr>
</tbody>
</table>

Calibration curves plotting Iₚa versus Concentration (mM) and Iₚc (mA) versus Concentration (mM) are shown in Figure B4 for the BASi Epsilon instrument, and Figure 5 for the student-built instrument. These calibration curves are consistent with the Randles-Sevcik equation, illustrating that the observed current is directly proportional to the concentration of the analyte. For the student-built instrument, the linear range of response was found to fall between 0.3 and 10 mM. Ferricyanide samples of unknown concentration were readily calculated from these calibration curves.

The formal potential (E°) is the mean of the anodic (Eₚa) and cathodic (Eₚc) peak potentials. For the ferri/ferrocyanide system, E° was found to be 185 mV using the BASi Epsilon instrument.
and 210 mV using the student-built instrument, versus Ag/AgCl, corresponding to 382 and 407 mV versus NHE respectively. These values were obtained from the instruments, without iR compensation. The lack of iR compensation contributed, in part, to the large peak-to-peak separation (ΔEp) away from the ideal theoretical Nernstian\textsuperscript{10-13} value of 59 mV. This peak difference is also influenced by the poor quality and imperfections in the active surface of the electrodes used. The determined $E^\circ$ is also highly dependent on the nature of electrolytes in the solution. Literature references\textsuperscript{14-18} provides values of 450 mV to 720 mV versus NHE at 25ºC for this system in a variety of common electrolytes; specifically, for a 1M KNO\textsubscript{3} electrolyte solution a value of 459 versus NHE at 25ºC is reported. The calculated potential values from this experiment using the student-built CV compare favorably with the values obtained from the commercial BASi Epsilon instrument, and with those reported in literature.

The effect of scan rate on the ferri/ferrocyanide redox potential and peak currents was also investigated. For the student-built instrument, the scan rate (mV/s) was adjusted manually using the frequency knob; and timing the full voltage sweep (the total voltage sweep of the voltammogram and dividing the time it took to make one full cycle by two). Complete CV plots when the scan rate is varied for a 2mM K\textsubscript{3}Fe(CN)\textsubscript{6} solution for both instruments are given in the SI. Pertinent CV data is provided in Table 4 for the BASi Epsilon instrument, and Table 5 for the student-built instrument.

**Table B4:** CV data from BASi Epsilon instrument, as the scan rate is varied for a 2mM K\textsubscript{3}Fe(CN)\textsubscript{6} solution. Potentials are versus Ag/AgCl.

<table>
<thead>
<tr>
<th>Scan Rate (mV/s)</th>
<th>$I_p$ (mA)</th>
<th>$E_p$ (mV)</th>
<th>$I_c$ (mA)</th>
<th>$E_c$ (mV)</th>
<th>$E^\circ$ (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>-0.0540</td>
<td>237</td>
<td>0.0609</td>
<td>129</td>
<td>183</td>
</tr>
<tr>
<td>50</td>
<td>-0.0789</td>
<td>239</td>
<td>0.0711</td>
<td>130</td>
<td>185</td>
</tr>
<tr>
<td>100</td>
<td>-0.1007</td>
<td>246</td>
<td>0.0902</td>
<td>125</td>
<td>186</td>
</tr>
<tr>
<td>150</td>
<td>-0.1200</td>
<td>249</td>
<td>0.1043</td>
<td>120</td>
<td>185</td>
</tr>
</tbody>
</table>
Table B5: CV data from student-built instrument, as the scan rate is varied for a 2mM K$_3$Fe(CN)$_6$ solution. Potentials are versus Ag/AgCl.

<table>
<thead>
<tr>
<th>Scan Rate (mV/s)</th>
<th>(Scan Rate)$^{1/2}$</th>
<th>$I_p$ (mA)</th>
<th>$E_p$ (mV)</th>
<th>$I_c$ (mA)</th>
<th>$E_c$ (mV)</th>
<th>$E^\circ$ (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>68</td>
<td>8.25</td>
<td>-0.093</td>
<td>261</td>
<td>0.093</td>
<td>151</td>
<td>206</td>
</tr>
<tr>
<td>86</td>
<td>9.27</td>
<td>-0.110</td>
<td>264</td>
<td>0.111</td>
<td>151</td>
<td>208</td>
</tr>
<tr>
<td>153</td>
<td>12.37</td>
<td>-0.134</td>
<td>260</td>
<td>0.134</td>
<td>140</td>
<td>200</td>
</tr>
<tr>
<td>200</td>
<td>14.14</td>
<td>-0.145</td>
<td>271</td>
<td>0.148</td>
<td>140</td>
<td>206</td>
</tr>
</tbody>
</table>

Plots of $I_p$ (mA) versus square root of the scan rate (mV/s) are provided in Figure 6 for the BASi instrument, and Figure 7 for the student-built instrument. The data illustrates that the redox potentials between the two instruments are quite comparable, and that the formal redox potential value ($E^\circ$ or $E_{1/2}$), calculated as the average of $E_p$ (reduction peak potential) and $E_c$ (oxidation peak potential), is independent of scan rate. Scan rate plots are consistent with the Randles-Sevcik equation, and show that the peak current ($I_p$ and $I_c$) increases with the square root of the scan rate, and is directly proportional to concentration of the analyte. The peak-to-peak separations ($\Delta E_p$) increase slightly from 110 to 131 mV when the scan rate is changed from 68 to 200 mV/s for the student-built instrument, this is very similar to what is seen for the BASi instrument, where a change from 108 to 129 mV can be observed when the scan rate is changed.
from 20 to 150 mV/s. The value is slightly larger than the theoretical value of $2.303 \frac{RT}{F} (= 59$ mV, at 25 °C) for a one-electron reversible redox$^{10-13}$ couple, and predominantly arises from uncompensated Ohmic ($iR_u$) drop, and the poor quality of the active surface of the electrodes in use. The overall correlation between the two instruments are very good, suggesting that the student-built instrument provides high quality data comparable to that obtained from the more expensive and sophisticated commercial instrument.

**Experiment #2: Quinone/Hydroquinone**

Quinones represent a class of compounds that are widely distributed in nature, and their basic structure is featured in many cofactors, coenzymes, and flavonoids; with numerous biological functions, including cellular respiration and as electron carriers in photosynthesis. As vitamins they represent a class of molecules involved in blood coagulation and treating illnesses such as osteoporosis and cardiovascular diseases.$^{19,20}$ The biological action of quinones is intimately linked to their electron transfer rates and redox potentials. The hydroquinone/ p-benzoquinone ($H_2Q/Q$) experiment developed for the student-built CV instrument focused on a well-studied$^{21-31}$ organic redox couple, and is an excellent system for illustrating the Nernst equation to students. The experiment also allows one to discuss the relationship of the Nernst equation to pH, and the key features that make this compound so useful and of such importance to the biological world.

Several known hydroquinone-buffer solutions at pH between 2 and 6 were analyzed with

![Figure B.6](image-url): (a) A typical voltammogram from the BASi Epsilon instrument, for 2mM hydroquinone at pH 3.0. Scanned from 0mV to 1000mV, then to -600mV, and back to 0mV, at a scan rate of 100 (mV/s). (b) A typical voltammogram from the Student-built CV, for 5mM hydroquinone at pH 3.0. Scanned from about 800mV to -100mV, and back to 800mV, at a scan rate of 74 (mV/s).
both a commercial BASi Epsilon and a student-built CV instrument, and data was collated in Table B6 and Table B7, respectively. In both cases a glassy carbon electrode (MF2012, BASi) was used as the working electrode. Complete cyclic voltammograms for the instruments are given in the SI. A typical voltammogram at pH3 (Figure B8) shows one cathodic peak in the negative-going scan attributed to the reduction of Q to H2Q, and one coupled anodic peak in the positive-going scan that corresponds to the oxidation of H2Q to Q. Both voltammograms are remarkably similar.

**Table B6.** CV data from the BASi Epsilon instrument, for 2mM hydroquinone solution at varying pH. Potentials are given versus Ag/AgCl.

<table>
<thead>
<tr>
<th>pH</th>
<th>E_p (mV)</th>
<th>E_c (mV)</th>
<th>E^o (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.0</td>
<td>482</td>
<td>206</td>
<td>344</td>
</tr>
<tr>
<td>3.0</td>
<td>448</td>
<td>167</td>
<td>308</td>
</tr>
<tr>
<td>4.0</td>
<td>405</td>
<td>128</td>
<td>267</td>
</tr>
<tr>
<td>5.0</td>
<td>379</td>
<td>113</td>
<td>246</td>
</tr>
<tr>
<td>6.0</td>
<td>321</td>
<td>78</td>
<td>200</td>
</tr>
</tbody>
</table>

**Table B7.** CV data from the student-built instrument, for 5mM hydroquinone solution at varying pH. Potentials are given versus Ag/AgCl.

<table>
<thead>
<tr>
<th>pH</th>
<th>E_p (mV)</th>
<th>E_c (mV)</th>
<th>E^o (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.0</td>
<td>499</td>
<td>176</td>
<td>338</td>
</tr>
<tr>
<td>3.0</td>
<td>471</td>
<td>103</td>
<td>287</td>
</tr>
<tr>
<td>4.0</td>
<td>436</td>
<td>44</td>
<td>240</td>
</tr>
<tr>
<td>5.5</td>
<td>398</td>
<td>-12</td>
<td>193</td>
</tr>
</tbody>
</table>

As the pH of the buffer solution increases, both the anodic and cathodic peak potentials shift to the more negative, indicating a decrease in the redox potential for the couple as is predicted by the Nernst equation. The observed cathodic shift illustrates that the redox potential is dependent on the equilibrium concentration of the redox species involved. Multiple scans at each pH show no change in the redox potentials or peak heights. A noticeable decrease in the I_p as the pH increases was observed in the data from both instruments, and is slightly more dramatic in the student-built instrument. We were careful in cleaning the working electrode between each different pH run, so the decrease in I_p is likely to be associated with some
decrease in the concentration of the reduced species and/or of the diffusion coefficient associated with the species. The higher than expected ΔE values from both instruments is most likely attributable to uncompensated Ohmic (IRu) drop, and perhaps some imperfections in the student-built electrodes; the value is clearly indicative of the considerable resistance the electrons encounter when travelling between the redox species and electrode surface. We are currently investigating the anodic current drop phenomenon, along with the influence of various possible contaminants and interferents.

The varying pH hydroquinone solutions data were analyzed further, and calibration curves of pH versus E° (mV) were generated, and illustrated in Figure B9. Plots from the BASi instrument and the student-built instrument are linear, with slopes of 37 ± 4 mV/pH and 43 ± 4 mV/pH, respectively. Although these values are not quite the ideal 0.059 V/pH unit that is expected for a 2-electron, 2-proton electrode process (Q/H2Q) redox system, they do clearly show the pH dependence of the hydroquinone/quinone redox potential, that the reaction involves about two electrons, and that the data obtained from the two instruments are comparable.

Additional quantitative information regarding the H2Q/Q couple was obtained from the peak heights according to the Randles–Sevcik equation. Calculation of the surface area (A) of the working electrode, together with the solution concentration and scan rate enabled calculation of the diffusion coefficient of the analyte (D) in the electrochemical system. The diffusion coefficient is a measure of how fast the analyte moves through the solution as a result of random

![Figure B.7:](image)

**Figure B.7:** (a) A calibration curve for a 2mM hydroquinone-buffered solution concentration for the BASi Epsilon instrument. E° versus pH, r² = 0.996, and Slope = 37 ± 4 mV/pH. (b) A calibration curve for a 5mM hydroquinone-buffered solution concentration for the Student-built instrument. E° versus pH, r² = 0.993, and Slope = 43 ± 4 mV/pH. Potentials are given versus Ag/AgCl.
collisions with other molecules. Using the voltammograms from the pH3 solutions, (Figure 8), the Ip (mean average) for the BASi Epsilon instrument was calculated to be 0.1358 mA (2 mM, 100 mV/s, 25°C), and for the student-built instrument to be 0.3531 mA (5 mM, 76 mV/s, 25°C). Given the glassy carbon working electrode surface area (A = 0.071 cm²), the diffusion coefficient of H₂Q was determined to be 1.58 x 10⁻⁵ for the BASi, and 2.25 x 10⁻⁵ for the student-built instrument. Although we were unable to find a value in literature dealing with an identical system, these values are comparable to each other, and certainly within range (10⁻⁴ to 10⁻⁶) to those obtained under different solvent and electrolyte conditions.²⁴,²⁷⁻³³

Experiment #3: Conversion of APAP to Benzoquinone

The third experiment developed for the student-built instrument dealt with the chemical and electrochemical mechanism of 4-acetaminophenol (APAP) as it is oxidized to benzoquinone (Scheme 1). The selection of APAP is not only based on its interesting redox properties, but also because students are quite familiar with this compound, and of course it is cheaply available. This experiment demonstrates that mechanistic information can be readily obtained from electrochemistry, for instance by looking at the effect of pH on the electrochemical signature of the analytes within a voltammogram. Although the data from commercial instrument have much better signal-to-noise, the student-built apparatus is clearly able to provide good data at a fraction of the cost. An additional aspect of this experiment used the CV technique to determine the amount of active ingredient in an over-the-counter medication. APAP (paracetamol, acetaminophen) is an analgesic and antipyretic. Its analgesic properties are comparable to those of aspirin, while its anti-inflammatory effects are significantly weaker. APAP is non-carcinogenic and an effective substitute of aspirin (acetylsalicylic acid) for those folks unable to tolerate aspirin. It is commonly used for minor aches and pains, and is a major ingredient in numerous cold and flu remedies such as Tylenol, Anacin, Acamol, and Panadol. Many techniques have been developed for the determination of APAP in pharmaceutical and biological samples.³⁴⁻³⁶ Since the molecule is readily oxidized at low potentials, electroanalytical methods³⁷,³⁸ are very well suited for the quantitative determination of APAP in over-the-counter cold remedies. The experiment described herein is a modified version of that given in literature.¹⁴,¹⁵,³⁷
Scheme B1: Proposed mechanism for the oxidation of APAP.

The oxidation of APAP to benzoquinone is pH dependent. The student-built CV instrument was able to monitor the shift in the redox potential, and the appearance/disappearance of intermediates as the pH was varied. The data generated by the student-built instrument at differing buffer solution pH is displayed alongside data from the commercial BASi Epsilon instrument in Figure B10. In both cases a glassy carbon electrode (MF2012, BASi) was used as the working electrode. Overall, the data is satisfyingly similar between the two instruments. Although the Ipₐ values appear distorted, the key features are apparent and comparable, The voltammogram from the student-built instrument contains some additional features, which arise from the much wider potential window.

The data shows that as the pH of the media is varied, chemical reactions involving APAP can be mapped out. At high pH (> 6) a reversible redox process is observed. At this pH, the N-

Figure B.8: (a) CV data from the BASi Epsilon for varying pH of APAP solutions (at 5mM). Scan segments were from about 900mV to 0mV, and then back to 900mV, at a scan rate of 100 (mV/s). (b) CV data from the student-built apparatus, for varying pH of APAP solutions (at 5mM). Scan segments were in general from about 1500mV to -500mV, and then back to 1500mV, at a scan rate of 102 (mV/s). Potentials are given versus Ag/AgCl.
acetyl-p-quinonimine (species 2 in Scheme B1) is stable, electroactive, and can be readily converted back to APAP (1) by reversing the sweep back to negative potentials. The large separation is in part due to the sluggish electron transfer kinetics and is exacerbated by the poor quality of the electrodes being used. As the pH is raised towards pH12, the oxidation process becomes less favorable. As the pH is decreased, the concentration of 1 steadily decreases, a change that is apparent in the cathodic current (Ip_c). At acidic pH, the redox wave also shifts to a more positive potential. As the pH drops below 3, the reduction (cathodic) waves are reduced; due to rapid protonation and hydration of 2 to yield the electrochemically inactive species 4 (see Scheme B1). The relatively fast scan rates used in the experiment also allow small amounts of 2 to be observed at lower pH values. Even at pH2 some features that are attributable to the initial redox couple were observed. In extremely acidic medium, the acetamide moiety in 4 is “chemically” lost to form the final benzoquinone product, 5. This mechanism is described as being an ‘ec’, where an electron transfer step produces a species that then undergoes a chemical reaction. At such low pH values, the redox couple associated with benzoquinone/hydroquinone is observed, albeit poorly defined. A standard pure solution of benzoquinone/hydroquinone can be used to verify these assignments.

The second part of this experiment involved use of the Randles-Sevcik equation to construct a calibration curve of Ip_a (mV) versus Concentration (mM), at a constant pH of 1.7. This linear calibration was then used to identify the concentration of an unknown APAP solution at this same pH. Because of its dependence upon the solution pH, the cathodic current (Ip_c) was

![Figure B.9](image-url): (a) A calibration curve for APAP-buffered solution concentration for the BASi Epsilon instrument. Ip_a versus concentration (mM). Solutions were buffered at pH 1.7, data gives r^2 = 0.988. (b) A calibration curve for APAP-buffered solution concentration Student-built instrument. Ip_a versus concentration (mM). Solutions were buffered at pH 1.7, data gives r^2 = 0.996.
not used for the quantitative analysis. The anodic peak current (Ip_a), however, was seen to be proportional to the concentration of the benzoquinone species (5) and therefore indicative of the quantity of the APAP (1) in the unknown samples (Figure B11). Complete CV data for both instruments are given in the SI. From these linear calibration curves, the concentration of an unknown sample of Tylenol Extra Strength Rapid Release was calculated to be precisely 5.00 mM, thus verifying the manufacturers claim on the label, and the accuracy and reliability of the student-built instrument.

B.5 Conclusions

The design and construction of a portable scanning potentiostat that can generate reliable CV data at a fraction of the cost of a commercial instrument was described. The performance, accuracy, and reliability of the student-built instrument have been successfully verified via a series of experiments of varying complexity. According to the experimental results, the student-built potentiostat can achieve similar results, with about the same accuracy as a commercially available potentiostat. Excellent current response data corresponding to the change of analyte concentration was achieved from the student-built potentiostat for the ferricyanide redox couple, the hydroquinone couple, and the 4-acetaminophenol experiments. Accordingly, a linear calibration curve was obtained, and the precise concentration (or pH in the case of the hydroquinone experiment) of unknown samples was obtained.

Although the voltammograms obtained from the student-made CV-potentiostat were not as smooth, clean, or pretty as those of the commercial instrument, the data generated was reproducible. In addition, the ability to achieve precise amperometric detection of analytes, and to measure and identify redox couples and redox potentials of various organic and inorganic systems using a three electrode CV protocol was confirmed. Overall, the student-built CV-potentiostat has the merits of moderate accuracy, small size, low weight, high portability, and very low cost. Student evaluation and feedback clearly suggests that the novel SMILE initiative (that continues to be developed) is a pedagogical success in providing a rigorous and memorable chemistry lab experience. It has been observed that our senior analytical chemistry students are considerably more engaged in the classroom and lab periods. Gathering data with this particular instrument has enabled our lower-level students to understand the concept behind the redox
process, to understand the relationship and measurement of current and potential using the CV technique, understand the importance of calibration curves and statistical analysis of data, far more clearly now than prior to the SMILE program.

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B.6 References


