Investigating Type I Collagen Self-Assembly Processes and End Products

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
Department of Chemistry
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Abstract

Segmental long spacing (SLS) collagen self-assembly was studied by analyzing aggregates formed from different nucleoside triphosphates at various protonation stages. Triple-negatively charged triphosphate groups were determined to be critical for SLS assembly, electrostatically bridging basic residues between collagen monomers. In the second part of this thesis, the nominal elastic modulus for each of the three forms of Type I collagen aggregate was measured and compared. Fibrous long spacing collagen, often associated with diseased tissues, exhibited lower stiffness in comparison to the other forms, native and SLS, suggesting decreased structural stability in diseased tissues. In the last section, a unidirectional pattern of native fibrils was assembled using mica as a template; the ability to customize and change the surface morphology was also demonstrated. For the first time, collagen monomers deposited on the mica were demonstrated to gain lateral mobility.
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<tbody>
<tr>
<td>AFM</td>
<td>Atomic force microscope/microscopy</td>
</tr>
<tr>
<td>Al</td>
<td>Aluminum</td>
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<tr>
<td>Arg</td>
<td>Arginine</td>
</tr>
<tr>
<td>Asp</td>
<td>Aspartic acid</td>
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<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
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<td>CTP</td>
<td>Cytidine triphosphate</td>
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<tr>
<td>dTTP</td>
<td>Deoxythymidine triphosphate</td>
</tr>
<tr>
<td>EM</td>
<td>Electron microscopy</td>
</tr>
<tr>
<td>FLS</td>
<td>Fibrous long spacing (collagen)</td>
</tr>
<tr>
<td>Glu</td>
<td>Glutamic acid</td>
</tr>
<tr>
<td>Gly</td>
<td>Glycine</td>
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<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
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<tr>
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<td>Potassium chloride</td>
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<td>Lysine</td>
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<td>Molecular mechanics</td>
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<td>NaCl</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
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<tr>
<td>Si</td>
<td>Silicon</td>
</tr>
<tr>
<td>SLS</td>
<td>Segmental long spacing (collagen)</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
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TPP  (Sodium) tripolyphosphate
UTP  Uridine triphosphate
Chapter 1

1 Introduction

1.1 Collagen self-assembly in vitro

Collagen is the most abundant structural protein in mammalian tissue and can be found in virtually all tissues. Despite the large amount of research on the different forms of collagen, the self-assembly mechanisms are still poorly understood. This thesis is concerned with further studying the aggregation of collagen monomers to form higher-order structures. In particular, how minor modifications in the self-assembly conditions result in distinct monomer alignment and final ordered aggregate formation. In this thesis, attention has been focused on the aggregation of Type I collagen, the most abundant and well-studied form.

Collagen monomers are the building blocks of higher order aggregates. Such self-assembly of distinct higher-order collagen aggregates occurs in vitro as well as in vivo, with final products virtually indistinguishable from each other.

The monomer is a triple helix of three polypeptide chains, with the amino acid sequence having a repeating pattern of Gly-X-Y. The X and Y amino acids point outwards, and are often Pro and Hyp residues. Some X and Y amino acids consist of charged amino acids, resulting in localized charges along the length of the monomer. In its final form, the monomer is semi-flexible (Shattuck et al., 1994) and is ~ 280 – 300 nm in length and ~ 15 nm in diameter (Boedtker and Doty, 1956).

The “normal” collagen aggregate found throughout the body is the native collagen, characterized by ~ 67 nm banding periodicity, with this distance termed a “D-period”. Current
models describe a one D-period stagger among monomers in the native fibril, a concept first introduced in the early 1960’s (Hodge and Petruska, 1963). *In vitro*, native collagen is assembled at pH ~ 7 and 37 °C, with initial end-to-end axial association of monomers followed by lateral association to form mature fibrils with D-periodic banding (Gale et al., 1995). The assembly of native collagen is dependent on pH, temperature and ionic strength, with a variance in any of the three parameters resulting in partial or absence of D-periodic banding (Kil, 2007). Of particular relevance to this thesis, Silver et al. discussed the *in vitro* assembly of native collagen as well as its mechanical function (Silver et al., 2003). Such topics will be further discussed throughout this thesis.

The two other forms of higher-order aggregates for Type I collagen are segmental long spacing (SLS) collagen and fibrous long spacing (FLS) collagen. SLS aggregates have lengths similar to that of a monomer, suggesting lateral alignment of monomers. This assembly occurs *in vitro* under acidic conditions in the presence of adenosine triphosphate (ATP). SLS collagen will be discussed in detail in Chapter 2, and readers may also consult an excellent review paper by Kuhn for SLS collagen in the literature (Kuhn, 1982). FLS collagen was first described in 1950 (Highberger et al., 1950) and is often associated with diseased tissues. FLS assembled with α1-acid-glycoprotein *in vitro* is characterized by a ~ 250 nm banding along the fibril. FLS collagen will be further discussed in Chapter 3. However, readers may consult numerous FLS articles from the Goh Research Group for detailed background on the subject (Paige et al., 1998; Lin and Goh, 2002; Rainey et al., 2002).
1.2 Thesis Overview

As discussed, SLS collagen is characterized by a lateral alignment of monomers, resulting in a similar length to that of a collagen monomer, \(~300\text{ nm}\). SLS is known to assemble in acidic conditions in the presence ATP molecules, where the ATP is thought to be integrated directly into the SLS aggregate. In Chapter 2, experimental protocols with several triphosphate polyanions at various protonation states were used to shed light on the role of polyanions in SLS self-assembly. Combined with theoretical simulations data in previous works (Anderson, 2005), the interaction between collagen and polyanions in SLS assembly will be described.

Collagen is the most abundant structural protein in mammalian tissue. As such, understanding the mechanical properties of collagen is of enormous importance. Although much work have been done on studying the mechanical strength of the most physiologically significant aggregate, native collagen, information is lacking for the other two form of Type I collagen aggregates: SLS and FLS. In particular, FLS collagen is often associated with diseased tissues, thus understanding the mechanical changes in such diseased tissue has vast implications for the treatment of associated symptoms. In Chapter 3, the mechanical properties of the three types of Type I collagen aggregates are measured via nanoindentation by atomic force microscopy (AFM). Results for native collagen will be compared to values in the literature. Furthermore, differences in mechanical properties among the three aggregates will be discussed as well.

In Chapter 4, a procedure will be described where a mica surface was used as a template for ordered collagen assembly. A uniform film of discrete banded native fibrils will be described, and has great potential in applications for surface coating as well as research in cell mobility and proliferation. For the first time, collagen monomers on the mica surface will be demonstrated to gain mobility under favourable collagen self-assembly conditions. Furthermore, this study
introduces a novel method that may be used for controlling, customizing, and changing collagen surface morphology.
1.3 References


Chapter 2

2 Segmental Long Spacing Collagen Self-Assembly

2.1 Segmental long spacing collagen

As introduced in Chapter 1, Segmental Long Spacing (SLS) collagen is one of the three forms of collagen aggregates that can be self-assembled in vitro from Type I collagen monomers (Figure 2.1). SLS collagen was first described in 1953 using transmission electron microscopy (TEM; Schmitt et al., 1953). It was characterized by a block like structure with approximate same length as a collagen monomer: ~ 280 nm. In EM images, defined banding patterns perpendicular to the length of a SLS aggregate were observed to be closely correlated to positively charged regions in the known amino acid sequence of a collagen monomer (Von der Mark et al. 1971). In positively stained SLS EM images, the dark bands correspond to charged, polar amino acids, and in negatively stained samples, the light bands correspond to hydrophobic amino acids. For the above reasons, it is now generally accepted that SLS aggregates consist of monomers aligned laterally with the same polarity.

Due to this parallel alignment of monomers, and thus the alignment of amino acid residues, SLS collagen has been widely utilized as a tool for analyzing amino acid residues in monomers. It was used to determine the sequences of Type III and IV collagen (Wiedemann et al., 1975; Stark et al., 1971), compare the differences among collagen types (Timpl et al., 1978), study the precursor of collagen, procollagen (Hoffmann et al., 1976), and show homology of amino acid sequence among vertebrates (Gosh et al., 1976).
To this day, there is no known direct observation of SLS collagen in vivo. However, SLS aggregates were observed in the culture medium of fibroblasts and chondrocytes from various connective tissues that actively synthesize collagen (Bruns et al., 1979). As a result, it is now a common hypothesis that SLS collagen acts as a form of compact collagen storage and/or transport in and out of the cell.

In acidic conditions in vitro, ATP is known to induce self-assembly of SLS collagen (Schmitt et al., 1953). The negatively charged ATP molecules were hypothesized to act as a neutralization agent of electrostatic repulsion between similarly charged regions of monomers aligned in-register. However, in addition to the charge, the structure of polyanions was found to be critical as well (Paige and Goh, 2001). In particular, adenosine diphosphate (ADP) failed to assemble SLS aggregates, whereas the non-hydrolyzable ATP analogue, ATP-γ-S, successfully assembled SLS collagen. These two observations have two important implications: the assembly requires presence of positive charges in three phosphate groups, and that assembly is not activated by hydrolysis of a phosphate bond as in the assembly of actin filaments. Furthermore, successful assembly of SLS collagen by guanosine 5’triphosphate (GTP) and failure of other triply charged polyanions (cibacron blue 3GA and amaranth) showed that while small changes in the nitrogenous base was tolerable for SLS assembly, specific arrangement of the three negatively charged groups was necessary (Paige and Goh, 2001).

In molecular mechanics (MM) and molecular dynamics (MD) studies, the theoretical interaction between ATP and collagen monomers was simulated (Anderson, 2005). The α and γ phosphates on ATP were shown to bridge repulsive basic residues from two adjacent collagen monomers. In the case of colocalized acidic and basic residues in the collagen monomer, the
positively charged adenine ring was shown to screen the negatively charged acidic residues from electrostatic repulsion between the two monomers.

In this chapter, experimental investigations into the formation of SLS collagen by different types of nucleotide triphosphates (NTP) are discussed. This includes ATP, GTP, cytidine triphosphate (CTP), uridine triphosphate (UTP), deoxythymidine triphosphate (dTTP), and tripolyphosphate (TPP). Topics explored include the different pH ranges under which SLS assembles with different NTPs, the protonation states of the collagen monomer and each polyanion, and recommendations for the improvement of previously reported MM and MD simulations.

2.2 Protonation

2.2.1 Amino acid residues in collagen monomer

Amino acid residues in the collagen monomer undergo protonation at a wide range of pH. Of particular interest are charged species that undergo protonation at the SLS collagen relevant acidic pH range (Table 2.1 and Figure 2.2). At such pH range, all three basic residues (Arg, His, and Lys) are positively charged and do not undergo protonation. However, the acidic residues (Asp and Glu) undergo protonation from a negatively charged residue to neutral when pH drops below ~ 4. Many of such acidic residues are colocalized with basic residues in the collagen monomer, thus it may be reasonable to assume that the effective pKa deviates slightly from 4. Furthermore, pKa values of residues in proteins are acknowledged to vary widely depending on the chemical environment, thus additional error must be considered for the protonation pH of acidic residues.

Contrary to MM and MD conditions in previous simulation studies (Anderson, 2005), this study postulates that acidic residues are neutral in SLS assembly. With the absence of
positive charges in NTP protonation species above pH 2 – 4.6 (varying for each NTP), the negatively charged acidic residues throughout the collagen monomer may be a hindrance for SLS collagen assembly. In combination with the fact that native collagen may begin to form at pH above 4, this study proposes that SLS collagen assembly only occurs with pH below 4, where Glu and Asp residues are protonated and therefore neutral.

2.2.2 Polyanions

Similar to amino acids discussed in section 2.2.1, polyanions in this study also undergo protonation at the SLS collagen relevant acidic pH range. Based on the protonation state(s) of each polyanion in successful SLS assembly, we can determine critical sites of collagen-polyanion interaction as well as evaluate the proposed SLS collagen assembly mechanism as discussed in section 2.1.

The protonation sites for each NTP and TPP are similar, but their pKa values differ (Table 2.1; Sigel et al., 1987, 2001, 2005; Tribolet et al., 1985, 1988; Watters et al., 1956). In all cases, the first proton is released from one of the three primary sites (at equilibrium with each other) of the two-fold protonated triphosphate group (pH 1 – 2). After this first deprotonation, the triphosphate group becomes triple-negatively charged. At slightly higher pH (pH 3 – 4.6), the second proton is released from the base of the NTP, with the exception of UTP and dTTP. After this second deprotonation, the triphosphate group retains the three negative charges while the positively charged ATP, GTP, and CTP base become neutral (Table 2.1 and Figures 2.2 to 2.8).

Protonation pKa values are expressed in the form $pK_{H_3ATP^-}^H = 1.7$, where the pKa value of 1.7 indicates the pH at which half the species have the protonation form, $H_3ATP^-$, and half the species have the deprotonated form $H_2ATP^{2-}$. In other words, at pH below 1.7, the ATP
molecules (H₃ATP⁻) largely have three protons and an overall single negative charge (for ATP: 2 negative charges and 1 positive charge). At pH above 1.7, the ATP molecules (H₂ATP²⁻) largely have two protons and an overall double negative charge (for ATP: 3 negative charges and 1 positive charge).

Figure 2.3 illustrates the protonation states of ATP at various pH. From H₃ATP⁻, the first deprotonation occurs at the triphosphate group, at \( pK_{H₃ATP⁻}^H = 1.7 \). From H₂ATP²⁻, the second deprotonation occurs at the adenine ring, at \( pK_{H₂ATP²⁻}^H = 4.0 \). GTP, also a purine NTP as ATP, undergoes the first deprotonation (from the triphosphate group) at \( pK_{H₃GTP⁻}^H = 1.3 \) and the second deprotonation (from the guanine ring) at \( pK_{H₂GTP²⁻}^H = 2.9 \) (Figure 2.4). Analogous to the purine NTPs, CTP, a pyrimidine NTP, undergoes deprotonation at \( pK_{H₃CTP⁻}^H = 1.7 \) and \( pK_{H₂CTP²⁻}^H = 4.6 \) (Figure 2.5). The other pyrimidine NTPs, UTP (Figure 2.6) and dTTP (Figure 2.7), however, is unique from other NTPs. Neither UTP nor TTP accept protons at the pyrimidine ring sites, as such, deprotonation only occurs at the triphosphate group. Therefore, while H₂ATP²⁻, H₂GTP²⁻, and H₂CTP²⁻ refer to the protonation state of the NTPs with triply negative triphosphate group and neutral base, it is HUTP³⁻ and HdTTP³⁻ that describe this state. With this in mind, the first deprotonation at the phosphate group occurs at \( pK_{H₃UTP⁰⁻}^H = pK_{H₂dTTP⁻⁺}^H = 2.0 \). Similar to UTP and dTTP, TPP (Figure 2.8) has no base group for protonation; all deprotonations occur at the triphosphate group with the triple-negatively charged state present after the first deprotonation, with two protons remaining. The first deprotonation occurs from H₃TPP²⁻, at \( pK_{H₃TPP⁻}^H = 1.1 \), and the second deprotonation occurs at \( pK_{H₂TPP²⁻}^H = 2.3 \).
For all polyanions, the third deprotonation (second for UTP and dTTP) occurs at the triphosphate group, resulting in four negative charges in the triphosphate group. This occurs at pH (pKa) ~ 6.5 for all NTP and pH (pKa) = 6.3 for TPP. As SLS assembly occurs in the acidic range, this third deprotonation is largely unrelated to this study.

As mentioned in section 2.2.1, negatively charged acidic residues in the collagen monomer may hinder SLS assembly, thus SLS collagen is hypothesized to only assemble when such negatively charged residues are neutral at pH below 4. Furthermore, the proposed mechanism describes a bridging mechanism by triple-negatively charged polyanions between adjacent collagen monomers. Based on the above, the study hypothesizes that SLS assembly occurs only when the triphosphate group is triple-negatively charged and below pH of 4 (Figure 2.2).

As a result of the hypothesis, SLS collagen assembly with ATP would occur only at pH above $pK_{H,ATP}^H = 1.7$ and below $pK_{Asp&Glu} = 4.0$, where the triphosphate group is triple-negatively charged and the adenine ring is single-positively charged.

GTP gains the state of triple-negatively charged triphosphate group above $pK_{H,GTP}^H = 1.3$, and the guanine ring becomes neutral above $pK_{H,GTP} = 2.9$. Therefore, according to this hypothesis, there are two possible states of GTP in SLS assembly: triple-negatively charged triphosphate group and single-positively charged guanine ring between $pK_{H,GTP} = 1.3$ and $pK_{H,GTP} = 2.9$, and triple-negatively charged triphosphate group and neutral guanine ring between $pK_{H,GTP} = 2.9$ and $pK_{Asp&Glu} = 4.0$. 
CTP is similar to ATP with hypothetical assembly occurring between \( pK^H_{H_2CTP^-} = 1.7 \) and \( pK_{\text{App} & \text{Glu}} = 4.0 \), with the CTP protonation species having a triple-negatively charged triphosphate group and single-positively charged cytosine ring.

UTP and dTTP hypothesized assembly would occur between \( pK^H_{H_2UTP^-} = pK^H_{H_2dTTP^-} = 2.0 \) and \( pK_{\text{App} & \text{Glu}} = 4.0 \), with a triple-negatively charged triphosphate group and neutral base.

Lastly, TPP hypothesized assembly would occur between \( pK^H_{H_2TPP^-} = 1.1 \) and \( pK_{H_2TPP^-} = 2.3 \), where the triphosphate group is triple-negatively charged. Furthermore, this study proposes that the \( pK^H_{H_2TPP^-} = 2.3 \) to \( pK_{\text{App} & \text{Glu}} = 4.0 \) range might also allow for SLS assembly where the triphosphate group has four negative charges.

In summary, SLS collagen assembly should exclusively occur at the following protonation species, pH range, triphosphate charge, and base charge:

- \( H_2ATP^-: \) pH 1.7 – 4.0 (3-, 1+)
- \( H_2GTP^-: \) pH 1.3 – 2.9 (3-, 1+) and HGTP\(^3\^-: \) pH 2.9 – 4.0 (3-, 0)
- \( H_2CTP^-: \) pH 1.7 – 4.6 (3-, 1+)
- \( HUTP^3^-: \) pH 2.0 – 6.5 (3-, 0)
- \( HdTTP^3^-: \) pH 2.0 – 6.5 (3-, 0)
- \( H_2TPP^3^-: \) pH 1.7 – 2.3 (3-, n/a) and HTPP\(^4^-: \) pH 2.3 – 4.0 (4-, n/a)
2.3 Materials and methods

2.3.1 *In vitro SLS collagen assembly*

PureCol collagen monomer solution (3 mg/mL in 12 M HCl, Lot #: 1629346, Advanced BioMatrix) was used directly from the stock bottle. Solutions of polyanions at 10 mg/mL were prepared by dissolving the solid (ATP from RBI; GTP from BioShop; CTP, UTP, dTTP and TPP from Sigma) in 0.05% v/v acetic acid (BDH).

In a centrifuge tube, 40 µL of polyanion solution was combined with (127 minus x) µL of 0.05% v/v acetic acid and (x) µL of 5%, 10% or 33% v/v acetic acid or 0.1 M sodium hydroxide (VWR), then briefly vortexed. A 33 µL aliquot of the PureCol solution was then added to the mixture and again briefly vortexed. The final volume was 200 µL, yielding final collagen concentration of 0.5 mg/mL and final polyanion concentration of 2 mg/mL. Concentration of acetic acid and sodium hydroxide were varied depending on “x”, with varied volume (0 – 127 µL) and solution (acetic acid at different concentration and sodium hydroxide) to control the pH conditions of assembly. The solution pH was measured by narrow range pH paper (MicroFine pH 1.4 to 2.8 and 2.9 to 5.2, Hydrion) and incubated at room temperature for two hours.

For typical SLS collagen assembly with ATP, 122 µL of 0.05% v/v acetic acid and 5 µL of 5% v/v acetic acid were used. Final pH as measured by the pH paper was ~ 3.3.

After incubation, samples were deposited onto freshly cleaved mica (~ 1 x 1 cm²) in 20 µL aliquots, and allowed to incubate for ~ 30 seconds without the sample drying out. Samples were then rinsed with deionized water and dried with a gentle stream of nitrogen gas prior to imaging by AFM.
2.3.2 Atomic force microscopy

Since the invention of AFM by Binnig et al. in 1986, it has become a common tool for studying surface morphology at the nano and micro scales. Of particular use for our biological samples, AFM utilizes the forces between the probe and surface to generate a topographical image of the sample. While atomic resolution is possible for AFM at low temperatures, the resolution required for this study allow for imaging in air in ambient temperatures, minimizing perturbations to collagen in sample preparation and imaging.

AFM was chosen over optical and electron microscopy for its resolution and sample preparation techniques. While optical microscopy can be used to measure sample dimensions in the x-y plane, it is difficult to measure samples in the z-dimension. Furthermore, our samples are near the diffraction limit of light, making the higher resolution in AFM an obvious advantage. EM achieves similar resolution as AFM. However, EM requires staining of collagen for proper imaging, potentially introducing unpredictable effects from sample-stain interactions. Furthermore, AFM can be performed in ambient conditions rather than vacuum conditions in EM, making it a more suitable choice for such biological samples.

The imaging mode used in this chapter was intermittent contact (tapping) mode in air, where the probe is driven to vibrate near its resonance frequency. The amplitude of the vibration is kept constant by moving the entire probe up and down relative to the sample surface, thus producing a topographical image of the surface. Because the probe is only intermittently in contact with the surface, it lessens the damage done to the sample for softer materials.

Samples were imaged with a JPK NanoWizard II AFM (JPK Instruments, Berlin, Germany) with silicon probes having typical spring constants between 40 and 50 N/m (NCH
probes, Nanoworld Innovative Technologies, Neuchâtel, Switzerland). All imaging was performed in air, showing reproducible results with minimal sample perturbation. Height and lock-in amplitude images were taken simultaneously, with z-scale measurement and cross sections extracted from height images only.

In summary, AFM was chosen as an imaging tool for its resolution, ambient condition operation, minimal sample perturbation, and ease of sample preparation. For further technical details in AFM, readers are advised to consult with the numerous review articles available, including one excellent and relevant article published by Gould et al. (Gould et al., 1990).

2.4 Results and discussion

2.4.1 Characterization of SLS aggregates

Successful SLS collagen assembly resulted in a clear solution with minimal turbidity after two hours of incubation. The most consistent SLS collagen assembly with ATP was found to be at pH 3.3, with 100% reproducibility. After two hours of incubation, analysis of the mixture by AFM revealed an abundance of SLS aggregates. More than 30 SLS aggregates can typically be found in any given 10 x 10 µm² AFM scan (Figure 2.9).

With a scan size of 1 x 1 µm² with 512 x 512 pixel density, the height of an SLS aggregate can be measured via cross sectional analysis (Figure 2.10). The length of a SLS crystallite was consistently observed to be ~ 300 nm, roughly corresponding to the length of a collagen monomer (~ 280 nm) plus AFM probe geometry. The height of a SLS crystallite was found to correlate with the width, and was chosen to describe the size of the aggregate because of the more reliable z-axis measurement by an AFM than in the x-y dimension due to probe geometry. For the remainder of this chapter, the “size” or “height” of a SLS aggregate will refer
to the maximum height as measured in a cross section along the length of the aggregate (Figure 2.10 c).

The height of SLS aggregates was consistently observed to be shorter than the width. This was likely the result of electrostatic attraction between the aggregate and the negatively charged mica substrate, causing the hypothesized cylindrical aggregates in solution to spread out on the mica to maximize surface contact area. As seen in Figure 2.11 c, instead of a rectangular longitudinal cross-section, we often observed pronounced features at the ends of the aggregate. This consistent observation could be attributed to the folding in the ends of the collagen monomers or a build up of negatively charged ATP in the positively charged end regions of the monomers (Veis and George, 1994).

Mature SLS aggregates have typical heights of 30 – 50 nm. For the purpose of this chapter, a height of 25 nm is defined as the minimum for “maturity”.

2.4.2 Incubation time

A sample of collagen with ATP at pH 3.3 was allowed to incubate for 50 hours with aliquots taken for imaging at 2, 5, 6, and 50 hours. Figures 2.10, 2.11, 2.12, and 2.13 showed negligible change in SLS aggregate heights beyond two hours of incubation. Therefore, two hours was set as the standard for SLS incubation time as described in section 2.2.1.

2.4.3 SLS assembly with various polyanions

2.4.3.1 SLS with ATP

Mature SLS assembly was found to have high dependence on pH, with consistent results observed where final collagen and polyanion concentrations were at or above 0.5 mg/mL and 2
mg/mL respectively. As discussed in section 2.4.1, the ideal pH range for ATP was found to be at 3.3, resulting in consistent self-assembly of mature SLS aggregates (Figure 2.10).

Systematic investigation of SLS assembly at pH steps of 0.1 to 0.3 was carried out, with representative images selected at pH 2.9, 3.3 (ideal), 3.8, and 4.4 (Figure 2.14). Below pH 2.9, no collagen aggregates were observed in the sample solution. At pH 2.9 – 3.2, discrete mature SLS aggregates were observed at two hours, with decreasing amount of thinner discrete immature aggregates also present in the samples. At pH 3.3, sample solution at two hours yielded discrete mature aggregates. At pH 3.8, clumps of mature SLS aggregates were observed, with few discrete SLS aggregates visible. While no SLS collagen was observed above pH 4.1, there was an increasing population of native collagen fibrils, characterized by a ~ 67 nm banding periodicity along the fibrils. For SLS collagen assembly with ATP, a trend with increasing pH from below 2.9 to above 4.1 was observed: no SLS aggregation (pH < 2.9); discrete immature (thin) aggregates (pH 2.9 – 3.1); discrete mature aggregates (pH 3.3); clumps of mature aggregates (pH 3.5 – 4.1); no SLS aggregation and native collagen assembly (pH > 4.1).

At the lower pH range of SLS assembly, thin immature aggregates were observed. Such fibrils did not grow in size with increased incubation time, suggesting low bridging capacity of triple-negatively charged triphosphate group between adjacent collagen monomers. This is likely due to an increasing population of ATP gaining a proton as the pH approaches $pK_{H\text{,ATP}}^H = 1.7$, resulting in ATP molecules with double-negatively charged triphosphate groups. Also, due to the in-register collagen monomer alignment, like charges are aligned with each other, creating electrostatic repulsion. While the polyanions bridge some of such like-charge repulsion between basic residues, the remaining basic residues and all acidic residues are not shielded. As the size
of SLS aggregates increase, electrostatic repulsion among like charges, bridging capacity of polyanions, and surface energy reach equilibrium, capping the size of SLS aggregates.

At the upper pH range, mature aggregates form clusters of relatively large masses (Figure 2.14 d). This is attributed to increased bridging capacity between ATP and collagen, resulting in aggregation among SLS collagen aggregates. The analysis of such forces is outside the scope of the current study and will be carried on in future studies.

As proposed in section 2.2.2, SLS assembly with ATP occurs only with neutral acidic amino acids and triple-negatively charged triphosphate groups in ATP. The pH for such conditions were between $pK_{H_ATP}^{\text{ill}} = 1.7$ and $pK_{\text{Asp\&Glu}} = 4.0$. Therefore, the successful experimental SLS assembly pH range of 2.9 to 4.1 satisfied such two conditions. The higher end of the pH range (4.1) was above the theoretical acidic residue protonation pKa of 3.86 and 4.07 for Asp and Glu respectively, where clumps of SLS collagen aggregate were observed. However, pKa only indicates the pH where 50% of the species are protonated. Together with the previously discussed effect of acidic-basic residue colocalization and other environmental factors, this deviation is negligible.

In summary, ATP assembly of SLS collagen occurred only in a pH range (2.9 – 4.1) where Asp and Glu residues on the collagen monomer were largely neutral. Furthermore, the only protonation species for ATP that successfully bridged collagen monomers was $H_2\text{ATP}^{2-}$ where the triphosphate group was triple-negatively charged and the adenine ring was single-positively charged. Such experimental results agreed with the hypothesis as well as previously reported MM and MD simulations of collagen-ATP interaction (Anderson, 2005).
2.4.3.2 SLS with GTP

SLS collagen assembly with GTP was found to be successful, in agreement with previously reported results (Paige and Goh, 2001). The optimal assembly conditions was found to be at pH 2.3, forming mature SLS aggregates at 2-hour incubation (Figure 2.15). Similar to ATP-SLS assembly, systematic assembly conditions were carried out at 0.1 to 0.3 pH intervals, with representative results shown in Figure 2.16.

At pH 1.7, thin deformed aggregates of ~ 300 nm in length were observed, suggesting immature SLS aggregation occurred with minimally satisfied GTP-collagen bridging affinity. This was consistent with observations in ATP-SLS assembly where thin immature SLS aggregates (height of ~ 5 nm) were observed at the lower pH range of SLS assembly conditions. Above pH 1.7, average SLS aggregate size was observed to increase with increasing pH until pH ~ 2.3. At such optimal pH for GTP-SLS assembly, mature aggregates with heights of 25 nm or more were observed. A further increase in pH conditions to 2.5 showed increasing clumping of mature SLS aggregates similar to the effect observed in ATP-SLS samples at pH ~ 3.8. Between pH 2.5 and 2.9, no collagen aggregation was observed. At pH 2.9 and above, GTP-SLS aggregation was observed to yield unbanded fibrils, an expected indication of increasing favourable environment for normal native collagen self-assembly. As such, the pH range for successful GTP-SLS assembly was found to be 1.7 – 2.5 with optimal conditions at pH 2.3.

The successful assembly of SLS with GTP shows that while the triphosphate group is important for SLS assembly, small changes in the purine ring had little effect on NTP-collagen interaction. Specifically, the guanine ring from GTP possesses a carbonyl group that is absent in the adenine ring from ATP. Therefore, the carbonyl group was determined to be insignificant in SLS assembly.
Described in section 2.2.2, GTP-SLS was hypothesized to assemble between $pK_{H,GTP}^{H} = 1.3$ and $pK_{H,GTP}^{H-} = 2.9$ for the $\text{H}_2\text{GTP}^{2-}$ species while also assembling between $pK_{H,GTP}^{H-} = 2.9$ and $pK_{\text{A}_{\text{Glu}}^{H}} = 4.0$ for the $\text{HGTP}^{3-}$ species. With the successful experimental assembly pH range between 1.7 and 2.5, this hypothesis was disproven. The two GTP protonation species differ in the protonation state of the guanine ring, with $\text{H}_2\text{GTP}^{2-}$ having a single positive charge and $\text{HGTP}^{3-}$ being neutral in the guanine ring. The result that GTP with a positively charged base successfully formed SLS aggregates while GTP with a neutral base failed was unexpected given the proposed hypothesis that acidic amino acid side chains were neutral rather than negatively charged. It was surprising to learn that a neutral charge at the guanine ring possibly hinders SLS assembly compared to a positively charged base group. Nevertheless, this observation was consistent with ATP-SLS where $\text{H}_2\text{ATP}^{2-}$ with a positively charged base group yielded full SLS assembly. Assembly did not occur for ATP-SLS with a neutral ATP base. However, that might be due several reasons. Firstly, pH of above 4 approached conditions favourable for native collagen assembly. Secondly, the acidic residues on the collagen monomer became negatively charged above pH of $\sim 4$, creating additional electrostatic repulsion among in-register aligned monomers. Lastly, it might be possible that ATP with neutral adenine may be ineffective for bridging collagen monomers. However, the latter could not be experimentally proven without separation from the first two. With an unclear role of a charge in the base of the polyanion, the effects of positively charged base (CTP), neutral base (UTP and dTTP), and no base at all (TPP) will be further explored in the following sections.

In summary, GTP-SLS assembly was successful between pH 1.7 – 2.5, with optimal pH at 2.3. The positive charge in the guanine ring was found to be significant in GTP-SLS assembly, while the carbonyl group in the guanine ring was not. Furthermore, the three negative charges in
the triphosphate group were found to be necessary for SLS assembly, affirning the proposed bridging mechanism of nucleoside triphosphates.

2.4.3.3 SLS with CTP

CTP, a nucleoside triphosphate with a cytosine base, is similar to ATP and GTP in charge but different in structure (Figure 2.5). As discussed in section 2.2.2, $H_2CTP^{2-}$ is the protonation species with three negative charges in the phosphate group and a positive charge in the base. Similar to ATP, its protonation state with a neutral base only exists above $pK_{H_2CTP^{2-}}^H = 4.6$, falling in the region of negatively charged acidic residues in the collagen monomer. The proposed hypothesis was that CTP-SLS exclusively assembles between $pK_{H_2CTP}^H = 1.7$ and $pKa_{Asp&Glu} \approx 4.0$.

With experimental conditions similar to ATP-SLS and GTP-SLS, mature SLS assembly with CTP was unsuccessful. The optimal pH for CTP assembly was found to be near 2.1 (Figure 2.17), where thin (~ 3 – 5 nm height) SLS aggregates were observed. Representative results for assembly in other pH conditions are shown in Figure 2.18. At pH 1.6 to 2.1, CTP-SLS assembly resulted in similar aggregation as pH 2.1 samples. From pH 2.1 to 2.3, aggregates of similar size as above were observed in clusters. At pH above 2.3, no collagen aggregates were observed.

Incubation time for CTP-SLS solution at pH 2.1 was increased to up to 29 days, also at room temperature, but yielded no observable difference in assembly aggregates (Figure 2.19). Increased concentration of collagen and CTP up to three times those described in section 2.3.1 also yielded no observable improvement. Such experiments excluded the possibility of slower assembly kinetics with identical end products in comparison to ATP-SLS and GTP-SLS.
The thin malformed SLS aggregates observed in pH 2.1 were consistent with those found in the lower pH range of SLS assembly with ATP (pH ~ 2.9) and GTP (pH ~ 1.7). Mature aggregates (height > 25 nm) were found with GTP-SLS samples at pH above 2.1, therefore, the lack of mature CTP-SLS aggregate could not be solely attributed to pH-induced changes in collagen monomers. As such, CTP-SLS interaction must differ from that of other NTPs.

With lengths of ~ 300nm, the observed CTP-SLS aggregates had very pronounced ridges (~ 5 nm height) at the ends along the length of the aggregate while the middle section was dramatically thinner (~ 1 – 2 nm height). Although the location of ridges corresponded with ones observed in other SLS aggregates, the large difference in height suggests localization of many CTP molecules in the ridge region and very few in the midsections. The suspected lack of CTP in the midsection would result in relatively more electrostatic repulsion in the midsections as compared with other NTP-SLS aggregates, thus leading to smaller size capping and malformation of the aggregates. An alternative explanation for such pronounced ridges would be the folding of monomers at the ends of their lengths. Such observations and theory can be confirmed using electron spectroscopic imaging in future studies.

Clusters of aggregates at the upper pH range of assembly for ATP-SLS and GTP-SLS was also observed for CTP-SLS at the upper pH range of ~ 2.2 – 2.3. This demonstrates interaction among discrete SLS aggregates, which could be a result of CTP-collagen interaction or staggered collagen interaction observed in native collagen assembly. The determination of such interaction will be a subject for future MM and MD simulation work.

In summary, while CTP lacks the capacity to assemble fully formed mature SLS aggregates, its behaviour with changes in pH was similar to that observed in other NTP-SLS assembly. The pH range for assembly of immature CTP-SLS was found to be ~ 1.6 – 2.3. These
results demonstrated detrimental interaction with collagen when the purine base of the polyanion was replaced with cytosine, a pyrimidine base.

2.4.3.4 SLS with UTP

UTP, a pyrimidine nucleoside triphosphate, reflects a small structural change in comparison to CTP (Figure 2.6). The most significant functional difference of UTP as compared to ATP, GTP and CTP is that the uracil ring does not undergo protonation/protonation, and maintains a neutral charge at the base. This is in contrast with ATP, GTP, and CTP, with all three possessing a positively charged base group at SLS assembly pH. Section 2.2.2 showed the proposed UTP-SLS assembly pH range to be between $pK_{H,UTP}^+ = 2.0$ and $pK_{Asp,Glu} = 4.0$, where the triphosphate group is triple-negatively charged and the uracil ring is neutral.

Mature SLS assembly with UTP was found to occur at pH ~ 2.9 (Figure 2.20), with the same final morphology as those observed in successful ATP-SLS and GTP-SLS assembly. At pH 2.4 to 2.7, well-formed block-like UTP-SLS aggregates were observed, albeit with smaller sizes than those from the optimal pH of ~ 2.9 (Figure 2.21). UTP-SLS aggregates were found up to pH 3.8, with native fibrils emerging at pH above 4.0 (Figure 2.21).

The successful assembly of mature UTP-SLS demonstrates that while a positively charged guanine ring was necessary in GTP-SLS assembly, a neutral uracil ring in UTP also yielded positive results. As such, electrostatic interaction between the positively charged base ring and collagen was excluded as the sole critical stabilization factor in SLS assembly.

While CTP is structurally more similar to ATP and GTP than UTP, it yielded less successful assembly end products than UTP. Specifically, ATP, GTP, and CTP all possess a
primary amine group in their respective base rings, but UTP does not. Therefore, the primary amine group was determined to be a non-critical component of SLS assembly.

In summary, UTP was found to assemble mature SLS aggregates that were morphologically identical to those formed with ATP and GTP. The successful assembly pH range was found to be between pH 2.4 and 3.8, with optimal mature SLS assembly at pH 2.9. UTP-SLS assembly demonstrated that SLS assembly is not dependent on electrostatic interactions between collagen and the base ring of a nucleoside triphosphate. Additionally, the primary amine group in the base ring was excluded as a critical component for SLS assembly. Furthermore, UTP-SLS assembly reaffirmed the importance of three negative charges in the triphosphate group for SLS assembly.

2.4.3.5 SLS with dTTP

dTTP is structurally similar to UTP, with an additional methyl group in the thymine ring and the absence of a hydroxyl group in the deoxyribose sugar ring. As with UTP, dTTP exhibits a neutral base ring. For the above reasons, dTTP is an ideal candidate for studying the effect of the ribose sugar on SLS assembly as well as confirming the observation from UTP that the charge of the base in the polyanion is insignificant. The proposed hypothesis in section 2.2.2 predicted exclusive SLS formation in the pH range between $pK_a^{H_2dTTP^-} = 2.0$ and $pK_a^{App&Glu} \approx 4.0$, where the triphosphate group is triple-negatively charged and the base is neutral.

SLS aggregates formed with dTTP at the optimal pH range of 3.0 – 3.2 (Figure 2.22). Analogous to results observed with UTP-SLS, smaller and malformed SLS aggregates were observed down to a pH minimum of 1.7 (Figure 2.23). With increasing pH from 1.7 to 3.2, the
SLS aggregates increased in size and had more defined block-like shape. Above pH 3.6, no SLS aggregates were observed.

Although malformed dTTP-SLS aggregates were observed at pH 1.7, below the proposed range of 2.0 to 4.0, it does not immediately disqualify the hypothesis. Firstly the $pK_a^{H,H_2dTTP^{2-}}$ value of 2.0 as published in the literature was not directly measured (Sigel et al., 1987). Instead, it was assumed to be the same as that of UTP, $pK_a^{H,H_2dUTP^{2-}} = 2.0 \pm 0.1$. Secondly, environmental factors are known to shift pKa values. Thirdly, the pKa value only refers to 50% of the species undergoing protonation at that pH. Lastly, the trend of decreasing SLS aggregate size with decreasing pH agreed with the above factors. Therefore, although the minimum pH range for dTTP-SLS was lower than the proposed pH 2.0, it was reasonable to assume that the double-negatively charged triphosphate group in the protonated $H_2dTP^{2-}$ species was not responsible for SLS assembly.

Confirming results observed in UTP-SLS assembly, a neutral charge in the base of the nucleoside triphosphate was demonstrated to successfully assemble SLS aggregates. This is contrary to the GTP-SLS observation that the HGTP$^{3-}$ protonation species at pH 2.9 – 4.0 with neutral base failed to assemble SLS aggregates. In the absence of negatively charged amino acid side chains in the collagen monomer, the failure of GTP-SLS assembly in the pH range of 2.9 – 4.0 was likely due to intermolecular forces rather than electrostatic interaction. The subject of GTP protonation at the base will be explored in future MM and MD simulation work.

In summary, successful mature SLS assembly with dTTP once again demonstrated that electrostatic interaction between the base of a nucleoside and collagen was not a critical factor in SLS assembly. Furthermore, dTTP-SLS assembly demonstrated that the hydroxyl group in the
ribose ring played a minimal role in NTP-collagen interaction. Lastly, the pH range of assembly of 1.8 – 3.6 reaffirms the hypothesis that a triple-negatively charged triphosphate group is necessary for SLS assembly.

2.4.3.6 SLS with TPP

In sections 2.4.3.1 to 2.4.3.5, the electrostatic interaction between the base of a nucleoside triphosphate and collagen was shown to be non-critical for SLS assembly. Furthermore, successful SLS assembly with dTTP showed changes in the ribose ring resulted in no observable change in SLS assembly. To further determine the role of nucleosides, TPP was chosen as a candidate for SLS assembly. With only the triphosphate group present in TPP, it has three negative charges in the pH range of 1.7 – 2.3. In pH 2.3 – 4.0, TPP has four negative charges, a state only available to NTP molecules above pH 6.5.

Throughout the pH range from 1.7 to 4.1, well-formed block-like but undersized (~ 15 nm height) SLS aggregates were observed (Figures 2.24). At the high end of the pH range ~ 4.0, the SLS aggregates were slightly smaller (~ 5 nm height) but still exhibited the well-formed block-like structure comparable to that of mature ATP-SLS aggregates. When incubation time was increased to 3 days, no additional increase in aggregate size was observed (Figure 2.25).

Unlike CTP-SLS, although TPP-SLS never attained the defined mature size of 25 nm height, all aggregates were well-formed with pronounced ridge size comparable to those of mature SLS from other NTPs. Furthermore, the TPP-SLS aggregates were larger (~ 15 nm) compared to those observed in CTP-SLS (~ 5 nm). This suggests that TPP bridges collagen monomers much in the same fashion as ATP, GTP, UTP, and dTTP, albeit having lowering bridging capacity for each TPP-monomer interaction. Therefore, the base in nucleoside triphosphates must play a positive role in SLS assembly, likely due to weaker intermolecular
forces rather than electrostatic interactions. The comparison between TPP and CTP also elucidated that the cytidine ring in CTP inhibits SLS assembly, rather than having a neutral role.

Successful TPP-SLS assembly at pH 1.7 to 2.3 ($\text{H}_2\text{TTP}^{3-}$ species) but not below demonstrated the importance of the three negative charges in SLS assembly. The absence of SLS formation with double-negatively charged phosphate groups at pH below 1.7 was consistent with the inability of ADP to assemble SLS (Paige and Goh, 2001), with ADP only capable of supporting two negative charges at SLS relevant pH ranges.

In agreement with the proposed hypothesis, TPP with four negative charges at pH 2.3 to 4.0 ($\text{HTTP}^4^+$ species) also showed successful SLS assembly. At such pH, successful SLS assembly affirms the proposed mechanism where polyanions provide electrostatic bridging between adjacent collagen monomers.

Another implication for the success of SLS assembly with four negative charges in the triphosphate group lies in the pH range of above 6.5. At such pH, the triphosphate group of nucleoside triphosphates lose an additional proton, bringing the molecules to a state of having four negative charges in the triphosphate group and either a positive charge (ATP, GTP, CTP) or no charge (UTP, dTTP) at the bases. The fact that the quadruply charged triphosphate group in TPP formed SLS while the same state for NTP failed, demonstrated that at pH above 6.5, the absence of SLS aggregates might be due to the negatively charged Asp and Glu in collagen resulting in electrostatic repulsion and not due to the polyanions. Therefore, the restriction of SLS assembly to below pH of ~ 4 is likely due to the protonation states of the various amino acids in the collagen monomer, the preferential assembly of native collagen, or both.
In summary, TPP was demonstrated to assemble well-formed but under-sized SLS at pH range of 1.7 to 4.1. Experiments demonstrated that while three negative charges in the triphosphate group were necessary in SLS assembly, four negative charges also yielded success, reaffirming the proposed mechanism where polyanions bridge positively charged amino acid side chains from adjacent collagen monomers. Furthermore, while the triphosphate group was critical for SLS assembly, the base in nucleoside triphosphates was found to play a positive role, likely due to intermolecular forces rather than electrostatic interactions.

2.4.3.7 SLS assembly for all polyanions

A collection of the SLS assembly pH ranges for each polyanion and their respective protonation species is summarized in Figure 2.26. Firstly, all SLS assembly was limited to below pH of ~ 4, affirming the hypothesis that negatively charged acidic residues hinder SLS collagen assembly via electrostatic repulsion. Secondly, SLS exclusively occurred where the protonation species of each polyanion had three or more negative charges in the triphosphate group, affirming the hypothesis that three negative charges are necessary for electrostatic bridging between basic amino acids from adjacent collagen monomers. Finally, the occurrence of SLS assembly with protonation species that have either positively charged or neutral base suggests that the base contributes to the stabilization of SLS aggregates via non-electrostatic interactions.

2.5 Summary

SLS collagen assembly mechanism was studied via comparison of various polyanions at different protonation states. ATP, GTP, UTP, and dTTP all resulted in mature SLS aggregate assembly (> 25 nm heights) demonstrating that a positively charged nucleoside base was not critical for SLS assembly. The primary amine group, carbonyl group, and addition of a methyl group in the nucleoside bases were determined to be non-critical factors in SLS assembly as
well. CTP assembly with collagen resulted in thin malformed SLS aggregates with ~ 5 nm heights. The successful SLS assembly of the closely related UTP and dTTP polyanions ruled out steric effects as a cause for unsuccessful CTP-SLS assembly. With no clear answer for this observation, SLS assembly inhibition by the cytidine group in CTP will be studied in future MM and MD studies. TPP, lacking the nucleoside group compared to other polyanions, resulted in well-formed but under-sized SLS aggregates (~ 15 nm height). TPP-SLS assembly demonstrated that while the triphosphate group was critical for SLS assembly, the nucleoside group in NTPs played a favourable role, likely due to weaker intermolecular forces rather than electrostatic interactions. Furthermore, TPP-SLS assembly demonstrated successful SLS assembly with triphosphate groups having three but also four negative charges, affirming the proposed mechanism where the triphosphate group bridge adjacent monomers via electrostatic interactions. Lastly, the restriction of all SLS assembly to below pH of ~ 4 implied neutral side chains in Glu and Asp during SLS assembly. This and other results above will be used to refine and expand on previously reported ATP-SLS MM and MD simulations work (Anderson, 2005), further promoting the understanding of SLS collagen assembly mechanism.
2.6 References


Sigel, H., Tribolet, R., Malinibalakrishnan, R., Martin, R.B., 1987. Comparison of the stabilities of monomeric metal-ion complexes formed with adenosine 5'-triphosphate (ATP) and pyrimidine-nucleoside 5'-triphosphates (CTP, UTP, TTP) and evaluation of the isomeric equilibria in the complexes of ATP and CTP. Inorganic Chemistry 26, 2149-2157.


Figure 2.1: AFM images of typical SLS aggregate: a) lock-in amplitude image and b) height image.
Table 2.1: pKa values for a) amino acids and b) polyanions.

### a)

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<td>$\text{HTPP}^{4+}$ (4-, n/a)</td>
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</tr>
</tbody>
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**Legend:**

- [ ] Acidic amino acids
- [ ] Species (Triphosphate charge, base charge)
- [ ] (2-, 0/1+) Species with double-negatively charged triphosphate group
- [ ] (3-, 1+) Species with triple-negatively charged triphosphate group and positive base
- [ ] (3-, 0) Species with triple-negatively charged triphosphate group and neutral base
- [ ] (4-, n/a) Species with quadruple-negatively charged triphosphate group

**Figure 2.2:** Graphical pKa values: protonation state of amino acids and polyanions at different pH.
Figure 2.3: pKa for protonation of ATP.
Figure 2.4: pKa for protonation of GTP.
\[ pK_1 = 1.7 \pm 0.1 \]
\[ pK_2 = 4.55 \pm 0.03 \]

**Figure 2.5:** pKa for protonation of CTP.
Figure 2.6: pKa for protonation of UTP.
Figure 2.7: pKa for protonation of dTTP.
Figure 2.8: pKa for protonation of TPP.
Figure 2.9: AFM images of SLS collagen assembled with ATP at pH 3.3 after 2-hour incubation: a) lock-in amplitude image and b) height image.
Figure 2.10: AFM images of SLS collagen assembled with ATP at pH 3.3 after 2-hour incubation: a) lock-in amplitude image, b) height image, and c) cross-section from the height image.
Figure 2.11: AFM height images of SLS collagen assembled with ATP at pH 3.3 after 5-hour incubation: a) large scan size b) small scan size, and c) cross-section of image b.
Figure 2.12: AFM images of SLS collagen assembled with ATP at pH 3.3 after 6-hour incubation: a) lock-in amplitude image and b) height image.
**Figure 2.13:** AFM images of SLS collagen assembled with ATP at pH 3.3 after 50-hour incubation: a) lock-in amplitude image and b) height image.
Figure 2.14: AFM images of SLS collagen assembled with ATP at pH a) 2.9, b) 3.3, c) 3.8, and d) 4.4 after 2-hour incubation.
Figure 2.15: AFM images of SLS collagen assembled with GTP at pH 2.3 after 2-hour incubation: a) lock-in amplitude image, b) height image, and c) cross-section from the height image.
**Figure 2.16:** AFM images of SLS collagen assembled with GTP at pH a) 1.7, b) 2.3, and c) 2.9 after 2-hour incubation.
Figure 2.17: AFM images of SLS collagen assembled with CTP at pH 2.1 after 2-hour incubation: a) lock-in amplitude image, b) height image, and c) cross-section from the height image.
Figure 2.18: AFM images of SLS collagen assembled with CTP at pH a) 1.6, b) 2.1, and c) 2.3 after 2-hour incubation.
**Figure 2.19:** AFM images of SLS collagen assembled with CTP at pH 2.1 after 29-day incubation: a) lock-in amplitude image, b) height image, and c) cross-section from the height image.
**Figure 2.20**: AFM images of SLS collagen assembled with UTP at pH 2.9 after 2-hour incubation: a) lock-in amplitude image, b) height image, and c) cross-section from the height image.
Figure 2.21: AFM images of SLS collagen assembled with UTP at pH a) 2.3, b) 2.4, c) 2.9, and d) 4.0 after 2-hour incubation.
Figure 2.22: AFM images of SLS collagen assembled with dTTP at pH 3.2 after 2-hour incubation: a) lock-in amplitude image, b) height image, and c) cross-section from the height image.
Figure 2.23: AFM images of SLS collagen assembled with dTTP at pH a) 1.7, b) 1.8, c) 2.3, and d) 2.9 after 2-hour incubation.
Figure 2.24: AFM images of SLS collagen assembled with TPP at pH a) 2.1, b) 2.3, c) 2.8, e) 3.2, and f) 3.5 after 2-hour incubation, with d) cross section of image c.
Figure 2.25: AFM images of SLS collagen assembled with TPP at pH 3.3 after 3-day incubation: a) lock-in amplitude image, b) height image, and c) cross-section from the height image.
<table>
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<tr>
<th>Amino Acid</th>
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<th>Species (Triphosphate charge, base charge)</th>
<th>pH Range</th>
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<td></td>
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</tr>
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</tr>
<tr>
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<td>1-</td>
<td>Neutral</td>
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<tr>
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<td>HTPP⁺ (4-, n/a)</td>
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<td>TPP</td>
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<td>SLS assembly pH range for each polyanion</td>
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**Legend:**
- **Green**: Acidic amino acids
- **Light Blue**: Species (Triphosphate charge, base charge)
- **Blue**: (2-, 0/1+) Species with double-negatively charged triphosphate group
- **Light Green**: (3-, 1+) Species with triple-negatively charged triphosphate group and positive base
- **Green**: (3-, 0) Species with triple-negatively charged triphosphate group and neutral base
- **Dark Green**: (4-, n/a) Species with quadruple-negatively charged triphosphate group
- **Red**: SLS assembly pH range for each polyanion

**Figure 2.26:** Assembly pH range of SLS collagen with various triphosphate polyanions and their relation to protonation states of amino acids and the polyanions.
Chapter 3

3 Nanoindentation Study of Type I Collagen Aggregates

3.1 Mechanical role of collagen

Collagen is the most abundant protein in mammalian tissue, and plays a major structural role in all parts of the body including the skin, tendon, and bone. Because of its predominant role as mechanical support, the native collagen structure and mechanical properties is well studied (Silver et al., 2003; Rainey et al., 2002; Paige and Goh, 2001). Although much work had been carried out in studying Type I native fibrils, mechanical characterization of other Type I collagen aggregates, namely fibrous long spacing (FLS) collagen and segmental long spacing (SLS) collagen, is currently lacking. The three collagen aggregates have distinct morphologies (Figures 3.1, 3.2, and 3.3), indicating different monomer alignment, and thus are expected to have different mechanical properties. This study aims to determine the validity of this hypothesis by utilizing nanoindentation technique by AFM to measure the nominal elastic modulus of each aggregate.

The native fibril is the most-studied form of Type I collagen. It is the prevalent form of collagen found in normal tissue, and typically possess banding periodicity of ~67 nm (D-period banding, Figure 3.1), as characterized by TEM (Chapman et al., 1990) and AFM (Baselt et al., 1993; Revenko et al., 2994; Gale et al., 1995; Bowen et al., 2000). Native collagen is widely used as a scaffold in tissue engineering due to its inherent biocompatibility. Collagen networks in vitro and in vivo aids in cell movement and proliferation, hence the importance in understanding the mechanical properties of collagen for application in the biomedical sciences. Previous studies on the mechanical properties of collagen have shown native fibrils to be mechanically
homogeneous structures, with the outer shell and inner core exhibiting the same nominal elastic modulus (Strasser et al., 2007; Wenger et al., 2008). Nanoindentations on (band) and between (interband) the periodic bands did not yield any significant difference in the nominal elastic modulus between the regions (Wenger et al., 2008), possibly due to the relative large size of the AFM probe in comparison with the ~67 nm banding.

FLS collagen (Figure 3.2) has been observed in and often associated with diseased tissues. As such, understanding the mechanical properties of FLS collagen may lead to better understanding of the diseased tissues and improved treatment for symptoms in relation to the change in collagen alignment from native to FLS fibrils. Examples of such diseases include atherosclerotic plaques (Morris et al., 1978), Hodgkin’s disease (Nakanishi et al., 1978), myeloproliferative disorder (Kamiyama, 1982), and silicosis (Slavin et al., 1985). FLS collagen was first observed in vitro in experiments conducted on acidified connective tissue extracts (Highberger et al., 1950 and 1951). In an AFM study, the ~250 nm periodic banding observed on the outer shell was also apparent in the inner core, displaying no supercoiling (Wen and Goh, 2006). As such, proposed FLS models hypothesized the overlap and cross-linking of laterally aligned monomers ends by α1-acid glycoprotein (Rainey et al., 2002), forming band regions at the overlap and creating structural and molecular heterogeneity between the bands and interbands. For this reason, the nominal elastic moduli of the band and interband of FLS collagen were measured and compared in this study.

Discussed in depth in chapter 2, SLS collagen is characterized by a lateral alignment of monomers resulting in a 300 nm length aggregate. SLS has become an invaluable tool in collagen research. Because of the lateral alignment, SLS exhibited banding pattern in stained EM images that reflect positively and negatively charged amino acids, helping to determine
sequences of the polypeptides (Kuhn, 1982). In vivo, SLS is commonly thought to act as compact monomer storage and transport in and out of the cell. This theory arose from the observation that SLS was present in both active cells and culture medium (Bruns et al., 1979).

Early studies on the mechanical properties of collagen utilized laser-trap microrheometry on collagen gel used in tissue engineering (Velegol and Lanni, 2001). The shear elastic modulus of the gel was found to vary significantly at different locations of the gel, with gel density dependent on local collagen concentration (Mooney et al., 2005). Studies aimed at measuring the nominal elastic modulus of banded native collagen utilized excised tissue (Stolz et al., 2004), theoretical modeling of monomers (Lorenzo et al., 2005), and most recently, AFM nanoindentations on individual native fibrils (Heim et al., 2006; Wenger et al., 2007). Native fibrils extracted from the inner dermis of the sea cucumber, Cumumaria frondosa, were found to have a nominal elastic modulus of 1 – 2 GPa (Heim et al., 2006), but fibrils extracted from rat tail was reported to have nominal elastic modulus of 5 – 11.5 GPa (Wenger et al., 2007), demonstrating potential difference in the nominal elastic modulus for fibrils from various sources. However, such deviation between studies could also be a result of other systematic errors including sample hydration, AFM probe size estimation, and AFM probe spring constant measurement error.

The elastic modulus (E) is the tendency of a material to deform elastically when a force is applied, with a higher value representing stiffer materials. Putting the above values into context, rubber typically has an elastic modulus of 0.01 – 1 GPa while nylon is in the range of 2 – 4 GPa. For harder materials, magnesium metal has an elastic modulus of 45 GPa while glass is typically in the range of 70 – 90 GPa.
Although much work has been carried out for studying native collagen fibrils, mechanical characterization of FLS collagen and SLS collagen is still lacking. It is the goal of this study to measure and compare the mechanical properties for the three aggregates. Such information is not only useful for modeling and understanding the monomer packing structure, it also holds great potential in understanding the symptoms and implications of the presence of FLS in diseased tissues. For the current study, collagen assembled in vitro was derived from reconstituted calfskin collagen (PureCol, Lot #: 1629346, Advanced BioMatrix). Although measured native collagen nominal elastic modulus may differ from the above studies due to the differing source of collagen, results can be reliably compared among the three Type I collagen aggregates assembled from the same source of collagen monomers.

3.2 Methods

3.2.1 In vitro assembly of Type I collagen

3.2.1.1 Native collagen

PureCol collagen monomer solution (3 mg/mL dissolved in HCl) was used directly from the stock bottle. Phosphate buffer solution (0.2 M) at pH 7 was prepared using sodium dihydrogen phosphate (Sigma) and sodium hydroxide (VWR). Potassium chloride (KCl; 1.0 M) solution was prepared directly from the KCl solid (Sigma). All salts were dissolved in 18 mega ohm deionized water.

Following a protocol by R. W. Loo (Loo, 2010), 48 µL of 1.0 M KCl solution was mixed with 240 µL of 0.2 M phosphate buffer and 144 µL of deionized water in a centrifuge tube. After mixing, 48 µL of PureCol solution was added to the solution then briefly vortexed. The centrifuge tube was then sealed and allowed to incubate in a heat block (AccuBlock Digital Dry Bath, Labnet International, Inc) at 37 °C for three days.
3.2.1.2 FLS collagen

PureCol collagen stock solution was dialyzed (16 mm tubing diameter, MWCO 12 – 14 kDa, Spectra/Por, Spectrum Laboratories, Inc) against deionized water with two water changes over 24 hours. The collagen solution was then diluted to 1 mg/mL with deionized water and stored at 4 °C until use. The α1-acid-glycoprotein solution (1 mg/ml) was prepared by dissolving the solid (Sigma) in deionized water then dialyzed (same membrane as above) against deionized water independently and also stored at 4 °C until use.

Following a protocol by J. B. Goh (Goh, 2008), equal volumes (100 µL) of the collagen solution and α1-acid-glycoprotein were mixed together in a centrifuge tube, yielding a final concentration of 0.5 mg/mL for each reagent. The centrifuge tube was then sealed and allowed to incubate overnight at room temperature.

3.2.1.3 SLS collagen

SLS assembly protocols were discussed in detail in section 2.3.1. A brief description is included here for the sake of completeness.

In a centrifuge tube, 40 µL of ATP solution was combined with 122 µL of 0.05% v/v acetic acid and 5 µL of 5% v/v acetic acid and briefly vortexed. A 33 µL aliquot of PureCol solution was then added to the mixture and again briefly vortexed. The pH of the final solution as measured by narrow range pH paper was 3.3 and the solution was allowed to incubate at room temperature for two hours.
3.2.1.4 Sample preparation

After incubation, 20 uL of each solution was deposited on to freshly cleaved mica (~ 1 x 1 cm²). After ~ 30 seconds, without the solutions drying out, samples were rinsed with deionized water and dried with a gentle stream of nitrogen gas.

3.2.2 Nanoindentation technique in AFM

Nanoindentation by AFM is an established method for determining elastic modulus for biological samples such as cells (Franze et al., 2006), microtubules (Pablo et al., 2003), bacteriophage capsids (Ivanovská et al., 2004), cartilage (Stolz et al., 2004), and even for native collagen fibrils as discussed previously (Heim et al., 2006; Wenger et al., 2007). The elastic modulus of a sample was calculated from the force-distance curve obtained from AFM nanoindentation experiments.

A JPK NanoWizard II AFM (JPK Instruments, Berlin, Germany) was used for imaging collagen aggregates as well as nanoindentation experiments. After an initial imaging of the sample in intermittent contact mode, force spectroscopy was performed where the probe was lowered to the surface of the sample, indenting the sample for 1 – 5 nm, and retracted to non-contact range. Total approach and retract time was each set to 0.5 seconds, with no pause in between. Actual contact time is in the range of ~ 0.1 seconds (Figure 3.4). To ensure elastic contact, the same sample was imaged again after nanoindentation to ensure absence of permanent deformation.

The elastic modulus is dependent on the Poisson’s ratio (ν) of the material, which is the tendency of the material to deform in the direction perpendicular to the applied force. Because the Poisson’s ratio for collagen is unknown, the nominal elastic modulus (E*) was used for quantification, where (Eq. 1):
The infinitely hard spherical indenter and elastic cylinder Hertzian model was used to calculate the nominal elastic modulus from a force-distance nanoindentation curve (as used in Heim et al., 2006). This assumes that the indenter (AFM probe) is a perfectly rigid probe, and that the sample (collagen) is an elastic cylinder (Figure 3.5) (Eq. 2 & Eq. 3):

\[ E^* = \frac{E}{(1 - \nu^2)} \tag{Eq. 1} \]

\[ E^* = \sqrt{\frac{9F^2}{16R_e\delta^3}} \tag{Eq. 2} \]

\[ R_e = \sqrt{\frac{R_t^2 R_c}{R_t + R_c}} \tag{Eq. 3} \]

where \( F \) is the applied force of the indenter (AFM probe), \( \delta \) is the probe-sample separation (indentation distance), \( R_t \) is the AFM probe radius, and \( R_c \) is the collagen sample radius.

Force curves were fitted to the parabolic probe–infinite 2D sheet Hertzian model using the JPK Data Processing software (JPK Instruments, Berlin, Germany), and modified to the elastic cylinder Hertzian model via manual calculation.

Comparison of mechanical properties among the three Type I collagen aggregates were performed in two duplicate sets of nanoindentation experiments. In each set, the nominal elastic modulus for the three aggregates were measured using one single AFM probe within a 12 hour period, thus minimizing the effect of systematic errors such as AFM probe geometries, probe size estimation, and ambient humidity. With each of such systematic factors potentially varying the measured modulus significantly, data was deemed comparable within one set of experiment but not across different sets of experiments.

In the first study, at least 20 nanoindentations were performed on each aggregate, yielding a total of ~ 80 nanoindentations for Probe #1. In the second study, more than 45
nanoindentations were performed on each collagen type, with a total of ~ 170 nanoindentations for Probe #2. The number of nanoindentations was chosen to maximize the number of data points while minimizing the error due to probe damage and accumulation of debris on the probe.

Probe #1 (NP-S, probe B, Bruker), was a silicon nitride probe with a measured spring constant of 61.4 N/m and ~ 50 nm probe radius. The spring constant was measured via the thermal noise method (Hutter et al., 1993) and the probe radius was evaluated by imaging a sharp silicon grating. Probe #2 (NCH-50, NanoWorld) was a silicon probe with measured spring constant of 40.3 N/m. The probe radius was measured to be 115 nm by scanning electron microscopy (SEM; Figure 3.6). The AFM probe holder used in SEM imaging was designed by Eisenstein and Goh (Eisenstein and Goh, 2012).

In an attempt to restrict nanoindentation to within the elastic region, indentation distance was limited to below 5 nm for FLS and 2 nm for native and SLS. At such depths (less than 10% of sample diameter), elastic modulus convolution due to the underlying stiff surface was negligible (Clifford and Seah, 2006).

Only the approach curve data was used in the Hertzian modeling (Heim et al., 2006). The retract curve was not used in analysis due to the possibility of unexpected interactions such as probe-sample adhesion or difference in probe retraction and collagen recovery speed.

3.3 Results and discussion

3.3.1 Nanoindentation experiment #1

3.3.1.1 FLS collagen bands and interband

As discussed in section 3.1, FLS collagen consists of monomer overlapping end-to-end, stabilized at the overlap region (bands) by α1-acid glycoprotein. Differing nominal elastic
modulus between the bands and interbands would therefore support the proposed structural heterogeneity in FLS collagen. The measured nominal elastic modulus for on band and interband were measured to be $3.6 \pm 0.8$ GPa, and $2.9 \pm 0.8$ GPa respectively. An unpaired t-test on the two sets of FLS collagen data yielded a two-tailed P value of 0.011. The difference in the two means was 0.7 GPa with a 95% confidence interval of this difference from 0.2 to 1.2 GPa. However, such difference was not a conclusive evidence of a difference in nominal elastic modulus because the error in measured nominal elastic moduli of collagen fibrils was in a range of 1 to 6 GPa (Heim et al., 2006; Grant et al., 2008; Wenger et al., 2007). The major contributors to such errors included probe-sample interactions, contact geometry, and fitting the data to the model equations. Furthermore, the diameter of the fibril varied by up to 50 nm between the band and interband regions, which might account for some of the difference in the measured nominal elastic modulus. Our results revealed that despite geometrical (monomer overlap at the band) and molecular (collagen monomer-α1-acid glycoprotein cross-links at the bands) differences between the bands and interband, FLS fibrils were relatively mechanically homogeneous structures like native fibrils, with comparable nominal elastic moduli between the bands and interbands. As a subject for future work, this experiment may be repeated multiple more times to gain statistical significance.

3.3.1.2 Comparison of native, FLS, and SLS collagens

For experiment #1, nanoindentations were performed in the following order: native, FLS, and SLS collagens (Figure 3.7). A sample force curve fit is shown in Figure 3.8. Both native and FLS collagens (~ 1 – 5 nm nanoindentations) were observably identical in the before and after images, but nanoindentations on SLS collagen (~ 1 nm) resulted in permanent marks (Figure
3.9). This observation will be discussed in detail in section 3.3.2, but may be assumed to be insignificant at the moment for nominal elastic modulus results.

Native fibrils, SLS, and FLS collagens were measured to have nominal moduli with standard deviation of 9 ± 3 GPa, 10.3 ± 1.9 GPa, and 3.2 ± 0.9 GPa (all FLS values) respectively (Figure 3.10). The measured value for SLS collagen was found to be higher than that of native collagen but within error. However, FLS collagen was shown to have an approximately 65% lower nominal elastic modulus than that of native and SLS collagens. This decreased stiffness in FLS collagen is especially important in vivo because of the association of FLS to various diseased tissues.

3.3.2 Nanoindentation experiment #2

In the second experiment, nanoindentations were performed in the following order: SLS, native, FLS collagen (Figure 3.11). The change in order was important to evaluate the error due to contamination of the probe with increasing number of nanoindentations. In this study, native, SLS, and FLS collagens were measured to have nominal moduli of 0.67 ± 0.14 GPa, 0.72 ± 0.08 GPa, and 0.49 ± 0.10 GPa respectively (Figure 3.12). Results from nanoindentation experiment #2 agreed with those in nanoindentation experiment #1, suggesting insignificance of experiment order for the number of nanoindentations performed by a single probe in this study. In both experiments, SLS collagen was found to have slightly higher measured elastic modulus compared to that of native collagen, but within error. Similarly, FLS collagen was found to have decreased values compared to native and SLS collagens in both sets of experiments, with a ~ 65 % and ~ 30 % decrease in each of the two sets.

In nanoindentation experiment #2, nanoindentations for SLS collagen were performed on three separate SLS aggregates, with 15, 16, and 15 nanoindentations each. Nominal elastic
modulus was measured to be $0.72 \pm 0.09$ GPa, $0.68 \pm 0.08$ GPa, and $0.75 \pm 0.07$ GPa. For nanoindentations of SLS aggregate #1, no permanent scars were observed after $\sim 1$ nm nanoindentation except for 2 of the nanoindentations at the edges of the aggregate (Figure 3.13 a, b). In $\sim 1.5$ nm nanoindentations of aggregates #2 and #3, small indents were observed afterwards (Figure 3.13 c, d). However, despite the difference, all three aggregates had statistically similar results, indicating that the inelastic contribution to nanoindentation measurement is likely relatively small. Furthermore, $\sim 1 – 5$ nm nanoindentations on native and FLS yielded no observable deformation afterwards, suggesting local instabilities in SLS. Such potential instabilities might be due to the alignment of like-charges along the lengths of the in-register monomer. Unfortunately, the current AFM nanoindentation setup lacked the consistency and spatial resolution necessary to correlate measured nominal elastic modulus with the known collagen amino acid sequence.

### 3.3.3 Comparison with literature and sources of error

Nominal elastic modulus measurements are highly dependent on the degree of hydration in the sample. The nominal elastic modulus of native collagen fibrils varied by three orders of magnitude depending on the degree of hydration (Grant et al., 2008), possibly accounting for some of the differences in the measured nominal elastic modulus between the two sets of experiments performed on different days. To ensure all samples were similarly hydrated, all samples were stored in ambient conditions in the same location for one week prior to the nanoindentation procedure.

In previously reported results, the elastic modulus of native collagen was also varied with the source of collagen. The measured native fibril nominal elastic modulus in nanoindentation experiment #1 and #2 were 9 GPa and 0.67 GPa respectively, values that were comparable to
those of previous studies: 1 – 2 GPa for fibrils from Cucumaria frondosa (Heim et al., 2006), 5 – 11.5 GPa from rat tail (Wenger et al., 2007), and 0.93 – 2.67 GPa from in vitro assembled fibrils (Strasser et al., 2007). Depending on the source of the fibrils, extracted collagen fibrils bear different physiological loads in vivo and are cross-linked to different degrees. Similar to a previous study by Strasser et al. (Strasser et al., 2007), the current study utilized in vitro assembled native fibrils, thus having the least amount of cross-links of the different sources. It is however noted that Strasser et al. incorporated α1-acid glycoprotein in the assembly conditions, resulting in fibrils exhibiting native and FLS collagen bandings at different ends of a single fibril. Furthermore, Strasser et al. used collagen monomer stock dissolved from a powder compound (Sigma) whereas the current study used pepsin digested collagen monomers dissolved in hydrochloric acid. With the result of FLS collagen having nominal elastic modulus ~ 65% (nanoindentation experiment #1) and ~ 30 % (nanoindentation experiment #2) lower than that of native collagen, it is not surprising that the native fibril nominal elastic modulus reported in this study differed from that reported by Strasser et al.

Another critical error in this study was in the determination of the spring constant and probe radius. As discussed in depth by Hutter et al. (Hutter et al., 1993), the error in thermal noise method for calculating the spring constant of the probe was up to 20%. Therefore, while results between native, SLS, and FLS collagen in one set of experiment with the same probe were unaffected by this error, measured nominal elastic moduli could not be accurately compared to studies using different AFM probes. In nanoindentation experiment #1, the probe radius was measured by imaging a sharp silicon grating to deduce probe geometry. However, as silicon gratings could not be infinitely sharp, there was an error in the order of nanometers. In nanoindentation experiment #2, the probe shape was visualized with SEM. However, images of
similar samples with the same AFM probe after SEM characterization showed an increase in probe size (at least ~ 10 nm) compared to before SEM characterization. Such a damaged probe can be seen in Figure 3.6. This might be due to accidental damage to the probe during loading in and out of the AFM and SEM.

3.4 Summary

Collagen exists in most parts of the body in many different forms, and this study has examined the differences in nominal elastic modulus for the three Type I collagen aggregates: native, FLS, and SLS collagens. In each of the two sets of experiments, one single AFM probe was used to perform nanoindentations within a 12-hour period, thus eliminating the effects of systematic errors from probe geometry, probe size estimation, and ambient humidity. Due to the significance of the systematic errors, measured nominal elastic modulus values were compared within set of experiment but not across different sets. In the first set of experiments, the nominal elastic modulus for native, SLS, and FLS collagens were measured to be 9 ± 3 GPa, 10.3 ± 1.9 GPa, and 3.2 ± 0.9 GPa respectively. In the second set of experiments, the values were measured to be 0.67 ± 0.14 GPa, 0.72 ± 0.08 GPa, and 0.49 ± 0.10 GPa respectively. The measured nominal elastic modulus values of in vitro assembled native fibrils in each of the two sets of experiments were comparable to those of previous studies, with the differences most likely due to the source of the collagen fibril and other systematic errors mentioned above. In both sets of experiments, the measured SLS collagen nominal elastic modulus values were slightly higher than that of native collagen, but were within error. However, FLS collagen was found to have ~65% and ~30% lower nominal elastic modulus values compared to native and SLS collagens, highlighting the decrease in structural support in FLS-associated diseased tissues.
3.5 References


Figure 3.1: AFM images of native collagen: a) lock-in amplitude image, b) height image, and c) cross-section from the height image showing the characteristic ~ 67 nm banding periodicity.
Figure 3.2: AFM images of FLS collagen: a) lock-in amplitude image, b) height image, and c) cross-section from the height image showing the characteristic ~ 250 nm banding periodicity.
**Figure 3.3:** Also shown in Figure 2.11. AFM images of SLS collagen: a) lock-in amplitude image, b) height image, and c) cross-section from the height image showing the characteristic ~ 300 nm length.
**Figure 3.4:** Sample force-time curve for the approach and retraction of an AFM probe during a nanoindentation experiment.
**Figure 3.5:** Hertzian representation of the AFM probe as an infinitely hard sphere and the collagen as an elastic cylinder.
Figure 3.6: SEM images of AFM Probe #2 with different magnification of the same probe in a), b), and c).
Figure 3.7: AFM images of a) native, b) SLS, and c) FLS collagens in nanoindentation experiment #1, with corresponding cross sections for determination of aggregate heights.
Figure 3.8: Sample Hertz model data fitting, with the fit region indicated by a grey background shading. The fitted line (green) showed good correlation with the data (red).
Figure 3.9: AFM images of SLS collagen a) before and b) after nanoindentations in experiment #1, showing permanent deformation along the sites of nanoindentation.
Figure 3.10: Measured nominal elastic moduli for native, SLS, and FLS collagens in nanoindentation experiment #1: a) chart and b) graph.
Figure 3.11: AFM images of a) native, b) SLS, and c) FLS collagens in nanoindentation experiment #2, with corresponding cross sections for determination of aggregate heights.
Figure 3.12: Measured nominal elastic moduli for native, SLS, and FLS collagens in nanoindentation experiment #2: a) chart and b) graph.
Figure 3.13: Nanoindentation experiment #2: AFM images of SLS collagen a), c) before and b), d) after nanoindentations for aggregates a), b) #1 and c), d) #2 and #3. Yellow dots in image a indicate sites of nanoindentations. For the exception for indent 1 and 15, no permanent deformation was observed for aggregate #1, in contrast with those seen in aggregates #2 and #3.
Chapter 4

4 Surface Mediated Assembly of Collagen on Mica

4.1 Collagen – mica interaction

Native Type I collagen fibrils are tremendously useful in biotechnology due to its inherent biocompatibility (Lee et al., 2001). For example, in vitro assembled collagen is widely used as a scaffold in tissue engineering (Friedrichs et al., 2007). Furthermore, collagen is often coated on bio-incompatible materials for use in vivo or for functionalized surface patterning (Monroe et al., 2009). As such, the study of surface mediated assembly of collagen is necessary for the advancement of biomedical applications.

Since the invention of the AFM in 1986 (Binnig et al., 1986), the technique has been increasingly utilized as a characterization tool in collagen research (Gale et al., 1995). Mica, an atomically flat material, was often chosen as a substrate for collagen fibril imaging. However, a strong electrostatic attraction exists between the overall positively charged collagen and negatively charged mica. While collagen fibrils assembled in solution do not exhibit uniform fibrillar directionality, the strong collagen – mica interaction can lead to uniform fibrillar alignment on the mica surface. This interaction is not unique to collagen, and was demonstrated with the surface patterning of palladium-based nanowires (Wang et al., 2007). Below, I will discuss the brief history of collagen – mica interaction in the literature from 2004 to the present (2012).

Jiang et al. (Jiang et al., 2004) first noted collagen fibrillar alignment on mica under AFM imaging, but erroneously attributed the directionality to hydrodynamic flow. Utilizing the slower
kinetics of surface collagen assembly compared to solution assembly, the authors confirmed the nucleation and growth mechanism of native collagen as well as lateral association of microfibrils (intermediate species), as previously described (Gale et al., 1995). Furthermore, the authors noted a dependence of fibrillar alignment on potassium ion (K\(^+\)) concentration. This dependence was later confirmed, where an increased K\(^+\) concentration from 100 mM to 400 mM resulted in increasing alignment of collagen microfibrils (Loo and Goh, 2008).

Two years following the first account, the same collagen fibril alignment was demonstrated experimentally to be a direct result of interaction with the mica lattice (Cisneros et al., 2006). Furthermore, the authors observed lateral as well as longitudinal fusing of mature banded native fibrils, confirming similar observations in vivo during wound healing.

It was not until 2008 (Sun et al., 2008) that theoretical considerations were published. This was followed by a more detailed account three years later (Leow and Hwang, 2011). Up until the latter study in 2011, all literature reported the use of “mica” without specifying the type: muscovite versus phlogopite. While phlogopite mica exhibits hexagonal symmetry, muscovite mica has a comparable but slightly distorted lattice. Leow and Hwang were able to induce a triangular fibril pattern on phlogopite mica-mediated assembly and unidirectional fibril pattern on muscovite mica, showing that the more commonly available muscovite mica was used in all previously mentioned studies that assembled unidirectional fibrils. In the current study, only muscovite mica was used, and the term “mica” will be used to describe muscovite mica for the remainder of the chapter.

The discussed collagen alignment is derived from the alignment of fibrils along the surface mica lattice geometry. In addition to Van der Waals forces that exist in much of this
interaction, the positively charged basic amino acids on collagen are electrostatically attracted to negatively charged K$^+$ pockets exposed during cleaving of the mica by sticky tape (Leow and Hwang, 2011). Such pockets are the result of Si$^{4+}$ replacement by Al$^{3+}$, rendering the surface negatively charged. For this reason, increasing K$^+$ concentration in the environment decreases the number of pockets available, thus decreasing collagen-mica affinity and increasing the hypothetical monomer mobility. Such pockets are exclusive to K$^+$, and not other monocations (Leow and Hwang, 2011). The surface K$^+$ pocket occupancy was measured to vary from 50% to 100% with increasing KCl concentration from 10 to 500 mM (Schlegel et al., 2006). Furthermore, K$^+$ in solution preferentially bind to lattice sites with two Al atoms rather those with one, resulting in a stronger binding of K$^+$ on alternating rows of the mica lattices. As such, with increasing K$^+$, the collagen bidirectional alignment observed in section 4.2.2 increasing shift into a unidirectional alignment parallel to the row of preferred K$^+$ binding sites. This is in agreed with the previously reported results (Loo and Goh, 2008), where microfibril alignment improved with increasing KCl concentration from 50 to 400 mM.

Due to the electrostatic role of positively charged basic amino acids from the collagen monomer, the attraction with K$^+$ pockets is expected to decrease with increasing pH. The basic amino acids, His, Lys, and Arg, have side chain pKa values of 6.0, 10.5, and 12.5 respectively. As pH increases, the basic amino acid side chains are deprotonated, loosing their positive charges. Therefore, with increasing pH, the interaction between mica and collagen is expected to weaken.

On a side note, the term “D-period” refers to the characteristic ~ 67 nm banding observed in native collagen. In addition, all samples noted in this chapter have surface film or fibril heights of less than 5 nm, consistent with all studies noted above.
While static collagen and mica interaction is considered in both experimental and theoretical studies, the degree of collagen mobility on mica surface is unclear. In this study, collagen monomers deposited on a mica surface were demonstrated to move laterally along the surface. Furthermore, such monomers were shown to self-assemble into a uniform film of discrete aligned mature collagen fibrils with full ~ 67 nm (D-period) banding periodicity. This demonstrated a novel method for controlling surface morphology of collagen fibrils at the nanoscale, creating customizable surface morphology for biomedical application and research.

4.2 Surface assembly of collagen

4.2.1 Surface assembly of collagen in solution

4.2.1.1 Protocol in the literature

Based on the collagen surface assembly method by Cisneros et al. (Cisneros et al., 2005), an incubation solution was prepared with 50 mM glycine (BioShop), 200 mM KCl (Sigma), 12 µg/mL PureCol collagen (Lot #: 1629346, Advanced BioMatrix), and adjusted to pH 9.05 with sodium hydroxide (10 N, VWR) monitored with a pH meter (Semi-Micro Combo Electrode, Pinnacle 540 pH Meter, Corning Incorporated). In a modified setup, ~ 1 mL of this solution was deposited on freshly cleaved mica (~ 1 x 1 cm$^2$), sealed in a disposable petri dish with parafilm, and allowed to incubate for 1 hour at room temperature. The sample was then rinsed with deionized water and dried with nitrogen before imaging by AFM in air.

With 100% reproducibility, the above incubation resulted in a full film of banded native collagen (Figure 4.1), in agreement with results in the literature (Cisneros et al., 2005).
4.2.1.2 Modified protocol

The protocol in section 4.2.2 was repeated but with pH adjusted to 9.4 instead of 9.05. The increased pH is expected to result in decreased electrostatic attraction between collagen and mica due to the deprotonation of basic amino acids in the collagen monomer.

In four out of eight trials, this method resulted in discrete unidirectional native fibrils after one hour of incubation (Figures 4.2 and 4.3). Other samples resulted in a full film of aligned collagen indistinguishable from those observed at pH 9.05. The reason for low reproducibility is unknown and will be investigated in future studies. Possible variation among trials include ambient temperature, changes in collagen concentration due to the small measured volume in the preparation of the incubation solution (4 µL), and different rates of evaporation of the incubation solution due to ambient humidity and small incubation volumes (1 mL), thus altering the solution concentrations and pH.

Despite the low reproducibility, this result was consistent with theory and previous experimental observations, as follows. With a higher pH, weaker interactions between collagen and mica would allow of relatively higher interaction among collagen monomers, forming favourable discrete fibrils rather than a spread out film of collagen on mica. Furthermore, this result was consistent with previous experimental observations (Jiang et al., 2004), where increasing pH was correlated with increasing separation between aligned collagen fibrils.

In conclusion, despite low reproducibility in assembling discrete unidirectional D-period native collagens on mica at an increased pH of 9.4, this result was consistent with theory and experimental trends in the literature. Future investigations are necessary to identify the critical factor from the list of proposed experimental errors.
4.2.2 Microfibril deposition: dependence on potassium ions

As noted in section 4.1, increasing concentration of K\(^+\) results in increasing alignment of collagen along the mica lattice. In this section, a protocol by Loo and Goh (Loo and Goh, 2008) was used to deposit bidirectional microfibrils on mica, which would be used for further experiments in section 4.2.3.

A solution was prepared by mixing 1 mL of 100 mM KCl (Sigma) with 4 µL of 3 mg/mL PureCol collagen monomers in 12 M HCl (Lot #: 1629346, Advanced BioMatrix), with a final pH of ~ 4.3. The solution was allowed to incubate on freshly cleaved mica (≈ 1 x 1 cm\(^2\)) for 5 minutes at room temperature. The sample was then rinsed with deionized water and dried under a gentle stream of nitrogen before imaging by AFM (NanoWizard II, JPK Instruments, Berlin, Germany).

Consistent with previously reported results (Loo and Goh, 2008), a uniform surface of microfibrils exhibiting bidirectionality was reproducibly observed (Figure 4.4). This was also consistent with the previously reported theory that increasing K\(^+\) concentration up to 500 mM would enhance collagen directionality on the mica surface (Leow and Hwang, 2011), where. To confirm compare the current study to previous studies, the 100 mM KCl solution from above was replaced with 200 mM sodium chloride (NaCl). No collagen directionality was observed in the resulting sample (Figure 4.5), verifying agreement with previous studies.

4.2.3 Collagen mobility on mica surface

4.2.3.1 Method

This following study was performed to show the mobility of adsorbed collagen monomers when incubated in solution. The general approach involves depositing a bidirectional film of microfibrils on mica, as discussed in section 4.2.1, rinsing the surface with deionized
water to wash away collagen loosely adsorbed to the surface, and finally, incubating the mica surface in a buffer solution (described below) at 37 °C for ~ 20 hours.

In a modified protocol from native collagen assembly described in section 3.2.1.1, an incubation solution was prepared with a mix of 110 mM KCl and 110 mM sodium phosphate buffer. This solution, without any collagen monomers, will henceforth be termed “native incubation solution”. After the deposition of collagen monomers and rinse stage, ~ 2 mL of the native incubation solution was added to the mica surface. The mica sample was then sealed in a Petri dish with parafilm to avoid evaporation, and placed in an Isotemp Oven (Fisher Scientific) for incubation at 37 °C. After the ~ 20 hour incubation period, the mica was removed from the solution, rinsed with deionized water, dried with a gentle stream of nitrogen, and imaged under AFM.

4.2.3.2 Results

AFM images of samples from the above procedure conclusively demonstrated the mobility of collagen monomers on the sample surface. After incubating the bidirectional microfibril film (Figure 4.4) in the native incubation solution, a uniform surface with discrete unidirectional native fibrils was observed. As seen in Figures 4.6 and 4.7, all fibrils were at least 1 µm in length with less than 5 nm height (comparable to initial microfibril film), and showed the characteristic D-period banding seen in mature native fibrils. Bumps with a few nanometers in height were observed in the gaps between the native fibrils, and can be attributed to salts commonly seen in AFM images in the absence of the rinse step. Such salts may be washed away with extended rinsing with deionized water. However, caution must be exercised as the force of the water stream (and the nitrogen stream during drying) can break the thin native fibrils,
overcoming the collagen–mica adsorption, and uplifting entire sections of the collagen film (Figures 4.8 and 4.9).

The formation of such discrete native fibrils might be attributed to the innate collagen–collagen D-period alignment and the mobility of collagen monomers on the mica surface. In the presence of 110 mM K$^+$, ~60% of the K$^+$ pockets on the mica surface were filled (Schlegel et al., 2006), weakening the overall electrostatic attraction between collagen and mica. As a result, the collagen-mica stabilization force became overpowered by the collagen–collagen interaction, leading to the formation of the energetically favourable native fibril form. As seen in previous studies (Gale et al., 1995), in vitro assembled native collagen solutions often resulted in a mixture of banded native fibrils and unbanded fibrils. Pure samples of banded native fibrils only existed in a narrow range of ionic strength in the solution (Loo, 2011), suggesting high ionic strength sensitivity and shallow potential energy minimums in achieving the “perfect” D-period alignment of monomers in the formation of the characteristic ~67 nm banding. Therefore, there must be a delicate balance between the collagen–mica and collagen–collagen forces for the successful formation of such banded fibrils aligned along the mica lattice. In fact, while the rearrangement of microfibrils in the formation of fibrils was consistently observed, D-periodic fibrillar banding was not 100% reproducible, with varying degrees of success each trial.

With the same experimental protocol, discrete unidirectional fibrils were consistently observed. However, the D-period banding was not always present. Figure 4.10 demonstrates a sample with observable D-period banding but with less definition than those observed in Figures 4.6 and 4.7. Figure 4.11 demonstrates a similarly prepared surface that had no observable D-period banding but retained the same unidirectional fibrillar alignment. With less than 50% reproducibility in the formation of full D-period banding, this was attributed to several factors as
explained above. Firstly, the formation of banded native fibrils was highly sensitive to ionic strength. Secondly, there exists a delicate balance between collagen–collagen interaction and collagen–mica attraction as dictated by K⁺ concentration. Thirdly, as different mica samples were used for each trial, there might exist a variation in the Si-Al ratio on the surface of the mica lattice, resulting in a varied K⁺ affinity and K⁺ pockets available. Fourthly, the small amount of evaporation in the incubation solution might vary due to ambient humidity, although this factor was thought to be unlikely because repeat trials using ~ 20 mL of incubation solution (instead of ~ 2 mL) resulted in similar D-period banding reproducibility. Finally, the collagen concentration on the mica might vary as a result of the microfibril deposition and rinsing steps. Nevertheless, despite all these potential issues, this study successfully demonstrated the ability to assemble discrete unidirectional D-periodic native fibrils from laterally mobile collagen microfibrils on a mica surface.

One alternate explanation in place of the lateral mobility of collagen monomers on the mica surface is as follows: collagen monomers might have desorbed from the surface into the native incubation solution (initially collagen-free), followed by the deposition of such monomers in solution onto nucleation sites on the mica surface. This possibility was tested via one test trial where the remainder of the native incubation solution was deposited onto a new freshly cleaved mica surface. If there were a significant amount of collagen monomers in the solution, they would be deposited on the mica surface due to the collage-mica electrostatic attraction. In an AFM scan of the surface, no such collagen monomers or aggregates were observed. With the high resolution in an AFM capable of detecting one single monomer, this result indicated an insignificant amount of collagen monomers desorbing from the mica surface during incubation. Therefore, the theory of laterally mobile collagen monomers held true.
As mentioned above, one main source of variation among different trials was the concentration of collagen on the mica surface. The amount of deposited collagen was difficult to measure by AFM, due to the thickness (only ~ 2 – 3 nm height) and non-uniform surface morphology (Figure 4.4). However, in two trials, the end product was observed to have a significantly lower concentration of collagen compared to other samples (Figure 4.12). In these two extreme cases, the difference in the amount of deposited collagen compared to other cases (Figures 4.6 and 4.7) demonstrated the significance of this source of error. With varying concentration of collagen on the mica, the collagen-collagen separation distance and charge density over the entire surface was thus expected to differ, affecting the degree of monomer–monomer D-period alignment in the finally product.

4.3 Summary

Interaction between collagen and the mica surface was utilized to control the alignment of collagen fibrils thus creating customizable surface morphology at the nanoscale. Through a modified protocol from Cisneros et al. (Cisneros et al., 2006), a uniform surface of unidirectional discrete D-banded native fibrils was assembled from monomers solution. Despite low reproducibility (< 50%), the relation of the higher pH and increased distance between fibrils was consistent with previous experimental observations (Jiang et al., 2004). In acidic pH, collagen microfibrils were observed to self-orient along the mica lattice (bidirectional) in the presence of K⁺. Under incubation of native fibril assembly conditions, the microfibrils were demonstrated to assemble into discrete fibrils. While the formation of such fibrils was consistent, the emergence of D-period banding was not. The variation among trials was attributed to a combination of factors including shallow potential energy minimums for D-period monomer–monomer alignment, a delicate balance of forces between collagen–collagen and collagen–monomer
interactions, different sources of mica, and varying collagen concentrations on the mica surface. Despite such factors, discrete unidirectional D-periodic native fibrils were successfully assembled from non-patterned collagen monomers on the mica surface. This result demonstrated for the first time the lateral movement of collagen monomers along the surface of the mica lattice, as induced by favourable collagen–collagen interactions. Furthermore, this study demonstrated a novel method in controlling, customizing, and changing the collagen surface morphology. This has enormous potential in the field of cell and tissue research where collagen is often used as scaffolding and coating material for academic research and medical applications.
4.4 References


Figure 4.1: AFM height images of a uniform film of banded native fibrils assembled on mica with a pH 9.05 solution of 50 mM glycine, 200 mM KCl, and 12 μg/mL PureCol collagen, followed by 1 hour incubation at room temperature. d) The cross section at c) an edge of a mica with lower collagen density was used to measure the height of the film.
Figure 4.2: AFM height images of discrete banded native fibrils assembled on mica with a pH 9.4 solution of 50 mM glycine, 200 mM KCl, and 12 µg/mL PureCol collagen, followed by 1 hour incubation at room temperature.
Figure 4.3: AFM height images of discrete banded native fibrils assembled on mica with a pH 9.4 solution of 50 mM glycine, 200 mM KCl, and 12 μg/mL PureCol collagen, followed by one hour incubation at room temperature.
**Figure 4.4:** AFM height images of a patterned film of micrifibrils after 5 minutes of incubation at room temperature with the following solution: 100 mM KCl and 12 μg/mL PureCol collagen.
Figure 4.5: AFM height image of unpatterned film of micrifibrils after 5 minutes of incubation at room temperature with the following solution: 200 mM NaCl and 12 µg/mL PureCol collagen.
**Figure 4.6:** AFM a) lock-in amplitude and b) height images of collagen sample on mica after ~ 20 hours of incubating the sample from Figure 4.4 in the following conditions: 110 mM KCl, 110 mM NaH$_2$PO$_4$, pH 7, 37°C. Note the D-periodic banding in the discrete fibrils.
**Figure 4.7:** Same preparation as Figure 4.6. AFM height images of collagen sample on mica after ~20 hours of incubating the sample from Figure 4.4 in the following conditions: 110 mM KCl, 110 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 7, 37°C. Note the D-periodic banding in the discrete fibrils.
**Figure 4.8:** Same preparation as Figures 4.6 and 4.7. AFM a) lock-in amplitude and b) height images of collagen sample on mica after ~20 hours of incubating the sample from Figure 4.4 in the following conditions: 110 mM KCl, 110 mM NaH$_2$PO$_4$, pH 7, 37°C. Note the breakage in the film, likely due to washing or drying steps.
Figure 4.9: Same as Figure 4.8: breakage of the collagen film. AFM a) lock-in amplitude and b) height images of collagen sample on mica after ~20 hours of incubating the sample from Figure 4.4 in the following conditions: 110 mM KCl, 110 mM NaH₂PO₄, pH 7, 37°C.
**Figure 4.10:** Same preparation as Figures 4.6 and 4.7. AFM a) lock-in amplitude and b) height images of collagen sample on mica after ~ 20 hours of incubating the sample from Figure 4.4 in the following conditions: 110 mM KCl, 110 mM NaH₂PO₄, pH 7, 37°C. Note the observable but faint D-periods compared to Figures 4.6 and 4.7.
Figure 4.11: Same preparation as Figures 4.6, 4.7, and 4.10. AFM height images of collagen sample on mica after ~20 hours of incubating the sample from Figure 4.4 in the following conditions: 110 mM KCl, 110 mM NaH$_2$PO$_4$, pH 7, 37°C. Note the absence of D-periodic banding.
Figure 4.12: Same preparation as Figures 4.6, 4.7, 4.10, and 4.11. AFM height images of collagen sample on mica after ~ 20 hours of incubating the sample from Figure 4.4 in the following conditions: 110 mM KCl, 110 mM NaH$_2$PO$_4$, pH 7, 37°C. Note the low concentration of collagen.