5-2012

The Effects of Coated Gold Nanorods on Porcine Atrioventricular Valve Tissues Using Atomic Force Microscopy

Heather L'ecuyer

_Clemson University_, hlecuye@g.clemson.edu

Follow this and additional works at: [https://tigerprints.clemson.edu/all_theses](https://tigerprints.clemson.edu/all_theses)

Part of the [Biomedical Engineering and Bioengineering Commons](https://tigerprints.clemson.edu/all_theses)

Recommended Citation


[https://tigerprints.clemson.edu/all_theses/1393](https://tigerprints.clemson.edu/all_theses/1393)

This Thesis is brought to you for free and open access by the Theses at TigerPrints. It has been accepted for inclusion in All Theses by an authorized administrator of TigerPrints. For more information, please contact kokeefe@clemson.edu.
THE EFFECTS OF COATED GOLD NANORODS ON PORCINE
ATRIOVENTRICULAR VALVE TISSUES USING ATOMIC FORCE MICROSCOPY

A Thesis
Presented to
The Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Master of Science
Bioengineering

by
Heather Michelle L’Ecuyer
May 2012

Accepted by:
Dr. Delphine Dean, Committee Chair
Dr. Goldsmith
Dr. Simionescu
ABSTRACT

In this Master’s thesis we examine the effect of surface modified gold nanorods (AuNRs) coated with polyelectrolyte multilayers (PEMs) on the mechanical properties of porcine mitral valve tissue ex vivo. The presence of healthy mitral valve tissue in the body is important to ensuring unidirectional flow of blood through the systemic circuit. Unfortunately, due to its anatomic complexity and unique mechanical behavior, pathological mitral valve tissue remains difficult to treat with conventional methods. We hope that the knowledge gleaned from these studies could be useful for developing new and novel treatments treating pathological mitral valve mechanical properties.

Specifically, we examine the localized changes in apparent elastic modulus and stress relaxation behavior for the porcine mitral valve leaflets treated with the AuNRs with the atomic force microscope (AFM). We study these effects specifically because there is little literature available that examines the unique changes in mechanical properties of tissues treated with nanorods. Nanorods are used exclusively because they have already been shown to have a general effect on the properties of collagen gels, which are a good model for atrioventricular valve tissues. Our studies indicate that the presence of these AuNRs initiate an increase in the apparent stiffness of the valvular tissue while leaving the observed stress relaxation behavior unchanged.
In addition to examining the overall effect of the AuNRs in the tissue, we also examine the effect of differing surface charges on the apparent elastic modulus and stress relaxation of the tissue by using two types of PEM coated AuNRs. The first type of PEM coated AuNR possesses a negatively charged terminal layer, while the second type of PEM coated AuNR possesses a positively charged terminal layer. These AuNRs are used at differing concentrations to better understand how many nanorods are necessary to observe an effect on the apparent elastic modulus and stress relaxation behavior. Overall, there was little difference in effect between the two types of AuNRs and the two concentrations of AuNRs.
DEDICATION

This thesis is dedicated to my parents, Mark and Renee L’Ecuyer, as well as my fiancé Mitchell Plyler, all of whom have been a constant source of support, encouragement, and stress relief. They have given me the drive and discipline necessary to complete this task, as well as the love and inspiration to do it well.
ACKNOWLEDGEMENTS

I am very grateful to have both Dr. Dean and Dr. Goldsmith as my wonderful advisors all this time; I would like to personally thank Dr. Dean for her positive attitude, constant reinforcement, patience, and assistance provided, as well as Dr. Goldsmith for her assistance, her knowledge about papers, presentations, and cell isolations, as well as her patience and positive attitude. I would also like to thank Dr. Simionescu for agreeing to be on my committee, and I would like to acknowledge Sandy Deitch for all her help with the AFM, Alex Lindburg for all his help and answers to my questions, Scott Wood for all his help and helpful advice, Snow Creek Slaughterhouse for their porcine hearts, and Jacob Hammers for his wonderful assistance with Matlab and pleasant personality.
Table of Contents

TITLE PAGE........................................................................................................................................... i

ABSTRACT.................................................................................................................................................. ii

DEDICATION.............................................................................................................................................. iv

ACKNOWLEDGEMENTS............................................................................................................................. v

LIST OF FIGURES ....................................................................................................................................... ix

LIST OF TABLES........................................................................................................................................ xi

CHAPTER 1: THE MITRAL VALVE.................................................................................................................. 1

1.1 GROSS ANATOMY ............................................................................................................................... 1

1.2 MICROSTRUCTURES ........................................................................................................................... 2

1.3 PHYSIOLOGY ....................................................................................................................................... 4

1.4 MECHANICAL PROPERTIES ............................................................................................................... 5

1.5 PATHOLOGY ....................................................................................................................................... 6

CHAPTER 2: GOLD NANORODS .................................................................................................................. 8

2.1 RELEVANCE AND USE ...................................................................................................................... 8

2.2 OPTICAL AND PHYSICAL PROPERTIES ........................................................................................... 8

2.3 INTERACTIONS WITH CELLS & TISSUES ....................................................................................... 9
2.4 CURRENT THERAPEUTIC RESEARCH ................................................................. 12

CHAPTER 3: THE ATOMIC FORCE MICROSCOPE ............................................. 14

3.1 RELEVANCE & USE .................................................................................. 14

3.2 THE HERTZ MODEL .................................................................................. 15

3.3 THE QLV MODEL ...................................................................................... 16

CHAPTER 4: MATERIALS AND METHODS .......................................................... 18

4.1 GOLD NANORODS – SYNTHESIS [18] ....................................................... 18

4.2 GOLD NANORODS – COATING [38] ........................................................... 21

4.3 SAMPLE PREPARATION – TISSUES ........................................................ 23

4.6 AFM – NANOINDENTATION .................................................................... 24

4.7 AFM – STRESS RELAXATION .................................................................. 29

4.8 DATA ANALYSIS ..................................................................................... 29

4.9 MICROSCOPY - SECTIONING ................................................................ 30

CHAPTER 5: RELEVANT RESULTS .................................................................. 32

CHAPTER 6: CONCLUSION & DISCUSSION ...................................................... 52

6.1 GOLD NANORODS ..................................................................................... 52

6.2 SAMPLE PREPARATIONS ......................................................................... 53

6.3 ATOMIC FORCE MICROSCOPY OF SAMPLES ........................................ 58
6.3.1 USING THE AFM.............................................................................................................. 58
6.3.2 DATA COLLECTION........................................................................................................ 63
6.3.3 DATA ANALYSIS .......................................................................................................... 66
6.4 MICROSCOPY .................................................................................................................. 68
CHAPTER 7: RECOMMENDATIONS..................................................................................... 69
APPENDICES ....................................................................................................................... 72
APPENDIX A – DISSECTING A HEART..................................................................................... 72
APPENDIX B – MATLAB SCRIPTS....................................................................................... 79
APPENDIX C – EXTRA METHODS............................................................................................. 81
REFERENCES ........................................................................................................................ 87
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1: Illustrated diagram depicting the trilaminar architecture of the mitral valve.</td>
<td>3</td>
</tr>
<tr>
<td>Figure 2: Analog representations of the viscoelastic models [38]</td>
<td>17</td>
</tr>
<tr>
<td>Figure 3: Diagram illustrating how samples were tested.</td>
<td>33</td>
</tr>
<tr>
<td>Figure 4: Images of the AuNRs used acquired via transmission electron microscopy.</td>
<td>34</td>
</tr>
<tr>
<td>Figure 5: A typical force curve for the AFM nanoindentation technique</td>
<td>35</td>
</tr>
<tr>
<td>Figure 6: A typical force curve for the AFM nanoindentation technique fit to the Hertz Model.</td>
<td>36</td>
</tr>
<tr>
<td>Figure 7: Bar graph displaying values for apparent elastic modulus of a particular condition.</td>
<td>37</td>
</tr>
<tr>
<td>Figure 8: Bar graph displaying values for apparent elastic modulus of the control samples.</td>
<td>38</td>
</tr>
<tr>
<td>Figure 9: Bar graph displaying values for apparent elastic modulus of samples injected with PDADMAC rods.</td>
<td>39</td>
</tr>
<tr>
<td>Figure 10: Bar graph displaying values for apparent elastic modulus of samples injected with PSS rods.</td>
<td>40</td>
</tr>
<tr>
<td>Figure 11: A typical force curve for the AFM stress relaxation technique.</td>
<td>47</td>
</tr>
<tr>
<td>Figure 12: A typical force curve for the AFM stress relaxation technique fit to the QLV model.</td>
<td>48</td>
</tr>
<tr>
<td>Figure 13: Bar graph displaying values for the percent relaxation by all samples.</td>
<td>49</td>
</tr>
<tr>
<td>Figure 14: Bar graph displaying values for the percent relaxation by the two control samples.</td>
<td>50</td>
</tr>
<tr>
<td>Figure 15: Bar graph displaying values for the percent relaxation by the PDADMAC treated samples.</td>
<td>50</td>
</tr>
<tr>
<td>Figure 16: Bar graph displaying values for the percent relaxation by the PSS treated samples.</td>
<td>51</td>
</tr>
<tr>
<td>Figure 17: Typical nanoindentation force curve altered by excess cyanoacrylate glue.</td>
<td>57</td>
</tr>
<tr>
<td>Figure</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>------</td>
</tr>
<tr>
<td>Figure 18: Illustrations of a pyramidal (A) and a spherical (B) AFM tips.</td>
<td>60</td>
</tr>
<tr>
<td>Figure 19: A porcine heart</td>
<td>72</td>
</tr>
<tr>
<td>Figure 20: The exposed ventricles of a porcine heart</td>
<td>73</td>
</tr>
<tr>
<td>Figure 21: The exposed valves of the porcine heart</td>
<td>74</td>
</tr>
<tr>
<td>Figure 22: The mitral leaflet within the heart with the chordae tendinae removed</td>
<td>75</td>
</tr>
<tr>
<td>Figure 23: The left ventricle with the mitral valve removed</td>
<td>76</td>
</tr>
<tr>
<td>Figure 24: The excised mitral valve</td>
<td>77</td>
</tr>
<tr>
<td>Figure 25: Samples selected from the mitral valve for testing</td>
<td>78</td>
</tr>
</tbody>
</table>
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 1: Outline of the experimental model used</td>
<td>33</td>
</tr>
<tr>
<td>Table 2: Calculated values for AuNR average length and average aspect ratio</td>
<td>34</td>
</tr>
<tr>
<td>Table 3: Analysis of Variance table</td>
<td>40</td>
</tr>
<tr>
<td>Table 4: Effects tests for the Analysis of Variance table</td>
<td>41</td>
</tr>
<tr>
<td>Table 5: Least square means tables</td>
<td>42</td>
</tr>
<tr>
<td>Table 6: Contrast between PDADMAC and Control i samples</td>
<td>43</td>
</tr>
<tr>
<td>Table 7: Contrast between PDADMAC and Control ii samples</td>
<td>44</td>
</tr>
<tr>
<td>Table 8: Contrast between PSS and Control i samples</td>
<td>45</td>
</tr>
<tr>
<td>Table 9: Contrast between PSS and Control ii samples</td>
<td>46</td>
</tr>
</tbody>
</table>
CHAPTER 1: THE MITRAL VALVE

1.1 GROSS ANATOMY

The overall structure and function of the heart is well known in the medical and medical research community. The heart is a vital organ which lies posteriorly to the sternum, anteriorly to the vertebral column, and medially to the lungs [1]. Specifically, the heart rests upon the diaphragm so that the base of the heart is just beneath the 2nd rib while the apex is at the 5th intercostal space [1]. Within the heart there are 4 chambers comprised of a left and right ventricle as well as a left and right atria; at the entrances and the exits to these chambers are the right and left atrioventricular valves, as well as the right and left semilunar valves, both of which are anchored to a fibrous ring, called the annulus [2]. Of particular interest to this thesis is the anatomy of the atrioventricular valves, especially the left atrioventricular valve, or the mitral valve. The mitral valve is focused on in this thesis due to the high number of diseases and pathologies affecting it. The mitral valve, also referred to as the bicuspid valve, can be found separating the left ventricle and the left atria and is identified by the presence of 2 flap-like leaflets [2]. Projecting from the free edges of the mitral leaflets on the ventricular side are thin fibrous strings called chordae tendinae; these chordae tendinae attach to the papillary muscles found in the ventricle [2] and are often classified according to the point of origination on the leaflets.
1.2 MICROSTRUCTURES

The word microstructure used in this paper will be used to describe how cells, ECM proteins, fibers, and other factors contribute to overall tissue properties, mechanical behavior and physiological function. While there is currently an overwhelming amount of research on the subject of heart valves, especially the aortic valve; pathological valvular microstructures, multi-scale valvular mechanics, and pathological bio-molecular processes are still areas of current research interest. However, thanks to the diligent work of many people, we have a fairly detailed knowledge of healthy mitral valve microstructure.

At a glance, the mitral valve is comprised of 4 primary components, that is, the annulus, the leaflets, the chordae tendinae and the papillary muscles [2]. Closer inspection of each leaflet with a microscope reveals a trilaminar architecture, with each layer being identified by a unique combination of extracellular matrix and valvular interstitial cells. A diagram of these layers is illustrated in figure 1; from proximal to distal, the layers are the atrialis layer, the spongiosa layer, and the fibrosa layer [2]. The atrialis layer can be identified by prominent elastic fibers as well as a radial arrangement of collagen [3, 4] and is continuous with the sub-endocardium of the preceding atria [2]. The spongiosa layer can be identified by the abundance of proteoglycans and some loosely associated valvular interstitial cells [3, 4], which give the layer a loose spongy appearance [2]. Finally, the fibrosa layer can be identified by densely packed circumferentially arranged collagen fibers and some fibroblast cells; the fibrosa is continuous with the annulus and
extends through the full length of the leaflet to the chordae tendinae and the papillary muscle tips. That is, the chordae tendinae originate from the fibrosa layer, insert into the papillary muscles present within the ventricles, and are composed largely of parallel collagen bundles, some glycosaminoglycans, and some elastin [2, 5, 4]. In the human mitral valve, the fibrous tissue located close to the annulus may also contain capillaries, smooth muscle cells, and some cardiomyocytes. It should be noted that over all blood contacting surfaces of the heart, including the chordae tendinae, lies a continuous layer of simple squamous endothelial cells [2]. Overall, the dry weight composition of the AV valve is approximately 60% collagen, 10% elastin, and 20% proteoglycans [4, 3].

Figure 1: Illustrated diagram depicting the trilaminar architecture of the mitral valve. Starting at the top of the diagram is the atrialis layer, marked as A. Next is the spongiosa layer, marked as S. Finally, the fibrosa layer is marked as F, and attaches to the chordae tendinae, marked as CT, which attach to the papillary muscle, marked as PM. It should be noted that the first layer encountered at any surface is the monolayer of endothelial cells.
1.3 PHYSIOLOGY

Overall, the heart is responsible for pumping about 1,850 gallons of blood a day blood throughout the pulmonary and systemic circuits [1]. The mitral valve present within the heart prevents backflow of blood from the left ventricle into the atria during ventricular systole, thus helping to enforce efficient and unidirectional blood flow to the body. Typically, the mitral apparatus encounters physiological pressures of 100 to 200 mmHg during daily cyclic function [6].

To meet these demanding mechanical needs created by a fully functioning heart, each of the structures associated with the atrioventricular valves have developed to meet unique functions. The fibrous annulus to which the mitral valve attaches provide support for the valve, as well as help provide a route for the cardiac conduction system [2]. The trilaminar structure of the leaflets and the unique arrangement of fibers and GAGs within each layer both help to resist various modes of material failure [3]. Specifically, it is believed that the proximal layer, the elastin rich atrialis layer, resists permanent deformation, fatigue failure, and prolapse into the atria [3]. It is believed that the medial layer, the GAG rich spongiosa provides shock absorbing capabilities, permits leaflet deformation, and provides a route for nourishment to reach the valvular interstitial cells [2, 3]. Finally, it is believed that the distal layer, the collagenous fibrosa layer, provides rigidity and strength to the valve [2, 3]. The chordae tendinae projecting from the fibrous layer helps reinforce the strength of the valve and prevent prolapse into the atria during
ventricular systole [1], while the layer of endothelial cells provides a non-thrombogenic surface [2, 7].

Since biology has so effectively combined structure and function, studies examining the mechanical function and mechanical properties of cells, tissues, and organ function simultaneously are important to better understanding physiological function. However, human mitral valve tissue is difficult to obtain, thus porcine mitral valve tissue is used. Porcine mitral valve tissue is commonly used in research due to similarities in composition [8] as well as size [9], and stability in saline storage with no significant changes in mechanical behavior for up to 5 days after dissection from the porcine heart [6].

1.4 MECHANICAL PROPERTIES

Much of the mechanical testing previously done on the mitral valve includes traditional macro-scale techniques and mathematical modeling techniques. While these techniques provide valuable information, the traditional testing techniques are destructive to the samples, while both traditional testing techniques and mathematical modeling techniques may not fully account for the contributions of the many individual components within the valve. Data from multi-axial mechanical testing has revealed some values for the elastic modulus of whole porcine mitral valves based on the orientation of fibers, circumferentially or radially, within the mitral valve; these values are summed up by May-Newman et al. Correlating these elastic moduli with the orientation of fibers suggested that testing a cross-fiber orientation yields lower elastic moduli than testing a
fiber orientation [6, 10]. The data has also revealed that valve leaflets, which are thin, are also nearly incompressible; this incompressibility is described by a Poisson’s ratio of 0.5, although many mathematical modeling studies will use a value of 0.45 for computational requirements [11].

1.5 PATHOLOGY

Many valve diseases, especially AV valve diseases, start with altered cell behavior and altered micro-organization in localized environments [3]. This is not a new concept, in materials engineering, it is accepted that the arrangement of atoms, grains, grain boundaries, and some other factors, also referred to as microstructures, are significant contributors to the bulk mechanical behavior of the materials. In the case of the mitral valve, many of the diseases are due to gradual changes in the valvular microstructure and cellular phenotype. Diseases such as these can be classified as either stenosis, in which blood flow into the ventricle is reduced, or insufficiency, in which blood is allowed to flow back into the atria during ventricular systole [12]. Some examples of these diseases include degenerative mitral valve disease, in which pathological changes to the leaflets, annulus, and chordae tendinae prevent complete closure of the mitral valve [13]; myxomatous valves, in which a disorganized array of fibers and an excess of GAGs permits prolapse of the mitral valve into the atria [13]; and finally, stenotic valves, in which rheumatic fever or some other trigger induces stiffening of the leaflet tissue over a slow period of time [12]. However, there are other diseases, such as congenital mitral valve disease, which are present in children that may not be due to slow changes, but
rather morphological abnormalities present at birth. In all of these diseases, death and a reduced quality of life is common, and Lincoln et al does a very nice job summarizing the various vital components found in connective tissue ECM, the regulatory role these components play, and the resulting diseases that develop when these regulatory components are disrupted or absent.
CHAPTER 2: GOLD NANORODS

2.1 RELEVANCE AND USE

The emergence of nanotechnology and nanostructure manipulation has led to many new improvements in various traditional technologies [14]. In the case of gold nanostructures, most commonly nanoparticles, or AuNPs, and nanorods, or AuNRs, specific sizes and shapes can be selected during synthesis to achieve distinct optical, electronic, and chemical properties [15]. These properties can then be exploited in the design of better imaging systems, biological sensors, and therapeutic approaches [14]. This flexibility has led to many studies examining the behavior of such nanostructures for many different applications; however, studies examining the effect of these nanostructures on the mechanical behavior of tissues is lacking. For its simplicity and high yield, this thesis will focus on knowledge concerning AuNRs synthesized via wet chemical seed-mediated growth method [14, 16], although there are many other methods available to synthesize AuNRs [16].

2.2 OPTICAL AND PHYSICAL PROPERTIES

Gold nanorods have been used in many imaging applications due to their unique optical behavior [14]. This optical behavior is described as arising from the spatial limitation of the motion of electrons from a bulk material down to a nano-sized material [14]; that is, at nano-sizes surface effects become important and the properties of the material change. In noble metals especially, photons become confined within the nano-size particles and
induce a phenomena known as plasmon oscillations of electrons. This confinement can greatly enhance resonantly coupled light and lead to many different interesting optical and physical properties which are summed up by Huang et al. Most interestingly, however, is how the shape of the gold nanorods can cause not one, but rather two, plasmon oscillations. The first plasmon is due to oscillation in the short axis, or the transverse axis, and is found in the visible light spectrum [14]. The second plasmon is due to oscillation in the long axis, or longitudinal axis, and is found in the near infrared, or NIR, spectrum [14]. It has been found that this second plasmon can be easily controlled by altering the aspect ratio and surface coating of gold nanorods [14]. Many imaging modalities and diagnostic techniques have been explored as a result of this unique interaction, some of which are discussed below.

2.3 INTERACTIONS WITH CELLS & TISSUES

Since the sizes of AuNRs are often similar to the sizes of various biological molecules, the AuNRs can interact with cells in ways that are similar to these molecules [15]. Generally, AuNRs themselves are not cytotoxic [17]; Alkilany et al determined experimentally that it is often the residues left over from the synthesis process contaminating the cellular environment which leads to cell death, not necessarily the presence, shape, or charge of the AuNRs themselves. It should be noted that Alkilany et al used a method of synthesis similar to this thesis, which is known to leave behind a residue of positively charged cetyltrimethylammonium bromide, or CTAB, on the surface of the rods [16, 18]. Specifically, free floating CTAB incubated with human colon cancer
cells showed similar cytotoxic results to cells incubated with AuNRs left unmodified. Further studies using AuNRs coated with either positively charged Poly-Allylamine Hydrochloride, or PAH, and negatively charged Poly-Acrylic Acid, or PAA, eliminated this cytotoxic effect. Finally, various AuNRs exposed to growth media containing serum all exhibited changes in surface charges after 5 minutes; whereas rods exposed to growth media without serum exhibited no such change. These results suggest that proteins contained within the serum desorb the CTAB from the surface of the unmodified rods, and it this free floating CTAB which contributes more to cytotoxicity than any difference in surface charge or general presence of AuNRs do [14, 17]. Studies later done by Sisco et al verified that AuNRs coated with polyelectrolyte multilayers, or PEMs, such as poly(styrene sulfonate), or PSS, and poly(diallyldimethylammonium chloride), or PDADMAC also exhibited no cytotoxic effects [19].

The uptake of surface modified or coated AuNRs by cells is strongly dependent on size and shape [15, 20, 21]. Mammalian cells in particular preferentially take up particulate structures 50 nm in size [15]; this uptake becomes diminished at sizes other than 50 nm and at shapes other than spherical [15, 21]. Furthermore, the maximum uptake of either rods or particles by cells occurs within the first 2 to 4 hours of application. At any time after 5 hours, cellular uptake plateaus and reaches an equilibrium value [15]. Previous studies have shown that the AuNRs used in this study will not be taken up by the cells, but rather will associate primarily with the collagen in the ECM [19, 22], or else remain on the cell membrane. Several studies investigating the mechanisms of uptake for
nanoparticles and nanorods indicated that these nanostructures enter the cell via receptor mediated endocytosis and clathrin mediated processes [17, 20, 15], and that these results are similar for both AuNPs and AuNRs. UV-Visible spectrophotometry and Fourier Transform Infrared Spectroscopy, or FTIR, of AuNRs following exposure to cell media verified the presence of adsorbed proteins on the surface of the rods, indicated by a shift in the plasmon resonance of the AuNRs. It was determined by these studies that the physical parameters of the AuNRs largely affect the time it takes the cellular membrane to engulf a structure, or the membrane wrapping time. Larger structures will bind to greater numbers of cell surface receptors, known as receptor crosslinking, resulting in reduced receptor diffusion and a greater binding energy each receptor. A study comparing the uptake of AuNRs covered with non-specific proteins to the protein transferring resulted in a decreased cellular uptake of AuNRs coated with the transferrin protein. Thus, with the number of receptors available to bind to each rod diminished, the ability of a cell to engulf a rod is also diminished. The crosslinking of these receptors may then result in altered downstream behavior and expression of proteins [20].

Additionally, it has been shown that gold nanorods will interfere will the expression of cellular phenotypes. Studies using polyelectrolyte coated AuNRs in collagen gel models with porcine valvular fibroblasts showed that the gold nanorods will interfere with the changes in fibroblast phenotype from a normal fibroblast to a myofibroblast [19, 22]. Since valvular fibroblasts are responsible for the organization and deposition of collagen in the atrioventricular valves, then this change in cellular phenotype will usually result in
altered collagen matrix organization [22]. Further study tentatively examining the shear
moduli of the altered collagen gels with rheology indicated the mechanical properties of
the gels will change based on the addition of the gold nanorods [22].

2.4 CURRENT THERAPEUTIC RESEARCH

Because nanotechnology is still in its infancy, the literature concerning much of the
current therapeutic applications is limited. Despite this, many have focused on the
possible applications of AuNRs and AuNPs towards improving targeted drug delivery
systems, as well as therapeutic and diagnostic imaging modalities [14]. One therapy of
current interest involves using AuNRs to initiate tumor cell death via optical
hyperthermia [23]. This method is made possible by the AuNRs ability to convert light
into heat during plasmon oscillation [23]. The AuNRs are immobilized either in the cell
or on the cell membrane, and a near infrared laser is scanned across the cells. As the
AuNRs oscillate, they initiate irreversible blebbing of the cellular membrane and cell
death [23]. Another area of current research interest uses AuNRs to improve various
microscopy methods, such as dark field microscopy and transmission electron
microscopy, or TEM [24]. In darkfield microscopy, light enters the objective when
scattered by a sample, and the dense nanorods have been used to provide contrast in thin
transparent samples. The same AuNRs can then be used as a contrast agent in
transmission electron microscopy without any additional alterations. This multi-modal
use lends itself well to targeted optical research methods by conjugating the AuNRs with
proteins and other materials unique to cellular processes of interest [24]. Since the
AuNRs can be used to enhance both optical and electron microscopy methods, another area of research interest involves conjugating AuNPs and AuNRs with proteins and other markers to investigate specific cellular processes. One particular study successfully conjugated AuNPs for uptake by the cellular nucleus using a simian virus [25].
CHAPTER 3: THE ATOMIC FORCE MICROSCOPE

3.1 RELEVANCE & USE

Atomic Force Microscopy, or AFM, is a mechanical microscopy method whereby mechanical testing and surface scanning of samples are possible by use of a very small, but scalable, probe commonly known as a tip. Two particular mechanical tests of interest discussed here are nanoindentation and stress relaxation. The nanoindentation test, or in some cases microindentation test, is used to provide information on the apparent stiffness of the valvular extracellular matrix (ECM). The stress relaxation test is used to provide information on the time dependent mechanical behavior of the valvular ECM. Creep testing is not included with atrioventricular valve tissues because these tissues have already been shown to exhibit negligible creep behavior [26].

The AFM is used in this thesis over traditional mechanical testing methods for a variety of reasons. The AFM is capable of making localized mechanical measurements, possesses good sensitivity, can be used with extremely small samples, is customizable to the parameters of interest, and possesses a superior force resolution [27, 28, 29]. Indeed, both the customizability and versatility of the AFM are arguably some of its greatest advantages. Multiple imaging or testing modes such as contact mode, non-contact mode, and tapping mode can all be employed to execute a various number of other experiments such as force spectroscopy, topographic imaging, magnetic force microscopy, electric force microscopy, etc., besides the techniques described here. Biological and non-
biological samples alike can be tested with minimal sample preparation in various liquid or air environments [27, 30, 29]. Also, the AFM permits the investigation of samples too small for standard scale mechanical testing, such as tissue samples from various transgenic murine models. Certainly this protocol can be easily adapted to allow for testing of tissues much smaller than porcine heart valves, such as murine valves.

3.2 THE HERTZ MODEL

When analyzing the nanoindentation data collected by the AFM, there are several models with which to interpret the data. That is, one can fit the data to the Hertz model, the Johnson-Kendall-Roberts model (JKR), or the Derjaguin-Muller-Toporov (DMT) model; Butt et al presents a table summarizing the equations for all three of these models. For our purposes, the Hertz Model, which is the most widely used in literature [31, 27, 32], and which is useful for comparative purposes, is applied to the nanoindentation force curves collected. The Hertz equation is equal to \( \frac{4E}{3(1-\nu^2)}R^{\frac{1}{3}}\delta^{\frac{2}{3}} \), where \( E \) is the apparent stiffness, \( \nu \) is Poisson’s ratio, and is assumed to be 0.5 [11] \( R = \) probe radius = 2.5 um, \( \delta \) = indentation height, and \( F \) = deflection of the tip. It is important to note that the Hertz model makes several important assumptions that affect both how data is collected and which AFM tips are chosen for use in experimentation. Dintwa et al summarizes these assumptions; that is, the Hertz model assumes that all materials involved in contact are both isotropic and homogeneous, that applied loads are elastic in nature, that the material behavior is linearly elastic, that the deformations in the samples are small, that the contacting surfaces are smooth and flat, and that the dimensions of the contacting
materials are much larger than the dimensions of the contacting surfaces. Based on these assumptions, Dintwa et al concluded that one of the biggest contributors to error in the calculation of apparent stiffness is the presence of large deformations. Indeed, many sources recommend indenting into the sample no more than half the size of the probe used [32, 33, 34] as deformation larger than the tip dimensions requires the use of a nearly linear material for the assumptions made to be valid [34]. Thus, with this equation, the model is fit to the first 500 nm of indentation data, which is a large enough indentation depth to ensure that the apparent stiffness is at a constant value, yet small enough to ensure agreement with the assumptions of the Hertz model [34].

3.3 THE QLV MODEL

When analyzing the stress relaxation behavior, there are a plethora of viscoelastic models that can be applied to the data to determine the time dependent mechanical behavior. Among these models are included the Standard Linear Solid Model, or SLS model, the Generalized Maxwell model, and the Quasilinear Viscoelastic model, or QLV model. These models can be represented by simple circuit analogies, as shown in figure 2, where each has specific advantages and limitations depending on the samples tested [35]. Of these models, the QLV model will be used in this thesis; the primary advantage of using the QLV model is that it better describes complex time dependent mechanical behavior, and is thus a good model for most tissues [36], including heart valve tissues. The Generalized Maxwell model, while used in modeling cellular mechanics [35], is generally not suited for describing tissues. The SLS model, while commonly used due to
its simplicity and few fitting parameters [35], is also not suited for describing tissues and is the least accurate of the models. The QLV model can be described mathematically by the equation
\[ \sigma(t) = G(t) \ast \sigma^e(\varepsilon) \]
where \( G(t) \) represents the reduced relaxation function [37] \[ G(t) = \frac{1 + c \int_{\tau_1}^{\tau_2} e^{-\frac{t}{\tau}} d\tau}{1 + c \int_{\tau_1}^{\tau_2} d\tau} \] and \( \sigma^e(\varepsilon) \) is the instantaneous elastic response to a step input of strain (\( \varepsilon \)).

**Figure 2:** Analog representations of the viscoelastic models [38]. The model shown in A depicts the Standard Linear Solid model; B depicts the Generalized Maxwell model; C depicts the Quasilinear Viscoelastic model.
CHAPTER 4: MATERIALS AND METHODS

4.1 GOLD NANORODS – SYNTHESIS [18]

4.1.1. Clean all glassware, including a 500 mL volumetric flask with lid and an
large bottom Erlenmeyer flask, with aqua regia acid wash comprised of 1
part Nitric Oxide: 3 parts HCl and rinse thoroughly with deionized water.
Cover and let the glassware set overnight.

4.1.2. Prepare 10 mL of 0.1 M Ascorbic Acid in deionized water in a 15 mL
conical tube. This has a 24 hour shelf life.

4.1.3. Prepare 25 mL of 0.1 M Chloric Acid [HAuCl4] in deionized water
in a 50 mL conical tube. This can be stored and used as a stock solution for later
nanorod synthesis.

4.1.4. Prepare 10 mL of 0.01 M Chloric Acid [HAuCl4] in deionized water from
the 0.1 M Chloric Acid solution in a 15 mL conical tube.

4.1.5. Prepare 500 mL of 0.1 M of hexadecyl trimethylammonium bromide
[[(C_{16}H_{33})N(CH_{3})_{3}Br], or CTAB, in deionized water in the 500 mL
volumetric flask. The solution will need to sit in a hot water bath for
approximately 5 minutes with stirring to ensure the CTAB dissolves fully.

4.1.6. Prepare the AuNR seed.

4.1.6.1. Combine 9.75 mL of 0.1 M CTAB with 250 µL of 0.01 M Chloric
Acid in a 50 mL conical tube; stir the solution on a very low setting
with a sterile stir bar.
4.1.6.2. Prepare two 15 mL conical tubes in an ice bath. One tube will be filled with 9 mL ice cold deionized water and labeled A; the second will be filled with 10 mL ice cold deionized water and labeled B. Set these tubes close to the AuNR seed.

4.1.6.3. Prepare two pipettes; one set at 1000 µL and the second set at 600 µL, and set beside the AuNR seed. These will be used to make the next steps go more quickly.

4.1.6.4. Make 10 mL of 0.01 M Sodium Borohydride [NaBH4] solution using the previously prepared conical tubes filled with ice cold deionized water. Do not prepare the sodium borohydride ahead of time.

4.1.6.4.1. Withdraw 1 mL of water from tube B and quickly dissolve sodium borohydride into tube A to make a 0.1 M solution. Invert tube A to mix.

4.1.6.4.2. Quickly withdraw 1 mL of solution from tube A and add to tube B to make 0.01 M solution. Invert tube B to mix.

4.1.6.5. Quickly add 600 µL of the 0.01 M ice cold sodium borohydride solution (tube B) to the tube with the AuNR seed and continue to stir the AuNR seed for 2-10 minutes.

4.1.7. Prepare the vessels to be used in the AuNR synthesis.

4.1.7.1. Fill two 15 mL conical tubes, labeled 1 and 2, with 9 mL of 0.1 M CTAB, 250 µL of 0.01 M Chloric Acid, and 50 µL of 0.1 M Ascorbic Acid and mix thoroughly.
4.1.7.2. Fill the wide bottom Erlenmeyer flask, previously cleaned, with 90 mL of CTAB, 2.5 mL of 0.01 M Chloric Acid, and 500 µL of 0.1 M Ascorbic Acid and mix thoroughly.

4.1.7.3. The solutions in the prepared vessels should be completely colorless; if not, add 20 µL aliquots of 0.1 M Ascorbic Acid until colorless.

4.1.7.4. Remove the lids from the vessels and set aside.

4.1.8. Prepare a timer and a 1 mL pipette; the timer should be set for 15 seconds and 30 seconds.

4.1.9. After mixing, add 1 mL of the AuNR seed to the 15 mL conical tube labeled 1. Cap quickly and invert tube 1 to mix.

4.1.10. Start the 15 second timer.

4.1.11. After mixing, add 1 mL of the solution from tube 1 to the tube labeled 2. Cap quickly and invert tube 2 to mix.

4.1.12. Start the 30 second timer.

4.1.13. After mixing, add the contents of tube 2 to the prepared Erlenmeyer flask and mix thoroughly.

4.1.14. Cover the Erlenmeyer flask and let the reaction continue overnight. The final solution should turn a deep shade of purple.

4.1.15. The next morning, gently warm the Erlenmeyer flask in hot water for 5 minutes to dissolve any crystalized CTAB; do not rock, shake, or tilt the Erlenmeyer flask, as this can disturb the nanorods.
4.1.16. Slowly pour the supernatant from the Erlenmeyer flask into the appropriate waste container; the brown film in the bottom of the flask contains the nanorods.

4.1.17. Re-suspend the nanorods in 10 mL of deionized water for 2X concentration, or in 20 mL of deionized water for a 1X concentration.

4.1.17.1. The rods can be stored in deionized water for 3 months or in CTAB for longer storage.

4.1.18. Centrifuge the rods at 4000 rcf (4500 rpm) for 5 minutes and re-suspend the rods in 10 mL of deionized water. This will remove nanoparticles and prevent the nanorods from aggregating.

4.2 GOLD NANORODS – COATING [38]

4.2.1. Prepare 10 mL of 10 mM sodium chloride [NaCl] in a 15 mL conical tube.

4.2.2. Prepare 50 mL of 1 mM Sodium Chloride [NaCl] in a 15 mL conical tube.

4.2.3. Prepare 10 mg/mL of Poly- (Styrene Sulfonate) or (PSS) in 10 mL of 1 mM NaCl in a 15 mL conical tube.

4.2.4. Prepare 10 mg/mL of Poly-(Diallyldimethylammonium Chloride) (PDADMAC) in 10 mL of 1 mM NaCl in a 15 mL conical tube.

4.2.5. Centrifuge 1.5 mL aliquots of 2X AuNR concentration at 4000 rcf (4500 rpm) for 5 minutes.

4.2.6. Re-suspend the AuNRs in 1 mL of deionized water; the nanorods are now ready to coat.
4.2.7. Add 100 µL of 10 mM NaCl and 200 µL of 10 mg/mL of PSS to the 1 mL of AuNRs.

4.2.8. Shake AuNR solution for 30 minutes.

4.2.9. Once solution is done mixing, centrifuge the AuNR solution for 5 minutes.

4.2.10. Remove the supernatant and re-suspend the AuNRs in 1 mL of deionized water.

4.2.11. Verify the charge on the AuNRs. The synthesis process leaves the AuNRs with a positively charged surface. The PSS polymer coat possesses a negative charge which adheres well to the positively charged surface and can be easily verified by measuring the zeta potential.

4.2.12. Add 100 µL of 10 mM NaCl and 200 µL of 10 mg/mL of PDADMAC to the 1 mL of AuNRs.

4.2.13. Shake AuNR solution for 30 minutes.

4.2.14. Centrifuge the AuNR solution for 5 minutes.

4.2.15. Remove the supernatant and re-suspend the AuNRs in 1 mL of deionized water.

4.2.16. Verify the charge on the AuNRs; the PDADMAC polymer coat possesses a positive charge which can also be verified by measuring the zeta potential.

4.2.17. The PDADMAC coated AuNRs are ready to use.

4.2.18. Repeat steps 2.4 to 2.8 to obtain PSS coated AuNRs.

4.2.19. The PSS coated AuNRs are ready to use.
4.3 SAMPLE PREPARATION – TISSUES

4.3.1. Sterilize all dissection materials and instruments needed prior to the day of experimentation.

4.3.2. Obtain fresh porcine hearts; keep on ice during transit to prevent tissue degradation, but do not freeze the hearts as this may alter the reported modulus of the tissues. [29]

4.3.3. In a dissection hood, remove the apex of the heart with a sharp scalpel to expose the two ventricles.

4.3.4. Make vertical incisions through the ventricular septum, the left outer wall, and the right outer wall of the heart; stop at approximately 1 cm from the base of the atrioventricular valves so as not to cut the leaflets or chordae. The heart should fold out to expose the valves and chordae tendinae.

4.3.5. Use surgical scissors to trim the chordae tendinae from the surface of the leaflets; try to get as close as possible without cutting the surface of the leaflet itself.

4.3.6. Separate the leaflet from the base of the valve while holding it gently but firmly on the free edge.

4.3.7. Place the dissected leaflet into a labeled container of sterile Hank’s Balanced Salt Solution warmed to 37°C while isolating the remaining desired leaflets.

4.3.8. Once all the leaflets have been separated, take a 5 mm biopsy punch and remove small circular samples from the leaflets using a sterile cutting
surface in a sterile hood. Isolate spots that look uniform in thickness, and avoid points of attachment for the chordae tendinae.

4.3.9. Place circular samples into flat containers filled with cell culture media composed of DMEM, 20% FBS, and 1% antibiotics/antifungal solution warmed to 37°C; place the containers into a sterile incubator at 37°C and 5% CO₂ while cleaning up and setting up the AFM.

4.3.10. All excess tissue and tissue related waste should be wrapped up and disposed of properly.

4.6 AFM – NANOINDENTATION

4.4.1. Set-up the instrument.

4.4.1.1. Assemble all materials and turn on all components that are necessary, including computer, microscope, vibration table, camera, laser, software, etc. Our lab uses an Asylum BioMFP-3D AFM.

4.4.1.2. Center the sample stage and all other positional components on the AFM; it makes finding the AFM tip much easier.

4.4.1.3. Place a hard surface, in our case a clean glass microscope slide, under the spot where samples will be tested. Make sure the surface will not move during testing.

4.4.1.4. Select an appropriate AFM tip for use. A V-shaped silicon nitride cantilever with a borosilicate microsphere probe of a radius of 5 um is preferred for testing heterogeneous and thin tissue samples. [30] A tip
with a spring constants of 0.06-0.12 N/m is preferred for testing soft samples [25,31].

4.4.1.5. Assemble an AFM tip holder that is suitable for use in liquids; this usually consists of the tip, the tip holder, and any matching hardware, such as a screw. To start, locate the upper side of the tip; this can be determined by looking for triangular cantilever. Place this side facing away from the holder’s electronics and in the center of the holder. Make sure to grab the tip by the sides using beak nosed tweezers during placement to avoid damaging the cantilever.

4.4.1.6. The AFM has a stand to house the tip holder that must be adjusted to maximum height prior to use; this is done to avoid breaking the tip. Remove the stand and clip in the AFM tip holder, making sure to match the electronics together. Replace the stand and slowly lower it using a level, stop when the tip is above the surface of the glass, but not yet touching.

4.4.1.7. There are two ways to find the tip when using liquid; one can first find the cantilever in air and then add liquid to the tip; or one can add the liquid to the tip first and then find the tip. Do not let liquid come into contact with any of the electronic components.

4.4.1.8. Find the tip using the setup’s camera or viewing eyepiece. Center the tip in the screen to examine the condition of the cantilever. A good cantilever will show up as dark on screen, except for the point at which
the laser is centered, which will shine brightly. A bent cantilever will show up as a white “V” on screen. If the cantilever appears bent, check its position first to make sure that it is not in contact with anything, or else adjust the optics of the system. If the cantilever is in fact bent, replace it with a new tip.

4.4.1.9. There are two options for the detection of cantilever deflection, interferometry and beam deflection. [32] Our setup uses beam deflection, and thus the AFM’s laser must be centered over the position of the spherical probe to detect accurate deflection.

4.4.1.10. Calibrate the AFM according to manufacturer’s instructions. The spring constant of a tip can be determined using thermal tuning [33]; a method for determining the value of the deflection sensitivity for a tip is described below. You will need to determine the deflection sensitivity prior to determining the spring constant of a particular tip.

4.4.2. 3.1) If a heated stage or sample holder is not being used to maintain sample temperature at 37°C during testing, then the samples will need to be removed from the incubator and allowed to come to room temperature prior to use; this will reduce thermal drifting. [34]

4.4.3. 3.2) Calibrate for the deflection sensitivity.

4.4.3.1. Add a drop of HBSS between the tip and a blank glass coverslip; select “contact mode” for a single point in the software so that there
will be movement only in the Z-direction. Then, slowly lower the stand until the tip just barely touches the glass surface.

4.4.3.2. Adjust the Z-Height and the tip approach speed. Our lab typically uses a 1-10 µm/s speed over a 3 µm/s ramp for this step. This speed is slow enough (<10 µm/s) so that hydrodynamic effects are negligible, even though the calibration step is fairly rate insensitive. Engage the tip after first zeroing the tip displacement; then, collect a single force-displacement curve.

4.4.3.3. Calculate the slope of the contact region of the curve to determine deflection sensitivity. The contact region is the linear portion of the curve after the tip contacts the hard surface (See Figure 1). Use this deflection sensitivity for all curves collected with the same tip with the same laser position. The deflection sensitivity must be recalibrated if the laser is moved at any time during an experiment.

4.4.4. Adjust the stand height so that the tip is no longer touching the surface of the glass, and remove the HBSS from the surface of the glass with a Kim-Wipe.

4.4.5. Collect a sample’s indentation data.

4.4.5.1. Use a Kim-Wipe to gently remove excess surface moisture from the sample for mounting; carefully lay the sample out flat on a glass coverslip with the fibrosa side of the valve facing up. Samples can be adhered to glass coverslips using small amounts of cyanoacrylate glue.
placed far from the region(s) of interest. Once the glue is fully dry, wipe the sample surface clean of any residue. Add 50-150 µL of media between the sample and the tip until a column of liquid forms. This step should be done quickly to avoid significant drying out of the sample.

4.4.5.2. Adjust the indentation depth to a value between 1 and 3 µm; this size ensures disengagement of the tip from the sample even if there is adhesion. Adjust the indentation speed to 1 µm/s; the speed can be varied to test the rate dependence of mechanical properties. Generally, indentation rates of ~1 µm/s or less will tend to minimize hysteresis and rate dependent stiffening effects [#].

4.4.5.3. Zero the tip deflection; engage the tip, and collect a force-displacement curve for the point of interest (Figure 2). If the tip does not come into contact with the sample on the first curve, then correct the tip height on either the AFM stand or in the AFM software. Once a desirable curve is obtained, repeat the indentation procedure as necessary to get representative data. Generally, obtaining 5 nanoindentation curves per location on a sample is a good measure of variability on point measurements.

4.4.6. Note: when using the AFM nanoindentation technique, it is important not to indent the tip further than the tip radius to obtain the most accurate results.
4.7 AFM – STRESS RELAXATION

4.5.1. Using information from the nanoindentation curves, determine a suitable indentation depth at which to collect a stress relaxation curve; set this depth as the “trigger” and the “absolute” distance. Our lab typically uses a 500 nm – 1 µm depth.

4.5.2. Set the system to “dwell” towards the sample in the software. Our lab uses a 1 µm/s indentation speed to ensure consistency with the indentation force measurements (section 3), but the speed can be varied to model different tissue responses. A faster indentation speed of 10 µm/s can be used to model a step response.

4.5.3. Set the desired amount of time you want the tip to “dwell” at the indentation depth; this time is usually 1 minute for heart valves, but can be as small as 10s. Collect a stress relaxation curve.

4.8 DATA ANALYSIS

4.6.1. The elastic modulus of the curves obtained may be determined using the Hertz Linear Elastic Analytical Model for spherical indentation [33, 26] as modeled by the equation $F = \frac{4}{3} \frac{E}{(1-\nu^2)} R^2 \delta^2$. Since the Hertz model is only valid for indentations much smaller than the tip radius, only the first 500 nm of indentation will be considered in our calculations. Here, $\nu$ is assumed to be 0.5, $R$ is the radius of the probe size or 2.5 µm, $\delta$ = indentation height, and $F$ = deflection of the tip. This model is useful due to its simplicity, but
more complex models, such as viscoelastic and proelastic, can be used to suit the exact tissue that is being tested, if necessary.

4.6.2. Fit the stress relaxation curves to the mechanical model of interest; popular models include the Standard Linear Solid Model (SLS) and Quasi-Linear Viscoelastic Model (QLV). Normal atrioventricular valve tissues typically exhibit quasi-viscoelastic behavior [26].

4.9 MICROSCOPY - SECTIONING

4.7.1. At each time point, select a random sample from each treatment condition for frozen sectioning.

4.7.2. Transport each sample to a frozen sectioning machine, our lab used a Cryostat, and follow the manufacturer’s instructions for embedding the sample in the appropriate embedding media.

4.7.2.1. Our lab used a red OCT and a white OCT; the white OCT was applied to the sample holder first and allowed to freeze.

4.7.2.2. The sample was then placed on the white OCT, covered with the red OCT, and allowed to freeze. This was done to make it easier to identify the sample in the freezing media.

4.7.2.3. Five samples at 10 µm in thickness were taken with a 30 µm space in between each sample.
4.7.3. Our lab used a CytoViva Hyperspectral imaging system to examine the dispersion of the nanorods throughout the sample. Since the AuNRs autofluoresce, staining of the samples is optional.
CHAPTER 5: RELEVANT RESULTS

Table 1 is provided below to give a clear picture of how the experimental results are organized. Generally, a sample size of $n=50$ force curves for each of the 24 experimental units was used in the nanoindentation experiments. A sample size of $n=5$ forces curves for each of the 24 experimental units was used in the stress relaxation experiments. As stated in the abstract, the purpose of these experiments are to examine the changes in apparent elastic modulus, the changes in time dependent mechanical behavior, the changes in observed response due to differing surface charge, and the changes in response over a period of time. For the sake of consistency and sample size, valves were cut into small 5 mm sections, and any treatments were applied to the center of the sample. Since we were originally unsure if the rods would have a localized effect, the rods were injected into the center of the sample and any mechanical testing began at this point of injection. From the center, 3 to 5 different testing points were selected in linear fashion as illustrated in figure 3.
Table 1: Outline of the experimental model used; Samples were either left plain (Control i), injected with a sterile saline solution (Control ii), injected with PDADMAC coated AuNRs, or injected with PSS coated AuNRs. For each type of rods used, a “high” concentration of rods and “low” concentration of rods was used. At each concentration and control condition, 4 time points (T = 24, 48, 72, 96) were tested for a total of 24 different experimental conditions.

<table>
<thead>
<tr>
<th>Treatment Applied</th>
<th>PDADMAC</th>
<th>PSS</th>
<th>Control i</th>
<th>Control ii</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration</td>
<td>High</td>
<td>Low</td>
<td>High</td>
<td>Low</td>
</tr>
<tr>
<td>Hour</td>
<td>24</td>
<td>48</td>
<td>72</td>
<td>96</td>
</tr>
<tr>
<td>Experimental Unit</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
</tbody>
</table>

Figure 3: Diagram illustrating how samples were tested.
**Figure 4:** Images of the AuNRs used acquired via transmission electron microscopy; the image on the left depicts PDADMAC coated AuNRs while the image on the right depicts PSS coated AuNRs.

**Table 2:** Calculated values for AuNR average length and average aspect ratio.

<table>
<thead>
<tr>
<th>Coated Gold Nanorod Dimensions</th>
<th>PDADMAC Rods</th>
<th>PSS Rods</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Length</td>
<td>Aspect Ratio</td>
</tr>
<tr>
<td>Average</td>
<td>367.73</td>
<td>15.53793542</td>
</tr>
<tr>
<td>St. Dev.</td>
<td>113.8246</td>
<td>5.904061116</td>
</tr>
</tbody>
</table>

Images of the AuNRs used taken via the transmission electron microscope are shown in figure 4; the tabulated values for the average rod length and aspect ratio, as well the standard deviations for these values, are shown in table 2. As expected, the synthesis process yielded long rods with a high aspect ratio [18]. The differences in values may be
attributed variation in the synthesis and coating processes used to make and prepare the rods.

**Figure 5**: A typical force curve for the AFM nanoindentation technique indicating the position of the tip with respect to the sample during curve collection. Illustration “A” corresponds with the curve’s horizontal portion indicating the tip is far from the sample. Illustration “B” corresponds with the change in slope of the extension curve indicating the tip has made contact with the sample. Illustration “C” corresponds with the linear portion of the extension curve indicating the tip indenting into the sample. Illustration “D” corresponds with the near-linear portion of the retraction curve indicating the tip withdrawing from the sample. Finally, Illustration “E” corresponds to the negative portion of the retraction curve indicating the tip is sticking to the sample.
**Figure 6:** A typical force curve for the AFM nanoindentation technique; the calculations from the Hertz model used to determine the apparent elastic modulus are plotted alongside the data.

Figure 5 is provided to illustrate a typical force curve collected via the nanoindentation technique and the behavior of the tip at each region within the force curve. Figure 6 represents properly collected nanoindentation data, and the fit of the extension curve data with the Hertz model. Generally, the hertz model will fit well to most of the data, as seen by the close match of the “data” and the “calculated” lines shown in figure 6. When fitting the hertz model to the data, the value for apparent elastic modulus is calculated from the Hertz equation. Immediate examination of the values for apparent elastic modulus shown in figure 7 indicates a trend towards increasing stiffness with the addition of the AuNRs, regardless of the type of AuNR, PDADMAC or PSS coated, added to the samples. Closer examination of this trend under each condition, that is Control i, Control
ii, HPDADMAC, LPDADMAC, HPSS, and LPSS, broken down into the four time points tested indicates differences in time dependent trends. Generally, high concentrations of the rods are necessary to observe an effect in the data, and the final surface charge on rods will dictate the trend in behavior. Overall, the positively charged PDADMAC rods will not be statistically significant from the control samples, while the negatively charged PSS rods will initiate an increase in the stiffness of the tissue samples. This is verified by the statistical analysis recorded in tables 3 through 6.

**Figure 7:** Bar graph displaying the values for apparent elastic modulus of a particular condition averaged over all time points.
Figure 8: Bar graph displaying the values for apparent elastic modulus of the two types of control samples averaged over each time point.
Figure 9: Bar graph displaying values for apparent elastic modulus of the samples injected with high or low concentrations of PDAMAC coated AuNRs averaged over each time point.
**Figure 10:** Bar graph displaying values for apparent elastic modulus of the samples injected with high or low concentrations of PDAMAC coated AuNRs averaged over each time point.

**Table 3:** Analysis of Variance table indicating there is some significance in the results. This particular test compares a null hypothesis assuming all means are equal to an alternative hypothesis assuming there is some difference between the means.
Table 4: Effects tests for the Analysis of Variance table shown in table 3. The p-values indicate statistical significance in the simple factor of condition, meaning there are some differences between conditions, whereas there is no statistical significance in the simple factor of day, meaning there may not be some differences between the days. The p-values also indicate statistical significance of the interaction between the simple factors of day and condition, meaning that the trends across the days for each condition may vary.

<table>
<thead>
<tr>
<th>Source</th>
<th>Nparm</th>
<th>DF</th>
<th>Sum of Squares</th>
<th>F</th>
<th>Prob &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Condition</td>
<td>5</td>
<td>5</td>
<td>3458.541</td>
<td>9.624</td>
<td>&lt;.0001*</td>
</tr>
<tr>
<td>Day</td>
<td>3</td>
<td>3</td>
<td>553.3476</td>
<td>2.566</td>
<td>0.0533</td>
</tr>
<tr>
<td>Condition * Day</td>
<td>15</td>
<td>15</td>
<td>7514.38</td>
<td>6.97</td>
<td>&lt;.0001*</td>
</tr>
</tbody>
</table>
Table 5: Least square means tables indicating the average values for apparent elastic modulus with the standard errors for each of the simple factors (Day and Condition). The values for each condition are calculated irrespective of the day whereas the values for day are calculated irrespective of condition.

<table>
<thead>
<tr>
<th>Effect Details</th>
</tr>
</thead>
</table>

**Condition**

<table>
<thead>
<tr>
<th>Least Squares Means Table</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Level</th>
<th>Least Sq Mean</th>
<th>Std Error</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPDAD</td>
<td>5.277729</td>
<td>0.6071042</td>
<td>5.7683</td>
</tr>
<tr>
<td>HPSS</td>
<td>10.58194</td>
<td>0.7161612</td>
<td>10.3532</td>
</tr>
<tr>
<td>i</td>
<td>4.796492</td>
<td>0.7021873</td>
<td>4.5705</td>
</tr>
<tr>
<td>ii</td>
<td>4.80348</td>
<td>0.7690569</td>
<td>5.2091</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Day</th>
<th>Least Squares Means Table</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Level</th>
<th>Least Sq Mean</th>
<th>Std Error</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.791792</td>
<td>0.5262247</td>
<td>6.33254</td>
</tr>
<tr>
<td>2</td>
<td>7.231909</td>
<td>0.5434445</td>
<td>7.78712</td>
</tr>
<tr>
<td>3</td>
<td>5.672196</td>
<td>0.6028011</td>
<td>5.55174</td>
</tr>
<tr>
<td>4</td>
<td>7.542391</td>
<td>0.7038164</td>
<td>7.92033</td>
</tr>
</tbody>
</table>
Table 6: Contrast examining statistical difference between PDADMAC coated AuNRs and the control I condition. The non-significant p-values indicate the addition of the PDADMAC AuNRs yields values for apparent elastic modulus which are not statistically different from the untreated samples.

<table>
<thead>
<tr>
<th>Test Details</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>HPDAD</td>
<td>0.5</td>
</tr>
<tr>
<td>HPSS</td>
<td>0</td>
</tr>
<tr>
<td>i</td>
<td>-1</td>
</tr>
<tr>
<td>ii</td>
<td>0</td>
</tr>
<tr>
<td>LPDAD</td>
<td>0.5</td>
</tr>
<tr>
<td>LPSS</td>
<td>0</td>
</tr>
</tbody>
</table>

| Estimate | Std Error | t Ratio | Prob>|t| | SS |
|----------|-----------|---------|-------|----|
| 1.2061   | 0.8658    | 1.3931  | 0.1639| 139.48|

<table>
<thead>
<tr>
<th>SS</th>
<th>NumDF</th>
<th>DenDF</th>
<th>F Ratio</th>
<th>Prob &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>139.5</td>
<td>1</td>
<td>925</td>
<td>1.9407</td>
<td>0.1639</td>
</tr>
</tbody>
</table>
Table 7: Contrast examining statistical difference between PDADMAC coated AuNRs and the control ii condition. The non-significant p-values indicate the addition of the PDADMAC AuNRs yields values for apparent elastic modulus which are not statistically different from the saline injected samples.

<table>
<thead>
<tr>
<th>Contrast - Comparing both concentrations of PDADMAC coated AuNRs to Control i</th>
</tr>
</thead>
<tbody>
<tr>
<td>Test Details</td>
</tr>
<tr>
<td>HPAD</td>
</tr>
<tr>
<td>HPSS</td>
</tr>
<tr>
<td>i</td>
</tr>
<tr>
<td>ii</td>
</tr>
<tr>
<td>LPAD</td>
</tr>
<tr>
<td>LPSS</td>
</tr>
</tbody>
</table>

| Estimate | Std Error | t Ratio | Prob>|t| | SS |
|---|---|---|---|---|
| 1.1991 | 0.9208 | 1.3022 | 0.1932 | 121.87 |

<table>
<thead>
<tr>
<th>SS</th>
<th>NumDF</th>
<th>DenDF</th>
<th>F Ratio</th>
<th>Prob &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>121.9</td>
<td>1</td>
<td>925</td>
<td>1.6957</td>
<td>0.1932</td>
</tr>
</tbody>
</table>
Table 8: Contrast examining the difference between the PSS coated AuNRs and the control I condition. The significant p-values indicate the addition of the PSS AuNRs yields values for apparent elastic modulus which are statistically different from the untreated samples.

<table>
<thead>
<tr>
<th>Contrast - Comparing both concentrations of PSS coated AuNRs to Control i</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Test Detail</strong></td>
</tr>
<tr>
<td>HPDAD</td>
</tr>
<tr>
<td>HPSS</td>
</tr>
<tr>
<td>i</td>
</tr>
<tr>
<td>ii</td>
</tr>
<tr>
<td>LPDAD</td>
</tr>
<tr>
<td>LPSS</td>
</tr>
</tbody>
</table>

| Estimate  | Std Error | t Ratio | Prob>|t| | SS        |
|-----------|-----------|---------|--------|----------|
| 4.0797    | 0.8777    | 4.6481  | 3.8E-06| 1552.8   |

<table>
<thead>
<tr>
<th>SS</th>
<th>NumDF</th>
<th>DenDF</th>
<th>F Ratio</th>
<th>Prob &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>1553</td>
<td>1</td>
<td>925</td>
<td>21.6044</td>
<td>&lt;.0001*</td>
</tr>
</tbody>
</table>
Table 9: Contrast examining the difference between the PSS coated AuNRs and the control ii condition. The significant p-values indicate the addition of the PSS AuNRs yields values for apparent elastic modulus which are statistically different from the saline injected samples.

| Test Detail | Estimate | Std Error | t Ratio | Prob>|t| | SS |
|-------------|----------|-----------|---------|------|-----|
| HPDAD 0     | 0        | 0         | 0       | .235 | .816|
| HPSS 0.5    | 0.5      | 1         | 2.12    | .041 | .041|
| i 0         | 0        | 0         | 0       | .235 | .816|
| ii -1       | -1       | 0         | -1      | .409 | .645|
| LPDAD 0     | 0        | 0         | 0       | .235 | .816|
| LPSS 0.5    | 0.5      | 1         | 2.12    | .041 | .041|

<table>
<thead>
<tr>
<th>SS</th>
<th>NumDF</th>
<th>DenDF</th>
<th>F Ratio</th>
<th>Prob &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>1372</td>
<td>1</td>
<td>925</td>
<td>19.0923</td>
<td>&lt;.0001*</td>
</tr>
</tbody>
</table>

Contrast - Comparing both concentrations of PSS coated AuNRs to Control ii
**Figure 11:** A typical force curve for the AFM stress relaxation technique indicating the position of the tip with respect to the sample during curve collection. Illustration “A” corresponds with the curve’s horizontal portion indicating the tip is far from the sample. Illustration “B” corresponds with the change in slope of the extension curve indicating the tip has made contact with the sample. Illustration “C” corresponds with the linear portion of the extension curve indicating the tip indentering into the sample. Illustration “D” corresponds with the classic relaxation curve indicating the tip is holding position in the sample. Illustration “E” corresponds with the retraction curve indicating the tip is withdrawing from the sample.
Figure 12: A typical force curve for the AFM stress relaxation technique; the calculations for the QLV model’s reduced relaxation function, or $G(t)$, with $c = 0.08$, $\tau_1 = 0.11$, and $\tau_2 = 4.46$ are plotted alongside the data.

Stress relaxation data can be represented in a normalized fashion for easy comparison; that is, the stress relaxation curves, minus the extension and retraction curves, are made to start at time = 0 seconds and a maximum value of 1. This value is taken to be the maximum stress in the curve, and the resulting values after 1 are taken to be a percentage of remaining stress. One way to interpret this information is to pick values for the percentage of relaxation from the normalized data and compare them. Another way to analyze the data is to fit this normalized curve to the QLV model’s reduced relaxation function to determine the values for $c$, $\tau_1$, and $\tau_2$. 
For our purposes, the values at time = 5 seconds were chosen and compared. This value was selected on the basis that it was not located in the initial slope of the stress relaxation curve yet remained a good time point to compare among all the curves collected. Examination of the values for percentage of relaxation at time = 5 seconds indicates no major difference in relaxation behavior. Closer inspection of the data for each condition by each time point reveals no underlying trends in the data. Examination of the fit of the QLV curve to a typical normalized stress relaxation curve in figure 12 indicates that the stress relaxation data fits well with QLV model ($R^2 = 0.98$). This is to be expected since the QLV model was developed for soft tissues with complex time dependent behavior not accurately described by a single time constant, that is, in figure 12 we can clearly see both a short term and a long term stress relaxation behavior.

![Figure 13: Bar graph displaying values for the percentage of relaxation by samples of a particular condition averaged over all time points at time = 5 seconds.](image)
**Figure 14:** Bar graph displaying values for the percentage of relaxation by the two control samples organized by time point at $t = 5$ seconds.

**Figure 15:** Bar graph displaying values for the percentage of relaxation by the PDADMAC treated samples organized by time point at $t = 5$ seconds.
Figure 16: Bar graph displaying values for the percentage of relaxation by PSS treated samples organized by time point at t = 5 seconds.
CHAPTER 6: CONCLUSION & DISCUSSION

6.1 GOLD NANORODS

The synthesis and coating of gold nanorods often requires finesse and patience to obtain a satisfactory yield. For example, the method of synthesis used by this thesis is extremely sensitive to stir rate, whereby an increased stir rate can negatively affect the yield of the nanorods. Likewise, the process of removing the nanorods from the container in which the rods were synthesized may result in some loss of rods. Additionally, the size of the rods during synthesis can be adversely altered by the time taken to complete the fast steps; generally, finishing the steps within the time specified yields a better batch of rods.

Aside from the synthesis process, the coating of AuNRs with PEM can also be fraught with complications. Prior to coating, the AuNRs are centrifuged and the supernatant replaced with sterile deionized water to remove the excess free CTAB floating in solution and prevent AuNR aggregation during the coating process. The layer-by-layer method of coating relies on the assumption that the previous coating covers all surfaces; should some areas of the AuNR be left uncoated, there remains the possibility of the next coating interacting unfavorably with the uncoated surface resulting in the entire layer by layer assembly crashing off. Measuring the surface charge between coatings ensures full coverage of the polymer coat ensuring the rods are ready for use.
6.2 SAMPLE PREPARATIONS

Sample preparation methods have developed of the constraints posed by the various testing equipment, the desire to collect better and more accurate data, the desire to normalize data and make experiments reproducible, concern for the safety of the researcher, and the desire to preserve limited samples for storage and later study. Of the many sample preparation methods, we will only consider the effect of a few on the results collected concerning the study of AV valves using AFM techniques, specifically freezing samples for later study and immobilizing samples for mechanical testing. As stated previously, AFM offers wonderful flexibility in terms of the condition of samples studied, the environment in which the samples are studied, and the modes with which the samples are studied [29]. However, since the study is on such a small scale, the effects of the various forms of accepted sample preparation methods can adversely affect the final results in both topographical studies and mechanical studies. Thus, the use of freshly prepared samples is the often the best way to obtain the most accurate results.

Preparing samples properly for nanoindentation and stress relaxation begins with the timely dissection of a freshly acquired porcine heart. Multiple hearts may be dissected at once, but due to the time constraints posed by using the AFM, one is usually sufficient. Usually, the hearts are transported on ice and in sterile PBS treated with antibiotics. Following transportation, a hood dedicated to dissection can be used to prevent contamination of the sterile cell culture hood. Once the desired valve tissue was isolated,
transfer of the tissue to a sterile hood can be done for further processing under more sterile conditions.

Generally, isolation of the atrioventricular valve tissues can be a simple process or a difficult process depending on the desired outcomes. That is, the procedure outlined in the protocol attempts to isolate the atrioventricular valves of the porcine heart in a quick and straightforward manner, while preserving the semi-lunar valves for further dissection; however, the procedure makes no further effort to preserve the cardiac wall, the ventricular septum, and the chordae tendinae for study. Furthermore, by merit of removing the sample from its natural environment, damage can be done to the sample, as seen in the case of dissecting AV heart valves from the heart for study. One particular study noted that the dissection process was prone to causing the loss of the endocardial cells on the valves, resulting in exposure of the underlying valvular trilaminar layer [7]. There are more sophisticated surgical methods that may be learned to preserve the basic structure of the heart [9]; however, these techniques are not used here because they require a considerable amount of skill and time and are otherwise unnecessary. Indeed, there are instances when this damage may not matter or may even be beneficial. A study done that looked at the supramolecular structure of collagen in all of the heart valves with a combination AFM and FTIR approach were able to adequately see the underlying collagenous matrix due to the loss of the endocardial cells.
It should be noted that not all parts of porcine atrioventricular valve leaflets are ideal samples for atomic force microscopy. The portion of the mitral valve adjoining the aortic valve, indicated by the arrows in figure 22, is generally unsuitable for both valvular interstitial cell isolation and AFM studies due to the inherent difference in mechanical properties. Remnants of the chordae tendinae present on the ventricular surfaces renders the leaflets non-uniform in both thickness and surface texture. While non-uniformity may exist in any sample, this fact is more disadvantageous with leaflets since the performance of the tip is best with a flat and unobstructed surface [29] and the sloping geometry tends to interfere with normal force measurements. Moreover, the leaflets tend to possess time dependent viscoelastic behavior [26], as opposed to the purely elastic behavior assumed by the Hertz model. This behavior necessitates the use of a consistent indentation velocity throughout the testing to minimize variation, although, a large amount of natural variation can be expected in the results due to the heterogeneous nature of the tissue. The development and use of consistent criteria for selecting testing spots was used to help to reduce some of the variation in and between samples.

In an effort to better control the amount of variation between the valve samples tested, 5mm circular sections are cut from a single valve leaflet. This step is used to isolate areas of uniform thickness, and ensure that the samples used are free of chordae tendinae remnants. Statistically, making the shape of the samples to be used equivalent makes it easier to examine the effects of various treatments on a single leaflet, as well as help
ensure that any possible treatment effects are not due to variations in the size of the samples.

While there are several advantages associated with using these smaller samples, there are also several disadvantages. Small samples are less mechanically stable and may float in the liquid environment. Since the AFM is a mechanical microscope, any movement during testing has the potential to damage the tip or significantly hamper the collection of data. Parts of the tissue sample can be bonded to a hard surface with cyanoacrylate glue to prevent movement, but the utmost care should be taken so as not to secure the sample near the regions of interest. It should be noted that the process of bonding a sample to a hard surface may alter the apparent stiffness, especially for very thin samples [33], and this fact applies to the use of double sided tape, as well. Generally, gently wiping off any residual cyanoacrylate glue from the surface of the tissue sample will minimize this artifact in the data.
Figure 17: Typical nanoindentation force curve altered by excess cyanoacrylate glue. Note the increased values for extension force and the vertical slope of the extension curve.

While it may seem advantageous to freeze freshly isolated samples for later use, the process of freezing a sample will generally cause the formation of ice crystals. These crystals have the potential to disrupt the organization of collagen fibers, resulting in altered values for apparent elastic modulus [39]. Indeed, in previously frozen samples a marked increase in the values for apparent elastic modulus was observed (results not shown). Additionally, the cyanoacrylate glue will cause a marked increase in the values for apparent elastic modulus if not applied carefully. Typically, using as little glue as possible and removing any remaining residue will ensure that the values for apparent elastic modulus recorded are due to the sample and not any artifact from the glue. A curve that has been significantly altered by the presence of excess cyanoacrylate glue is
shown in figure 17. It should be noted that curves altered by excess cyanoacrylate glue will not fit to the Hertz model.

6.3 ATOMIC FORCE MICROSCOPY OF SAMPLES

6.3.1 USING THE AFM

In these experiments it should be noted that the AFM tip characteristics, and its interactions with the samples, are the primary factors that dictate the overall nature and success of a particular AFM experiment. The cantilever shape, the probe shape, the cantilever size, the probe size, the cantilever spring constant, the cantilever material, the probe material, and overall tip surface coatings can all be chosen to suit the needs of a particular experiment [39, 29, 31, 27]. Unfortunately, with so many options available it may sometimes be difficult to determine which type of tip to start with, especially since the quality and characteristics of the tip selected can have a huge impact on the data collected [27, 31]. Of these many characteristics, the probe shape of the AFM tip possesses the biggest impact on the force curves collected for mechanical tests, while other characteristics such as cantilever shape, material, and spring constant have the biggest impact on the calculated values for apparent stiffness and other mechanical properties of tissue samples [27, 32].

While there are many probe shapes available, two well-known options, shown in figure 6, are sharp pyramidal and spherical. Typically, pyramidal robes are used in topographic
studies [39, 28, 29] while spherical probes are used in mechanical testing applications [39, 28, 30, 40]. Pyramidal probes, while providing excellent resolution for surface topography studies, make a poor probe for mechanical testing. The sharp point of the probe can damage soft tissue structures, and the exact probe size and shape is often difficult to determine. Furthermore, the probe geometry is likely to violate certain simplifying assumptions used to determine the mechanical properties of the tissue studied [27, 32]. Similarly, the extremely small radius of curvature between the contacting surface of the probe and tissue can result in large deformations in tissue [32], consistent overestimation of tissue stiffness in nanoindentation studies [32], and detection of heterogeneity in the tissues studied resulting in highly variable data [32]. On the other hand spherical probes are poorly suited high resolution topographic imaging studies, but remain an excellent choice for mechanical testing and experimentation. When used to test soft biological samples, spherical probes ensure not only accurate and reproducible data collection, but also proper fit with many of the simplifying assumptions used to determine mechanical properties of soft tissues [32]. Additionally, the size and structure of the spherical probes attached to the cantilever is better controlled making quantitative analysis with the probes, as is the case with nanoindentation and stress relaxation, easier and more reliable [27, 32].
Besides the probe shape, the overall “force resolution” of the cantilever must be considered. If a cantilever is too soft, it will deflect without indenting into the sample. However, if a cantilever is too stiff, it will fail to be deflected by the sample. Cantilever spring constant is a function of several factors, such as material properties of both the probe and cantilever and the overall shape of the cantilever [27, 31]. Thus, determining the exact spring constant of the cantilever is extremely important to knowing a tips force resolution, as the values reported in the manufacturer’s final specifications are often slightly incorrect [27]. Cantilever width, length, thickness, any surface or other bulk material defects, the attached probe weight, and any surface coatings all will affect the final value of cantilever stiffness [27, 41] as well as any intermolecular interactions between the tip and the sample [27, 41]. Unfortunately there is no universal process with which to determine the spring constant, but there is a commonly used method most often known as “thermal tuning” that measures thermal noise fluctuations in the tip [31]. Since
each AFM system is different, the manufacturers should have instructions for performing a thermal tune of a tip listed in the user’s manual. Unlike the cantilever spring constant, the cantilever shape is easily determined by looking at the tip specifications. Generally, there are only two different shapes to choose from; a simple plank shape and a V-shape. Of the two, the V-shape is more commonly used with soft tissues due to the superior torsional stiffness and mechanical sensitivity than its plank shaped counterpart [29].

There are many commercially available tips with spherical probes for the mechanical testing of biological materials, though, a silicon nitride V-shaped cantilever with a 2 or 5 um borosilicate spherical probe and a spring constant varying between 0.06-0.15 N/m is typically used with atrioventricular valve tissues [42, 43]. Nonetheless, there have been nanoindentation experiments conducted with probes as large as 25 um [39] since the size of the surface features present on the sample of interest will generally determine the size of the probe used. Likewise, materials other than borosilicate glass, such as polystyrene [32], have been used to form the spherical probe; especially in cases where the surface interactions between the tip and the sample are large enough to become a concern in the results. In such cases or otherwise, it may be a good idea to clean the tip with a drop of ethanol between samples to prevent the accumulation of materials that may cause the tip to “stick” during subsequent tests. The sticking of a tip to a sample is doubly problematic since it will alter the force curve that is recorded and if strong enough, may result in permanent deformation to the cantilever rendering the tip virtually useless for further mechanical applications. Factors such as moving the tip too quickly across a sample,
deflecting the tip beyond acceptable limits, or storing the tip in extreme temperatures can likewise cause permanent deformation to the cantilever [29, 34].

Aside from knowing the cantilever shape, probe shape, probe size, and overall spring constant, other information such as deflection sensitivity, zero force, and zero distance must be known to calculate the apparent stiffness from a force curve for nanoindentation. This information is often obtained after the tip has been properly installed in the AFM tip holder and the optics properly aligned. As stated previously, the deflection sensitivity of the tip should be determined prior to verifying the spring constant. This will ensure the accuracy of the values reported for the spring constant and the functional condition of the tip prior to actual use. Once these values have been determined, zero distance and zero force can be determined from each individual force curve. Zero force may sometimes be referred to as “baseline,” and is the horizontal region in a graph where the tip is far away from the sample [28]. Zero distance is taken to be the beginning of the linear region when the tip “jumps” into contact with the sample, and may sometimes be difficult to determine [28]. A set of criteria for determining the zero distance should be developed and used consistently to reduce the error when calculating the apparent stiffness of a sample.

Since deflection is determined via laser, it is important to ensure that all of the optical components are lined up correctly in the experimental environment, here, a liquid environment. The laser used should be focused onto the reflective backside of an un-
deflected cantilever at the position of the probe; if done correctly, this will result in a maximum intensity, or maximum “sum,” seen by a position sensitive photo-detector or some other sensor used to measure deflection [27, 28, 44]. Often, troubles with the tip and tip deflection can be solved by double checking the position of the tip in the tip holder, the position of the laser on the tip, the deflection of the tip, and the general condition of the tip. It is not unusual for a good amount of time to be spent ensuring the laser optics and tip are properly aligned since the position of the laser is critical to accurately determining the cantilever deflection.

6.3.2 DATA COLLECTION

A fluid cell may be used to test the samples; however, a clean glass microscope slide or coverslip works well, too. It is possible to test the atrioventricular valves in a dry environment [43] to prevent floating, but a liquid environment is used here to simulate a physiologically relevant setting [28]. The liquid is also used to better control the molecular interactions between the tip and the sample [28], especially since the tip will have a strong tendency to stick to the sample. Hank’s Buffered Salt Solution (HBSS) or Cell Culture Media (CCM) are used here since they are readily available and can be used to remove excess cyanoacrylate residues from the samples. If a fluid cell is not used, then the liquid surface tension is exploited to prevent the liquid from spilling over into expensive electronics. To achieve this, a prepared sample is rinsed, dried, and centered below the AFM tip, which is subsequently lowered until sample and tip are just barely touching. A needle and syringe is then used to add a precise amount of liquid, usually
100-150 µL, between the tip and the sample. This amount is enough to connect the sample and the tip with a column of liquid, but not so much as to allow liquid to spill over. The AFM tip can then be further lowered to begin collecting force measurements. Generally, it is a good idea to keep the samples as hydrated as possible throughout the entirety of the experiment to prevent degradation of the proteoglycans and glysoaminoglycans present in the spongiosa layer of the valves.

Collecting nanoindentation and stress relaxation force takes about an hour and a half per sample. Initial force curves collected via nanoindentation can be used to optimize the probe velocity. Generally, increasing the probe velocity will increase the amount of hysteresis seen in a force curve. Hysteresis can be calculated by subtracting the area under the retraction curve from the area under the extension curve [45]. This area can then be normalized and plotted to determine the optimal probe velocity, that is, a velocity that is slow enough to minimize hysteresis, yet fast enough to capture the number of desired force curves over the course of the experiment. A velocity of 1 um/s is consistently used in our work.

During testing, it is not uncommon to spend a good deal of this time ensuring that the indentation depth of the tip into the sample for each force curve is kept constant. Thermal drifting of the AFM tip and piezoelectric components, especially in a liquid environment [31], is often the biggest obstacle in keeping the indentation depth constant. Drifting is a process that results in the tip “spontaneously” moving towards or away from
a sample, thus changing the indentation depth. This phenomenon can be seen in the collection of consecutive nanoindentation force curves, as well as in a stress relaxation curve. However, this drifting is especially detrimental to the collection of a stress relaxation curve, which by nature assumes that the position of the sample and the tip is kept constant. While there is not much that can be done to stop it entirely, steps can be taken to minimize it. Often, temperature changes in liquids during testing, or even temperature changes in the room during testing, can exacerbate the problem [29, 31]. Thus, the temperatures around the tip and the piezoelectric components must be kept stable. If the drifting becomes a serious problem, it is often best to check the stability of the sample, or else quit and try again at a later time.

A stress relaxation curve can be obtained prior to, or immediately following, the collection of several nanoindentation force curves, depending on the capabilities of the system available. Generally, the nanoindentation curves are used to determine the stability and constancy of the tip’s indentation depth, as well as determine the stress relaxation “trigger.” The trigger is the desired amount of cantilever deflection, or voltage, which must be met for the tip to hold position. If the desired stress relaxation trigger is previously known, and the system is capable of automatically “finding” the sample, then the collection of nanoindentation curves can be skipped. Once the tip has been “triggered,” it will hold in that position for a pre-determined amount of time to collect the stress relaxation curve. A typical stress relaxation curve will last 60 s, although longer or shorter times may be used if desired.
6.3.3 DATA ANALYSIS

Many AFM systems plot nanoindentation force curves as a function of cantilever deflection versus the z-sensor or piezoelectric position [28], both of which can be determined via different methods based on the AFM system used. Since each system is different, the default units on these curves may change. It is a good idea to consult the user’s manual prior to use to verify these default units and avoid unnecessary calculations later. Stress relaxation force curves may also be plotted this way and thus must be converted to cantilever deflection versus time to observe the meaningful results. Plotting extension force versus time is also acceptable. Generally, cantilever deflection is detected via laser, while the z-sensor position is controlled by the computer and software used.

Once the curves are plotted appropriately, the model of interest can be fit to the data to determine the parameters of interest. In the case of nanoindentation, the Hertz model is used to determine the apparent elastic modulus of the tissue. In the case of stress relaxation, the primary curve of interest is normalized to a value of 1 and a time of 0 seconds. This normalized data can then be used in a couple of ways. One way is to determine the percent relaxation at a specific time point; another is to determine the dimensionless “c” constant, as well as the time constants τ1 and τ2. It should be noted that when fitting to the models, both the Hertz and the QLV, the appropriate values for the various parameters, such as tip radius, poisson’s ratio, etc. must be used. Using incorrect values will generally result in overestimation or underestimation of the apparent elastic modulus, improper fit of the QLV model, and faulty results.
As can be seen in the results, primary effect of the PDADMAC coated AuNRs differs from the primary effect of the PSS coated AuNRs. Statistical analysis of the data confirmed that PSS samples were generally statistically different from both control conditions while the PDADMAC AuNR treated samples were not spastically different from both control conditions. This is in agreement with the previous literature [22] and is expected. Generally, the rods are injected into the center of the sample, and usually into the spongiosa layer. Since the spongiosa layer is comprised of a large majority of negatively charged glycosaminoglycans, it is not surprising that the positively charged AuNRs, or the PDADMAC AuNRs, will have a different effect from the negatively charged AuNRs, or the PSS AuNRs.

Visual inspection of the data indicates a large amount of variation; it could be that the large variation seen in all the samples are due to the variation in sample composition. The tissue samples were tested on the fibrosa side of the valve, which traditionally is more prone to disease than the atrialis side of the valve. However, the fibrosa side of the valve is also where the chordae tendinae insert, and thus these remnants of chordae may affect the value obtained for the elastic modulus. Aside from the chordae tendinae, residues from the cyanoacrylate glue left on the surface of the sample may interact with the tip or alter the samples also leading to highly variable data, and this effect was in fact seen in samples not properly rinsed of residue.
6.4 MICROSCOPY

The Cytoviva Hyperspectral Imaging System is used to provide a spectral analysis of nano-scale materials; the microscope is a type of dark field optical microscopy which operates in the visible to near infrared spectrum, but is tailored to image nanorods and nanoparticles specifically. This technique provides a spectral analysis of the PEM coated AuNRs used in this thesis to confirm the presence of the AuNRs in the tissue. Initial examination of the samples under the Cytoviva (results not shown) verified the presence of the AuNRs in the thin edges of the samples. Unfortunately, the center of the samples effectively scatters too much light due to the thickness, thus a spectral image could not be taken for regular samples. Despite this, the AuNRs could be visualized at the thinner and more transparent edges of the tissue; which is why the samples are frozen sectioned into 10 µm slices and set aside for imaging later.
CHAPTER 7: RECOMMENDATIONS

Based on our results, there are several directions in which the project, and the knowledge gained from projects similar to it, can be furthered. Firstly, the project used PEM coated AuNRs exclusively due to the previous reports of the qualitative changes in collagen gels containing the AuNRs. Thus, in the short term, it would be advantageous to better explore the changes in mechanical properties in collagen gels and mitral valve samples due to the presence of other types and shapes gold nanoparticles. Specifically, either collagen gels or mitral valve samples doped with various PEM coated gold nanoparticles could better elucidate the dependence of shape versus surface coating on the alteration of mechanical behavior. Additionally, in the interest of using the coated AuNRs to help correct valvular pathologies, it would be advantageous to conduct studies constructing collagen gels with pathological cells. Specifically, valvular interstitial cells taken from stenotic valves would better elucidate the interaction of AuNRs in altering cellular phenotype. Following these studies, it would be advantageous to compare our results to traditional mechanical testing methods. Since the AFM is testing the cross-fiber direction, or the “mat” of fibers constructing the ECM of the mitral valve, it would be necessary to determine the effect of the AuNRs on the mechanical properties of the radial and circumferential orientation of collagen fibers.

Secondly, it would be useful to explore new methods of AuNR application, besides an injection based method. Indeed, the effective immobilization of surface modified AuNRs
into the tissue of interest would open many new levels of research and clinical applications. While the injection based method has been useful for our purposes, it may not be practical for therapeutic applications to the mitral valve. The mitral valve is constantly moving, and the blood flow throughout the body may not carry the AuNRs to the desired location. Thus, the development of a delivery system exploiting the charges present on the surface of the AuNRs, or exploiting the unique resonant properties of the AuNRs, could be used to force the AuNRs into the tissue of interest. Specifically, many recently developed biodegradable gels, glues, and other materials doped with the AuNRs could be used to deliver the AuNRs to the atrioventricular annulus, the primary support structure for the atrioventricular valves.

Thirdly, the exploration of other surface modification methods to allow for the immobilization of various proteins and other markers could be used to improve studies involving cell membrane receptors. That is, the morphology of AuNRs lends well to the crosslinking of the cellular surface receptors. Their large shape and ability to be easily functionalized could potentially bind to the many receptors presented on the cellular membrane. In theory, these properties could be exploited towards the improvement of laser induced hyperthermia of cancer cells, as one study in particular proved the effectiveness of the therapy increased following immobilization of the rods on the surface of the cells, rather than in the interior of the cells [23]. Likewise, the development of an effective delivery method coupled with effective functionalization methods taken together in a clinical setting could allow for chelation agents and other medications to be
delivered to calcified valves. Chelation agents have the possibility of breaking up calcium clusters, and once used, the AuNRs have the potential to reverse pathological phenotypes within the mitral valve. Thus, studies examining the efficacy of surface modified AuNRs mediating the breakup of calcium deposits would also be useful.
Figure 19: A porcine heart; the green line indicates where the first incision is made.
Figure 20: The exposed ventricles of a porcine heart; the green line indicates where the second incision is to be made. The green “A” indicates the right ventricle; the green “B” indicates the interventricular septum; the green “C” indicates the left ventricle.
Figure 21: The exposed valves of the porcine heart; the green “A” indicates the right atrioventricular valve, or the tricuspid valve. The green “B” indicates the interventricular septum, and the green “C” indicates the mitral valve. Note how the direction of the incision allows the valve leaflets to be pulled taught.
Figure 22: The mitral leaflet within the heart with the chordae tendinae removed.
Figure 23: The left ventricle with the mitral valve removed.
**Figure 24:** The excised mitral valve; the area indicated by the arrows is generally not used in sample selection cell isolation protocols.
Figure 25: Samples selected from the mitral valve for testing; The circular samples designated “A” and “B” are good examples of acceptable samples; The samples designated “C” and “D” are good examples of unacceptable samples.
APPENDIX B – MATLAB SCRIPTS

B.1 Relaxation.m

function [percentrelaxation,data_final,r2,fit1,coef1] = Relaxation()
%UNTITLED Summary of this function goes here
% Detailed explanation goes here
DataMatrix = xlsread('C:\Users\hlecuye\Documents\MATLAB\Heather\StressR Data PSS Rods.xlsx','LPSS');
[m,n] = size(DataMatrix);
row = 1;
col = 2;
LowPeak = 600;
tens = 10000;
percentrelaxation = [];
dataset = 1;
max = 0;
timedif = 0;
normalizer = [];
datasets = n/2;
OutputMatrix = [];
while dataset <= datasets,
    while row <= m,
        if DataMatrix(row,col) > max,
            max = DataMatrix(row,col);
            timedif = DataMatrix(row,col-1);
        end
        row = row+1;
    end
    normalizer(dataset,1:2) = [max,timedif];
dataset = dataset+1;
row = 1;
col = col+2;
max = 0;
end
datast = 1;
col = 2;
row = 1;
while dataset <= datasets,
    while row <= m,
        DataMatrix(row,col-1) = DataMatrix(row,col-1)-normalizer(dataset,2);
        DataMatrix(row,col) = DataMatrix(row,col)/normalizer(dataset,1);
    end
end
row=row+1;
end

dataset = dataset+1;
col = col+2;
row = 1;
end
dataset = 1;
col = 2;
row = 1;
while dataset <= datasets,
    while row <= m,
        if DataMatrix(row,col) == 1,
            timestart = row;
        end
        if DataMatrix(row,col-1) >= 0 && DataMatrix(row,col) < LowPeak,
            if row <= timestart+tens,
                LowPeaktime = DataMatrix(row,col-1);
                LowPeak = DataMatrix(row,col);
            end
        end
        row = row+1;
    end
    row = 1;
end
percentrelaxation(dataset,1) = (1-LowPeak)*100; %#ok<*AGROW>
OutputMatrix(1:tens+1,col-1:col) = DataMatrix(timestart:timestart+tens,col-1:col);
percentrelaxation(dataset,2) = LowPeaktime;
midpointrelaxation = OutputMatrix(5000,dataset*2);
percentrelaxation(dataset,3) = (1-midpointrelaxation)*100;
col = col+2;
dataset = dataset+1;
LowPeak = 600;
end
data_final = [];
dataset = 1;
col = 2;
while dataset <= datasets,
    data_final(:,col-1)=OutputMatrix(:,col);
    data_final(:,col)=OutputMatrix(:,col-1);
col = col+2;
dataset = dataset+1;
end
[r2,fit1,coef1] = QLVFit(data_final);
end
B.2 QLVFit.m

```matlab
function [R2, fit, coefEsts] = QLVFit(data)
    StartingVals = [1 1 1];
    coefEsts = nlinfit(data(:,2), data(:,1), @TheGFunction, StartingVals);
    xgrid = data(:,2);
    plot(xgrid, data(:,1), '.')
    plot(xgrid, TheGFunction(coefEsts, xgrid), 'r');
    hold on
    fit = TheGFunction(coefEsts, xgrid);
    R = corr2(fit, data(:,1));
    R2 = R^2
```

B.3 TheGFunction.m

```matlab
function G = TheGFunction(coefEst, xgrid)
    G = 0*xgrid;
    C = coefEst(1);
    tau1 = coefEst(2);
    tau2 = coefEst(3);
    dtau = (tau2 - tau1)/500;
    taus = linspace(tau1, tau2, 500)';
    for i = 1:length(xgrid)
        int1 = sum(exp(-xgrid(i)/taus)./taus)*dtau;
        int2 = sum(1./taus)*dtau;
        G(i) = (1 + C.*int1)/(1+ C.*int2);
    end
```

APPENDIX C – EXTRA METHODS

C.1 Sample Preparation - Isolating Valvular Fibroblast Cells

1.1.1. Sterilize all dissection materials, tools, and other instruments needed the day prior to isolation.

1.1.2. Prepare the necessary solutions.
1.1.2.1. Prepare 1 to 2.5 L of cold and sterile PBS containing 50 Units/mL Nystatin, 2.5 μg/mL Amphotericin B, 50 μg/mL Gentamycin, 100 Units/mL Penicillin G, and 100 μg/mL Streptomycin.

1.1.2.2. Prepare Porcine Valvular Interstitial Cell (VIC) Media consisting of DMEM, 10 % Fetal Bovine Serum (FBS), 50 Units/mL Nystatin, 2.5 μg/mL Amphotericin B, 50 μg/mL Gentamycin, 100 Units/mL Penicillin, and 100 μg/mL of Streptomycin.

1.1.2.3. Prepare 500 mL of KRB solution containing 3.45 g of NaCl, 0.145 g of MgSO4, 0.175 g of KCl, 1.05 g of NaHCO3, 0.08 g of KH2PO4, and 0.95 μL of Phenol Red in sterile deionized water. The final solution should have a pH of 7.4.

1.1.2.4. Prepare 250 mL of KRB II containing 5 g of Bovine Serum Albumin (BSA) Fraction V, 0.5 g of Glucose, 100 μg/mL Streptomycin, and 100 Units/mL Penicillin in the previously prepared KRB solution.

1.1.2.4.1. Gas the KRB for 30 minutes with 5 % CO2 and balanced air prior to adding the BSA and glucose if the pH is not at 7.4.

1.1.2.4.2. Sterile filter the entire solution and store at 4 degrees Celsius until use.

1.1.2.5. Prepare 100 mL of 600 Units/mL Collagenase II with 0.4 % HS in the previously prepared KRB II solution; do not prepare the Collagenase II until you are ready to use it.
1.1.3. Obtain 3 porcine hearts weighing between 275 and 400 g; transport the hearts in most of the previously prepared ice cold PBS containing the antibiotics. Keep the hearts and solution cold during transport.

1.1.4. Follow the dissection protocols listed in 3.3 to 3.6 to obtain the mitral and tricuspid valves; be especially careful to avoid obtaining parts of chordae tendinae, the septal portion of the valves, and any muscle tissue.

1.1.5. Place the valves into separate containers containing the rest of the previously prepared ice cold PBS with antibiotics during the completion of the dissection procedure.

1.1.6. When all valves have been isolated, pour off the cold PBS and transfer the valves to 2 sterile Erlenmeyer flasks with lids.

1.1.7. Add 10 mL of 600 Units/mL Collagenase warmed to 37°C to the valves.

1.1.8. Shake the valves and Collagenase for 10 minutes at 37°C.

1.1.9. Pour off the first digestion into a waste beaker.

1.1.9.1. This digestion contains mostly endothelial cells.

1.1.9.2. Use a sterile cannula to help remove the collagenase.

1.1.10. Add 10 mL of 600 Units/mL Collagenase warmed to 37°C to the valves.

1.1.11. Shake the valves and Collagenase for 15 minutes at 37°C.

1.1.12. Pour the Collagenase into a 50 mL conical tube and add equal volume of KRB II.

1.1.13. Repeat steps 4.10 to 4.12 for a total of 3 more times.
1.1.14. Combine all mitral valve digests and tricuspid valve digests together before centrifugation.

1.1.15. Centrifuge the remaining 50 mL conical tubes at 800 rpm for 8 minutes.

1.1.16. Slowly remove the supernatant, the cell pellet will loose and “fuzzy;” re-suspend the cells in a total of 5 mL of Porcine VIC Media.

1.1.17. Plate all of the mitral cells in one 100 mm cell culture dish; repeat for the tricuspid cells.

1.1.18. Change cell media daily; the cells take approximately 1-1/2 to 2 weeks to grow to confluence.

**C.2 Sample Preparation - Creating Collagen Gels**

1.1.1. Determine the number of collagen gels to be made; this will determine the volume of each solution to be prepared. The collagen gels use a 1:1:8 ratio of 0.2 N Hepes : 10X MEM : Bovine Collagen Type I; the gels here are 1 µL in volume and made in 96 well plates.

1.1.2. Prepare all necessary solutions.

1.1.2.1. Prepare the necessary amount of 10X MEM; this is to be kept ice cold.

1.1.2.2. Prepare the necessary amount of 0.2 N Hepes; this is to be kept ice cold.

1.1.2.3. Prepare the necessary amount of Bovine type I collagen; this is to be kept ice cold.
1.1.2.4. Prepare the necessary amount of a high and low concentration of PSS coated AuNRS in deionized water.

1.1.2.5. Prepare the necessary amount of a high and low concentration of PDADMAC coated AuNRs in deionized water.

1.1.2.6. Warm trypsin and either sterile HBSS or sterile PBS to 37 °C.

1.1.2.7. Warm the porcine VIC media used previously to 37 °C.

1.1.3. Prepare eppendorf tubes of 0.2N Hepes, AuNRs, and 10X MEM; keep these tubes on ice with the collagen.

1.1.3.1. Centrifuge the necessary amount of AuNRS and remove the supernatant.

1.1.3.2. Re-suspend the AuNRs in the 0.2 N Hepes; add the necessary amount of 10X MEM and set aside.

1.1.4. Prepare the porcine valvular interstitial cells.

1.1.4.1. Remove the cells from the incubator, and remove the cell media from the cells. Rinse the cells with sterile HBSS or PBS.

1.1.4.2. Add 2-3 mL trypsin to cells and return the cells to the incubator for 5 minutes; check the cells under a microscope to ensure the cells are completely off the plate.

1.1.4.3. Add 2-3 mL of media to the cells, and transfer the cells to a centrifuge tube.

1.1.4.4. Centrifuge the cells at rpm for 8 minutes.
1.1.4.5. Draw off the supernatant while being careful not to disturb the cell pellet; resuspend the cells in 3 mL of media.

1.1.4.6. Count the cells; the collagen gels use approximately 20,000 cells per collagen gel. The final cell count should allow for 20,000 cells to be suspended in 2-5 µL of media; anything larger than 5 µL will need to be removed from the amount of 10X MEM added.

1.1.4.7. The cells are now ready to be used.

1.1.5. Label the 96 well plate with the desired treatment conditions; add 20,000 cells suspended in media to each well.

1.1.6. Add the necessary amount of collagen type I to Hepes/AuNR/10X MEM mixtures in the eppendorf tubes.

1.1.7. Add 100 µL of the final collagen solution to the cells and mix with a pipette. Use a new pipette for every well to prevent contamination in the collagen solution.

1.1.8. Place 96 well plate(s) containing the collagen gels into an incubator set at 37°C and 5% CO² for 1 hour.

1.1.9. Add 100 µL of Porcine VIC media to each gel; the gels are now ready to use.
REFERENCES


