4-3-2014

Solvent Dependent Molecular Mechanics: A Case Study Using Type I Collagen

Heather Harper
University of South Florida, hmharper@mail.usf.edu

Follow this and additional works at: https://scholarcommons.usf.edu/etd

Part of the Biophysics Commons

Scholar Commons Citation

This Dissertation is brought to you for free and open access by the Graduate School at Scholar Commons. It has been accepted for inclusion in Graduate Theses and Dissertations by an authorized administrator of Scholar Commons. For more information, please contact scholarcommons@usf.edu.
Solvent Dependent Molecular Mechanics: A Case Study Using Type I Collagen

by

Heather McRae Harper

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy
Department of Physics
College of Arts and Sciences
University of South Florida

Major Professor: W. Garrett Matthews, Ph.D.
Myung Kim, Ph.D.
Martin Muschol, Ph.D.
Sagar Pandit, Ph.D.

Date of Approval:
April 3rd, 2014

Keywords: Atomic Force Microscopy, Ion Condensation, Tissue Engineering, Cornea

Copyright © 2014, Heather McRae Harper
DEDICATION

I would like to dedicate this dissertation to my grandfather, PawPaw. When I was young, he told me that getting an education and gaining knowledge was the most important thing I can do. He said that you can lose, or someone can take away, anything in your life: a job, a car, a house, even a family member. But, you can never lose your education.
ACKNOWLEDGMENTS

This research was supported in part by the National Science Foundation through CBET RAPD Award No. 0854023 and the East Asia and Pacific Summer Institute (EAPSI) Program. The Japan Society for the Promotion of Science (JSPS) provided funding for research in Japan as part of the JSPS Summer Program 2013.

I would like to thank Professor Toru Asahi, Professor Naoya Sawamura, Mr. Kenta Nakagawa, Waseda University, and TWIns for their support, resources, knowledge, and the opportunity to conduct research in Japan. I would also like to thank the following people for their assistance: Ms. Erin Brownell, Mr. Joey Foley, Mr. Udagawa, and my committee for all of their hard work and patience.
# TABLE OF CONTENTS

List of Tables iii

List of Figures iv

Abstract vi

References ix

Chapter One: Introduction 1

Type I Collagen 3

Collagen Molecules and Fibrils 3
Collagen Membranes 6

Atomic Force Microscopy 7

Polymer Mechanics 9

Optical Properties of Collagen Membranes 12

Birefringence 13

Dichroism 14

Polarized Light Microscopy and G-HAUP 16

References 19

Chapter Two: Persistence Length of Molecular Type I Collagen in DI Water, PBS, and a Fibril Forming Buffer 27

Abstract 27

Introduction 28

Theoretical Basis 29

Materials and Methods 31

Results 33

Discussion 38

Conclusions 43

References 43

Chapter Three: The Effects of Ionic Species and Concentration on the Conformations of Molecular Type I Collagen 49

Abstract 49

Introduction 50

Materials and Methods 51

Measurement Techniques and Data Analysis 52

Surface Equilibration Control Experiment 55
pH Measurement 57

Results 57
<table>
<thead>
<tr>
<th>Chapter 4: The Effects of Temperature on the Stability of Molecular Type I Collagen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
</tr>
<tr>
<td>General Procedure</td>
</tr>
<tr>
<td>Materials and Methods</td>
</tr>
<tr>
<td>Results</td>
</tr>
<tr>
<td>Discussion</td>
</tr>
<tr>
<td>References</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Chapter 5: Fabricating and Measuring the Non-Linear Optical Properties of Type I Collagen Membranes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
</tr>
<tr>
<td>Introduction</td>
</tr>
<tr>
<td>Materials and Methods</td>
</tr>
<tr>
<td>Results</td>
</tr>
<tr>
<td>Discussion</td>
</tr>
<tr>
<td>Conclusions</td>
</tr>
<tr>
<td>References</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Appendix A: Investigation of Viable Surfaces for Atomic Force Microscope Imaging of Molecular Collagen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glass and Silicon</td>
</tr>
<tr>
<td>Graphite</td>
</tr>
<tr>
<td>References</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Appendix B: Copyright Permissions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polarized Light Microscopy Images</td>
</tr>
<tr>
<td>Biopolymers</td>
</tr>
<tr>
<td>ChemComm</td>
</tr>
</tbody>
</table>
LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 2.1:</td>
<td>Results of WLC fitting to the type I collagen molecules deposited from suspension in nanopure water, Fibril Forming Buffer, and Phosphate Buffered Saline along with the number of molecules measured.</td>
<td>37</td>
</tr>
<tr>
<td>Table 3.1:</td>
<td>Fluorescence spectroscopy results showing approximate pH of varying collagen solutions as compared with control pH solutions.</td>
<td>58</td>
</tr>
<tr>
<td>Table 3.2:</td>
<td>Results for all salt dilutions.</td>
<td>59</td>
</tr>
<tr>
<td>Table 3.3:</td>
<td>The results of all Langmuir isotherm fits from Figure 3.3.</td>
<td>60</td>
</tr>
<tr>
<td>Table 5.1:</td>
<td>Fabrication conditions for the collagen membranes presented in this study.</td>
<td>80</td>
</tr>
<tr>
<td>Table 5.2:</td>
<td>Linear birefringence, LB measurements of common materials from the literature.</td>
<td>88</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

Figure 1.1: Type I collagen fibrils imaged in tapping mode with AFM. 4
Figure 1.2: Schematic representation of the hierarchy of collagen. 5
Figure 1.3: The flow casting method for creating collagen membranes. 7
Figure 1.4: Schematic representation of an AFM. 8
Figure 1.5: A representative image of how linearly polarized light is rotated in a circularly birefringent material. 13
Figure 1.6: A representative image of the change in the angle of polarization, of linearly polarized light, in a circularly birefringent material. 14
Figure 1.7: A representative image of how linearly polarized light is transformed into elliptically polarized light by a material exhibiting circular dichroic properties. 15
Figure 1.8: A representative image of how linearly polarized light is transformed into elliptically polarized light in a circularly dichroic material 16
Figure 1.9: A schematic representation of the G-HAUP system. 18
Figure 2.1: AFM images illustrating the deposition of molecular type I collagen on mica from a) Nanopure water, b) Fibril Forming Buffer, and c) Phosphate Buffered Saline. 33
Figure 2.2: Zoom in from a 1µm X 1µm AFM image of a collagen molecule illustrating the extraction of the end-to-end distance and contour length. 34
Figure 2.3: Plot of squared end-to-end distance versus contour length for collagen molecules deposited from Nanopure water. 35
Figure 2.4: Plot of squared end-to-end distance versus contour length for collagen molecules deposited from Fibril Forming Buffer. 36
Figure 2.5: Plot of squared end-to-end distance versus contour length for collagen molecules deposited from Phosphate Buffered Saline. 36
Figure 3.1: An illustrative example of the trace along a single collagen molecule.

Figure 3.2: Contour length and squared end-to-end distance data for the 0.01M KCl dilution.

Figure 3.3: Persistence length of type I molecular collagen deposited onto mica from a DI water dilution followed by incubation in 0.1M NaCl for varying times at room temperature.

Figure 3.4: Fluorescence spectroscopy curves for six collagen dilutions and four pH control samples.

Figure 3.5: Persistence length as a function of molarity for all salts tested.

Figure 3.6: Persistence length as a function of molarity for all monovalent salts tested.

Figure 3.7: Persistence length as a function of molarity for all divalent salts tested.

Figure 4.1: The average molecular density of type I collagen molecules as a function of extended heating.

Figure 5.1: A schematic illustration of the collagen flow-casting process.

Figure 5.2: Polarized light microscope images of collagen membranes.

Figure 5.3: A schematic representation of the G-HAUP apparatus.

Figure 5.4: Atomic force microscope, AFM, images of collagen membranes.

Figure 5.5: Wavelength dependences of LB (a), LD (b) and ORP (c) of the collagen membranes.

Figure A.1: An AFM image of molecular type I collagen deposited onto a premium glass coverslip.

Figure A.2: A large-scale AFM image of type I molecular collagen deposited onto cleaved graphite.

Figure A.3: An AFM image of molecular collagen deposited onto freshly cleaved graphite.
ABSTRACT

Being the most abundant protein in the body, by mass, type I collagen provides the building blocks for tissues such as bone, extra-cellular matrix, tendons, cornea, etc[1-3]. The ability of a single protein to create structures with such various mechanical properties is not fully understood. Before one can engineer and assemble a complex tissue, such as cornea, the mechanisms underlying the formation and assembly, mechanical properties, and structure must be investigated and quantified. The work presented herein contains an extensive study of Type I collagen from the molecular to the tissue level.

The engineering of collagenous tissues that mimic the mechanical and optical properties of native human cornea have been performed by a number of groups[4-7]. In all of these studies, the corneal-mimicking tissues have been created using a number of methods including repeated flow casting. To date, the ability to create self-assembled corneal tissue has not been achieved. Understanding the mechanisms of formation of native cornea will not only bring us closer to achieving self-assembled transplantable corneal tissue but will also aid in the engineering of all collagenous tissues and other structures comprised of filamentous units.

Recently, the study of type I collagen has primarily focused on the tissue, fiber, and fibril scale[2, 8-21]. Grant, et al.[20] measured the elastic modulus of collagen fibrils in various
solutions and found that by increasing ion concentration, in the solution around the fibril, the elastic modulus increased. The solution dependent behavior of the elastic modulus of collagen fibrils was measured but the cause of the dependence was unknown. Grant et al. state that due to the complex nature of the interactions between collagen fibrils and aqueous solutions, the exact cause of this effect is difficult to determine. Through work presented herein, not only do we show that this behavior is seen at the molecular level but also quantify the relationship between ionic concentration and molecular stiffness for a variety of ionic species.

Studies of collagen mechanics, on the molecular level, are brief[22-26]. The most prominent of these studies in recent years was performed by Sun, et al.[27] wherein a persistence length of 14.5nm, for human type I procollagen, was measured. The persistence length of the molecule, which is a measure of flexibility, is a highly debated topic with quoted values of 14.5nm[27], 57nm[28], 130nm[29], 175nm[30], 308nm[31], and 544nm[32]. The broad range of values indicates that the flexibility of the collagen molecule is a complex question.

It became apparent that the disagreement of the persistence length of molecular collagen in the literature may be due to the use of different ionic solutions. To address this, an initial atomic force microscope, AFM, study of the persistence length of molecular collagen diluted in DI water and two ionic solutions was conducted. This study showed that there is a strong solution dependence to the flexibility of the molecule. The ionic solutions presented molecules with a large persistence length, a straightened configuration, while the DI water dilution resulted in a persistence length that was a factor of 10 smaller.

Because two different complex ionic solutions in the initial study showed different persistence lengths, an evaluation of the effect of each individual salt was performed. To elucidate the effects of individual ionic species on the conformations and persistence length of
Type I collagen varying concentration of monovalent and divalent salts with different cations and anions were tested. It was found that increasing ionic concentration for all species types resulted in a higher persistence length but the rate of change in persistence length as a function of concentration is unique to each species.

In 2002 Leikina, et al.[33] suggested that Type I molecular collagen is unstable at body temperature using differential scanning calorimetry. To examine these results, an AFM study was performed that imaged the collagen molecules after being held at body temperature for varying times. The density of molecules deposited onto mica, above a 200nm length cutoff, was calculated and it shows that the number of molecules above 200nm in length decreases with increasing incubation time.

These environmental studies were performed with an aim to understanding the role of environment in creating a corneal mimicking tissue. Currently, the most promising method of collagen membrane fabrication for corneal replacement was developed by Tanaka, et al.[4]. This unique repeated flow casting method allows for the manufacturing of transparent collagen membranes with controllable thickness and fibrillar alignment. Using the repeated flow casting technique, orthogonally oriented collagen membranes were created and their optical properties were measured using the Generalized High Accuracy Universal Polarimeter, G-HAUP. When engineering a tissue for the eye, the optical properties of the tissue are of the utmost importance. Appropriately for corneal tissues, the measurements for linear birefringence and linear dichroism were negligible.

It was clear, from the literature, that a fundamental understanding of molecular type I collagen was not available. In this work, the mechanical properties and environmentally sensitive behavior of bovine dermal type I molecular collagen is studied. The exploration into
the unique behavior of these systems begins with documenting the rich ionic species and concentration dependent flexibility of molecular type I collagen and the temperature dependence on the stability of the molecule is tested. The study concludes with the construction of corneal mimicking tissues using the repeated flow casting method and measuring the complex optical properties of these tissues.

References


CHAPTER ONE:
INTRODUCTION

Any investigation into the self-assembly process of collagen-based tissues starts by looking at the fundamental properties of the smallest unit, the collagen molecule. The basic physical properties of the molecule, such as the length, diameter, sequence of amino acids, etc., are well-known[1]. The amount of literature focusing on the study of collagen is massive, but has been primarily focused on the fibril, fiber, and tissue scales. Knowledge of the mechanical properties of collagen on the molecular scale is harder to find and presents a wide discrepancy in the measured flexibility, or persistence length, of the molecule. Published values for the persistence length of an ~300nm long type I collagen molecule range from ~15nm[2] to ~544nm[3]. Our analysis of the collagen molecule and its properties begins with an investigation and explanation of this discrepancy.

A search of existing literature suggested an apparent explanation for the disparate results of the persistence length of collagen, which is the lack of a control. All of the studies we found were performed using type I collagen suspended in different ionic solutions during measurement. The body of work herein begins with an investigation into the persistence length of type I collagen molecules in phosphate buffered saline (PBS), a fibril forming buffer (FFB), and a DI
water control. This initial study shows that the molecules present relatively straight conformations in the complex ionic solutions present in PBS and FFB. Conversely, in DI water, the molecules are highly flexible and have a low persistence length, on the order of that measured by Sun et al.\[2\]. Though both the PBS and FFB solutions present molecules with high persistence length, they were not equal. This indicates that the roles of individual ionic species are important and should be investigated further.

To examine the effects of individual ionic species on the persistence length of collagen, we exposed the molecules to varying concentrations of NaCl, KCl, KBr, CaCl\(_2\), and MgCl\(_2\). Investigating each of these salts individually allowed the effects of monovalent salts, divalent salts, changing cations, and changing anions to be evaluated. It was found that in all cases tested, an increase in ionic concentration corresponded to an increase in persistence length. Interestingly, these rates of increase are species dependent. Combined with other studies, see Chapter 3, these results show that preferential ion binding causes a direct change in the conformations, and thus measured persistence length, of the type I collagen molecule.

Quantifying the effects of ions on the physical properties of the molecule showed that environment plays an important role in the molecules’ behavior. Various groups have studied the effects of pH on collagen, during fibril formation[4-7]. But, a new aspect to the role of environment on molecular collagen was published by Leikina et al. which suggested that molecular collagen is unstable at body temperature[8]. The Leikina study used ultra-slow scanning calorimetry and circular dichroism to track the unfolding of the protein, but the individual molecules were not directly imaged. Using a bioheater stage for an AFM, we were able to corroborate those results using bovine dermal type I collagen. We found that the number of intact collagen molecules decreases with time when held at 37°C.
All of these studies contained herein, on the physical properties of molecular collagen, were performed from an initial desire to understand the mechanisms of collagen self-assembly and the creation of collagen-based corneal tissue. The final step in this journey was to then create artificial corneal mimicking membranes. Using the repeated flow-casting method developed by Duncan and Tanaka[9, 10], collagen membranes, with an orthogonal fibril alignment between layers, were manufactured. The optical properties of the membranes were then measured with the Generalized-High Accuracy Universal Polarimeter (G-HAUP) to examine the membranes’ non-linear optical properties.

Studying the mechanical properties and tissue engineering of collagen requires a basic understanding of type I collagen, the equipment used in the studies, and the mathematical models used to describe the results. We begin with a general outline of type I collagen on the molecular, fibril, and membrane scales followed by an overview of atomic force microscopy, polymer mechanics, and the optical properties of collagen membranes.

Type I Collagen

COLLAGEN MOLECULES AND FIBRILS

Molecular type I collagen, or tropocollagen, is composed of three polypeptide chains that intertwine to form a right-handed triple-helical structure measuring ~1.5Å in diameter and
~300nm in length[1]. To make larger structures, molecular collagen aligns in a quarter-staggered pattern to form collagen fibrils[11]. Due to the nature of the packing, type I collagen fibrils present a banding pattern, called the D-band, measuring ~67nm (see Figure 1.1). Higher order structures are formed by fibrils fusing together to form fibers, sheets, and 3-dimensional mesh structures as in Figure 1.2.

![Image of Type I collagen fibrils imaged in tapping mode with AFM.][12]

**Figure 1.1** Type I collagen fibrils imaged in tapping mode with AFM. [12]

Of the three polypeptide chains in the collagen molecule, there are two α1(I) chains and one α2(I) chain. In both types of polypeptide chains, the sequences contain a glycine, Gly, at
every third residue[13]. The small physical dimensions of the Gly residues allow for the close intertwining of the chains when forming the triple helix.

All of the α-chains in fibril forming collagens can be expressed as sequences of amino acid triplets of the form of Gly-X-Y[13]. In both the α1(I) and α2(I) chains, the X position is dominated by proline, Pro, and the Y position is dominated by hydroxyproline, Hyp[14]. Even though Pro and Hyp are the dominant residues, the Gly-Pro-Hyp tripeptide only comprises ~44% of the molecule[14, 15]. There are over 15 other residues known to occupy X and Y positions, most notably: alanine, arginine, and glutamine.

Figure 1.2 Schematic representation of the hierarchy of collagen.
COLLAGEN MEMBRANES

One of the unique aspects of the corneal stroma is the orientation and lamellar structure of the collagen fibrils. The cornea is comprised of layers of collagen fibrils of varying orientations that run in the plane of the lamellae[16-18]. Near the center of the cornea, the fibrillar orientations between lamellae are orthogonal.

There are many research groups who have developed techniques for engineering collagen membranes to mimic the mechanics and optics of human cornea[19-23]. Most of these methods are difficult, expensive, require specialized equipment, or are highly time intensive. Importantly, any successful method for creating artificial corneal stroma tissue must be able to control the direction of fibrils from layer to layer.

One of the most novel ways for creating collagen membranes for corneal tissue replacement was developed by Tanaka, et al.[9, 10]. The membranes are manufactured by flowing a collagen solution over a substrate, incubating to form fibrils, drying at low temperature, and then flowing the next layer (see Figure 1.3). This technique allows for precise fibrillar orientation control in each layer as well as easy control of layer and membrane thickness.

After the lamination process is completed, the membranes can be chemically cross-linked. The chemical cross-linking process increases the transparency of the membranes. Chemical cross-linking is achieved by soaking the laminated membrane in a solution of 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide, EDC, and N-Hydroxysuccinimide, NHS. The membranes are then cleaned with DI water and dried at low temperature.
**Figure 1.3** The flow casting method for creating collagen membranes[24]. See Chapter Five for specific processing details.

**Atomic Force Microscopy**

In 1986 Binnig, Quate, and Gerber[25] published the invention of the atomic force microscope, AFM, to address the inability of an STM to achieve an atomic resolution image of an insulating surface. Since then, the AFM has seen innumerable advances in its technology and abilities. Not only can an AFM achieve atomic resolution images but it can also be used to manipulate the surface[26-28], stretch molecules[29-33], and perform indentation measurements[34-36].

The basic concept of the AFM is quite simple. A thin cantilever with a sharp tip is used to probe the surface (see Figure 1.4). As the tip comes in contact with the surface, the cantilever
bends which causes the angle of the reflected laser beam to change. This change in the laser spot is recorded by the photo-detector, which, through a feedback loop, causes the cantilever to retreat from the surface.

There are two basic imaging modes of AFM: contact mode and tapping mode. In contact mode, the AFM tip is kept in contact with the imaging surface and through the feedback loop applies a constant force as it drags the tip across the surface. Tapping mode, which is used in this research, obtains the topographical information by probing the surface point-by-point at intervals determined by the user. Tapping mode imaging was chosen for this work to minimize any possible deformation of the molecules by the tip. The actual movement of the AFM

**Figure 1.4** Schematic representation of an AFM[37].
cantilever and sample stage is controlled by manipulating piezoelectric material on all three axes of movement[25].

**Polymer Mechanics**

The study of polymer and chain statistics started heavily developing in the 1950’s and 1960’s with large and significant contributions from Flory[38-41]. Through this time, many models were created, tested, and modified. The outcomes of this research presented with two of the most significant polymer models: the Freely Jointed Chain Model, FJC, and the Worm-like Chain Model, WLC.

The Ideal Chain or Freely Jointed Chain Model, FJC Model, describes the mechanics of a polymer chain with \( N \) rigid segments that are allowed to freely rotate around their connecting joints[42]. The segments are considered to be orientationally independent and non-interacting. It has been shown that the FJC Model fails to describe the elasticity of DNA at all except extremely low extension forces[43].

When a polymer, or molecule, is imaged with AFM, the conformation of the molecule on the surface is directly related to the flexibility of the molecule. By measuring the length of the molecule and the distance from one end point to the other, one can calculate a persistence length using the Worm-like Chain, WLC, model. The WLC model has become the standard model used to quantify the mechanics of collagen[2, 44-46], DNA[47-49], and other proteins[50, 51].
The FJC model assumes that the molecule, or polymer, is made up of rigid rods that are free to rotate around their connecting joints. One of the biggest drawbacks of the FJC model is that it does not allow for elasticity of the molecule. The WLC model treats the molecule as a relatively rigid homogeneous elastic chain[47, 48].

The persistence length of a molecule, as calculated by the WLC model, is dependent on whether the molecule being measured is equilibrated in 2 or 3 dimensions. For molecules that are 3 dimensionally equilibrated, the WLC model states that the average ‘end-to-end’ distance, \( R \), can be expressed as a function of the persistence length, \( P \), and the contour length of the molecule, \( L \), as follows.

\[
\langle R^2 \rangle_{3D} = 2PL \left( 1 - \frac{P}{L} \left( 1 - e^{-\frac{L}{P}} \right) \right)
\]

The persistence length calculated by the WLC can be directly related to the Young’s Modulus of the molecule by

\[
YI = k_B TP
\]

where \( Y \) is the Young’s modulus, \( k_B \) is Boltzmann’s Constant, \( T \) is the temperature, and \( I \) is the moment area of inertia. Using these relationships, one can easily convert the measurable conformational values of \( R \) and \( L \) to the Young’s modulus.

To image the molecules with AFM, they are deposited from solution to a flat mica substrate. This forces the molecules from a 3 to a 2-dimensional environment. During this
process, there are two situations that can occur: the molecules can equilibrate in two dimensions on the surface or they remain equilibrated in three dimensions and become ‘kinetically trapped’.

In the case of two dimensional equilibration, the WLC model is expressed as follows[47].

\[
\langle R^2 \rangle_{2D} = 4PL \left(1 - \frac{2P}{L} \left(1 - e^{-\frac{L}{2P}}\right)\right)
\]

Kinetic trapping occurs when a molecule does not equilibrate on the two dimensional surface. When imaging such a molecule with AFM, the resulting picture is a two dimensional projection of the object. The projected WLC model equation is below.

\[
\langle R^2 \rangle_{proj} = \frac{2}{3} \langle R^2 \rangle_{3D} = \frac{4}{3}PL \left(1 - \frac{P}{L} \left(1 - e^{-\frac{L}{P}}\right)\right)
\]

If the persistence length of the molecule is known \textit{a priori}, these equations can be used to determine the conditions of kinetic trapping. If the presence or absence of 2D equilibration is known \textit{a priori} then one can easily determine the persistence length. In the case of molecular collagen, there is no consensus on the persistence length of the molecule and studies of molecular equilibration have not been performed. It is because of this that all of the data presented herein is fit to both the 2D and projected forms of the WLC model.

It will be shown, that for collagen molecules presenting more straightened conformations, the values calculated for the persistence length show highly unphysical results when using the projected form of the model. This indicates that the molecules are equilibrating on the surface.
In the case of highly flexible molecules, the persistence length is quite small and as $P \to 0$ the 2D and projected models display similar results.

**Optical Properties of Collagen Membranes**

When engineering and manufacturing a membrane intended to be used as a corneal stroma replacement, the optical properties of the membrane are of extreme importance. These optical properties need to mimic that of native cornea or the membrane will not be viable for transplant. Not only is the transparency of the membrane important, due to the nature of the corneal stroma being comprised of helical proteins, the non-linear optical properties must also be measure. The non-linear optical properties of collagen and other proteins is well studied[8, 52-58].

The Generalized-High Accuracy Universal Polarimeter, or G-HAUP, is a polarized light microscopy device that simultaneously measures linear birefringence (LB), linear dichroism (LD), circular birefringence (CB), and circular dichroism (CD). HAUP was invented by Kobayashi and later expanded to the current G-HAUP system by Asahi[59-61]. Since then, it has been used to measure the optical properties of many materials[62-67].

In this study, the G-HAUP apparatus is used to measure the non-linear optical properties of manufactured collagen membranes and calculate the LB, LD, and optical rotary power, ORP. Optical rotary power is the ability of a material to rotate a plane of polarized light.
In a birefringent medium, the index of refraction of the medium is dependent on the direction of polarization[68]. When linearly polarized light passes through a material with birefringent properties, the light gets split into two mutually perpendicular rays called the ordinary and the extraordinary rays. These rays travel through the medium at different angles causing double refraction.

![Figure 1.5](image)

**Figure 1.5** A representative image of how linearly polarized light is rotated in a circularly birefringent material. Linearly polarized light (blue) can be deconstructed into right (green) and left (red) handed circularly polarized rays. In a circularly birefringent material, the speed of light through the material is dependent upon the polarization, causing the right and left circularly polarized rays to travel at different speeds. This causes the plane of polarization of the resulting light to be shifted. Image reproduced with permission, see Appendix B[69].

For a circularly birefringent material, incoming polarized light can be thought of as a superposition of right-handed and left-handed polarized light rays[69]. These two circularly
polarized rays will experience different indices of refraction. Upon exiting the material, the two circularly polarized rays ‘recombine’, but are now, in essence, out of phase. This results in a linearly polarized product whose angle has shifted. Simply, a circularly birefringent medium will rotate the plane of polarization of linearly polarized light.

**Figure 1.6** A representative image of the change in the angle of polarization, of linearly polarized light, in a circularly birefringent material. These are the two square areas represented in Figure 1.5. The image on the left shows the linearly polarized light (blue) as it enters the material. The image on the right shows the resulting linearly polarized light has been rotated. Image reproduced with permission, see Appendix B[69].

**DICHROISM**

Dichroism is similar to birefringence in that it is dependent upon the angle of the polarization of the light entering the material. Instead of the light experiencing varying speed as
a function of polarization angle, it experiences varying absorbance. Light rays polarized in one direction will experience a different absorbance than light polarized in a different direction in a dichroic medium[68]. The amplitude of the polarized light ray is dependent on the dichroic properties of the material.

In the case of circular dichroism, linearly polarized light can be thought of as a superposition of left and right-handed polarized rays of equal amplitude. After travelling through the medium, the amplitudes of each circularly polarized ray are modified by the material’s circular dichroic properties. When recombined, due to the change in amplitude, the resulting ray is no longer linearly polarized. In the case of circular dichroism, a material will transform linearly polarized light into elliptically polarized light[69].

**Figure 1.7** A representative image of how linearly polarized light is transformed into elliptically polarized light by a material exhibiting circular dichroic properties. Linearly polarized light (blue) can be deconstructed into right (green) and left (red) handed circularly polarized rays. In a circularly dichroic material, the absorbance of light in the material is dependent upon the polarization. This causes the resulting amplitudes of the circularly polarized light to change. Because of the change in amplitude of the circular components, the resulting light is now elliptically polarized. Image reproduced with permission, see Appendix B[69]
A typical polarized light microscope consists of a polarizer, sample stage, and analyzer. Incident light travels through the polarizer which blocks all light rays except those that are polarized in one specific direction. The polarized light then travels through the sample. After traveling through the sample, the light passes through the analyzer, which works like the polarizer by only letting through light rays polarized in a specific direction.

The polarizer and analyzer are set at crossed-Nicols’ position. Crossed-Nicols’ position is when the angle of the polarized light, allowed through the polarizer, is set at a 90° difference from the angle of polarization allowed through the analyzer. At this configuration, all of the
light is blocked by the analyzer, and no light gets through the system. When a sample with birefringent properties is placed between the polarizer and analyzer, it will rotate the plane of polarization allowing a portion of the light to pass through the analyzer. By examining the intensity of the light and the angles between the sample, polarizer, and analyzer, one can quantify the optical properties of the sample.

A traditional polarized light microscope was used to image and locate the optic axis of the collagen membranes. The optic axis is the axis at which light passing through the sample experiences little to no retardation. In a perfect system, this would mean that no light passes through the analyzer at crossed Nicol’s position. When the optic axis of the membranes is located, the sample is placed in the G-HAUP machine so that the scanning will occur centered on this axis.

G-HAUP uses a sophisticated method of analyzing polarized light passing through a sample. Both the polarizer, P, and analyzer, A, are rotated simultaneously and the analyzer is rotated independently through a chosen angle about the optic axis of the sample. The intensity of the light passing through the analyzer is recorded as a function of both the angle of the polarizer from the extinction position, $\theta'$, and the angle of the analyzer with respect to the polarizer, $Y'$, for each wavelength of light in the scan range (see Figure 1.9).

From these values, and the thickness of the sample, $d$, one can calculate the LB, LD, ORP, and CD of the sample as,

**Equation 1.5** \[ LB = \Delta \cdot \lambda 2\pi d \]

**Equation 1.6** \[ LD = E \cdot \lambda 2\pi d \]
Equation 1.7 \[ ORP = 2\pi LB \cdot k \lambda \]  

Equation 1.8 \[ CD = \Delta \cdot k' \cdot \lambda \pi d \]  

where \( \Delta \) is the measured retardation, \( E \) is the absorption, \( \lambda \) is the wavelength, and \( k \) and \( k' \) are not directly measured, but they are calculated by eliminating the systematic error parameters.

**Figure 1.9** A schematic representation of the G-HAUP system[70]. Light enters the system through the polarizer, passes through the sample, and finally passes through the analyzer. In the G-HAUP system, the angles of rotation of the polarizer and analyzer are controlled.
References


12. Image courtesy of Erin Brownell


37. Image courtesy of Garrett Matthes, PhD.


69. Szilagyi, A. [cited 2014; Available from: http://www.enzim.hu/~szia/cddemo/edemo0.htm.]

70. Image courtesy of T. Asahi.
CHAPTER TWO:

PERSISTENCE LENGTH OF MOLECULAR TYPE I COLLAGEN IN DI WATER, PBS,
AND A FIBRIL FORMING BUFFER

Abstract

Type I collagen is a fibril-forming protein largely responsible for the mechanical stability of body tissues. The tissue level properties of collagen have been studied for decades, and an increasing number of studies have been performed at the fibril scale. However, the mechanical properties of collagen at the molecular scale are not well established. In the study presented herein, the persistence length of pepsin digested bovine type I collagen is extracted from the conformations assumed when deposited from solution onto two-dimensional surfaces. This persistence length is a measure of the flexibility of the molecule. Comparison of the results for molecules deposited from a fibril forming buffer (FFB), phosphate buffered saline (PBS), and a DI water control allow for the study of the effect of the solutions on the conformations of the molecule and provides insight into the molecule’s behavior in situ.

∗ This study has been accepted for publication in Biopolymers, 2014. It is reproduced here with permission, see Appendix B.
Introduction

Collagen, the most abundant protein in the body by mass, forms hierarchical structures that provide for the mechanical stability of tissues and organs[3]. On the smallest scale, molecular type I collagen self-assembles into a quarter-staggered pattern to form collagen fibrils[4]. These fibrils then arrange into larger fibers leading eventually to three-dimensional scaffolding structures such as tendons, ligament, and cornea[3, 5, 6]. Collagen has been studied, both experimentally and theoretically, at the tissue, fiber and fibril levels for many years[6-18], indicating the importance of understanding the mechanics of these materials. Notably lacking are investigations of the mechanics of single molecules. Modifications to the mechanics at this scale would ripple through the higher ordered structures, affecting both the mechanical properties and assembly of these structures[19].

Only a few mechanical studies have been directly performed on single type I collagen molecules. The most prominent of these investigations was performed by Sun et al[17]. In this work, optical trapping was used to extend single human type I procollagen molecules between dielectric spheres. The results of these experiments demonstrated that, by fitting to the worm-like-chain (WLC) model, the type I collagen monomer has a persistence length of 14.5nm. This result indicates a high degree of flexibility. Subsequently, Bozec et al.[20] used an atomic force microscope (AFM) to image type I collagen molecules after deposition onto a substrate, followed by forcibly extending the molecules. They also fit their stretching data to the WLC model, though no persistence length was extracted. In addition to these recent direct measurements, many researchers have reported persistence lengths or elastic moduli for collagens of varying sources. Their results span a broad range including 57nm[21], 130nm[22], 175nm[23],
308nm[24], and 544nm[25]. This range of over an order of magnitude highlights the need for further study – these persistence lengths predict that the ~300nm long molecule behaves as anything between a flexible random coil to essentially a rigid rod.

To further our understanding of the mechanics of type I collagen, herein we present an independent study of the flexibility of molecular collagen. We quantify this flexibility by extracting the persistence length through the analysis of molecular conformations on two-dimensional surfaces as characterized by AFM. To investigate the sensitivity of molecular collagen to solvent conditions, the persistence length was measured after suspension in two different buffer solutions plus a nanopure water (18MΩ) control. This approach allowed us to directly examine the effect of salts on the conformation of the molecule.

**Theoretical Basis**

The persistence length of a molecule is defined as the length “over which the orientations of the bonds become uncorrelated”[26]. The persistence length, \( P \), can be directly related to the Young’s Modulus, \( Y \), of the molecule by[27]:

\[
\text{Equation 2.1} \quad YI = k_BT P
\]

where \( I \) is the area moment of inertia – dependant on the fourth power of the molecular radius when using a cylindrical model, \( T \) the temperature, and \( k_B \) is the Boltzmann constant. It is clear
from this relation that, at constant temperature and molecular dimensions, a lower persistence length indicates a more flexible molecule.

Images of type I collagen molecules adsorbed from solution onto mica were used to extract a measurement of the persistence length of the molecule. Assuming that the molecules equilibrate on the surface, the conformation of the molecules can be described through the two-dimensional equilibrated worm-like-chain (2D-WLC) model[28]:

\[
\langle R^2 \rangle_{2D} = 4PL \left[ 1 - \frac{2P}{L} \left( 1 - e^{-\frac{L}{2P}} \right) \right]
\]

In this equation, \( L \) is the contour length of the molecule and \( R \) is the end-to-end distance.

Alternatively, if the molecule does not equilibrate on the surface, after adsorption it assumes a kinetically trapped conformation that results from the transition from three to two dimensions. In this case, the mean square end-to-end distance will be two-thirds of the equilibrated mean square end-to-end distance for the three-dimensional worm-like-chain (3D-WLC) model[28]:

\[
\langle R^2 \rangle_{proj} = \frac{4}{3} PL \left[ 1 - \frac{P}{L} \left( 1 - e^{-\frac{L}{P}} \right) \right]
\]

From AFM images, the contour length and the end-to-end distance can be measured directly for individual molecules. Thus, fitting the above equations to plots of \( R^2 \) versus \( L \) requires only one fitting parameter, and the persistence length may be determined.
In the past, this type of experiment has been performed using a known persistence length value[28], thus the WLC model equations were used to determine whether the molecule equilibrates on the surface. In this study we do not know \textit{a priori} either the persistence length or the equilibration state. Consequently, we tested both the 2D equilibrated model and the projected 3D-equilibrated model against the data. Results that were physically improbable were rejected on this basis, and we report the remaining measured persistence lengths.

\textbf{Materials and Methods}

A stock solution of 0.51 wt.% bovine type I collagen in 0.01M HCl was obtained from MiMedx, Inc. (Tampa, FL). This collagen stock was extracted from bovine dermis using a pepsin digestion step in the purification process. This stock was then diluted to make three collagen suspensions: (1) a 1:1000 dilution of collagen stock solution in nanopure water, (2) a 1:8000 dilution of the collagen stock solution in fibril forming buffer (FFB; 105mM NaCl and 44mM NaH$_2$PO$_4$ in water), and (3) a 1:1000 dilution of the collagen stock solution in phosphate buffered saline (PBS, Fisher Scientific, 137mM NaCl, 10mM Na$_2$HPO$_4$, 2mM KH$_2$PO$_4$, and 2.7mM KCl in water). FFB was selected for comparison with fibrillogenesis experiments, and PBS was selected because it is a common physiological buffer. The high dilutions insured non-interacting molecules in solution. Since single molecules were desired for the present study, inhibition of fibril formation was achieved through the greater dilution in FFB.
The samples to be used for imaging were prepared by depositing 50µL of the collagen/nanopure water dilution, the collagen/FFB dilution, or the collagen/PBS dilution onto freshly cleaved mica surfaces for varying times. The deposition times for each solution varied, having been determined by the need for well-dispersed molecules on the surface to facilitate analysis of the conformation and minimization of molecule-molecule interactions. The deposition time for the collagen/water sample was 20s, for collagen/FFB 5s, and for collagen/PBS 20s. After the incubation period, the samples were briefly rinsed with nanopure water to remove excess salt depositions and gently dried under a nitrogen stream. The samples then were imaged in intermittent contact mode using an Asylum Research MFP-3D AFM (Asylum Research, Santa Barbara, CA). The silicon cantilevers used had either nominal 5.0N/m spring constant and 160KHz resonant frequency or 40N/m spring constant and 170KHz resonant frequency (μMasch USA, San Jose, CA). The contour length and end-to-end distance of each molecule was measured using Igor Pro software (WaveMetrics Inc., Lake Oswego, OR, USA).

The data were taken from 1µm x 1µm height trace images collected with a 512 x 512 pixel resolution for each image. With these imaging parameters, height information was taken approximately every 2nm x 2nm. Typical images used for analysis are shown in Figure 2.1. Only molecules that were completely imaged in the scan area were included in the data sets. Example data extraction is shown in Figure 2.2.

The contour length and end-to-end distance data collected from the images were plotted and fit to both the 2D and projected 3D WLC model equations as shown in Figure 2.3 using Igor Pro software. A value for the persistence length was obtained for type I collagen molecules in each of the three solutions as deposited on mica. All subsequent statistical analyses also were performed using Igor Pro.
Figure 2.1 AFM images illustrating the deposition of molecular type I collagen on mica from a) Nanopure water, b) Fibril Forming Buffer, and c) Phosphate Buffered Saline. Notice the more extended structures observed when deposited from the salt containing solutions (b and c).

Results

It was apparent by inspection of the AFM images that a significant change in the conformation of the molecules occurred, depending upon which solvent was used for the dilution (see Figure 1). In all images collected, the collagen molecules were observed to adopt a more condensed structure when suspended in water and a straightened conformation when suspended in either FFB or PBS.

From the AFM images, contour lengths and end-to-end distances were measured for each molecule falling entirely within the scan range. A representative example of this data collection is shown in Figure 2.2. Figures 2.3-2.5 show both the raw and binned data plotted as squared end-to-end distance versus persistence length, allowing a least-squares fitting of Equations 2.2 and 2.3 to be done. Figures 2.3-2.5 also show the resulting fitted curves. Given a persistence length and contour length, the WLC model equations present an average squared end-to-end
distance within these parameters. To this end, the WLC model equations were fit to both the raw
and binned data (see Table 2.1).

Figure 2.2 Zoom in from a 1µm X 1µm AFM image of a collagen molecule illustrating the
extraction of the end-to-end distance and contour length. The end-to-end distance is measured
directly from a line section (shown in blue) of the imaged molecule at the points indicated by the
white arrows. The molecule then is traced as shown by the red line drawn along the molecule.
Inset: The height versus distance along the molecule is plotted. The contour length is the
distance between the white arrows as extracted from these plots.
The bins were chosen to have a width of 25% of the standard deviation of the contour length. Within each of these bins, the average squared end-to-end distance was calculated. The only fitting parameter was the persistence length $P$, and the value returned for this parameter in each solvent for each model is summarized in Table 2.1. The resulting persistence length values from the raw and binned data agree for all solutions tested.

![Plot](image)

**Figure 2.3** Plot of squared end-to-end distance versus contour length for collagen molecules deposited from Nanopure water. Both the raw and binned data are shown. The solid line is a least squares curve fit to equation 2.

For type I collagen molecules suspended in nanopure water, we cannot say with certainty whether the molecules were equilibrated on the surface or adsorbed without surface rearrangement. Thus we report that the persistence length was between ~12 and 40nm as determined from the 2D-WLC model and the 3D-WLC projected model, respectively.
Figure 2.4 Plot of squared end-to-end distance versus contour length for collagen molecules deposited from Fibril Forming Buffer. Both the raw and binned data are shown. The solid line is a least squares curve fit to equation 2.

Figure 2.5 Plot of squared end-to-end distance versus contour length for collagen molecules deposited from Phosphate Buffered Saline. Both the raw and binned data are shown. The solid line is a least squares curve fit to equation 2.
By comparison with the data for collagen suspended in the two buffered salt solutions (see Table 2.1), again it was clear that the type I collagen molecule was substantially more flexible in the absence of the salts.

**Table 2.1** Results of WLC fitting to the type I collagen molecules deposited from suspension in nanopure water, Fibril Forming Buffer, and Phosphate Buffered Saline along with the number of molecules measured. Persistence length values are given for both the raw and binned data. Values are given as mean ± standard deviation.

<table>
<thead>
<tr>
<th>Dilution</th>
<th>Style</th>
<th>Average Contour Length(nm)</th>
<th>P(nm) 2D-WLC</th>
<th>P(nm) Proj. 3D-WLC</th>
<th>Number of Molecules</th>
</tr>
</thead>
<tbody>
<tr>
<td>DI water</td>
<td>Raw</td>
<td>257.8 ± 13.4</td>
<td>11.785 ± 1.0</td>
<td>37.6 ± 3.47</td>
<td>145</td>
</tr>
<tr>
<td></td>
<td>Binned</td>
<td></td>
<td>12.474 ± 3.0</td>
<td>40.1 ± 10.5</td>
<td></td>
</tr>
<tr>
<td>FFB</td>
<td>Raw</td>
<td>255.6 ± 14.1</td>
<td>135.1 ± 11.9</td>
<td>18038 ± 1870</td>
<td>104</td>
</tr>
<tr>
<td></td>
<td>Binned</td>
<td></td>
<td>134.99 ± 14.4</td>
<td>24482 ± 1330</td>
<td></td>
</tr>
<tr>
<td>PBS</td>
<td>Raw</td>
<td>260.0 ± 20.7</td>
<td>161.87 ± 16.3</td>
<td>32844 ± 599</td>
<td>101</td>
</tr>
<tr>
<td></td>
<td>Binned</td>
<td></td>
<td>164.65 ± 20.8</td>
<td>37966 ± 776</td>
<td></td>
</tr>
</tbody>
</table>

The measured persistence length for type I collagen on freshly cleaved mica was ~135nm for the suspension in FFB and ~160nm for the suspension in PBS. These values were determined from the analysis using the 2D-WLC model. The values predicted by the projected 3D-WLC model (see Table 2.1) were rejected on physical grounds by considering the elastic modulus implied by the result when applied using Equation 2.1. For example, a persistence length of ~18µm (midway between the lowest and highest projected 3D-WLC persistence length values) would imply an elastic modulus of 19GPa for a cylindrical rod with a 1.5nm diameter, appropriate for collagen. For comparison, recent reports for the elastic modulus of bovine
femoral trabecular bone, obtained through nano-indentation experiments, under no macroscopic compressive strain, is \( \sim 20 \text{GPa}[29] \). The mineralized bone would be expected to be significantly more rigid than the unmineralized collagen molecules.

**Discussion**

We imaged molecular type I collagen, as deposited onto freshly cleaved mica, from nanopure water and two different buffered salt solutions. We then used these images to extract persistence lengths for collagen under these conditions. That the molecules assume a more rigid structure in the presence of salt was clear from both qualitative inspection of the images (Figure 2.1) and quantitative analysis of the conformations (Table 2.1).

The measured persistence length of type I collagen in water was between \( \sim 12 \text{nm} \) and 40nm, and the contour length was \( \sim 258 \text{nm} \). We report the persistence length as a range because the manner in which the molecules adsorb cannot be determined unambiguously, unlike the cases in which buffered salts were used (see Results). We also note that the measured persistence length of collagen for the nanopure water suspension, arguably, was slightly higher than the true value. A small systematic error was introduced during the analysis of the experimental data. Molecules can become so tightly wound that it became unclear as to the exact shape of the molecule, and thus such molecules were not included in the data set if the exact trace was uncertain. This eliminated the possibility of measuring the most tightly wound specimens, leading to a slightly elevated estimate for the persistence length. This phenomenon occurred...
rarely enough (<5% of the molecules observed) that the effect on the final value for the persistence length should be rather small.

The persistence length values for type I collagen diluted in FFB was ~135nm, and the contour length was ~256nm. When diluted in PBS, the persistence length was ~165nm, and the contour length was ~260nm. The values resulting from the projected 3D WLC model were rejected in both cases for reasons described above in the Results section. The persistence lengths for molecules suspended in these two solutions are quite similar, with both solutions leading to a straightened conformation with a large persistence length. Likewise, the contour lengths for molecules in all three solvents are similar – the largest difference is between collagen suspended in PBS and collagen in FFB, and this difference was 1.7%. However, the persistence length of type I collagen suspended in pure water was significantly different from that measured in either of the buffered salt solutions. Indeed, the persistence length increases by over an order of magnitude in the salt containing solutions.

The mechanism behind the significant increase of the persistence length when moving from pure water to a salt containing solvent is likely to be an electrostatic effect. There are at least three potential sources for these electrostatic interactions:

As a first explanation for the observed behavior, the fixed charges associated with the peptides must be considered. In polyampholytes such as type I collagen, and indeed most proteins, there exists a mixture of positive and negative fixed charges along the polymer backbone, and the distribution of these charges is dependent upon the peptide sequence. The resulting Coulomb interactions along the molecule would be mixed attractive and repulsive, with the attractive interactions being offset not only by the repulsive but also by the rigidity of the backbone itself. A broad range of behaviors would be expected for differences between charge
distributions and chain stiffnesses. The addition of salt generates ions that partially shield the fixed charges dispersed along the polymer backbone. In the absence of salt, the electrostatic interactions would be significantly larger.

The screening effects of salt typically are described by the Debye screening length[30]:

\[
\kappa^{-1} = \left[ \frac{\varepsilon \varepsilon_0 k_B T}{\sum n_i z_i^2 e^2} \right]^{\frac{1}{2}}
\]

where \( \varepsilon \) is the dielectric constant of water, \( \varepsilon_0 \) the permittivity of free space, \( k_B \) the Boltzmann constant, \( e \) is the electronic charge, \( n_i \) is the ionic density of species \( i \), and \( z_i \) is the valency of species \( i \). The Debye screening lengths were calculated for both buffers and were found to be 0.314nm for PBS and 0.328nm for FFB. Given that this length is close to that expected for the length of a single amino acid in the backbone, the FFB and PBS cases are likely to be very nearly fully shielded, and the persistence length measured in these solutions should be close to that of the backbone in the absence of charge. Thus, it is not at all surprising to find that the observed persistence lengths in FFB and PBS are so similar. That the screening length of PBS is larger than that of FFB, and the resulting persistence length in PBS is larger than the persistence length in FFB taken together gives further support for this interpretation of the data.

A second potential explanation incorporating electrostatic effects must be included: the results could also be explained by selective ion adsorption along the molecular backbone, resulting in an increased charge density that overwhelms the screening effects. The effect of specific ion-binding was studied and quantified by Weinstock, et al.[31]. In this study, Type I
collagen was suspended in solution containing varying amounts and species of ions. The ions were then dialyzed out of the solution and the remaining ions, being bound to the collagen molecules, were measured. In this situation, increasing the ion concentration leads to the occupation of the adsorption sites, eventually tending toward saturation. The Coulomb interactions of the adsorbed ions would act to stiffen the polymer. Again, if ions of equal valence are occupying the same sites, then the same persistence length would be expected for the relatively high salt concentrations found in FFB and PBS.

A last interpretation of these results is that the addition of ions in the solution changes the solvation of the collagen molecules. At lower salt concentrations the molecules preferentially interact with themselves rather than with the water, reducing the solvation and condensing the molecules. Conversely, at higher salt concentrations the molecule/salt/water complex achieves a lower free energy by increasing the interactions with water, causing the molecule to adopt a more extended conformation. Resolution of these alternate interpretations will require further experimentation.

It is now appropriate to address how these results fit within the current literature. Both of the persistence length values in the absence of salt were in reasonable agreement with the 14.5 nm persistence length determined by Sun et al.[17] for type I procollagen. We note that, while their collagen molecules were suspended in a complex buffer differing from those examined in this paper, it did include relatively high salt concentrations. It also should be noted that the procollagen molecules used in this previous study include non-helical domains that normally are cleaved before fibrils can be formed. These domains have the potential to be quite flexible; thus the procollagen molecule might be thought of as two soft springs bounding the ends of a stiff spring. Under forcible extension, deformation of the soft springs would dominate. The
persistence length probed primarily would be that of the non-helical domains. Therefore, it is reasonable that they would observe a highly extensible (small persistence length) collagen molecule, even when suspended in a high salt solution.

The results presented here are in better agreement with the 130nm found by Utiyama et al.[22] and the 175nm found by Sasaki and Odajima[32]. Utiyama was investigating rat skin tropocollagen by viscosity measurements, and Sasaki was investigating bovine Achilles tendon collagen in situ by X-ray diffractometry. Though the collagen sources in these investigations were different from the present study and the measurement techniques varied, both sets of experiments were performed on type I collagens in saline solutions comparable to those used in the current work. The agreement of our results with these prior studies implies that the nature of the ions in the solvent might be a more important determining factor of the persistence length of type I collagen than the source of the material.

The importance of these results lies in understanding both fibril formation and the development of the mechanical properties of the higher ordered collagen structures. The aggregation of collagen molecules into fibrils would be facilitated if the molecules assume extended structures. Such a conformation would expose sites needed to properly align the molecules, whereas a condensed structure would hide these sites, delaying or preventing fibril formation. Several groups are working to develop a theory describing fibril mechanics based upon knowledge of the molecule’s mechanical properties[8, 9, 15, 19, 33-38]. The values generated by this current study can be used by those investigators.

Using Equation 2.1, one can convert the persistence length of the 1.5nm diameter molecule, a common figure [5, 8, 33, 38, 39], to a value for the Young’s Modulus. At room temperature, our data show Young’s Modulii ranging from ~14 to 40 MPa. This range in solvent
dependent modulus is not only seen at the molecular level. In 2009 Grant, et al. studied the change in elasticity of collagen fibrils as a function of solvent conditions[39]. It was found that the elastic modulus of collagen fibrils ranges from 2 to 200 MPa depending on solvent conditions. Grant et al. state that the increase in modulus as a function of increasing salt was the most difficult result to rationalize. We present, for the first time, the observation of a correlation between ionic interactions and the Young’s Modulus of collagen on the molecular scale. It appears that this increase in modulus, while occurring at the molecular scale, continues to affect collagen mechanics at the fibril scale and likely up through macroscopic (whole tissue) scales.

Conclusions

The persistence length of type I collagen derived from bovine dermis was measured in three solvents that varied in ionic content. The persistence length was observed to increase significantly, by over an order of magnitude, with increased ion concentrations. The results were in good agreement with a subset of the values previously reported in the literature. These results will be of interest to those investigating fibrillogenesis and extracellular matrix mechanics.
References

1. Department of Physics, University of South Florida, Florida, USA.

2. University of South Florida, Florida, USA.


CHAPTER THREE:
THE EFFECTS OF IONIC SPECIES AND CONCENTRATION ON THE MOLECULAR CONFORMATIONS OF TYPE I COLLAGEN[1]

Abstract

In this work, we show that condensation of ions on type I molecular collagen caused molecular-scale conformational changes that were dependent upon both ion species and concentration. These changes resulted in a straightening of the structure, implying at least local stiffening of the molecule. Given the importance of the fibrillar collagens, especially type I collagen, to the mechanical integrity of body tissues, such ion-induced modifications are likely to have important local consequences within these systems. Specifically, in this study bovine dermal type I collagen molecules were suspended in solutions varying in both ionic species and concentration, after which the conformations of the molecules were characterized. It was found that with all ionic species tested, increasing the ionic concentration corresponded with a straightening of the molecule, characterized herein as a persistence length. The dependence of persistence length on ionic species correlated with the degree of ionic condensation reported in previous results. However, the rate at which the persistence length increased with ion
concentration was not attributable to simple charge shielding. At the salt concentrations used here, 0.001-0.1M, the data fit well to the Langmuir Adsorption Isotherm, implying a simple adsorption mechanism. The salt species chosen for this work aimed to elucidate the effects of mono- versus divalent salts and the effects of changing the anion and cation species.

**Introduction**

Collagen is the key contributor to the structural and mechanical properties of mammalian tissues, including ligament and tendon, cornea, skin, and bone. However, these tissues vary widely in their structure and mechanics. From a bioengineering perspective, the ability of collagen to form such disparate structures of broad mechanical properties is both curious and of great importance[2-5].

The fibril forming collagens, including types I, II, V, XI, XXIV, and XXVII, are part of a hierarchally organized system: at the nanoscale, the triple helical tropocollagen molecular monomer aggregates in a quarter staggered fashion to form the fibrils, which in turn assemble into the more macroscopic hierarchal forms including ropes, sheets, or open meshes. At this macroscopic scale, the structure and mechanics of tissues have been investigated for decades, providing a great deal of information.

The study of the mechanical properties of collagen on the fibril scale has seen great attention over the last few years both experimentally and theoretically[6-15]. Most relevant for the present work, Grant et al. studied the effects of various buffer solutions on the mechanical
properties of collagen fibrils and found that the presence of salts increased the elastic modulus[16].

The single molecule scale has been less investigated. Sun et al. have quoted a persistence length of 14.5nm for molecular type I collagen obtained through stretching experiments in a single buffer[17]. Previous work by Lovelady et al., presented in Chapter 2, has shown that the persistence length of molecular collagen ranges between ~12nm in DI water and ~150nm in PBS, as deposited on clean mica, indicating that the range in previously reported results might be related to solvent conditions[18]. Indirect measurements using rheology and light scattering have provided data ranging from 57nm to 544nm[19-23].

The fact that salt affects the mechanical properties of collagen, on both the molecular and fibril scale, is apparent. The previously mentioned study by Lovelady et al. was the first direct measurement of how solvents play a role in the mechanics of molecular collagen. However, the specifics of how various salts affect the persistence length of collagen and the mechanisms of this interaction have not been fully explored. The experiments reported herein sought to provide insight into this process.

Materials & Methods

A stock solution of 0.51 wt.% pepsin digested bovine dermal type I collagen in 0.01M HCl was obtained from MiMedx, Inc. (Tampa, FL). The collagen stock was added to varying salt solutions in a volume concentration of 1 part collagen stock to 1000 parts salt solution. Five
different salts were used in this study: NaCl, KCl, KBr, CaCl$_2$, and MgCl$_2$. This group of salts allows for comparison between mono- and divalent ionic species and the effects of changing the anion and cation species. Ionic concentrations ranged from 0.001 to 0.1M.

All collagen/salt dilutions were prepared for imaging with an atomic force microscope (AFM) in the same manner. 50µL of the collagen/salt solution was applied to freshly cleaved mica for 10s, briefly rinsed with DI water, and dried under a gentle Ni stream.

The mica depositions were imaged in intermittent contact, AC, mode using an Asylum Research MFP 3-D AFM (Asylum Research, Santa Barbara, CA). Silicon cantilevers were used that have nominal spring constants of 4.5, 7.5, and 14 N/m and nominal resonant frequencies of 150, 210, and 315 kHz respectively (µMasch USA, San Jose, CA). From each 1µm x 1µm height trace image, the contour length and end-to-end distance of each molecule was measured and recorded by tracing the molecules, see Figure 3.1. Each image was taken using 512 x 512 pixels, which gave a data point at approximately every 2nm. Only molecules completely imaged in the scan area were measured.

**Measurement Techniques and Data Analysis**

The persistence length, $P$, of a molecule is directly related to the Young’s Modulus, $Y$, by[24]:

$$ YI = k_B TP $$

**Equation 3.1**
where $I$ is the area moment of inertia, $T$ the temperature, and $k_B$ is the Boltzmann constant. There are two main methods to calculate the persistence length of a molecule using AFM. One method uses force spectroscopy and forcibly extends the molecule with the AFM tip. The persistence length then is extracted from the applied force and stretching data[25]. The second method, employed in this work, is to deposit the molecules onto a surface, measure the contour length and end-to-end distance of each molecule, and fit these data to the Worm-like Chain, WLC, Model. This method has recently been used to characterize both collagen [18] and DNA[26].

When molecules suspended in solution are deposited onto a flat surface, such as mica, they are transitioning from a three- to a two-dimensional environment. If the molecules equilibrate on the surface in two dimensions their conformations can be described by the 2D WLC Model equation below.

\[
\langle R^2 \rangle_{2D} = 4PL\left(1 - \frac{2P}{L}\left(1 - e^{-\frac{L}{2P}}\right)\right)
\]

where $R$ is the end-to-end distance, $P$ is the persistence length, and $L$ the contour length. If the molecules do not equilibrate on the surface they become ‘kinetically trapped’[26]. Due to the nature of AFM, the images of a 3D equilibrated protein do not achieve a true 3D representation of the molecules but rather a 2D projection of a three dimensionally equilibrated structure. In terms of the WLC Model, such molecules are represented using Equation 3.3 below.
Equation 3.3

\[
\langle R^2 \rangle_{3Dproj} = \frac{4}{3} PL \left(1 - \frac{P}{L} \left(1 - e^{-\frac{L}{P}}\right)\right)
\]

Without knowing \textit{a priori} whether the molecules will equilibrate on the surface, all data are fit to both WLC Model equations.

Figure 3.1 An illustrative example of the trace along a single collagen molecule. This type of trace is used for extracting the contour length and end-to-end distance of the molecule. Inset shows the height values along the line trace.
For each dilution, the contour length and end-to-end distance data were plotted and fit to both the two and projected three-dimensional WLC model equations, Equations 3.2 and 3.3, and values for the persistence length were calculated. All data analysis was performed in Igor Pro (WaveMetrics Inc., Lake Oswego, OR, USA). As an example, Figure 3.2 shows the molecular conformation data for the 0.1M KCl dilution along with the WLC model fit.

**Figure 3.2** Contour length and squared end-to-end distance data for the 0.01M KCl dilution.

SURFACE EQUILIBRATION CONTROL EXPERIMENT

To investigate effects of surface interactions between the mica substrate and the collagen molecules and whether the molecule could diffuse on the surface, collagen molecules from a DI water dilution were deposited onto a freshly cleaved mica substrate for 20s, briefly rinsed with DI water, and dried under gentle Ni flow. The collagen treated mica substrates were then
submerged in a solution of 0.1M NaCl for varying times (10, 20, 45, 90, and 120 minutes), briefly rinsed with DI water, and dried under gentle Ni flow. These samples were imaged and contour length and end-to-end distance data are analyzed, using the same methods as described above, to extract a persistence length for each soaking time.

It has been previously shown that collagen suspended in a DI water solution and deposited onto a mica substrate has a persistence length of ~12nm [18]. When these samples were then exposed to a 0.1M NaCl solution, after being deposited onto the mica substrate, it was seen that the molecules assumed a more straightened configuration over time, see Figure 3.3. It is expected that the molecules will continue the current trend until reaching the full persistence length of ~105nm as observed with a 0.1M NaCl dilution. This provided direct evidence of molecular diffusion and equilibration on the surface, further implying a two dimensionally equilibrated structure.

![Figure 3.3](image)

**Figure 3.3** Persistence length of type I molecular collagen deposited onto mica from a DI water dilution followed by incubation in 0.1M NaCl for varying times at room temperature.
PH MEASUREMENT

In this current study, pH was not directly controlled. To adequately control the pH of the collagen solutions, a buffer containing relatively high concentrations of salts would be required. This would impede the desired analysis of molecular conformations at low salt concentrations. Furthermore, increasing pH promotes fibril formation. Even though the pH was not controlled, it was measured with fluorescence spectroscopy, see Table 3.1 and Figure 3.4.

10 µM concentrations of the pH sensitive fluorescent dye 2',7'-Bis-(2-Carboxyethyl)-5-(and-6)-Carboxyfluorescein, BCECF Acid, (Life Technologies, Carlsbad, CA) was used. The control pH samples were 20mM HEPES solutions. The counts and ratio, for each solution in Table 3.1, were collected as an average of 6 measurement runs over 2 different samples.

The counts ratio between excitations at 440 and 490nm for each sample and control buffer were calculated. The ratio shows that all of the samples tested were close to a pH value of ~4. The data also show that there is no significant change in pH between the samples. The pH of mono- and divalent samples at varying concentrations was tested. Thus, we have concluded that the conformational changes seen in this study were not attributed to changes in pH.

Results

A persistence length for each collagen dilution on mica was calculated using both the 2D and 3D Proj. WLC model equations, see Table 3.2. In all cases, the Projected 3D WLC model
Table 3.1 Fluorescence spectroscopy results showing approximate pH of varying collagen solutions as compared with control pH solutions. The collagen solutions contain 1 part collagen stock to 1000 parts ionic solution. The control pH samples are 20mM HEPES solutions.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Counts ratio</th>
<th>Counts at 440nm x10^6</th>
<th>Counts at 490nm x10^6</th>
</tr>
</thead>
<tbody>
<tr>
<td>DI</td>
<td>1.085</td>
<td>5.53</td>
<td>6.00</td>
</tr>
<tr>
<td>0.1M NaCl</td>
<td>1.210</td>
<td>4.40</td>
<td>5.32</td>
</tr>
<tr>
<td>0.01M NaCl</td>
<td>1.049</td>
<td>4.44</td>
<td>4.66</td>
</tr>
<tr>
<td>0.005M NaCl</td>
<td>1.057</td>
<td>4.20</td>
<td>4.44</td>
</tr>
<tr>
<td>0.001M KCl</td>
<td>1.036</td>
<td>4.14</td>
<td>4.28</td>
</tr>
<tr>
<td>0.1M MgCl2</td>
<td>1.111</td>
<td>3.98</td>
<td>4.42</td>
</tr>
<tr>
<td>pH 3.97</td>
<td>0.861</td>
<td>3.19</td>
<td>2.74</td>
</tr>
<tr>
<td>pH 4.65</td>
<td>1.137</td>
<td>3.42</td>
<td>3.89</td>
</tr>
<tr>
<td>pH 5.01</td>
<td>1.277</td>
<td>3.58</td>
<td>4.57</td>
</tr>
<tr>
<td>pH 5.60</td>
<td>1.508</td>
<td>3.31</td>
<td>5.00</td>
</tr>
<tr>
<td>pH 6.49</td>
<td>2.116</td>
<td>6.22</td>
<td>13.2</td>
</tr>
</tbody>
</table>

Figure 3.4 Fluorescence spectroscopy curves for six collagen dilutions and four pH control samples\(^1\).

\[^1\] The fluorescence spectroscopy data were collected with assistance from Joey Foley in the laboratory of Dr. Martin Muschol.
Table 3.2 Results for all salt dilutions.

<table>
<thead>
<tr>
<th></th>
<th>Concentration</th>
<th>Number of Molecules</th>
<th>2D WLC Persistency Length (nm)</th>
<th>3Dproj WLC Persistency Length (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>NaCl</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.001 M</td>
<td>71</td>
<td>21.3 ± 1.9</td>
<td>73.7 ± 7.8</td>
<td></td>
</tr>
<tr>
<td>0.005 M</td>
<td>151</td>
<td>37.4 ± 3.0</td>
<td>156.3 ± 19.6</td>
<td></td>
</tr>
<tr>
<td>0.01 M</td>
<td>150</td>
<td>52.3 ± 4.5</td>
<td>294.7 ± 54.4</td>
<td></td>
</tr>
<tr>
<td>0.025 M</td>
<td>149</td>
<td>65.7 ± 5.4</td>
<td>560.9 ± 151</td>
<td></td>
</tr>
<tr>
<td>0.05 M</td>
<td>139</td>
<td>84.7 ± 8.0</td>
<td>4604.8 ± 8920</td>
<td></td>
</tr>
<tr>
<td>0.1 M</td>
<td>132</td>
<td>105.4 ± 9.1</td>
<td>18526 ± 1760</td>
<td></td>
</tr>
<tr>
<td><strong>KCl</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.001 M</td>
<td>194</td>
<td>31.8 ± 2.6</td>
<td>124.6 ± 14.6</td>
<td></td>
</tr>
<tr>
<td>0.01 M</td>
<td>103</td>
<td>79.2 ± 8.4</td>
<td>1214.6 ± 775</td>
<td></td>
</tr>
<tr>
<td>0.05 M</td>
<td>19</td>
<td>176.3 ± 20.9</td>
<td>45198 ± 857</td>
<td></td>
</tr>
<tr>
<td>0.1 M</td>
<td>90</td>
<td>224.3 ± 23.8</td>
<td>74602 ± 150</td>
<td></td>
</tr>
<tr>
<td><strong>KBr</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.001 M</td>
<td>73</td>
<td>25.2 ± 3.3</td>
<td>90.9 ± 15.2</td>
<td></td>
</tr>
<tr>
<td>0.005 M</td>
<td>54</td>
<td>54.7 ± 7.8</td>
<td>331.8 ± 110</td>
<td></td>
</tr>
<tr>
<td>0.025 M</td>
<td>71</td>
<td>97.0 ± 11.1</td>
<td>19961 ± 1800</td>
<td></td>
</tr>
<tr>
<td>0.1 M</td>
<td>62</td>
<td>198.7 ± 28.2</td>
<td>63430 ± 236</td>
<td></td>
</tr>
<tr>
<td><strong>MgCl₂</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.001 M</td>
<td>76</td>
<td>57.9 ± 7.0</td>
<td>364.3 ± 107</td>
<td></td>
</tr>
<tr>
<td>0.005 M</td>
<td>55</td>
<td>70.5 ± 9.2</td>
<td>745.8 ± 399</td>
<td></td>
</tr>
<tr>
<td>0.025 M</td>
<td>65</td>
<td>84.5 ± 12.5</td>
<td>1972.8 ± 2680</td>
<td></td>
</tr>
<tr>
<td>0.1 M</td>
<td>70</td>
<td>101.0 ± 13.0</td>
<td>20160 ± 2100</td>
<td></td>
</tr>
<tr>
<td><strong>CaCl₂</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.001 M</td>
<td>83</td>
<td>26.3 ± 3.3</td>
<td>95.9 ± 15.9</td>
<td></td>
</tr>
<tr>
<td>0.005 M</td>
<td>127</td>
<td>45.1 ± 4.3</td>
<td>234.8 ± 44.0</td>
<td></td>
</tr>
<tr>
<td>0.01 M</td>
<td>155</td>
<td>57.7 ± 4.8</td>
<td>369.8 ± 75.6</td>
<td></td>
</tr>
<tr>
<td>0.05 M</td>
<td>153</td>
<td>55.1 ± 4.7</td>
<td>331.7 ± 65.0</td>
<td></td>
</tr>
<tr>
<td>0.1 M</td>
<td>85</td>
<td>68.8 ± 7.7</td>
<td>651.2 ± 265.0</td>
<td></td>
</tr>
</tbody>
</table>
equation produced unphysical results for the highest concentrations. This result was interpreted as indicating that the molecules equilibrated on the surface, and will be accurately described by the 2D WLC Model. In the limit of a small persistence length both the 2D and 3Dproj WLC models predict equivalent molecular conformations.

The calculated 2D WLC persistence length was plotted as a function of salt concentration, for all ionic species tested, see Figures 3.5-3.7. Within this range of concentrations the persistence length data fit well to a modified Langmuir Adsorption Isotherm[27], Equation 3.4.

\[
P = a + \frac{QKc}{1 + Kc}
\]

In Equation 3.4, \(a\) is an offset, \(c\) is the molarity, \(Q\) is the maximum adsorption capacity and \(K\) represents the affinity of the ions to adsorb onto the protein[28]. The offset, \(a\), was added to the standard form of the isotherm for physical reasons: a persistence length of 0nm is unlikely, even at 0 concentration. Table 3.3 displays the values from the Langmuir isotherm fits for all ionic species studied.

<table>
<thead>
<tr>
<th>Ionic Species</th>
<th>a</th>
<th>Q</th>
<th>K</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>20.8 ± 4.6</td>
<td>107.4 ± 10.2</td>
<td>32.7 ± 10.9</td>
</tr>
<tr>
<td>KCl</td>
<td>26.0 ± 1.5</td>
<td>286.5 ± 5.5</td>
<td>22.4 ± 1.3</td>
</tr>
<tr>
<td>KBr</td>
<td>28.1 ± 10.4</td>
<td>314.0 ± 94.7</td>
<td>11.8 ± 7.9</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>56.7 ± 4.5</td>
<td>51.4 ± 6.8</td>
<td>55.4 ± 32.5</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>10.6 ± 22.3</td>
<td>54.2 ± 20.4</td>
<td>402.1 ± 464.0</td>
</tr>
</tbody>
</table>
Figure 3.5 Persistence length as a function of molarity for all salts tested. The data are fit to a modified Langmuir Isotherm, Equation 3.4.

Figure 3.6 Persistence length as a function of molarity for all monovalent salts tested. The data are fit to a modified Langmuir Isotherm, Equation 3.4.
Discussion

The persistence length of type I molecular collagen increased with all ionic species tested. Interestingly, each ionic species caused a different rate of persistence length increase. If the measured persistence lengths had collapsed onto a single curve, when plotted as a function of ionic strength, the interpretation of the data would be through shielding of native charges along the protein’s length. That is to say, as the concentration of ions in solution increases, the ‘intrinsic’ persistence length of the polymer backbone (that in the absence of charges distributed along its length and prescribed purely by the mechanics of the material) would emerge. However, this expected outcome was not observed. Instead, the effects of the ions in solution were found to depend upon the ionic species. This dependence was found to follow Langmuir

Figure 3.7 Persistence length as a function of molarity for all divalent salts tested. The data are fit to a modified Langmuir Isotherm, Equation 3.4.
isotherms where the fitting parameters were, again, species dependent. That the data follow the Langmuir isotherm implies adsorption of ions on the molecules, but the different fitting parameters indicate that this occurred in a selective manner. Similar effects have been previously documented[29, 30]. Though these papers found that ions bind selectively to collagen, the present work was the first to directly demonstrate the effect these ions have on a physical property of the molecules.

For type I collagen, the effects of ions on the persistence length were dominated by the cation in the solution. This observation is made clear by comparing the persistence lengths in NaCl, KCl, and KBr solutions: 1. With Cl\(^-\) ions appearing in both NaCl and KCl solutions, K\(^+\) had a much greater effect on the end-to-end distance of the molecule than does Na\(^+\), even though Na\(^+\) and K\(^+\) have the same valence charge; 2. With K\(^+\) ions appearing in both KCl and KBr solutions, little effect was caused by the Cl\(^-\) and Br\(^-\) ions.

These results were strikingly different from the Hofmeister series[31], which orders salts by their ability to salt proteins in or out of aqueous solutions. In the present experiments, maximal persistence length would correspond with maximal ‘salting in’. While the Hofmeister series would predict Ca\(^{++}\) > Na\(^+\) > K\(^+\) (in order of the ‘salting in’ effect), instead the observed order was K\(^+\) > Na\(^+\) > Ca\(^{++}\)[32]. One interpretation of the series is that certain ions in solution interact strongly with proteins, affecting the protein’s interaction with water[33]. In this sense, the adsorption of ions to the collagen molecules is consistent with the idea of increased solubility and increased persistence length.

In 1967 Weinstock et al. [34] reported that type I molecular collagen showed preferential ion binding. In that study it also was shown clearly that divalent salts bind significantly less than the monovalent salts tested. Our results agree with the ion binding data presented by Weinstock.
Unfortunately, the Weinstock et al. study did not test KCl or KBr, the species in which we found the most dramatic increase in effect.

There are potential biological and biomedical consequences to the persistence length of collagen being affected by ions in solution. Diseases that have associated modifications in extracellular ion concentrations, such as kidney disease or Addison’s disease, would cause changes in the mechanics of the extracellular matrix (ECM), as would more acute conditions such as dehydration. The response of collagen to the ions in solution also provides the body with another mechanism by which local changes in the ECM might be created. Additionally, researchers developing scaffolds for tissue engineering might be able to use the concentrations of ions in the bathing solutions to fine-tune the mechanics in their systems.

**Conclusions**

In conclusion, it was found that ions bind to type I collagen in a preferential manner and that the differences in bound ionic species, produce measurably different conformational changes in the molecules. These conformational changes were reflected in the persistence length of the molecules, and thereby in their mechanical properties. Given the key role of the fibrillar collagens in determining the mechanics of the extracellular matrix, these observations have implications for ECM biology. This study also lends explanation to the large discrepancy in persistence length values of type I collagen present in the literature.
References

1. Portions of this research are being submitted for publication to XXXX


CHAPTER FOUR:

THE EFFECTS OF TEMPERATURE ON THE STABILITY OF MOLECULAR TYPE I COLLAGEN

Abstract

Leikina et al. published a study in 2002 that demonstrated that type I collagen is thermally unstable at body temperature[1]. Since then, numerous studies have expanded on this idea and investigated temperature effects on the fibril and tissue scales[2-5] or have expanded the study using methods that do not directly image the molecules[6-8]. Temperature stability of the collagen molecule and how that relates to the molecular conformations are of great importance.

Previous to Leikina et al., studies on temperature dependence focused on melting and denaturing temperatures[9-11]. However, these studies do not have direct measurements of the molecular conformations leading up to and during the denaturing process. Probing the denaturing process and thermal stability of type I collagen will aid in our understanding of the complex behavior of the molecule.

In this study, molecular type I collagen will be held at 37 degrees Celsius for varying times. The molecules will then be deposited onto mica and imaged with atomic force
microscopy. This ‘proof of principle’ study shows that the number of molecules in solution decreases over time while being held at body temperature.

**General Procedure**

The initial goal of this study was to image molecular collagen with AFM while submerged in the ionic solution. Once stable imaging was achieved, the temperature would be slowly increased to body temperature as the molecules were continuously imaged. This would allow for real-time tracking of molecular conformational changes, unraveling of the triple helix, and other degradation of the molecule.

Stable and clear images of molecular collagen in liquid could not be achieved. The molecules were not chemically bound to the surface because this would eliminate the possibility for conformational change. Without being bound to the surface, the molecules were too mobile to image with any clarity.

Without being able to image the process in real-time, a ‘snapshot’ approach was taken. The idea is that the molecules will be brought to and held at body temperature for varying times at which the snapshots are taken. The snapshots are taken by removing the heated solution containing the collagen molecules, depositing onto mica, drying, and imaging. The resulting imaged conformations and molecular densities are representative of the molecules in solution at that time. Another advantage of this method is that the molecules remain in the three
dimensional environment until deposition, and not restricting the conformational changes to two dimensions.

**Materials and Methods**

A stock solution of 0.51 wt.% bovine type I collagen in 0.01M HCl was obtained from MiMedx, Inc. (Tampa, FL). This collagen stock was extracted from bovine dermis using a pepsin digestion step in the purification process. The resulting molecule, called tropocollagen, consists only of the collagen triple helix. The stock solution was diluted into a 1000:1 ratio of 0.1M NaCl to collagen stock.

5mL of the refrigerated collagen dilution was deposited into an Asylum Research Bioheater Stage (Asylum Research, Santa Barbara, CA). A glass cover was placed over the stage to minimize evaporation. The collagen solution was heated to 37.0 degrees Celsius at a ramping rate of 2 degrees per minute. The Asylum Research Bioheater Stage has a temperature control accuracy of 0.1 degrees Celsius.*reference.*

Samples were taken after holding at 37 degrees Celsius for 0, 30, 45, 60, 75, and 90 minutes. Each sample was taken by removing 50µL of the heated collagen solution, immediately depositing onto freshly cleaved mica for 20s, briefly rinsing with DI water to halt the deposition process, and gently drying under Ni flow.

The mica samples were imaged in AC mode with an Asylum Research MFP-3D AFM. For each sample 3µm X 3µm images were taken at a resolution of 512 X 512 pixels. All
molecules that were completely imaged within the scan area were counted. Only molecules measuring above 200nm in length were counted.

Results

The molecular density of type I collagen molecules, as deposited on freshly cleaved mica, decreased as the molecule-rich solution was held at body temperature, see Fig. 4.1. As a control, the 0 minute samples were taken when the solution reached 37 degrees Celsius. This preliminary study shows that the density of type I collagen molecules decreases by over 50% after 90 minutes.

![Graph](image)

**Figure 4.1** The average molecular density of type I collagen molecules as a function of extended heating. The error bars on this graph represent the standard deviation.
Discussion

This ‘proof of principle’ study has demonstrated that the affect of temperature on molecular type I collagen is an area that warrants further investigation. The next step would be to measure molecular density as a function of time, while being held at 37 degrees Celsius, of molecular collagen in a physiological or fibril forming buffer. This would give a more accurate representation of molecular collagen in situ. Going further in depth into the effects of temperature could show a change in the persistence length of the molecule over time, enhanced imaging may be able to show unraveling of the triple helix, and investigating the effects of temperatures beyond 37 degrees Celsius could provide further information on the structural changes of collagen at elevated temperatures.

References


CHAPTER FIVE:
FABRICATING AND MEASURING THE NON-LINEAR OPTICAL PROPERTIES OF
TYPE I COLLAGEN MEMBRANES[1]

Abstract

A novel fabrication method for creating anisotropic collagen membranes has been
developed[2, 3]. By employing a unique flow-casting method, collagen membranes are
engineered layer-by-layer. The human corneal stroma is comprised of collagen fibril lamella
whose orientation varies from layer to layer[4-6]. Near the center of the cornea, the fibrillar
orientations between lamella are orthogonal. The flow-casting method employed herein easily
allows for precise control of fibrillar alignment in each layer.

Evaluation and measurement of the optical properties and structural characteristics of
artificial collagen membranes are necessary when engineering collagen-based tissues such as
artificial cornea. In this study, linear birefringence (LB), linear dichroism (LD), and the optical
rotatory power (ORP) of artificial anisotropic collagen membranes were measured with the
generalized-High Accuracy Universal Polarimeter (G-HAUP). Absorbance measurements were
also conducted with a conventional UV-Vis spectrophotometer. As a result, optical
transparency, optical anisotropy, and optical activity in artificial collagen membranes were successfully evaluated.

**Introduction**

Many natural polymers and their synthetic analogues have been used as biomaterials in the field of tissue engineering. In particular, the use of collagen as a biomaterial has been widely developed because of its biological characteristics such as biodegradability and weak antigenicity. Collagen has attracted much attention as a primary resource for medical application. Collagen-based biomaterials cover a wide range of clinical applications such as reconstructions of bone, blood vessels, corneal stroma, and regenerations of peripheral nerves[7-11].

Collagen is also an important protein in the human body. Its molecules form aggregates such as microfibrils, fibrils, fibers, and bundles to maintain mechanical and functional properties in tissues and organs[12]. The orientation and structure of collagen molecules/aggregates plays an important role in the properties of tissues[8, 11, 13-16]. For example, the mechanical and optical functions of corneal stroma are strongly related to the unique orientation and lamella structure of the collagen fibrils[4-6]. The development of anisotropic collagen membranes, for artificial corneal applications, requires mimicking the complex orientation and lamella structure of native corneal stroma.
High strength magnetic field application, electro-spinning, and dip-pen nanolithography techniques have enabled the fabrication of anisotropic collagen membranes[17-19]. However, these techniques often require large, expensive, and complex equipment that may not be suitable for practical use. Moreover, these techniques typically do not allow the ability to control the degree of orientation of collagen fibrils or to easily vary the number and thickness of the lamella for mimicking the three-dimensional structures of tissues and organs[20]. Versatility in controlling fibrillar orientation, lamella thickness, and overall membrane thickness are of great importance.

Recently, we developed a novel fabrication method of creating anisotropic collagen membranes[3]. Each membrane was constructed layer-by-layer and the thickness and fibril orientation of each lamellae was controlled. The laminated collagen membranes, fabricated using this method, are good candidates for evaluating the orientation and structure of the collagen molecules/fibrils for the following two purposes: first, to develop an evaluation method of collagen membranes with preferred orientation and second, to clarify differences in orientation, structure, and optical properties between collagen membranes with parallel and orthogonal lamination.

As a useful method to evaluate the degree of preferred orientation and molecular structure of collagen membranes, we focused on the simultaneous and accurate measurements of optical anisotropy including linear birefringence (LB), linear dichroism (LD), and chiro-optical properties such as optical rotatory power (ORP) and circular dichroism (CD) of the membranes. However, the simultaneous measurement of LB, LD, ORP and CD in anisotropic materials is extremely difficult. The observed ORP and CD signals are overwhelmed by LB and LD signals because they are $10^3$-$10^4$ times greater than the ORP and CD signals[21, 22]. Therefore,
conventional optical instruments, such as polarimeters and CD spectrophotometers, cannot accurately measure the ORP and CD signals of anisotropic materials except in a specific case: only along the optic axis. Thus, the simultaneous measurement of optical anisotropy and chiro-optical properties in anisotropic collagen membranes remains a crucial issue to be solved.

In 1996, Kobayashi and Asahi[24, 25] extended the principle of the original HAUP method[23] to measure anisotropic absorbing materials. We can now simultaneously measure LD and CD in addition to LB and ORP with this extended HAUP method, G-HAUP. The G-HAUP is a universal chiro-optical spectrophotometer. It differs substantially from the original HAUP method in both measurement theory and optical systems. This expansion, developed by Kuroda and coworkers[26], has allowed G-HAUP to also measure the ORP and CD of anisotropic materials.

In this study, the optical anisotropy and optical activity, i.e. ORP, in manufactured collagen membranes with parallel and orthogonal lamination were evaluated with the extended HAUP method, G-HAUP.

**Materials and Methods**

The collagen membranes were fabricated using the method outlined in Tanaka, et al.[3]. The fabrication method was divided into four steps: flow-casting, fibril formation, dehydration, and lamination. In the flow-casting step, a 3% atelocollagen solution (Atelocollagen Implant, Koken, Tokyo, Japan), which is a pepsin-treated collagen dissolved in phosphoric acid buffer
solution, was centrifuged onto a SiO substrate (Oken, Tokyo, Japan). The substrate was mounted on a handcrafted stage with a swing-type centrifugal machine (Himac CF16RX, Hitachi Koki, Tokyo, Japan). The centrifugation was performed at low temperature, 4°C, to prevent fibril formation as the solution is spread into a thin layer, see Figure 5.1.

Figure 5.1 A schematic illustration of the collagen flow-casting process. A collagen solution is applied to the top of a glass substrate, placed in the hand-crafted stage, centrifuged at 4°C, incubated at 37°C to form fibrils, and dried at low temperature (4-8°C).

After centrifugation, the collagen formed a thin and confluent layer of molecules on the surface. In the fibril formation step, the substrate, with the thin molecular collagen layer, was incubated at 37 °C for 30 minutes. During the incubation process, the collagen molecules
aggregated and self-assembled into collagen fibrils. Due to spinning, the fibrils were oriented in the direction of flow.

In the dehydration step, the collagen fibril layer was refrigerated at 4 °C until dried. These steps were repeated many times to laminate the collagen layers. This characteristic fabrication method provides each collagen layer with a preferred orientation by turning, at each flow-casting step, the substrate. In this study, we fabricated five types of collagen membranes. The fabrication conditions of the collagen membranes are listed in Table 5.1.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Relative Centrifugal Acceleration$^a$ (xg)</th>
<th>Fibril Formation</th>
<th>Lamination Direction, # of Layers</th>
<th>Thickness (µm)$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>769</td>
<td>No</td>
<td>Parallel, 4 layers</td>
<td>8.63 ± 0.64</td>
</tr>
<tr>
<td>B</td>
<td>769</td>
<td>Yes</td>
<td>Parallel, 4 layers</td>
<td>8.73 ± 0.48</td>
</tr>
<tr>
<td>C</td>
<td>1968</td>
<td>Yes</td>
<td>Parallel, 4 layers</td>
<td>3.61 ± 0.30</td>
</tr>
<tr>
<td>D</td>
<td>769</td>
<td>No</td>
<td>Orthogonal, 4 layers</td>
<td>7.45 ± 0.46</td>
</tr>
<tr>
<td>E</td>
<td>769</td>
<td>Yes</td>
<td>Orthogonal, 4 layers</td>
<td>9.08 ± 0.21</td>
</tr>
</tbody>
</table>

$^a$ The unit of relative centrifugal acceleration is defined as the ratio of centrifugal acceleration to gravitational acceleration of the earth, g.

$^b$ Thickness of the collagen membranes were measured with the film thickness meter (Alpha-Step 500, KLA Tencor, Kanagawa, Japan). Presented is the average and standard deviation of 6 thickness measurements.

To clarify differences due to the fabrication conditions, we observed the fabricated collagen membranes by polarized light microscopy (DMLP, Leica, Hesse, Germany) with a λ or retardation plate. These polarized light microscopy images were necessary to determine the fast axis of the membranes. The fast axis is the axis along which the polarized ray experiences the largest index of refraction, and thus a faster speed through the material. The determination of the
fast axis was an important step because the material was placed into the G-HAUP apparatus based on the direction of the fast axis.

AFM images taken in tapping, or AC, mode (MFP-3D-IO-OLY, Asylum Research, California, USA) were performed to investigate the surface morphology of the membranes. Silicon AFM cantilevers with a nominal resonance frequency of ~60 kHz and a spring constant of about 2 N/m were used with an imaging scan rate of 1.0 Hz and scanning density of 512 points and lines per 20 micron scan.

<table>
<thead>
<tr>
<th>Sample</th>
<th>−45°</th>
<th>±0°</th>
<th>+45°</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
</tr>
<tr>
<td>B</td>
<td><img src="image4.png" alt="Image" /></td>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
</tr>
<tr>
<td>D</td>
<td><img src="image7.png" alt="Image" /></td>
<td><img src="image8.png" alt="Image" /></td>
<td><img src="image9.png" alt="Image" /></td>
</tr>
</tbody>
</table>

**Figure 5.2** Polarized light microscope images of collagen membranes. The details for the fabrication of the samples are in Table 5.1. Samples A and B are parallel and sample D is orthogonally oriented. Also, samples A and D do not include the fibril formation incubation step. The black scale bar represents 1mm and the arrows indicate collagen flow directions. The angles at the top of the figure are the angles through which the sample was rotated.
The G-HAUP measurements were performed by first aligning the polarizer and analyzer to the crossed Nicol’s position. See Figure 5.3 for a schematic representation of the G-HAUP apparatus. The crossed Nicol’s position was located by measuring the intensity of light passing through the analyzer. The crossed Nicol’s position was determined to be at the minimum. In a perfect system, the intensity of light passing through the analyzer at crossed Nicol’s would be 0.

**Figure 5.3** A schematic representation of the G-HAUP apparatus. Light enters the system through the polarizer, passes through the sample, and finally passes through the analyzer. In the G-HAUP system, the angles of rotation of the polarizer and analyzer are controlled.
The membrane samples were placed into the G-HAUP apparatus in a temperature controlled 25°C environment. The extinction point of the sample was located by passing 550nm wavelength light through the sample, rotating the analyzer ±5°, and measuring the intensity of light passing through the analyzer. The determination of the fast axis from the polarized light microscopy images gave a general starting point for finding the exact extinction position.

Basic HAUP measurements are taken by rotating the analyzer, about the extinction position, and by measuring the intensity of the transmitted light. This was done for wavelengths of light between 390 and 680nm at 20nm increments. The extended HAUP method performs the same intensity measurements as the general HAUP but also repeats these measurements at different initial positions of the polarizer.

The thickness of each sample was measured using an Alpha-Step 500 (KLA Tencor, Kanagawa, Japan). To achieve this measurement, part of the membrane was removed from the glass slide using a razor blade and tape. The Alpha-Step measured the change in height between the membrane surface and the exposed substrate.

Results

The polarized light microscope and AFM images of the collagen membranes are shown in Figures 5.2 and 5.4. Through the polarized light microscopy images, we can determine the optical character, i.e., the directions of fast and slow light rays in the collagen membranes with parallel lamination, Figure 5.2. The azimuth angles of -45 ° and +45 ° exhibit addition and
subtraction configuration, respectively. We found that the orientation of the fast axis is perpendicular to the flow direction of the collagen solution during membrane fabrication. Due to a lack of visible birefringence in the orthogonally oriented membranes, the fast axis could not be determined.

Figure 5.4 Atomic force microscope, AFM, images of collagen membranes. Both membranes are 4 layers of parallel orientation. The image on the left has not been incubated to form fibrils (sample A) and the image on the right has been incubated to promote collagen fibril formation (sample B).

The optical anisotropy and optical activity in collagen membranes were measured with the extended HAUP method. The wavelength dependences of the LB, LD, and ORP of the collagen membranes, measured at 25 °C, are shown in Figure 5.5 (a), (b), and (c). As Figure 5.5 (a) shows, the LB of the collagen membranes with parallel lamination was increased by both the incubation procedure at 37 °C and a higher relative centrifugal acceleration of 1968xg.
Therefore, our results indicate that the preferred orientation was increased by the incubation procedure.

LB of the collagen membranes with orthogonal lamination shows small values compared with parallel lamination samples. We regard the orthogonal lamination samples as almost optically isotropic membranes. This is supported by the results of the polarized light microscope images (Sample D of Figure 5.2).

**Discussion**

As Figure 5.2 shows, a homogeneous morphology was formed through the incubation procedure at 37 °C. Samples A and D were not incubated in order to prevent fibril formation. During the low temperature dehydration process, cracks were formed in the membranes. These cracks can be seen in the polarized light microscope images in Figure 5.2. Note that sample B does not exhibit drying cracks. This leads us to the conclusion that the formation of fibrils in the collagen membranes increased the structural integrity of the membrane.

It is important to note the stark difference between parallel and orthogonally oriented collagen membranes when imaging by polarized light microscopy. In the case of parallel orientation, the triple-helix of the collagen molecules and the direction of the collagen fibrils are all aligned parallel to each other with a rotation of 180° between each layer. The images of samples A and B in Figure 5.2 show that the fast axis of the parallel oriented collagen membrane is perpendicular to the direction of fibrillar orientation. The index of refraction of linearly
polarized light passing through the parallel oriented sample is highly dependent upon the angle of polarization.

Compared with the polarized-light-microscopy images of the orthogonally oriented membrane, sample D in Figure 5.2, the birefringence of the sample appears to be almost eliminated. This is due to the 90° rotation between each layer. Linearly polarized light will experience a similar optical environment at every angle.

Also, as Figure 5.5 (b) shows, absolute values of LD for the collagen membranes with parallel lamination were increased by the incubation procedure and higher relative centrifugal acceleration. According to experiments of UV-Vis spectra, there is little absorption in the wavelength region of this study. Therefore, these LD signals are not due to a difference in absorption between slow and fast light rays. More likely, the LD signals are a result of light scattering due to diameter inhomogeneity and density fluctuation of the collagen fibrils. LD and LB measurements of the collagen membranes with orthogonal lamination show small values compared with parallel lamination samples.

As Figure 5.5 (c) shows, ORP of the collagen membranes indicates positive normal dispersion in this wavelength region. This implies that right-handed circularly polarized light propagates faster than left-handed circularly polarized light. The collagen molecule contains three left-handed GLY-X-Y sequenced α-chains. The three left-handed α-chains combine to form a right-handed triple helix[8, 11, 15, 16]. Our present study of AFM images (Figure 5.4) combined with our previous study using SEM and TEM[3] indicate that the helical axes of collagen in the membranes are aligned along the direction of flow of the collagen solution during membrane fabrication.
We fabricated the collagen membranes by our method. The fabrication conditions of each flow-casting step were measured in Table 1. The thickness of the collagen membranes were measured with the film thickness meter TIP-202 (Oken, Tokyo, Japan), which is a pepsin-treated collagen dissolve in phosphoric acid solution. The polarized light microscope and AFM image of the collagen membranes are shown in Figs. 2 (a) and (b). Changes of the optical anisotropy and optical activities of each thin single-layer were observed in Figs. 2 (a) and (b). The optical anisotropy was measured by the extended HAUP method. Figure 3 shows the optical anisotropy of the collagen membranes. A black rhomboid, red square, blue triangle, green circle and purple rhomboid represent the Sample A, B, C, D and E, respectively.

**Table 1**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Preferred Orientation</th>
<th>Collagen Membranes</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>+ Parallel</td>
<td>8.63±0.64</td>
</tr>
<tr>
<td>B</td>
<td>- Parallel</td>
<td>8.73±0.48</td>
</tr>
<tr>
<td>C</td>
<td>+ Orthogonal</td>
<td>7.45±0.46</td>
</tr>
<tr>
<td>D</td>
<td>- Orthogonal</td>
<td>9.08±0.21</td>
</tr>
<tr>
<td>E</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Figure 2**

Schematic image of the flow-casting step of the fabrication method of the collagen membranes. The black lines represent the scale bar (1 mm) and the black arrows represent the flowing direction of each thin single-layer.

**Figure 3**

Wavelength dependences of LB (a), LD (b) and ORP (c) of the collagen membranes. A black rhomboid, red square, blue triangle, green circle and purple rhomboid represent the Sample A, B, C, D and E, respectively.
The collagen α-chains contain a unique repeated amino acid sequence, glycine-Xaa-Yaa, where Xaa and Yaa positions are often proline and hydroxyproline, respectively[8, 11, 15, 16]. According to Djerassi[30], the ORP of proline-rich proteins can be readily changed by heat addition. However, significant differences of ORP due to the incubation procedure were not observed. We therefore do not believe that the incubation procedure affects the molecular structure of collagen.

The measured LB for the orthogonally oriented membranes is in line with other reports. For the linear birefringence of human cornea, see Table 5.2. By orthogonally orienting the fibrils in the membrane layers, the effects of linear birefringence are reduced to almost 0.

Table 5.2  Linear birefringence, LB measurements of common materials from the literature. References for each value are listed in the table. The orthogonally oriented collagen membranes studied in this chapter have a measured LB that agrees with previously reported linear birefringence measurements of human cornea.

<table>
<thead>
<tr>
<th>Material</th>
<th>LB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nylon66 [31]</td>
<td>$6.0 \times 10^{-2}$</td>
</tr>
<tr>
<td>Polyvinyl chloride [31]</td>
<td>$1.02 \times 10^{-2}$</td>
</tr>
<tr>
<td>(Sheep) wool [31]</td>
<td>$1.0 - 1.1 \times 10^{-2}$</td>
</tr>
<tr>
<td>Rat-tail tendon [32]</td>
<td>$3.0 \times 10^{-3}$</td>
</tr>
<tr>
<td>Cat cornea [33]</td>
<td>$3.0 \times 10^{-3}$</td>
</tr>
<tr>
<td>Human cornea [34]</td>
<td>$1.59 \times 10^{-3}$</td>
</tr>
</tbody>
</table>
Conclusions

In conclusion, we have fabricated five types of collagen membranes using a novel method. The optical anisotropy and optical activity of these collagen membranes have been successfully evaluated with the extended HAUP method. As a result, we have found that measurement with the extended HAUP method is a powerful way to evaluate the orientation and structure of collagen molecules/fibrils of biomaterials. In the near future, We expect this novel technique to be applied to other biomaterials such as collagen/hydroxyapatite bone-like nanocomposite materials.

References


APPENDIX A:
INVESTIGATION OF Viable SURFACES FOR ATOMIC FORCE MICROSCOPE
IMAGING OF MOLECULAR COLLAGEN

All of the data presented in this work that resulted from atomic force microscope, AFM, imaging are obtained from depositing molecular collagen onto freshly cleaved mica substrates. Mica is a commonly used imaging substrate due to it’s atomically flat and easy to cleave surface[1-6]. Mica is a naturally occurring layered mineral substance whose surface becomes negatively charged in the presence of water[7]. The mica used in this research, obtained from Ted Pella, Inc., is V1 grade muscovite mica. It is primarily composed of silica (SiO$_2$), alumina (Al$_2$O$_3$), and potassium oxide (K$_2$O)[8].

The mica used in this study was imaged and the average surface roughness was measured. To measure the surface roughness, mica was freshly cleaved, and imaged with AFM in AC mode. Six 1µm x1µm images were taken at a resolution of 512x512 pixels. The RMS roughness was calculated for each image using the Asylum Research and Igor Pro software. The average RMS roughness of the mica sample was calculated to 0.0686nm. This is well below the 1.5nm diameter of type I collagen[9]. Other substrates were tested for imaging viability: glass,
silicon, and layered graphite. To be suitable for imaging, the substrates must have a flat surface and achieve good molecular deposition.

**Glass and Silicon**

Premium cover glass (Fisher Scientific, Waltham, MA) with a thickness ranging from 0.13 to 0.17 mm was obtained. Type I molecular collagen was deposited onto the premium cover glass for 20 seconds from a 0.001M NaCl solution. The resulting deposition clearly showed the molecules had deposited onto the surface, but the surface roughness is too high to achieve clear resolution and measurement of the molecules, see Figure A.1.

Along with the premium cover glass, glass slides, and economy cover glass were also tested for their substrate viability. The premium cover glass, seen in Figure A.1, provided the best results. Two grades of polished silicon wafers were tested for their surface roughness and collagen deposition viability and found to be similar to the premium cover glass results.

**Graphite**

A highly oriented pyrolytic graphite slab (HOPG) was obtained from Ted Pella, Inc. The slab provides an atomically flat surface for imaging and is easily cleavable with tape, similar to
mica. The graphite was freshly cleaved and a solution of 1000:1 0.001M NaCl:collagen was deposited onto the surface. Depositing collagen on the graphite was not a simple task. Long deposition times were required, 5 to 20 minutes, as opposed to the seconds it takes to deposit on mica. Deposition tests that included and excluded a DI water rinse were also performed.

It was found after deposition, that in all of the tests, the collagen molecules preferentially deposited on the cleave boundaries, see Figures A.2 and A.3. The molecules also appeared to ‘bunch up’ to minimize interaction with the graphite surface. It was determined that graphite was not a suitable candidate for imaging molecular collagen with AFM.

Figure A.1 An AFM image of molecular type I collagen deposited onto a premium glass coverslip.
Figure A.2 A large-scale AFM image of type I molecular collagen deposited onto cleaved graphite. 5µm x 5µm scan area with a resolution of 512x512 pixels.
Figure A.3 An AFM image of molecular collagen deposited onto freshly cleaved graphite. 5µm x 5µm scan area with a resolution of 512x512 pixels.

References


APPENDIX B:
COPYRIGHT PERMISSIONS

Polarized light microscopy Images

Image use and citing?

3 messages

Heather Harper <hmharper@mail.usf.edu>  Sat, Mar 8, 2014 at 10:46 PM
To: szilagyi.andras@ttk.mta.hu

Dr. Szilagyi,

I am writing my dissertation for a PhD in Applied Physics from the University of South Florida. I need a good visual aid to help describe circular birefringence and circular dichroism. Your web demonstrations are some of the best tools and images I have found.

Can I use images from your website in my dissertation? If so, how would you like me to cite them?

Thank you!

Heather Harper

Andras Szilagyi <szilagyi.andras@ttk.mta.hu>  Sun, Mar 9, 2014 at 9:13 AM
To: Heather Harper <hmharper@mail.usf.edu>

Dear Heather,

sure you can use the images. However, I suggest that you use screenshots from my EMANIM program instead of the animated gifs on the web site. The gif images are low resolution and probably do not look good in print.

To cite, just use URL of the page.

Best,
Andras

[Quoted text hidden]
This is a License Agreement between Heather Lovelady ("You") and John Wiley and Sons ("John Wiley and Sons") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by John Wiley and Sons, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

<table>
<thead>
<tr>
<th>License Number</th>
<th>3290840855868</th>
</tr>
</thead>
<tbody>
<tr>
<td>License date</td>
<td>Dec 16, 2013</td>
</tr>
<tr>
<td>Licensed content publisher</td>
<td>John Wiley and Sons</td>
</tr>
<tr>
<td>Licensed content publication</td>
<td>Biopolymers</td>
</tr>
<tr>
<td>Licensed content title</td>
<td>Solvent specific persistence length of molecular type I collagen</td>
</tr>
<tr>
<td>Licensed copyright line</td>
<td>Copyright © 2013 Wiley Periodicals, Inc., a Wiley company</td>
</tr>
<tr>
<td>Licensed content author</td>
<td>Heather H. Lovelady, Satish Shashidhara, W. Garrett Matthews</td>
</tr>
<tr>
<td>Licensed content date</td>
<td>Aug 1, 2013</td>
</tr>
<tr>
<td>Start page</td>
<td>n/a</td>
</tr>
<tr>
<td>End page</td>
<td>n/a</td>
</tr>
<tr>
<td>Type of use</td>
<td>Dissertation/Thesis</td>
</tr>
<tr>
<td>Requestor type</td>
<td>Author of this Wiley article</td>
</tr>
<tr>
<td>Format</td>
<td>Print and electronic</td>
</tr>
<tr>
<td>Portion</td>
<td>Full article</td>
</tr>
<tr>
<td>Will you be translating?</td>
<td>No</td>
</tr>
<tr>
<td>Total</td>
<td>0.00 USD</td>
</tr>
</tbody>
</table>

TERMS AND CONDITIONS

This copyrighted material is owned by or exclusively licensed to John Wiley & Sons, Inc. or one of its group companies (each a "Wiley Company") or a society for whom a Wiley Company has exclusive publishing rights in relation to a particular journal (collectively "WILEY"). By clicking "accept" in connection with completing this licensing transaction, you agree that the following terms and conditions apply to this transaction (along with the billing and payment terms and conditions established by the Copyright Clearance Center Inc., ("CCC's Billing and Payment terms and conditions"), at the time that you opened your RightsLink account (these are available at any time at http://myaccount.copyright.com).
Terms and Conditions

1. The materials you have requested permission to reproduce (the "Materials") are protected by copyright.

2. You are hereby granted a personal, non-exclusive, non-sublicensable, non-transferable, worldwide, limited license to reproduce the Materials for the purpose specified in the licensing process. This license is for a one-time use only with a maximum distribution equal to the number that you identified in the licensing process. Any form of republication granted by this license must be completed within two years of the date of the grant of this license (although copies prepared before may be distributed thereafter). The Materials shall not be used in any other manner or for any other purpose. Permission is granted subject to an appropriate acknowledgement given to the author, title of the material/book/journal and the publisher. You shall also duplicate the copyright notice that appears in the Wiley publication in your use of the Material. Permission is also granted on the understanding that nowhere in the text is a previously published source acknowledged for all or part of this Material. Any third party material is expressly excluded from this permission.

3. With respect to the Materials, all rights are reserved. Except as expressly granted by the terms of the license, no part of the Materials may be copied, modified, adapted (except for minor reformatting required by the new Publication), translated, reproduced, transferred or distributed, in any form or by any means, and no derivative works may be made based on the Materials without the prior permission of the respective copyright owner. You may not alter, remove or suppress in any manner any copyright, trademark or other notices displayed by the Materials. You may not license, rent, sell, loan, lease, pledge, offer as security, transfer or assign the Materials, or any of the rights granted to you hereunder to any other person.

4. The Materials and all of the intellectual property rights therein shall at all times remain the exclusive property of John Wiley & Sons Inc or one of its related companies (WILEY) or their respective licensors, and your interest therein is only that of having possession of and the right to reproduce the Materials pursuant to Section 2 herein during the continuance of this Agreement. You agree that you own no right, title or interest in or to the Materials or any of the intellectual property rights therein. You shall have no rights hereunder other than the license as provided for above in Section 2. No right, license or interest to any trademark, trade name, service mark or other branding ("Marks") of WILEY or its licensors is granted hereunder, and you agree that you shall not assert any such right, license or interest with respect thereto.

5. NEITHER WILEY NOR ITS LICENSORS MAKES ANY WARRANTY OR REPRESENTATION OF ANY KIND TO YOU OR ANY THIRD PARTY, EXPRESS, IMPLIED OR STATUTORY, WITH RESPECT TO THE MATERIALS OR THE ACCURACY OF ANY INFORMATION CONTAINED IN THE MATERIALS, INCLUDING, WITHOUT LIMITATION, ANY IMPLIED WARRANTY OF MERCHANTABILITY, ACCURACY, SATISFACTORY QUALITY, FITNESS FOR A PARTICULAR PURPOSE, USABILITY, INTEGRATION OR NON-INFRINGEMENT AND ALL SUCH WARRANTIES ARE HEREBY EXCLUDED BY WILEY AND ITS LICENSORS AND WAIVED BY YOU.

6. WILEY shall have the right to terminate this Agreement immediately upon breach of this
Agreement by you.

7. You shall indemnify, defend and hold harmless WILEY, its Licensors and their respective directors, officers, agents and employees, from and against any actual or threatened claims, demands, causes of action or proceedings arising from any breach of this Agreement by you.

8. IN NO EVENT SHALL WILEY OR ITS LICENSORS BE LIABLE TO YOU OR ANY OTHER PARTY OR ANY OTHER PERSON OR ENTITY FOR ANY SPECIAL, CONSEQUENTIAL, INCIDENTAL, INDIRECT, EXEMPLARY OR PUNITIVE DAMAGES, HOWEVER CAUSED, ARISING OUT OF OR IN CONNECTION WITH THE DOWNLOADING, PROVISIONING, VIEWING OR USE OF THE MATERIALS REGARDLESS OF THE FORM OF ACTION, WHETHER FOR BREACH OF CONTRACT, BREACH OF WARRANTY, TORT, NEGLIGENCE, INFRINGEMENT OR OTHERWISE (INCLUDING, WITHOUT LIMITATION, DAMAGES BASED ON LOSS OF PROFITS, DATA, FILES, USE, BUSINESS OPPORTUNITY OR CLAIMS OF THIRD PARTIES), AND WHETHER OR NOT THE PARTY HAS BEEN ADVISED OF THE POSSIBILITY OF SUCH DAMAGES. THIS LIMITATION SHALL APPLY NOTWITHSTANDING ANY FAILURE OF ESSENTIAL PURPOSE OF ANY LIMITED REMEDY PROVIDED HEREIN.

9. Should any provision of this Agreement be held by a court of competent jurisdiction to be illegal, invalid, or unenforceable, that provision shall be deemed amended to achieve as nearly as possible the same economic effect as the original provision, and the legality, validity and enforceability of the remaining provisions of this Agreement shall not be affected or impaired thereby.

10. The failure of either party to enforce any term or condition of this Agreement shall not constitute a waiver of either party's right to enforce each and every term and condition of this Agreement. No breach under this agreement shall be deemed waived or excused by either party unless such waiver or consent is in writing signed by the party granting such waiver or consent. The waiver by or consent of a party to a breach of any provision of this Agreement shall not operate or be construed as a waiver of or consent to any other or subsequent breach by such other party.

11. This Agreement may not be assigned (including by operation of law or otherwise) by you without WILEY's prior written consent.

12. Any fee required for this permission shall be non-refundable after thirty (30) days from receipt.

13. These terms and conditions together with CCC's Billing and Payment terms and conditions (which are incorporated herein) form the entire agreement between you and WILEY concerning this licensing transaction and (in the absence of fraud) supersedes all prior agreements and representations of the parties, oral or written. This Agreement may not be amended except in writing signed by both parties. This Agreement shall be binding upon and inure to the benefit of the parties' successors, legal representatives, and authorized assigns.

14. In the event of any conflict between your obligations established by these terms and conditions and those established by CCC's Billing and Payment terms and conditions, these terms and conditions shall prevail.
15. WILEY expressly reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this licensing transaction, (ii) these terms and conditions and (iii) CCC's Billing and Payment terms and conditions.

16. This Agreement will be void if the Type of Use, Format, Circulation, or Requestor Type was misrepresented during the licensing process.

17. This Agreement shall be governed by and construed in accordance with the laws of the State of New York, USA, without regards to such state's conflict of law rules. Any legal action, suit or proceeding arising out of or relating to these Terms and Conditions or the breach thereof shall be instituted in a court of competent jurisdiction in New York County in the State of New York in the United States of America and each party hereby consents and submits to the personal jurisdiction of such court, waives any objection to venue in such court and consents to service of process by registered or certified mail, return receipt requested, at the last known address of such party.

Wiley Open Access Terms and Conditions

Wiley publishes Open Access articles in both its Wiley Open Access Journals program [http://www.wileyopenaccess.com/view/index.html] and as Online Open articles in its subscription journals. The majority of Wiley Open Access Journals have adopted the Creative Commons Attribution License (CC BY) which permits the unrestricted use, distribution, reproduction, adaptation and commercial exploitation of the article in any medium. No permission is required to use the article in this way provided that the article is properly cited and other license terms are observed. A small number of Wiley Open Access journals have retained the Creative Commons Attribution Non Commercial License (CC BY-NC), which permits use, distribution and reproduction in any medium, provided the original work is properly cited and is not used for commercial purposes.

Online Open articles - Authors selecting Online Open are, unless particular exceptions apply, offered a choice of Creative Commons licenses. They may therefore select from the CC BY, the CC BY-NC and the Attribution-NoDerivatives (CC BY-NC-ND). The CC BY-NC-ND is more restrictive than the CC BY-NC as it does not permit adaptations or modifications without rights holder consent.

Wiley Open Access articles are protected by copyright and are posted to repositories and websites in accordance with the terms of the applicable Creative Commons license referenced on the article. At the time of deposit, Wiley Open Access articles include all changes made during peer review, copyediting, and publishing. Repositories and websites that host the article are responsible for incorporating any publisher-supplied amendments or retractions issued subsequently. Wiley Open Access articles are also available without charge on Wiley's publishing platform, Wiley Online Library or any successor sites.

Conditions applicable to all Wiley Open Access articles:

- The authors' moral rights must not be compromised. These rights include the right of "paternity" (also known as "attribution" - the right for the author to be identified as such) and "integrity" (the right for the author not to have the work altered in such a way that the author's reputation or integrity may be damaged).
- Where content in the article is identified as belonging to a third party, it is the obligation of the user to ensure that any reuse complies with the copyright policies of the owner of that content.

- If article content is copied, downloaded or otherwise reused for research and other purposes as permitted, a link to the appropriate bibliographic citation (authors, journal, article title, volume, issue, page numbers, DOI and the link to the definitive published version on Wiley Online Library) should be maintained. Copyright notices and disclaimers must not be deleted.
  - Creative Commons licenses are copyright licenses and do not confer any other rights, including but not limited to trademark or patent rights.

- Any translations, for which a prior translation agreement with Wiley has not been agreed, must prominently display the statement: "This is an unofficial translation of an article that appeared in a Wiley publication. The publisher has not endorsed this translation."

**Conditions applicable to non-commercial licenses (CC BY-NC and CC BY-NC-ND)**

For non-commercial and non-promotional purposes individual non-commercial users may access, download, copy, display and redistribute colleagues Wiley Open Access articles. In addition, articles adopting the CC BY-NC may be adapted, translated, and text- and data-mined subject to the conditions above.

**Use by commercial "for-profit" organizations**

Use of non-commercial Wiley Open Access articles for commercial, promotional, or marketing purposes requires further explicit permission from Wiley and will be subject to a fee. Commercial purposes include:

- Copying or downloading of articles, or linking to such articles for further redistribution, sale or licensing;

- Copying, downloading or posting by a site or service that incorporates advertising with such content;

- The inclusion or incorporation of article content in other works or services (other than normal quotations with an appropriate citation) that is then available for sale or licensing, for a fee (for example, a compilation produced for marketing purposes, inclusion in a sales pack)

- Use of article content (other than normal quotations with appropriate citation) by for-profit organizations for promotional purposes

- Linking to article content in e-mails redistributed for promotional, marketing or educational purposes;
Use for the purposes of monetary reward by means of sale, resale, license, loan, transfer or other form of commercial exploitation such as marketing products

Print reprints of Wiley Open Access articles can be purchased from: corporatesales@wiley.com

The modification or adaptation for any purpose of an article referencing the CC BY-NC-ND License requires consent which can be requested from RightsLink@wiley.com.

Other Terms and Conditions:

BY CLICKING ON THE "I AGREE..." BOX, YOU ACKNOWLEDGE THAT YOU HAVE READ AND FULLY UNDERSTAND EACH OF THE SECTIONS OF AND PROVISIONS SET FORTH IN THIS AGREEMENT AND THAT YOU ARE IN AGREEMENT WITH AND ARE WILLING TO ACCEPT ALL OF YOUR OBLIGATIONS AS SET FORTH IN THIS AGREEMENT.

v1.8

If you would like to pay for this license now, please remit this license along with your payment made payable to "COPYRIGHT CLEARANCE CENTER" otherwise you will be invoiced within 48 hours of the license date. Payment should be in the form of a check or money order referencing your account number and this invoice number RLNK501183111. Once you receive your invoice for this order, you may pay your invoice by credit card. Please follow instructions provided at that time.

Make Payment To:
Copyright Clearance Center
Dept 001
P.O. Box 843006
Boston, MA 02284-3006

For suggestions or comments regarding this order, contact RightsLink Customer Support: customercare@copyright.com or +1-877-622-5543 (toll free in the US) or +1-978-646-2777.

Gratis licenses (referencing $0 in the Total field) are free. Please retain this printable license for your reference. No payment is required.
Rights retained by journal authors

When the author signs the exclusive Licence to Publish for a journal article, he/she retains certain rights that may be exercised without reference to the RSC. He/she may:

- Reproduce/republish portions of the article (including the abstract)
- Photocopy the article and distribute such photocopies and distribute copies of the PDF of the article that the RSC makes available to the corresponding author of the article upon publication of the article for personal or professional use only, provided that any such copies are not offered for sale.

Persons who receive or access the PDF mentioned above must be notified that this may not be further made available or distributed.

- Adapt the article and reproduce adaptations of the article for any purpose other than the commercial exploitation of a work similar to the original
- Reproduce, perform, transmit and otherwise communicate the article to the public in spoken presentations (including those which are accompanied by visual material such as slides, overheads and computer projections)

The author(s) must submit a written request to the RSC for any other use than those specified above. All cases of republication/reproduction must be accompanied by an acknowledgement of first publication of the Work by the RSC. The acknowledgement depends on the journal in which the article was published.

- For New Journal of Chemistry the acknowledgement is:
  - [Original citation] - Reproduced by permission of The Royal Society of Chemistry (RSC) on behalf of the Centre National de la Recherche Scientifique (CNRS) and the RSC

- For PCCP the acknowledgement is:
  - [Original citation] - Reproduced by permission of the PCCP Owner Societies

- For Photochemical & Photobiological Sciences the acknowledgement is:
  - [Original citation] - Reproduced by permission of The Royal Society of Chemistry (RSC) on behalf of the European Society for Photobiology, the European Photochemistry Association, and RSC

- For all other journals the acknowledgement is:
  - [Original citation] - Reproduced by permission of The Royal Society of Chemistry (RSC)

The acknowledgement should also include a hyperlink to the article on the RSC website.

The author also has some rights concerning the deposition of the whole article, details of which are given on the Author Deposition pages.

Contact and Further Information

Gill Cockhead
Contracts & Copyright Executive
Tel: +44(0) 1223 432 134
Email: Gill Cockhead

© Royal Society of Chemistry 2014