ASSEMBLY MECHANISMS OF TYPE I COLLAGEN AGGREGATES

by

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A thesis submitted in conformity with the requirements for the degree of PhD.
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ASSEMBLY MECHANISMS OF TYPE I COLLAGEN AGGREGATES
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Abstract

The solution-phase association of biological molecules to form aggregates has been studied from a variety of perspectives, such as the flocculation of polymers, association of colloids and the aggregation of proteins. The latter subject has been the target of particular attention, mainly because of its importance in biomedical applications as well as its implication in diseases such as spongiform encephalopathies and Alzheimer's disease. Investigating protein aggregation at a fundamental level, however, is often complicated by the poorly defined structures of the aggregation products formed, as well as by the difficulty in working with biological systems.

In this thesis, studies of Type I collagen, a rod-like protein which can undergo in vitro aggregation into highly-ordered protein structures, have been carried out with a view towards determining the mechanisms by which protein aggregates form. Two particular aggregate structures have been investigated. The first, a block-like aggregate called segmental long spacing collagen, was formed by the addition of nucleotide triphosphates to collagen monomers. By analyzing aggregate structures, formation kinetics and growth thermodynamics, it was concluded that these aggregates form via a hierarchical growth mechanism involving the formation of a stable intermediate and subsequent fusion of these intermediates. The second form of aggregate investigated was a fibril referred to as fibrous long spacing collagen. In this case, particular insight
was made into elucidating the structure of these fibrils using the atomic force microscope. Results obtained again suggest that this aggregate forms via a hierarchical mechanism, first forming stable ‘protofibrils’, which then merge in a complex manner to produce the final structure. In the final part of this thesis, the capabilities of the atomic force microscope in performing imaging of dynamic, biological processes in real time was examined, and was used to image the enzymatic digestion of collagen fibrils by the protein collagenase. The utility of the atomic force microscopy for \textit{in situ} investigation of complex processes such as collagen formation is discussed with a view towards applying this technique to future work.
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- Matthew Paige
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AFM</td>
<td>Atomic force microscope</td>
</tr>
<tr>
<td>EM</td>
<td>Electron microscope (with precursor S for scanning, T for transmission)</td>
</tr>
<tr>
<td>STM</td>
<td>Scanning tunneling microscope</td>
</tr>
<tr>
<td>FLS</td>
<td>Fibrous long spacing collagen</td>
</tr>
<tr>
<td>SLS</td>
<td>Segmental long spacing collagen</td>
</tr>
<tr>
<td>NTP</td>
<td>Nucleotide triphosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine 5'-triphosphate (sodium salt)</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine 5'-diphosphate (sodium salt)</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine 5'-triphosphate (sodium salt)</td>
</tr>
<tr>
<td>UTP</td>
<td>Uracil 5'-triphosphate (sodium salt)</td>
</tr>
<tr>
<td>ITC</td>
<td>Isothermal titration calorimetry</td>
</tr>
<tr>
<td>DLVO</td>
<td>Derjaguin-Landau-Verwey-Overbeek</td>
</tr>
<tr>
<td>TMV</td>
<td>Tobacco mosaic virus</td>
</tr>
<tr>
<td>DLA</td>
<td>Diffusion limited aggregation</td>
</tr>
<tr>
<td>RLA</td>
<td>Reaction limited aggregation</td>
</tr>
<tr>
<td>FTIR</td>
<td>Fourier transform infrared spectroscopy</td>
</tr>
<tr>
<td>PMT</td>
<td>Photomultiplier tube</td>
</tr>
<tr>
<td>NSOM</td>
<td>Near field scanning optical microscope</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DPPC</td>
<td>Diphosphatidyl choline</td>
</tr>
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Chapter 1: Introduction to thesis

1.1 Introduction

This thesis is concerned with the aggregation of proteins to form structures of an ordered nature. In particular, attention has been focused on the aggregation of Type I collagen, a protein which is considered to be well suited for fundamental investigations. The primary interest in these studies is in obtaining a fundamental understanding of the mechanisms responsible for the formation of ordered structures, with a view towards controlling and ultimately directing the course of their assembly.

The organization of this thesis shall be as follows: there shall be an initial introduction to the topic of protein aggregation, followed by a review of some relevant principles of colloid chemistry, including a discussion of both kinetics and thermodynamics of aggregation, which are fundamental to this work. This will be followed by a general overview of the literature on systems which show aggregation properties which are similar to collagen, and finally a review of collagen aggregation itself. Subsequent chapters will contain a summary of the research carried out, with each chapter providing a more detailed introduction as it applies to the specific experimental work.

1.2 Protein aggregation

The statement that the aggregation of biomolecules, and proteins in particular, is an important field of research is beyond reproach. One can list a variety of areas in which protein aggregation plays a fundamental role. In disease pathology, aggregation gives rise to the formation of cataracts (1) and atherosclerotic plaques (2). For biomedical applications, protein aggregation and adsorption is generally thought of in a
negative sense, as it results in so-called ‘bio-fouling’, the impairment of function of an implanted prosthetic device because of adsorption of proteinaceous matter (3). In purely industrial terms, an oft cited example where protein aggregation plays a role is the clogging of milk pasteurization apparatus by milk proteins (4). Regardless of its exact nature, the aggregation of proteins is a topic that has ramifications in a number of vital applications.

The majority of the examples listed above share a common feature, that being that the protein aggregates consist of loosely packed, irregular structures. These structures can be easily modeled as fractal systems (5). Examples of proteins which are known to produce fractal structures include bovine serum albumin (6), and fibrin (7). The formation of these irregular aggregates, which shall, for lack of a better name, be referred to as non-specific aggregates, is generally considered to be caused by an unwanted side-reaction of protein folding. As reviewed by Kiefhaber (8), various models explaining this phenomenon have been suggested, including that aggregate formation results from a competition between the folding of denatured proteins and aggregation of the unfolded protein. Phrased differently, non-specific protein aggregation is a naturally occurring phenomenon brought about by proteins found in an unfolded state. An unfolded protein can either fold into its native conformation, or aggregate with other unfolded proteins to form a more thermodynamically stable structure. Further evidence for this has been forwarded by investigations of protein mutations, in which specific modifications of a protein have been shown to increase the tendency of proteins to unfold and aggregate (see review by Wetzel (9)). This has implications in a number of areas, including the highly intensive research in the field of Alzheimer’s disease. In this area of research, it has
become apparent that denaturation of proteins not normally associated with Alzheimer's can lead to the formation of amyloid-like plaques, the trademark of the disease (10, 11).

Not all protein systems form random, non-specific aggregates. Systems which form more complex structures, including neurotubules (12), the protein coats of viruses (13) and filaments (14) abound. It is these protein aggregates, which form in a highly-organised manner, which we are interested in.

A phrase traditionally used to describe the formation of organized structures is "self-assembly", though the expression has lost much of its significance, having been adopted by a huge number of research disciplines. Let us instead declare the term "ordered-aggregation" as being the phenomena in which we are interested. The prototypical example of ordered-aggregation is the formation of ribosomes, cellular organelles found in all eukaryotes. Ribosomes are composed of ribonucleic acids subunits. and can be degraded into these constituent subunits through a variety of techniques. When individual subunits are mixed with one another, under suitable conditions, they construct themselves back into a working ribosome. An apt analogy to this ordered-aggregation has been suggested by Engel (15), in which he likened the process to placing the components of a transistor radio in a box, randomly mixing the parts, then shaking the container, with the end result being the formation of a working radio. In effect, all the information needed to form the final structure is contained in its component building blocks. We shall hereafter define ordered-aggregation as being the formation of a highly ordered, often periodic and perhaps even functional entity from an initially random mixture of precursor units.
In this thesis, we are interested in ordered-aggregation that involves biological molecules in solution. Indeed, the majority of systems which show ordered-aggregation are biological in nature, and often involve proteins. As summarised by Oosawa (16): “Amino acids polymerise into polypeptides, and polypeptides form specific three-dimensional structures in order to function as proteins. Protein molecules make assemblies by intermolecular interaction, and these assemblies form various subcellular structures.” It is the formation of these assemblies, most commonly observed in subcellular structures, which we are interested. However, an intrinsic difficulty with investigations of biological systems is their innate complexity. When dealing with \textit{in vivo} (living) systems, a huge number of parameters over which one has little control may affect the aggregation process. As an experimental physical chemist, one is obliged to minimize the number of uncontrolled parameters in the system being studied, in order to isolate one effect over another. In many cases, this necessitates the separation of the precursor molecules from the biological system and carrying out investigations \textit{in vitro} (outside the living organism). While this topic shall be discussed in more detail at a later time, we shall make the comment that the conditions used in these investigations are not intended to emulate physiological conditions but rather are intended to simplify assembly conditions to the point that a minimal number of experimental parameters must be dealt with. Whether or not these results may be extrapolated to the \textit{in vivo} system is unclear. In the case of collagen, many of the structures formed are indistinguishable from those formed in living organisms (17), suggesting that \textit{in vitro} studies may be compatible with those carried out \textit{in vivo}. 
We shall furthermore refine the length scale over which the studies shall be carried out. While the folding of peptide chains into tertiary protein structures perhaps fits under the umbrella definition of ordered-aggregation, we shall concern ourselves with structures on a larger scale. Primary consideration will be of objects in the range of tens of nanometres up to tens of microns – the former of which, if not the latter, is the length scale over which interactions between particles is generally governed by the well-known principles of colloids.

1.3 Proteins as colloids

The philosophy of treating biomolecules and specifically proteins as colloids is hardly a new one. The topic has been discussed in numerous textbooks (18, 19). Colloids are generally considered to be particles with sizes in the range of 1 –1000 nm which are dispersed in solution. At these length scales, the ratio of total particle surface area to particle volume is large and surface effects tend to dominate the interactions between particles. Much of the work in the field of colloids has been dedicated towards understanding and regulating the forces between colloidal particles, which dictate whether the system will remain stable for an infinite time, or undergo aggregation. An important contribution to determining the fundamental causes of protein aggregation has been provided by theories describing intermolecular and interparticle forces, one of the more popular being DLVO (Derjaguin-Landau-Verwey-Overbeek) theory.

Originally produced to explain phenomenology in systems such as sols and silver halides. DLVO theory has now found application in a huge number of fields, including protein aggregation. A useful summary of the application of DLVO theory to protein aggregation has been provided by DeYoung (20). In short, DLVO theory accounts for an
electric double layer and van der Waals forces between charged particles, allowing calculation of surface potentials as a function of particle separation, for a given particle geometry. One of the predictions of DLVO theory is the dependence of particle interaction potentials upon the ionic strength of the surrounding medium, as illustrated in Figure 1.1. As shown, DLVO theory gives predictive power over the nature of particle association — that being whether association is dictated by kinetic or thermodynamic factors. At low salt concentrations, Figure 1 (a), electrostatic interactions between particles are large, resulting in a large free-energy barrier lying above the primary minimum. Overcoming this barrier is unfavourable, resulting in particle aggregation which is very slow. In the opposite case, at high electrolyte concentrations (Figure 1(c)) electrostatic charges are highly screened and van der Waals forces become dominant. Aggregation in this case is usually rapid. The primary difficulty encountered by theories such as DLVO, is that while surface potentials can be easily determined for particles of a simple geometry and uniform charge distribution, such as planes or spheres, more complicated structures make the relevant calculations problematic. Biomolecules often have complicated and flexible shapes, with non-uniform distributions of charge over their surfaces. Additionally, these parameters can fluctuate significantly with both time and the conditions of their surrounding medium. While simplified models do exist, calculating potentials for complicated biomolecular structures is a monumentally difficult challenge.

1.4 Protein aggregation: Kinetics & Thermodynamics

While theories such as DLVO grant some basic predictive power over the driving forces behind protein aggregation, they do not provide any real insight into the exact
mechanisms of aggregation nor into the geometry of the structures which they form. Both the formation mechanism and the structure of colloidal aggregates are intimately connected with the kinetics and thermodynamics of the aggregation process. Let us first consider the thermodynamic driving forces behind the formation of simple, ordered protein aggregates. Much of this material is summarised from the monograph by Oosawa (16).

1.5 Thermodynamics of ordered aggregation

In solution, dispersed monomers and aggregates (often referred to as polymers) coexist in equilibrium. Let us first consider the most simple case of aggregation, that of formation of linear polymers in which protein monomers are aligned end-on with one another. Defining an equilibrium number concentration of monomers (mol/solvent mol) as $C_1$ and of $i$-mers as $C_i$, we can define via mass action:

$$C_i = K^{-1}(KC_1)^i \quad [1]$$

where $K$ is an equilibrium / binding constant given by: $K=\exp(-\Delta G/kT)$ and $\Delta G$ is the Gibbs free energy for the formation of monomer-monomer bonds under the assumption that this value is independent of parameters such as the degree of polymerization. From [1] we can express the total number of protein molecules, either as free, dispersed monomer or bound into polymer, as:

$$C_0 = \sum iC_i = \frac{C_1}{(1-KC_1)^2} \quad [2]$$

The relationship between the total concentration of protein and the concentration of free monomer is plotted in Figure 1.2 a (16). In general, as the total concentration of protein increases, the amount of free monomer in solution increases and approaches the value $K^{-1}$ in the limit of infinite concentration. This indicates that as we raise the total
concentration of protein in the solution, we will increasingly observe the formation of large polymer strands. Let us furthermore define the average degree of polymerization as:

\[ <i> = \frac{\sum iC_i}{\sum C_i} = \frac{1}{(1 - KC_i)} \quad [3] \]

Again, as shown in Figure 1.2 a, this value increases, gradually, with increasing total protein concentration. As noted by Oosawa, the aggregation of the protein tropomyosin is well described by a linear aggregation scheme.

Let us now consider a somewhat more complex aggregation scheme, the formation of helical polymers. In this scheme, we shall define the aggregate structure such that each monomer is bound with four or more neighbouring monomers. We shall state that a linear trimer of three proteins will be deformed by the addition of a fourth monomer, to give a helical shaped polymer. Our initial aggregate 'core' shall consist of such a distorted tetramer, and aggregate growth will occur such that addition of a monomer to the end of the polymer results in the formation of bonds between two neighbouring monomers and a long, helical polymer is formed. On this basis, let us define the number concentration of i-mers in the helical aggregate as \( C_{ih} \) (i-mer in helical polymer).

On this basis, we can define:

\[ C_{3h} = \gamma C_3 = \gamma K^{-1}(KC_i)^3 \quad [4] \]

\[ C_{4h} = K_h C_{3h} C_1 = \gamma K^{-1} K^3 K_h C_1 \quad [5] \]

and in general:
\[ C_{h} = K_h C_{(t-1)h} C_1 = \gamma \left( \frac{K}{K_h} \right)^2 K_h^{-1} (K_h C_1) \]  
\[ \sigma K^{-1} (K_h C_1) \]  

where \( \sigma = \gamma \left( \frac{K}{K_h} \right)^2 \), \( \gamma = \exp(-\Delta G^*/kT) \) and \( K_h = \exp(-\Delta G_h / kT) \)

where \( \Delta G^* \) is the excess free energy required for the deformation of the linear trimer to give the curved, helical polymer and \( \Delta G_h \) is the change in free energy for the addition of a monomer to the growing polymer. In general, \( \gamma \) is a factor which is smaller than one as \( \Delta G^* \) for the monomer to trimer addition will be a positive value – deforming the initial monomer unit requires an input of energy. Also worthy of note is the fact that because there are more bonds per monomer in the helical model over the simple linear model, then \( K_h \) will be larger than \( K \). From equation [6], we can generate an expression for the total concentration of helical polymers \( C_h \) as:

\[ C_h = \sum_{i=2}^{\infty} i C_{i,h} = \frac{\sigma C_1}{(1 - K_h C_1)^2} - (\sigma C_1 + 2\sigma K_h C_1) \]  

For the concentration of monomers and linear polymers (dimers and trimers), we may use the previously developed expression in equation [2]. Hence, the total concentration of protein, \( C_0 \) is given by the sum of these two expressions:

\[ C_0 = \frac{C_1}{(1 - K C_1)^2} + \frac{\sigma C_1}{(1 - K_h C_1)^2} - (\sigma C_1 + 2\sigma K_h C_1) \]  

For cases where \( \sigma \ll 1 \), as is typically the case as the factor \( \gamma \) is much smaller than unity, and for the previously noted condition of \( K < K_h \), equation [8] can be simplified to:

\[ C_0 \approx C_1 + \frac{\sigma C_1}{(1 - K_h C_1)^2} \]  

[9]
Consider now the situation where the total protein concentration, $C_0$, is raised at constant values of $K_h$ and $\sigma$ (constant environmental conditions). Because the value of $\sigma$ is very small, the second term of equation [9] becomes insignificant, and the total concentration of protein is dominated by that of the monomer: the solution essentially consists of dispersed monomers only. However, as $C_0$ (and accordingly, $C_1$) approach the value of $K_h^{-1}$, the second term becomes non-negligible and the concentration of aggregates in solution accordingly becomes non-negligible. On this basis, one can define a critical concentration of monomers for the formation of polymers as:

$$C_c = K_h^{-1}$$  \[10\]

That is, below this critical concentration of monomers, polymer growth does not occur. Any excess amount of monomer above $K_h^{-1}$ are incorporated into polymers. As in the case of the simple, linear aggregation, we can define a degree of polymerization:

$$\langle i \rangle = \frac{\sum ic_i}{\sum c_i} = \frac{1}{1 - K_h C_c} = \left(\frac{C_h}{C_c}\right)^{\frac{1}{2}} \frac{1}{\sigma^2}$$  \[11\]

A plot illustrating the physical features of helical polymerization is shown in Figure 1.2b.

The existence of a critical concentration of monomers allows one to draw parallels between the formation of aggregates and the condensation of gas to liquid or the formation of crystals from a saturated solution. From a thermodynamic point of view, the formation of colloidal aggregates may be considered as the formation of a new, stable phase from an initially unstable or metastable system. Studies of homogeneous nucleation, nucleation in which a change of phase occurs in the absence of structural impurities, suggest that the formation of a new phase is initiated by the formation of multiple particle clusters which form via random fluctuations of system properties. The
multiple particle clusters may be thought of as a true heterogeneous system with the mother phase (21). Furthermore, this cluster is capable of further growth. Proposed by Volmer and Weber (22), the clusters (referred to as nuclei) can be treated in terms of equilibrium thermodynamics, and the free energies of formation of clusters may be calculated. From this point of view, the nucleation of colloidal aggregates is often likened to the condensation of a liquid droplet from the vapour phase. This is done primarily because, in the vapour phase, the mathematics is fairly simple; however, the basic principles derived here can be extended into the colloidal regime. Let us first consider \( n \) moles of a vapour at pressure \( P \), in equilibrium with its liquid form. One first defines an initial equilibrium between gas phase and bulk liquid:

\[
\text{n A}_{(g)} = \text{n A}_{(l)} \quad [12]
\]

From this we may define a \( \Delta G \) for transfer of \( n \) moles of vapour at pressure \( P \) to a growing liquid drop of radius \( r \), pressure \( P_0 \). The net \( \Delta G \) is given by the expression:

\[
\Delta G = -\frac{4\pi^3}{3} \left( \frac{\rho}{M_w} \right) RT \ln \left( \frac{P}{P_0} \right) + 4\pi r^2 \sigma \quad [13]
\]

where \( \rho \) is the liquid density, \( M_w \) is its molecular weight and \( \sigma \), the surface tension of the liquid droplet. This function is shown in Figure 1.3 (23). Differentiating, we find the a single maxima of this function, often referred to as the critical radius \( r_c \):

\[
\left( \frac{\partial \Delta G}{\partial r} \right) = 0 ; \ r_c = \frac{2\sigma \bar{V}}{RT \ln \left( \frac{P}{P_0} \right)} ; \ \Delta G_{\text{max}} = \frac{16\pi \sigma^3 \bar{V}^2}{3 \left( RT \ln \left( \frac{P}{P_0} \right) \right)^2} \quad [14]
\]

Adamson (23) has suggested the following mechanistic interpretation of these results:

For vapour pressures below ambient \( P_0 \), \( \Delta G \) increases with increasing cluster size. While all sizes of clusters would exist, only the smallest of nuclei would be present in any
significant number. The number of these clusters would be prone to random fluctuations. For pressures above $P_0$, there will also be fluctuations in the number of clusters with size less than $r_c$. However, when a cluster reaches the critical size $r_c$, favourable fluctuations cause it to grow indefinitely. Expressed more simply, objects smaller than the critical radius most frequently return to the vapour, while objects larger will tend to grow spontaneously.

As noted by Vold and Vold (24), the development of this field has not remained restricted to vapour-liquid nucleation. One may extend similar arguments to account for precipitate formation. Let us consider the formation of an initial nucleus, composed of $n$ molecules, each of volume $v$. We may write:

$$\Delta G = -nv\Delta G_v + K \frac{n^{2/3} v^{2/3} \sigma}{\Delta G_v}$$  \[15\]

where $\Delta G_{ve}$ is defined as the free energy of formation of the precipitate on a per unit volume basis. $\sigma$ is the surface tension and $K$ is a precipitate shape-dependent factor. By again maximizing this function, we can extract a critical nucleus size:

$$\frac{\partial \Delta G}{\partial r} = 0; \quad n^* = \frac{8 K^{3/2} \sigma^3}{27 \Delta G_v^3} \quad : \quad \Delta G^* = \frac{4K^3 \sigma^3}{27\Delta G_v^2}$$  \[16\]

Parallels between this case and that of vapour condensation are obvious: above a certain number of particles in the nucleus, objects will tend to grow in a spontaneous manner.

1.6 Enthalpy

If one is correct in describing protein aggregation in terms of reversible thermodynamics, than one can also ascribe enthalpies of formation to the aggregation process. However, until relatively recent times, calorimetric techniques have not been able to accurately measure the small quantities of heat evolved or absorbed in biological
aggregation processes (the difficulty tends to lie in the quantity of protein available for use; preparing large quantities of high-purity protein is often problematic, forcing one to work with very small amounts and as such the absolute amount of heat evolved from biological processes tend to fall below the detection limit of many calorimeters). As such, thermodynamic data is often extrapolated from kinetic data, using an Arrhenius approach to determine activation enthalpies and the like. As noted by Oosawa (16), the general result of such experiments has been to show that aggregation of proteins is, for the most part, an endothermic process. Examples of systems in which the aggregation is endothermic include formation of TMV protein coat (13), neurotubules (12) and actin (16).

1.7 Kinetics of ordered aggregation

A variety of models have been used to describe aggregation kinetics, both to fit experimental data, and for carrying out computer simulations.

From a purely computational point of view, two of the most commonly used methods for simulating aggregation are Diffusion Limited Aggregation (DLA) and Reaction Limited Aggregation (RLA). In the first approach, particles are constrained to a region of space and allowed to move about in a random walk. When particles encounter one another, they stick and remain frozen in the geometry of contact. The interaction between particles is so strong, the aggregation kinetics is limited only by the rate at which particles can diffuse through the medium. Simulated structures formed using DLA algorithms tend to be fractal and dendritic in shape, and as such have been used successfully to describe the formation of non-specific protein aggregates (20). The other commonly employed simulation model is RLA, in which it is the particle association
process which limits the rate of growth of the aggregate, as a large number of particle collisions are required before particles stick to each other. These two simulation methods have been used to describe a number of systems, such as colloidal gold (25), silica (26) and polystyrene latex (27). However, they have also been employed to describe ‘ordered-aggregation’, specifically in the formation of collagen (28), having met with mixed success. We shall explore this in more detail in later chapters.

The alternative approach to modeling aggregation kinetics is to fit experimental data to predictions from a proposed mechanism. For the aggregation of proteins, there are two broad classifications of mechanisms which are commonly used, with small variations of the themes being taken up as required. The two classifications are aggregation which occurs via nucleation and growth, as commonly seen in the formation of crystals (29), or a hierarchical type of growth.

An excellent example of the fitting of kinetic data to a nucleation and growth type model has been carried out by Tschopp and Engel (30) in their investigation of T4 bacteriophage tail sheath protein association to form polysheaths. In this system, the protein P18 aggregates with other P18 units to form a complex, larger structure termed a polysheath. The authors of this paper tracked the concentration of polysheaths via light scattering in a spectrofluorimeter, and fitted the experimental data in the following manner:

Let us define \( C_{p}^{*} \) to be the concentration of monomers incorporated into rod-like particles, \( C_{p} \) the concentration of nuclei and \( C_{A} \) to be the concentration of free monomers. Initially, \( x \) monomer units form a nucleus \( A_{x} \) with a forward rate constant \( k_{N} \):

\[
xA \rightarrow A_{x} ; \quad \frac{dC_{p}}{dt} = k_{N}C_{A}^{x} \quad [17]
\]
Growth then occurs by addition of monomer units to these nuclei, with a forward rate constant of $k$:

$$A_i + A \xrightarrow{k} A_{i+1} \quad [18]$$

As such the rate of increase of polysheath filament concentration goes as:

$$\frac{dCp^*}{dt} = k_i C_A^i C_p \quad [19]$$

Numerical integration of equations [17] and [19] allowed the generation of curves showing $Cp^*$ as a function of time, which were sigmoidal in shape. With appropriate selection of constants and fitting parameters, the agreement between the model and experimental results were excellent, as shown in Figure 1.4. One may make further refinements of the nucleation-and-growth type models such as taking into account reverse steps of the equilibrium, or growth steps with rate constants which depend upon the number of monomers in the aggregate. At this point, a word of caution is required. While it is true that the solutions to equations [17] and [19] yield a polymer mass which has a sigmoidal time-dependence, too often researchers tend to attribute sigmoidicity to aggregation which occurs via nucleation and growth, without any attempt to verify the mechanism by alternate methods. We shall discuss this topic more fully in Chapter 3.

The second general classification of growth mechanisms commonly used to described ordered aggregation is hierarchical or stepwise growth. The hallmark of hierarchical growth is the formation of stable, intermediate structures, which merge with one another to form the final product. A highly generalised reaction scheme for hierarchical growth is as follows: Let us define $A$ to be a basic monomer sub-unit. Monomer units can merge to form a stable intermediate, $B$: 
Further growth now occurs via interaction of the intermediates, B, to form the final product, C:

\[ A + A \xrightarrow{k_1} B \] [20]

\[ B + B \xrightarrow{k_2} C \] [21]

While the difference between this scheme and the simple nucleation and growth method may appear minimal, the kinetics of the assembly are profoundly different, as we shall discuss in Chapter 3. The most well-known system which undergoes growth by a hierarchical mechanism is the keratin intermediate filaments, which is generally believed to form via the initial production of a protein dimer, followed by merging of dimers into a tetramer. The tetramers coalesce into protofilaments, then protofibrils, which gather into groups of four to form the final intermediate filament (31).

1.8 Ordered aggregation in non-collagenous systems

As noted previously, there are a number of systems which fit under the description of ordered-aggregation. One of the more well-studied is the polymerization of muscle actin. Actin is a protein found in all eukaryotic cells, and plays a major role in the maintenance of cell shape and in the formation of contractile fibres in muscle (32). The form of actin which provides structural support to the cell, termed filamentary or F-actin, is a long (up to 200 μm) polymer composed of globular or G-actin molecules. The assembly and disassembly of actin filaments is important for a variety of diverse applications in cells, including locomotion, phagocytosis and cytokinesis (32). The formation of filamentary actin from G-actin is a complex process, likely involving a cooperative nucleation and growth process. This subject is reviewed by Engel (15) as well as by Oosawa and Kasai (33). In general, it is believed that the process starts with a
nucleation step, likely either the formation of a dimer or a trimer followed by propagation and elongation. The propagation step is thought to occur when G-actin, in association with a bound nucleotide such as ATP, undergoes polymerisation with a growing chain of F-actin, releasing inorganic phosphate (addition of a monomer to growing filament). Depolymerization of G-actin coupled to ADP occurs simultaneously with the polymerization reaction, giving rise to the steady-state scheme shown in Figure 1.5.

Another example of a system showing ordered aggregation is the formation of bovine epidermal keratin filaments, a fibrous protein found in both inner living cell layers and dead stratum corneum layers. In their seminal work on the topic, Steinert et al. (34) characterised the in vitro assembly process using a combination of light-scattering, x-ray diffraction and electron microscopy. It was observed that the seven basic α-keratin polypeptide building blocks which make up the keratin filaments in living tissue could be made to polymerise in vitro to form the same structures seen in living tissues. On the strength of x-ray diffraction measurements, the workers concluded that the final keratin filament polymers were comprised of a three-chained unit. From their light-scattering measurements, a mechanism of formation of the filaments was proposed, in which the aggregation involved an initial, rate-determining formation of a hexameric nucleus, followed by a rapid polymerisation stage. This mechanism was proposed on the basis of fitting sigmoidal kinetic curves to the gathered light-scattering data.

Investigations by Gaskin et al. (12) into the assembly and disassembly of porcine neurotubules are another example of an investigation of ordered-aggregation. Neurotubules, an important cytoplasmic structure in living tissues, are known to undergo assembly and disassembly in vitro. upon exposure of tubulin molecules to nucleotide
triphosphates and physiological temperatures. Again, using light-scattering techniques, the authors attempted to determine the mechanism of growth of the tubules. Despite extensive kinetic measurements, the authors could not confidently assign a mechanism to tubule formation. While sigmoidal growth curves were obtained and the authors believed a combination of initiation and propagation steps were responsible for the results obtained, they acknowledged a complexity to these stages that they were unable to resolve.

1.9 Type I Collagen

As stated previously, we have chosen to investigate the aggregation properties of the protein Type I collagen. Type I collagen is the most abundant protein found in animal tissues. It is present in tendons, bones, skin, cartilage and a wide range of other structural tissues. In tissues, collagen is most commonly found in the form of long, polymerised fibrils. The fibrils themselves are macromolecular aggregates, the basic building block of which is the collagen molecule, sometimes referred to as the collagen monomer.

Because of collagen's profound importance in living tissues, and also because of its widespread use as a biomaterial, the structure and biochemical properties of the monomer have been studied exhaustively. An excellent review on properties of the monomer and on collagen in general has been written by Nimni (35). The collagen monomer is a semi-flexible rod, with an approximate length of ~280 nm and diameter of ~1.5 nm. It consists of three amino acid chains which are interwoven to form a right-handed, triple helix. The hallmark of collagen is that almost every third amino acid in a chain is a glycine residue. The amino acid sequence -(Gly-X-Y)ₙ- is commonly
observed, with X and Y being the amino acids proline and hydroxyproline, respectively. The structure is such that every third amino acid of the chain is located in the centre of the triple helix. This central region is so sterically crowded that the only amino acid side chain that can fit into it is that belonging to glycine. Proline and hydroxyproline are notoriously inflexible and bulky residues, which grant the molecule a degree of structural rigidity (36). For Type I collagen, the three polypeptide chains within the monomer are not identical; there are two chains referred to as α(1) chain, and a second type termed an α(2). The two types differ in their exact amino acid sequence, though the same basic repeat unit is still present. A schematic representation of a segment of the collagen monomer is shown in Figure 1.6. When first synthesized in a living organism, the collagen monomer consists of the large, triple helical domain, with non-helical telopeptide and propeptide regions located at its ends. After secretion from the cells in which it is synthesized, collagen often undergoes N-proteinase cleavage of its propeptide and telopeptide segments, leaving the helical region intact. While the helical region is not perfectly uniform, and has, in fact, been further subdivided by some researchers into at least seven subdomains (37), it is often treated as being a single, uniform object. In this thesis, unless stated explicitly, this shall be the general philosophy taken.

In aqueous environments, most proteins are charged molecules and collagen is no exception. The amino acid sequence for type I collagen has been well characterized, and in conjunction with appropriate pKa data, has been used to generate plots showing the distribution of charge along the length of the monomer. A calculated charge distribution for the collagen monomer at physiological pH (~7) is shown in Figure 1.7 (38). Experimental evidence in support of these calculations exists and will be discussed in
greater detail in subsequent chapters. While the monomer shows both positive and negative charges, there is a net predominance of positive charge on one end of the monomer. This positive charge has profound implications for the aggregation properties of the collagen monomer, as will shortly become apparent. It is also known to play an important role in the binding of proteins and minerals to collagen molecules (21, 39).

A property of collagen that has made it an excellent system for study is that collagen monomers can be separated from animal tissues, and the assembly process can be made to occur under *in vitro* conditions. This has allowed for the controlled investigation of formation of the collagen assembly structures.

### 1.10 Collagen aggregation and fibril formation

In living organisms, Type I collagen monomers aggregate to form highly organized structures. The most commonly occurring structure formed by collagen is what is referred to as a native-type fibril. Native-type collagen fibrils, found in most animal tissues, are usually tens of nanometres in diameter, and many microns in length (35). The most striking feature of the fibril is a periodic banding pattern of ~67 nm running along its length. The distance 67 nm is often referred to as a 'D-spacing' in the collagen literature, with each collagen monomer being approximately 4.4D or ~300 nm in length. An electron micrograph of a typical native-type fibril is shown in Figure 1.8 a (40). Despite years of study, the exact structure of the native-type fibril is still not well defined, or at least not entirely accepted. The most widely used model for the collagen fibril structure is the so-called Hodge-Petruska model (41). In this model, fibrils are thought to consist of monomer units aligned in a partial stagger array along the long axis of the fibril. The dark regions on negatively stained EM micrographs correspond to 'gap-
zones. regions in the fibril which are not occupied by the collagen monomer because of the staggered nature of their packing (See Figure 1.8 b). Often misinterpreted, this model was initially generated to aid interpretation of electron micrographs of collagen fibrils and was not intended to give a full, three-dimensional description of the collagen fibril structure. More complete models have been proposed (e.g. see (42)), though these models are largely conjectural and none has of yet met with general acceptance.

The \textit{in vitro} ordered aggregation of type I collagen into fibrils, referred to in the literature as fibrillogenesis, has been studied extensively, with recent reviews having been produced by both Veis and George (38) as well as by Kadler et al. (43). Fibrillogenesis of collagen can be initiated in several ways. The most common approach is to use the so-called “cold-start” method by Holmes (44), in which a cold, acidified solution of collagen monomer is mixed with a neutral pH salt buffer, and the entire mixture is heated to a temperature in the range of \(-25-40^\circ\) C. The reaction conditions are such that one may adjust parameters, such as concentration of monomer, ionic strength and temperature, and characterise the effect upon the assembly process. An alternate preparation for collagen fibrils is to perform dialysis upon the acidified monomer, gradually raising its pH to neutrality. While offering the advantage of not using salt solutions, which can impede microscopic imaging of fibrils, the process is kinetically slower than the cold start approach and lacks control over the precise conditions under which the assembly takes place.

The mechanism by which collagen fibrils form \textit{in vitro} remains one of active debate. Two models of assembly have been used to describe the process, namely the previously discussed nucleation and growth theory, and the hierarchical or stepwise
Evidence in favour of both models has been obtained, though neither has been unequivocally accepted.

In their investigation of *in vitro* native-type collagen aggregation, Comper and Veis (45) claimed that collagen fibril growth can be divided up into a simple nucleation and propagation phase. Using turbidimetry to monitor the extent of aggregation, they observed sigmoidal-type growth curves, from which they extrapolated a nucleation and growth type mechanism. This work is supported by the results of Bernengo et al. (46), who used dynamic light-scattering techniques along with electrical birefringence to determine diffusion constants of collagen monomers and multimers during the course of the assembly process. These authors observed that at very early stages in the assembly only collagen monomers could be observed, and at later times, only very large aggregates (fibrils). No other species, such as dimers, trimers or other small intermediates could be found, despite them being easily resolvable if present. Based on this data, the authors supported a nucleation and growth related mechanism, in which the initial nucleation event involved some monomer-related event, such as internal stiffening or conformational transition. These results were supported by dynamic light-scattering experiments carried out by Payne et al. (47), who also concluded that significant numbers of dimers or trimers are not present during the early stages of fibril assembly.

Further evidence in favour of such a mechanism comes in the form of FTIR experiments carried out by Veis and Payne (48), which suggest that the collagen monomer does indeed undergo conformational rearrangements, albeit subtle ones, upon exposure to environmental conditions similar to those under which collagen assembly occurs. These authors observed that upon exposure to collagen assembly conditions, the
stretching bands associated the carbonyl groups in the Gly-X-Y triplet of collagen were shifted to higher wavenumbers, indicating a strengthening of the carbonyl bond or a weakening of the intrahelix hydrogen bond. It was postulated that these conformational changes in the monomer may “create the appropriate local conformations required for the intermolecular interactions that establish the ... assembly pattern” (38).

Evidence in favour of a hierarchical or step-wise assembly has also been presented by a number of authors. On the strength of turbidimetry measurements and electron microscope observations (49), Gelman proposed that the assembly should be divided up into three distinct stages: an initiation step, which likely involves the formation of what was termed a ‘short microfibril’, followed by a linear growth stage, in which the microfibrils grow longer but not wider, followed by a third, lateral growth step in which native, banded fibrils are formed via lateral association of microfibrils. A fundamental assumption in these investigations was that the increase in the turbidimetry signal measured can be attributed to the lateral aggregation of particles alone, an assumption we shall explore in greater depth in subsequent chapters.

Gelman provided further evidence in favour of this type of mechanism on the basis of dynamic light-scattering measurements (50). The three proposed stages of the assembly were found to correlate well with the existence of distinct aggregate sizes. Prior to starting the assembly, the collagen solutions were shown to contain only semi-flexible rods of dimensions ~ 300 nm x 1.2 nm, corresponding well to the expected size of the monomer. During the first ‘initialisation’ step, the average particle size increased slightly, to which the authors attributed the formation of stable intermediates with approximate dimensions >1500 nm length and < 8 nm in diameter. These intermediates
were postulated to contain between 5-100 monomer units, though the authors admitted to a fair amount of uncertainty in this matter. The next stage of the assembly, again, involved lengthening of these intermediates until a certain, 'critical' length was reached. After this stage, and into the lateral aggregation stage, dynamic-light-scattering measurements became unusable because of the size of the particles involved. though the existence of banded fibrils at this stage was noted through EM measurements.

Silver provided additional evidence in favour of a hierarchical assembly mechanism by using angle-resolved static light-scattering techniques (51, 52). In these investigations, the author determined the molecular weight of collagen species during the early stages of the assembly, and found that the lag phase of the assembly terminates with the formation of aggregates whose molecular weights exceed 9.3x10^5, a molecular weight consistent with the formation of a linear trimer. On the basis of a series of such measurements, Silver proposed the following to be the mechanism of assembly: Linear dimers and trimers form in solution during the early stages of the assembly process. Once a certain number of trimers are formed, lateral aggregation involving approximately 5 trimers occurs, resulting in the formation of a pentameric intermediate. Finally, this pentameric intermediate can grow by linear or lateral addition of other units.

In addition to experimental work, collagen assembly has been the target of more computational and theoretical investigations. Parkinson et al. (28) developed a model to investigate the aggregation of rod-like particles in two dimensions, making use of diffusion-limited aggregation. By assigning binding rules to the rods, such as making the rods behave as perfectly symmetrical objects which could adhere at any contact point, or giving them an asymmetry such that they could only adhere with one another if they
overlapped by an integral of D (~67 nm), the authors found they could simulate the formation of fibrillar structures. Because they could form fibrils without having to assign asymmetry to the rods, the authors suggested that the formation of fibrillar structures is a property inherent to rod-like aggregating particles, and that the detailed structure of the aggregating rod plays only a minor role in dictating the final structure of the aggregate. Wallace has developed a method for computing free energies of aggregates of Type I collagen, and used these to compute free energies between successive steps along assembly pathways (53). Using this approach, Wallace could account for the formation of D-staggered fibrils over non-staggered aggregates. This work also predicted that the dimers and trimers suggested to exist by Silver (51) and others are not major intermediates, though the introduction of an additional activation energy parameter into the calculations resulted in some situations in which such entities were the earliest forms of aggregates formed in the assembly.
1.11 Topics of investigation in this thesis

The review of collagen literature in the previous section illustrates the complexity of aggregation in the collagen system. However, all investigations discussed to this point have been concerned with formation of the 67nm spacing native-type fibril, primarily because of its ubiquity in nature. An issue which has not yet been discussed is the issue of polymorphism. That is, the native-type collagen fibril is not the only aggregate structure which can form \textit{in vitro}, and the mechanisms by which these structural polymorphs assembly remain unstudied. In this thesis, we shall discuss investigations of two structural polymorphs of type I collagen: the segmental long spacing (SLS) aggregate and the fibrous long spacing fibril. The exact nature of these structural polymorphs of collagen will be left to the specific chapters in which they are discussed. Here, it is sufficient to state that small variations in the conditions of the assembly can result in large variations of aggregate structure, as well as in the mechanisms by which they form. We have seen hints of this, mainly in section 1.5, where the intimate relation between aggregate structure and thermodynamics was reviewed. In this thesis, we shall employ the principles we have discussed in this introductory section to explore the ordered-aggregation of these polymorphic variants.


Figure 1.1: Summary of DLVO Theory from DeYoung (20). Interparticle free energy is plotted as a function of intermolecular separation. Curves a), b) and c) represent low, medium and high salt concentrations, respectively. Reprinted with permission from the American Chemical Society. Copyright 1993. American Chemical Society.
Figure 1.2: Reproduced from Oosawa and Asakura (16). Plot displays physical features of (a) linear and (b) helical polymerization. In a), the solid line represents the monomer concentration in equilibrium with linear polymers, while the broken line represents the degree of polymerization. The x-axis shows the total monomer concentration. In b), the line $C_1$ represents the concentration of monomers, $C_h$ the number of helical polymers and $<i>$ the average degree of polymerisation, all as a function of total concentration of macromolecules. (Copyright © 1975 by Academic Press)
Figure 1.3: Reproduced from Adamson (23). Plot showing Gibbs free energy vs nucleus radius for homogeneous nucleation of liquid from the vapour phase. Function reaches its maximum value at the critical radius, defined in equation [14].
Figure 1.4: Sigmoidal light scattering curves for the nucleation and growth of polysheath filaments (Tschopp and Engel(30)). Circles represent experimental data collected from light scattering experiments. Solid lines are model developed by numerical integration of equations [17],[19] and determining appropriate fitting parameters. Reprinted from ref. (30) with permission from Elsevier Science.
Figure 1.5: Steady state equilibrium process for growth of F-Actin from G-Actin (From Engel (15)). Step I involves an irreversible formation of the F-actin filament: step II, reversible dissociation of G-actin with associated ADP from the filament: step III, the reversible exchange of ADP against ATP for monomeric G-actin. (Copyright © 1994 by Academic Press).
Figure 1.6 a: Schematic illustration of a segment of a collagen monomer (Reproduced from Nimni(35)). Monomer consists of three amino acid chains which are interwoven to form a final, triple helical structure. For Type I collagen, two of the constituent chains are identical, referred to as $\alpha(1)$ chains, while the third, $\alpha(2)$, has a slightly different amino acid sequence.
Figure 1.7: Charge distribution along length of collagen monomer calculated at near-physiological pH. Bars indicate average net charge for every three amino acid triplets. Segments above center line represent positive charges, below, negative. Note predominance of positive charge near left-hand side of plot (the so-called H1 region of the molecule). (Reproduced from Veis (38)). (Copyright © 1994 by Academic Press).
Figure 1.8a: Electron micrograph of negatively stained native-type collagen fibril formed in vitro (reproduced from Kadler (40)). Scale bar represents the approximate length of one collagen monomer (~300 nm).
Figure 1.8 b: Schematic illustration of the Hodge-Petruska model as applied to interpretation of negatively stained EM collagen fibrils (36). Light regions in images correspond to so-called ‘gap regions’, consisting of spaces which exist between the aligned collagen molecules.
Chapter 2: Segmental Long Spacing Collagen

2.1 Introduction

During investigations of collagen fibril structures by electron microscopy, Schmitt et al. (1) discovered an entirely new type of collagen aggregate structure. These authors found that a neutral pH collagen monomer solution buffered in phosphate and dialysed against an acidic citrate buffer gave rise to a white precipitate. When this precipitate was examined under the electron microscope, it was found to contain thin filaments, unstructured ‘non-specific’ aggregates and a more ordered, block-like aggregate, which showed evidence of an internal banding structure. Further refinement of their sample preparation, namely the addition of ~ 0.2% w/v adenosine 5' triphosphate (ATP) to acidified collagen monomer solutions, resulted in the near exclusive production of this ordered aggregate. The lengths of the aggregate corresponded remarkably well with the ~ 300 nm length of individual collagen molecules. These new structures were dubbed ‘segmental long spacing’ or SLS aggregates. An electron micrograph of SLS aggregates, taken from the original publication by Schmitt, is shown in Figure 2.1.

Since their initial discovery, SLS collagen aggregates have become enormously important in collagen research. It is now generally accepted that the aggregates consist of a bundle of collagen molecules, aligned laterally and with the same polarity. As reviewed by Kuhn (2), the aforementioned EM banding pattern observed on SLS aggregates (Figure 2.2) reflects the distribution of amino acids along the polypeptide chains of the constituent collagen monomers. By careful analysis of the banding pattern, one can extrapolate amino acid sequences of the collagen molecules. Dark bands of positively stained SLS correspond to locations on the collagen monomer occupied by
charged, polar amino acids, whereas in negatively stained samples, the light bands correlate with the locations of hydrophobic amino acids. The validity of this technique was confirmed when the first continuous stretches of the collagen monomer amino acid sequence were determined and compared with the EM results (3). Since this discovery, analyses of SLS banding patterns have proven to be useful for a variety of purposes: SLS band analysis has been used to determine sequences of Type III and IV collagen (4, 5), to help characterize the endproducts of the enzymatic degradation of collagen (6) and in the characterization of procollagen, the precursor form of collagen prior to enzymatic cleavage of its non-helical extensions (7). Ghosh et al. (8) have used the banding patterns in SLS to compare collagen types amongst different vertebrates, providing a general view of homology in amino acid sequences in animals.

While originally thought to exist only as 'artificial structures' which are only formed in vitro, SLS collagen is now believed to exist in living organisms. SLS-like aggregates have been observed in cultures of connective tissue cells which are actively synthesizing collagen (9). SLS was observed in both the active cells and the culture medium, suggesting that SLS was being transported in and out of the cell. It was suggested that forming SLS is a way of storing large quantities of collagen monomer in a compact manner, possibly for transport out of the cell.

Effort has been applied by several researchers to determine the exact role of the nucleotide ATP in the formation of SLS. Schmitt et al. (1) originally postulated that the ATP may be incorporated into the structure of the aggregate, though the evidence for this was minimal. Stronger evidence that this might be the case was provided by Kobayashi et al. (10), who performed an electronmicroscopical analysis of SLS without using heavy
metal staining, as the latter may introduce artifacts into the imaging. While the banding patterns observed were weak, they were sufficiently strong to allow adequate analysis. As was the case for stained aggregates, the banding patterns that were observed could be correlated with the amino acid sequence of the collagen monomer. The authors postulated that the banding patterns in the unstained aggregates were enhanced by ATP that was bound to positively charged basic residues of collagen. It was suggested that the ATP was located inside the aggregate, acting as bridging units between basic residues of adjacent monomers. This model is illustrated in Figure 2.3. These studies also indicated the presence of a distinct positively charged region near the N-terminus of monomer, in agreement with calculations by Veis and George (11) based upon the known collagen amino acid sequence (Chapter 1. Figure 1.7). It has been suggested that the role of the ATP in SLS formation is to inhibit the electrostatic repulsion between similarly charged regions of monomers aligned in register, which are preventing aggregation.

While the majority of research involving SLS has been in applying it to amino acid analysis of collagen, little has been performed on studying the mechanism by which it forms. In the literature, SLS aggregates are often termed ‘crystallites’ (e.g.,2)), suggesting that they form by the lateral addition of monomer units into an orderly lattice. However, there is no direct evidence for this. The existence of SLS aggregates present an important opportunity for the investigation of ordered aggregation. The final morphology of the aggregate is much simpler than that of the native-type collagen fibril, making analysis of structures much more simple. The reaction conditions required for the aggregation process are amenable to a controlled, in vitro study, with all of the required chemical components being available from commercial sources. In the following two
chapters. investigations into the formation of SLS crystallites are discussed, with the goal of determining the exact mechanism of their growth. The experiments described made use of a combination of techniques, including light scattering for kinetic measurements, atomic force microscopy for structural characterization and isothermal titration calorimetry for thermodynamic data.

2.2 Materials and Methods

2.2.1 General Overview of Techniques:

In the following section, a review of the basic methods of analysis used in the experiments for this thesis will be provided. A review of a method common to all of the experimental sections, atomic force microscopy, will be included, along with a discussion on laser light scattering. More specific details about the experiments will be included in a secondary section in this thesis and in the ‘Materials and Methods’ section of subsequent chapters.

2.2.2 Atomic Force Microscopy:

Since their initial invention in the early 1980s by Binnig, scanning probe microscopes such as the scanning tunneling microscope (STM) (12) and the atomic force microscope (AFM) (13) have become common tools for surface analysis. These instruments have proven to be useful for a wide range of applications, including studies of metals, semiconductors, polymer surfaces and biological samples. Numerous reviews of scanning probe techniques have been written – excellent ones have been produced by Sarid (14) and Gould et al. (15).

Of particular use to those performing measurements on soft polymer and biological samples is the AFM. Originally invented in 1986, this method makes use of
forces exerted between a probe tip and a sample surface to provide topographical information about the surface (13). While originally created to study surface features in the nanometer range (16), modern instruments are now capable of measuring features whose dimensions range from the atomic scale to sizable fractions of a millimeter.

In the AFM's simplest form of operation, often referred to as 'contact mode imaging', a sharpened probe tip, which is mounted upon a cantilever arm, is brought into contact with a sample surface and raster scanned. The probe tip tracks the surface of the sample, and topographical variations result in deflections of the cantilever. By tracking these deflections, typically by monitoring the movement of a diode laser reflected off the backside of the cantilever, one can construct an image of the surface over which the tip is scanned. The basic principles of contact mode imaging are illustrated in Figure 2.4.

The AFM offers several advantages over more traditional microscopy techniques, such as optical and electron microscopy. While optical microscopes can be used to measure object dimensions in the x-y plane, they generally cannot provide information about feature sizes in the z-dimension. Additionally, the best resolution achievable by optical methods are typically on the order of a micron, the lower bounds being set by the diffraction limit of light (17). The AFM can provide high-resolution imaging of all three spatial dimensions, and, as it does not make use of light for measurements, is not limited by diffraction.

An advantage of the AFM over electron microscopy is that no sample staining is required, which precludes possible artifacts from sample-stain interactions. Also, imaging can be performed under ambient conditions, negating the need for vacuum chambers and related equipment. Unlike scanning tunneling microscopy, samples imaged in the AFM
need not be conductive, as the mechanism of contrast is not related to the tunneling of electrons. One of the greatest advantages of AFM, however, which has only been truly realized in the past few years, is its ability to image samples under fluid which offers the unique potential for imaging biological samples in their native, hydrated states. The latter will be a topic explored in more detail in Chapter 5.

The resolution of the AFM is superior to optical microscopes and is comparable with that of EM. A comparison of the AFM with optical and scanning electron microscopy is shown in Table 2.1. Modern commercial AFMs boast a vertical resolution on the order of ~ 0.01 nm, and a lateral resolution of ~ 0.1-3 nm. In the latter case, resolution is limited by the so-called 'convolution effect', in which the image produced by the AFM is actually a combination of both the tip and sample geometry. This effect is illustrated in Figure 2.5. In general, the resolution of the technique is limited by size of the probe tip. Most commercial probes have a tip radius of ~50 nm (18), though smaller tips can be produced by a variety of methods (eg. (19)).

Many variations of contact mode imaging have been developed. Some examples of these include intermittent contact imaging (Tapping Mode®) in which the tip is brought into contact with the sample in a periodic fashion, reducing the lateral forces which are applied to the sample. lateral force imaging in which the frictional forces between the surface and tip are measured (20) and chemical force imaging in which the interactions between chemical groups on a functionalized tip and surface are used to generate contrast (21). These imaging modes, along with a myriad of others, allow for more than simple structural elucidation of surfaces to be performed. AFM can now be
used to elucidate a variety of mechanical, electrical and even chemical properties of surfaces.

In summary, the AFM is a versatile instrument, capable of probing surface topographies at high resolution under a variety of environmental conditions. It offers advantages to optical and electron microscopy, in terms of both resolution and ease of sample preparation. In addition to its ability to probe topographical features, the AFM can also provide information such as mechanical and chemical properties of a surface.

2.2.3 Laser Light Scattering

Light scattering is a classical method of analysis for measuring a range of physical properties of molecules. While scattering methods can be used to analyze samples in solid, gaseous or solution phases, they have been applied most extensively to studies of macromolecules in solutions. With the advent of the laser, a wide range of types of light-scattering methodologies have been produced and have been used to extract an equally wide range of macromolecular properties. For example, light scattering measurements can provide molecular weights of molecules in solutions, diffusion coefficients and particle sizes to name a few.

The basic principle of light scattering is that when electromagnetic radiation impinges upon a collection of molecules, light is scattered from the sample at all angles. The scattered light can be used to extract information about the physical properties of the scattering sample. In this thesis, the primary concern is with the scattering of light from solutions of macromolecules that are both polydisperse and evolving in size and shape as a function of time. We shall now derive some fundamental relations between observable properties of scattered light (intensities) and properties of the scattering solution. Much
of the following section is compiled from the work of Brown, Kratochvil and Johnson (22-24).

Let us first consider a single, isolated atom in the gas phase. We shall expose this atom to a beam of unpolarised electromagnetic radiation, the electric field of which shall be expressed as:

$$\vec{E} = \bar{E}_0 \cos(k_0 y - \omega_0 t) \quad [1]$$

where $k_0 = 2\pi/\lambda_0$, $\omega_0 = 2\pi \nu_0$, and $\nu_0$, $\lambda_0$ are the frequencies and wavelength of the light, respectively. The electric field causes the electrons in the atom to vibrate and generate an oscillating dipole. In general, the magnitude of the induced dipole will be proportional to the magnitude of the incident field, with a proportionality constant termed the polarizability:

$$\mu = \alpha \bar{E} \quad [2]$$

where $\alpha$ is a scalar for isotropic molecules, a tensor for anisotropic.

The oscillating dipole fluctuates in time, giving rise to an oscillating electric field. As the field strength is proportional to the acceleration of the oscillating electrons, one may write:

$$\frac{\partial^2 \mu}{\partial t^2} = -\bar{E}_0 (4\pi^2 \nu_0^2) \cos(k_0 y - \omega_0 t) \quad [3]$$

Now, if one defines a detector position at some distance $r$ and an angle $\theta$ from the scattering centre, take the intensity ($I_s$) of scattered light at this point to be proportional to the square of the scattered field, $\bar{E}$, and normalize against $\bar{E}_0$ one can write:

$$\frac{I_s}{I_0} = \frac{8\pi^4 \alpha^2}{r^2 \lambda^4} \bar{E}_0 (1 + \cos^2 \theta) \quad [4]$$
where $I_s$ is the intensity of scattered light, and $I_0$ the intensity of incident light. The $\lambda^{-4}$ dependence of scattering intensity is often referred to as Rayleigh scattering, in honour of this pioneer in the field.

Such is the case for a single particle. The situation is somewhat more complicated for a collection of particles, but not intractably so. For a collection of ideal gas particles, one must take into account the molecular weight of the gas as well as the effect of concentration upon the index of refraction of the gas. The equivalent expression for equation [4] for an ideal gas is:

$$I_s = I_0 (1 + \cos^2 \theta) \frac{2\pi^2}{r^2 \lambda_0^4 N_A} x \left( \frac{dn}{dc} \right) M c$$  \[5\]

where $N_A$ is Avogadro’s number, $n$ is the refractive index of the gas, $c$ is concentration in units of mass/volume and $M$ is the molecular weight of the scattering specie. On the basis of this equation, it is often common to define the so-called Rayleigh ratio:

$$R_o = \frac{I_s}{I_0} x \frac{r^2}{(1 + \cos^2 \theta)}$$  \[6\]

This expression is useful because it eliminates intensity changes which are dependent upon parameters of the apparatus. In terms of the Rayleigh ratio, equation [5] simplifies to:

$$R_o = \frac{2\pi^2 n^2}{\lambda^4 N_A} \left( \frac{dn}{dc} \right)^2 M c$$  \[7\]

While expressions of this type work well to describe scattering from gasses, they are not sufficient to describe the behaviour of macromolecules in condensed phases. In such situations, one must take into account a number of different parameters. Foremost amongst these is intraparticle interference of scattered light. When particles are much
smaller than the wavelength of light used – dimensions around $\lambda/20$ is the typical cutoff quoted – they essentially behave like point scatterers of light. However, when particles approach this limit, light which scatters off different parts of the molecule will reach the detector with markedly different phases. The light from these different sources will interfere with one another and the resulting intensity arriving at the detector will be reduced. Of course, this interference effect shows a strong dependence on the angle of the detector; it can be shown, both experimentally and through calculation, that the phase shift between light scattered by different parts of the particle will increase with increasing angle of observation (23). This is illustrated in Figure 2.6.

To account for this angular dependence in scattered intensity, one introduces the so-called particle scattering function, sometimes referred to as the particle structure factor. This function is defined as:

$$P(\theta) = \left[ \frac{R_\theta}{R_0} \right]$$  \[8\]

where $R_\theta$ and $R_0$ are the Rayleigh ratios at the angle $\theta$ and angle 0, and $c$ is the concentration of macromolecule. For small particles, the Rayleigh ratio has no angular dependence and as such, the particle structure factor is unity for all angles of observation. For larger particles, $P(\theta)$ is smaller than one, indicating a lessening in scattered intensity.

Mathematical relationships between the particle structure factor and the shape of a scattering particle can be determined. The general methodology for doing so is to treat each particle as being a collection of individual scattering centres. The electric field generated at the detector is determined by summing over contributions from each scattering centre and determining the net intensity. One must also account for all
possible orientations of the particle in solution. As in solution, it must be assumed that the particle orients itself with equal probability in all directions. While a difficult problem, a general solution for all particle shapes has been formulated. This is given as:

\[ P(\theta) = \frac{1}{\sigma^2} \sum_{i=1}^{a} \sum_{j=1}^{a} \sin \frac{\mu h_{ij}}{\mu h_{ij}} \]  

[9]

where \( h_{ij} \) is the distance between the i-th and j-th scattering centre, \( \mu = (4\pi/\lambda) \sin(\theta/2) \) (scattering vector) and \( \theta \) is the angle between the incident and scattered beam. While not generally solvable for any shape, equation [9] can be solved for some simple shapes, such as a thin rod, Gaussian coil and a sphere.

Returning now to the issue of generating a usable expression for scattering off solutions of macromolecules, the next issue to be accounted for is one of polydispersity. As noted, equation [7] assumes a uniform particle size. In reality, most macromolecular solutions contain a wide distribution of sizes, which must be accounted for. This can be done by replacing the molecular weight, \( M \), in equation [7] by an averaged molecular weight \( M_w \). This is generally defined as:

\[ M_w = \frac{\sum c_i M_i}{\sum c_i} \]  

[10]

where \( c_i \) and \( M_i \) are the concentration and molecular weight of the i-th component. However, a wide range of particle shapes and sizes in solution means one must also take into account the effect of polydispersity on the particle scattering function. Again, an averaged value must be used, which shall be defined as:

\[ \overline{P}(\theta) = \frac{\sum c_i M_i P_i(\theta)}{cM_w} \]  

[11]
Taking all of these factors into account, the final expression for the measurement of scattered light intensity for a polydisperse solution of macromolecules is:

\[
R(\theta) = \frac{2\pi^2 n^2_0}{\lambda^4 N_A} \left( \frac{dn}{dc} \right)^2 \left( \sum_i c_i M_i P_i(\theta) \right) \left( \sum_i \frac{c_i}{cM_i} \right) c \quad [12]
\]

or

\[
R(\theta) = K c M_\omega \overline{P}(\theta) \quad \text{where } K = \frac{2\pi^2 n^2_0}{\lambda^4 N_A} \left( \frac{dn}{dc} \right)^2 \quad [13]
\]

These expressions will prove to be useful in later chapters.

2.2.4 Preparation of Segmental Long Spacing collagen aggregates

a) In vitro SLS collagen assembly

Type I calf-skin collagen (Sigma) was dissolved over ice in 0.05% acetic acid, with occasional sonicating to facilitate the breakdown of collagen aggregates. The mixture was centrifuged at 10,000 rpm for 60 minutes at 4°C. After centrifugation, the supernatant was filtered through 0.45 μm Millipore filters (Sigma) and mixed with the appropriate polyanion solution. Solutions of the polyanions were prepared by dissolving the solid (ATP, ADP, GTP, Amaranth, Cibacron 3GA from Sigma; ATP-γ-S from Calbiochem) in 0.05% acetic acid. Collagen and polyanion solutions were combined at room temperature to yield mixtures with final collagen concentrations typically of ~ 0.5 mg/mL, polyanion concentrations of ~ 2 mg/mL and a final pH of 3.5. The mixtures were allowed to equilibrate overnight at room temperature. A series of dilutions ranging from 10-1000 fold were prepared by adding aliquots of the reaction mixture to an appropriate volume of Millipore water. The diluted samples were deposited onto freshly
cleaved sheets of mica in 20 µL aliquots and allowed to dry for an hour prior to imaging in the AFM.

b) Atomic Force Microscopy

Samples were imaged with a Nanoscope III instrument (Digital Instruments, Santa Barbara, CA), typically using a square pyramidal silicon nitride tip of nominal spring constant of ~ 0.58 N/m. Tips produced by electron beam deposition were also used in attempts to improve resolution. Images were taken in contact mode, in air. Images obtained were consistently reproducible, showing minimal perturbation from the probe tip. Both height and force mode images were taken simultaneously, with height measurements being taken from height mode images only.
2.3 Results & Discussion:

2.3.1 Structural characterization of SLS aggregates

When monomeric collagen solutions were combined with ATP, ATP-γ-S or GTP according to the preparation described above, turbid solutions were obtained. Analysis of the resulting mixtures by AFM revealed an abundance of SLS aggregates. A typical AFM image of a 100-fold diluted sample obtained from an ATP-collagen preparation is shown in Figure 2.7 a,b. The AFM images of SLS aggregates were consistent with the EM results described by Schmitt et al. (1). The aggregates appeared rectangular, and were formed in a range of sizes. The lateral dimensions were approximately ~360 nm by 200-400 nm, with a noticeable large spread in the latter value. Note that because AFM images are a convolution of the sample and probe tip geometry, the lateral dimensions are an overestimate of the actual ones. Particle diameters were 30-50 nm in height above the mica substrate. As the AFM allows particle heights to be measured without convolution effects, the distribution of aggregate diameters shall be used to provide a general measure of average aggregate size for investigations in subsequent parts of this thesis. As a reference, a typical distribution of aggregate diameters from a standard preparation of SLS is shown in Figure 2.8.

A sectional analysis of a typical aggregate is shown in Figure 2.9 a,b. The dimension with less variance (~360 nm) shall be ascribed to the long axis of the monomer (plus tip geometry), in accordance with previous models of monomers arranged in register. Measurements taken along this collagen monomer axis will be referred to as the longitudinal section (Figure 2.9 a), while perpendicular measurements (shown in Figure 2.9 b) will be called the cross section. It should be noted that the shortest
dimension (30-50 nm). shown in Figure 2.9c is always that above the substrate. This could be the result of a positive interaction between the aggregate and the mica substrate, causing the aggregates to always lie flat in order to maximize the area of contact with the substrate.

The various sections clearly indicate that the aggregates are not cylindrically symmetric. Closer examination of Figure 2.9a shows that while the longitudinal section is almost rectangular with a steep slope, this can be ascribed to the ends of the aligned monomers. The cross section, however, shows a less steep slope, with an obvious rounding at the apex. If one were to view the SLS aggregate as a small crystal, faceting would be expected, instead of this rounded cross section. One possibility is that the interaction between SLS and the mica substrate is much too strong, and is causing a deformation of the weakly-crystalline aggregate.

With the use of electron beam deposited tips of high aspect ratio, finer structural features on the surface of an aggregate could be resolved. The longitudinal-section shows the presence of a series of minor grooves on the surface of the aggregate. The most pronounced feature consisted of a higher ridge on one end of the aggregate, which is \(~2.5\) nm in height above the average aggregate surface. While these ridges may be caused by folding of the collagen monomer, it seems more likely that they are caused by the accumulation of ATP molecules at the end of the monomer. In SLS aggregates, all molecules are believed to have the same polarity, that is, the N and C-terminus of the protein are aligned in the same direction (2). Presumably, the end of the aggregate which is occupied by the H1 region on the molecules (see Figure 1.7) will contain a high local concentration of ATP because of the net positive charge on the monomer. This will
result in the buildup of non-negligible quantities of ATP at the H1 terminus of the aggregate, resulting in the formation of a pronounced ridge.

In addition to the primary ridge on the end of the aggregate, herein referred to as the H1 region of the aggregate, a series of smaller ripples along its length are also present. The resolution of these fine structural details is limited by the finite width and aspect ratio of the AFM tip. The presence of these ultrastructural features are in accord with the results from EM (2), and are presumably caused by the alignment of corresponding amino acids within the monomers. Unfortunately, the resolution needed in order to make a quantitative comparison could not be obtained; AFM probes with much smaller tip radii, such as those constructed from carbon nanotubes (Dai et al. 1996) may be useful for this characterization but these are currently difficult and expensive to produce.

2.3.2 Ability of different polyanions to induce SLS aggregation

As stated previously, the general view is that the negative charges of ATP are thought to neutralize the positive charges on the collagen monomers, which serve to counteract aggregation (11). Hence, addition of any substance which increases the overall ionic strength of the solution should give rise to SLS formation. However, attempts to induce SLS aggregation by the addition of a variety of polyanionic reagents, including inorganic phosphate (in the form of KH2PO4 and calcium triphosphate) and ADP, met with uniform failure, indicating that simple charge neutralization is not the sole requirement for this effect. On the other hand, the addition of GTP to monomeric collagen produced aggregates which were structurally very similar, albeit with a somewhat larger diameter, from those obtained with ATP, as shown in Figure 2.10. It is
also worth noting that with the GTP induced aggregates, the dimension which corresponds to the length of the monomer is not necessarily the largest dimension. In Figure 2.10, an arrow indicates an aggregate in which the 'monomer length' dimension is shorter than the other lateral dimension. The H1 region is readily apparent in the longitudinal section (Figure 2.11a), but the distance between the edges of the aggregate are much smaller than those taken perpendicular to this length (Figure 2.11b). Peculiar structures were formed by the addition of UTP to collagen monomers, some of which are shown in Figure 2.12a,b. The aggregates had some morphological properties in common with the SLS aggregates, particularly the presence of a major ridge on one end of the aggregate. However, the structures were much thinner in diameter than normal SLS (diameter distribution shown in Figure 2.13) and in some cases, had a significantly greater length. Inspection of a longitudinal cross-section (Figure 2.14) reveals that several of these structures have multiple H1 ridges, indicating they consist of multiple SLS-like subunits aligned in an end-on fashion. The growth of these objects was found to occur much more slowly than that of normal SLS, though this is a qualitative observation only.

These results with GTP, ATP, UTP and ADP may suggest that the aggregation is activated by the hydrolysis of the phosphate bond, similar to the case for the ATP-assisted assembly of actin filaments (25). In general, nucleotide triphosphates (NTPs) in biological systems play the role of an energy source; that is they are consumed in processes requiring an input of energy. To examine the possible importance of NTP hydrolysis in SLS aggregation, we used ATP-γ-S, a non-hydrolyzable ATP analogue (the structure of this polyanion, and that of all others used in these experiments, is shown in
Figure 2.15). This preparation produced SLS aggregates with no observable structural difference from those produced by the addition of ATP or GTP, indicating that the role of the polyanion is not to provide assistance with the energetics of the assembly by means of exothermic degradation.

To further investigate the role of charged polyanions in the assembly, we examined the effect of two dyes, Cibacron blue 3GA and Amaranth. Like ATP and GTP, both of these dyes possess three negatively charged groups. In both cases, SLS aggregation was not induced. although a heavy, fibrous precipitate of indeterminate structure was formed. indicating that while a high local concentration of negative charge can yield aggregation. SLS formation is by no means inevitable; the type and amount of charge is clearly an important prerequisite for SLS formation. but the manner in which it is distributed within a polyanion appears to also play a crucial role.

The results obtained suggest that not only are three negatively charged groups needed. but that they have to be arranged in a particular way with respect to one another. A simple linear chain of negative charges is not sufficient. as indicated by the failure of calcium triphosphate to induce SLS formation. Both the phosphate functionality and the nitrogenous base of the nucleotide triphosphate play a role in the aggregate formation. as evinced by the general effect of nitrogenous base type (e.g. guanidine. adenine. uridine) on aggregate diameter. It is also possible that the ability of the polyanion to participate in hydrogen bonding may play a significant role as well. since adenine. guanidine and uridine functionalities are well-known for this ability.
2.3.3 Role of ATP in aggregate structure:

While it has been generally assumed that the role of ATP in SLS is incorporation into the aggregate, evidence in the literature which proves this is far from unequivocal. In the following experiment, the proof that such is the case has been provided by making use of UV-Vis spectroscopy.

SLS aggregates were prepared in the manner described previously. After preparation, the sample was centrifuged at 10,000 rpm for 15 minutes to remove SLS from solution, and the pellet was retained. A UV-Vis spectrum of the supernatant solution is shown in Figure 2.16. Unlike most proteins, Type I collagen does not have a strong absorbance band in the region 250-300 nm because of its lack of tyrosine and tryptophan residues. The absorbance peak in Figure 2.16 is therefore caused exclusively by the presence of excess ATP in solution. The absorbance maxima for ATP occurs at ~270 nm with an extinction coefficient of ε=14x10^3 cm^-1 M^-1 (34).

After collecting a spectrum of the initial supernatant, the pellet was washed thoroughly with acetic acid, then sonicated and vortexed. The sample was re-centrifuged and the supernatant was separated. Spectra of the supernatant were taken at both 260 and 270 nm. This wash/sonicate/vortex cycle was repeated three times, by which point the absorbance of the supernatant at the chosen wavelengths decreased to ~ 0.1. At this point, it was decided that all excess ATP had been removed from the sample. The collagen pellet was then re-dissolved by leaving it overnight in 0.05% acetic acid at 4°C, with occasional agitation. A UV-Vis spectrum of the resulting solution was taken and compared with the previous spectra. A summary of the resultant UV-Vis absorbance
measurements are shown in Figure 2.17. and a summary of the procedure is shown in Figure 2.18.

Dissolution of the SLS aggregates into their constituent monomers resulted in the liberation of ATP into solution, as indicated by the marked increase in absorbance at both wavelengths. This shows that ATP must be bound within the aggregate, in agreement with the suggestion by Kobayashi (10, 26). One can estimate the molar ratio of collagen monomer to ATP bound within the aggregates by making use of the extinction coefficient of ATP and the concentration of the stock collagen solution. From the Beer Lambert Law:

\[ A = \varepsilon C l \quad [14] \]

where \( A \) is absorbance, \( C \) is concentration and \( l \) is the path length. One may calculate a final ATP concentration in the supernatant, and accounting for the sample volume, determine the number of moles of ATP in solution. Dividing by the known number of moles of collagen in the sample, yields a final estimate of 25 moles of ATP per mole of collagen monomer. Note, error estimates have not been provided for this value, as it should be taken to be correct for around an order of magnitude only. This degree of uncertainty occurs because some collagen is inevitably lost during the centrifugation/vortex/sonication cycle, and the calculations performed here necessarily assume no loss of collagen. If anything, the value of 25 will be somewhat of an underestimate. The number seems reasonable, in light of the model proposed by Kobayashi (10) in which the ATP molecules are associated with the many basic residues lying along the length of the collagen monomer.
2.3.4 Mechanism of growth by AFM

During the course of studying SLS aggregates formed via addition of ATP to collagen, images which provide direct insight into their mechanism of formation were obtained. In the occasional sample, prepared in the manner described previously, one could observe aggregates before they have become fully developed SLS crystallites. Some examples of these partially developed aggregates are shown in Figure 2.19 a.b. While not observed in every sample, partially developed aggregates were observed in a reasonable number of preparations, and presumably if one were capable of imaging a large enough area on the mica substrate one would observe such structures for every sample.

Consider, now, an image of such partially formed aggregates in greater detail. In both Figure 2.19 a and b, one observes various collagen assembly products, ranging from discrete fibrous objects to fully mature SLS crystallites. These assembly products are more readily apparent Figure 2.20, a zoom of Figure 2.19 a. For ease of analysis, Figure 2.20 has been divided into eighteen distinct regions and labeled accordingly. The labeled figure and magnified views of more crowded regions are shown in Figures 2.21-2.30. While measurements of every object in each region was impractical, a representative proportion were measured for heights. Lateral measurements were often made impractical because of the rather entangled nature of the entities involved. However, in some cases, when possible, both height and lateral measurements were taken. Height measurements of the objects measured are summarized in Table 2.2, along with a brief qualitative description of the structures.
An attempt to describe all the data in this figure will not be made, as the sheer volume is best expressed in a tabular format. However, several representative examples are worth exploring in greater depth. Let us first consider region (1), described in Table 2.2 as being a single aggregate with height 22.7 nm. A sectional analysis of this aggregate, shown in Figure 2.31 a,b shows several interesting features. In Figure 2.31a, one observes the distinctive longitudinal section of an SLS aggregate, complete with characteristic H1 ridge. With this aggregate, lateral measurements could be taken; along the longitudinal direction, the lateral dimension was ~390 nm and along the cross-section. (Figure 2.31 b) the distance was ~450 nm. While the longitudinal section corresponds well in both length and shape to a fully formed SLS aggregate, the diameter and cross-section indicate this is a partially formed aggregate. That is, the aggregate is rather flattened - it is spread out in the lateral (cross-section) dimension and has a markedly reduced height.

Region ten contains a large number of highly-entangled fibrous objects. Measurement of nine of these objects which could be reasonably distinguished, yielded fibre diameters ranging from 3.6 - 10.7 nm, with a mean of approximately 7.5 nm. These dimensions clearly indicate that the objects cannot be collagen monomers, which have diameters of ~1.5 nm. Rather, these objects are oligomers, likely consisting of two or more monomer units. There appears to be some long-range ordering of the oligomers present; while they are distinct, independent entities, a definite lateral alignment amongst them is evident.

Consider now, the objects contained in region seven, a magnified image of which is shown in Figure 2.21. This region contains a number of fibrous objects of the type
observed in region 10. Again, as tabulated in Table 2.2, a number of the objects have been counted and measured, yielding an average diameter of around 11.2 nm. As is apparent, these oligomers are in the process of aligning themselves in a lateral fashion. A sectional analysis of one of these oligomers, shown in Figure 2.32, reveals the by now familiar longitudinal section of a mature SLS aggregate. While the ends of this intermediate are somewhat entangled, its lateral size is estimated to be ~380 nm, in reasonable accord with our previous measurements ascribed to the collagen monomer length.

Regions 15 and 16 (region 15 shown in magnified form in Figure 2.28) contain a number of interesting objects for study. In both regions, measurements have been carried out on three objects having average heights of 15.7 and 17.1 nm, respectively. These intermediates are larger than those observed in other regions. The intermediates are also quite closely packed with one another, and in the case of the objects in region 15, are beginning to fuse together. The AFM tip can no longer penetrate between the intermediates and reach the underlying substrate, suggesting the intermediates which are on their way to forming a single, discrete SLS aggregate.

In region 5, one observes a single aggregate, with a height of 30.8 nm. As evinced by the characteristic longitudinal section and dimensions, this is a fully formed, mature SLS aggregate.

On the basis of these measurements, one can postulate that the growth of SLS crystallites occurs in a hierarchical or step-wise manner, similar to the formation of keratin filaments (27). The initial stage in the process is some as yet unobserved step in which collagen monomers merge to form stable, fibrous oligomers. These oligomers
then associate laterally with one another, first over a long distance, as in Figure 2.24, but are gradually drawn together (regions 7, 15 and 16) and compacted, ultimately fusing and forming the final crystallite (region 5). This mechanism is illustrated, schematically, in Figure 2.35.

In light of this interpretation, however, several issues arise which need to be addressed. The first is one of sample preparation. In the preparation used in these experiments, the collagen assembly mixture is dried onto a mica substrate prior to imaging in the AFM. It is possible that the drying process itself can alter the structures under investigation, an occurrence dubbed ‘drying-artifacts’ by those in the field. However, drying artifacts are usually quite obvious to the experienced AFM user. They typically manifest themselves as circular patterns of amorphous material as a result of the gradual drying of round water droplets on the mica substrate. Samples which show this sort of effect are generally discarded because not only are any structures which can be observed considered to be unreliable, it is difficult to observe any objects at all as they often form featureless agglomerates. In the case of the samples examined here, no obvious drying artifacts were observed.

Another issue worthy of consideration is one of reversibility. The aggregation of SLS is a reversible, dynamic process, although to induce ‘degradation’ of SLS collagen aggregates to monomers to any significant extent requires the removal of all excess ATP from solution and periodic agitation of the mixture. One must consider the possibility that the structures in Figure 2.20 may well be in the process of de-aggregating instead of forming the final aggregate. Unfortunately there is no easy way to determine which of these two scenarios is actually occurring in these images. Dynamic AFM imaging of the
aggregation process would go a long way towards addressing this issue, however the solution conditions required for the assembly to proceed are not well suited for in-situ imaging. In order to be able to image this process dynamically, it is necessary to have the collagen adsorbing firmly to the surface and yet mimic the general reaction conditions for the aggregation. In this case, the two are not compatible – the acidic aggregation conditions (pH 3.5) prevent the collagen from sticking firmly enough to the mica substrate to allow imaging.

2.4.1 Summary

Segmental long spacing collagen has been used as a model for studying protein aggregation, with a view towards gaining an understanding of the mechanisms by which aggregation occurs. SLS collagen aggregates have been formed in vitro and studied by means of the AFM. Measurements of the aggregate yield typical longitudinal and cross sections of and 360 nm and 200-300 nm, respectively, which are consistent with results obtained from electron microscopy. Electron beam-deposited AFM tips have revealed small ultrastructural features on the surface of the aggregate, but the small size of these features have made them difficult to resolve. Images of SLS aggregates in the early stages of the formation process have been obtained. The aggregates appear to form by the lateral addition of collagen oligomers which are ~ 2.5 nm in height, followed by the packing and fusing together of these intermediates. This suggests that the assembly of SLS collagen proceeds by means of a stepwise mechanism, with the oligomers being stable intermediates in the process.
2.5 References


Figure 2.1: Transmission electron microscope image of SLS aggregates formed in vitro by the addition of ~0.2% ATP to collagen monomer (Reproduced from Schmitt et al (1)).
Figure 2.2: Positively stained SLS aggregate imaged using transmission electron microscopy. Cross striation patterns reflect the amino acid sequence along the length of the constituent collagen monomers. Monomer alignment in the aggregate is represented by the thick arrow. Reproduced from Kobayashi et al. (31)
Figure 2.3: Schematic illustration of role of ATP in SLS aggregate structure. Reproduced from Kobayashi et al. (10). ATP is thought to act as a bridging unit, holding collagen monomers together in the final aggregate.
Figure 2.4: Schematic illustration of the AFM's operating principles. A sharpened tip is raster scanned across a surface, with topographical features on the surface giving rise to deflections of the cantilever upon which the tip is mounted. The end result is a three dimensional representation of the surface, usually in a digital format, which is amenable to image analysis.
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<th>Optical Microscope</th>
<th>SEM</th>
<th>SPM</th>
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<td>vacuum</td>
<td>ambient liquid vacuum</td>
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<tr>
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<td>sample must be vacuum compatible, not build up charge</td>
<td>sample must not have excessive variations in surface height</td>
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**Table 2.1:** Comparison of different types of microscopy (18)
**Figure 2.5:** Schematic illustration of tip convolution effect. If a rounded object were imaged with an ideal, atomic sized AFM tip, the trace shown in (a) would result. The actual convoluted image produced by the path of the gray, realistically shaped tip in (b) is shown by the trace in (c). In (d), the additional area produced by the tip convolution is shaded darkly. The inwardly curving area beneath the widest point of the sample is completely inaccessible to the tip, meaning that the more lightly shaded area in (d) can never be imaged by the AFM.
Figure 2.6: Schematic representation of intraparticle interference, with light being scattered from different points on the same particle. O, A, B and C are the reference planes. I$_0$ is the incident beam. $X_i$, $X_j$ are two general scattering points of the particle and $\theta_k$'s are the angles of observation. (Reproduced from Kratochvil (23))
Figure 2.7 a,b: Deflection mode AFM images of SLS collagen aggregates formed via addition of ATP to acidified collagen monomers. Product was diluted by 100X prior to imaging. For a), image size is 15 x 15 µm, for b), image size is 5 µm x 5 µm.
Figure 2.8: Plot showing distribution of SLS aggregate diameters from a standard preparation of SLS aggregates (ATP=1.7 mg/mL. pH=3.5. room temperature). Fitted Gaussian mean = 53 nm
Figure 2.9: Sectional analysis of SLS aggregate. Figure a) shows longitudinal section of SLS aggregate b) cross section c) aggregate diameter.
Figure 2.10: Deflection mode AFM image of SLS aggregates formed by the addition of GTP to collagen monomers. Arrow indicates an aggregate chosen for cross-sectional analysis, shown in Figure 2.11. Image size is (15x15 μm).
Figure 2.1: Sectional analysis of SLS aggregate formed by the addition of GTP to SLS collagen monomers.

a) longitudinal section
b) cross section
Figure 2.12: AFM images of SLS-like aggregates formed by the addition of 2 mg/mL UTP to acidified collagen monomers. Aggregates appear to consist of multiple SLS units aggregated end-on.

a) 15 x 15 μm
b) 4 x 4 μm
**Figure 2.13:** Particle diameter distribution for SLS-like aggregates formed by the addition of UTP to acidified collagen monomers (UTP = 2mg/mL, pH = 3.5, room temperature). Fitted Gaussian mean = 6.6 nm

**Figure 2.14:** Longitudinal section of an SLS-like aggregate formed by the addition of UTP to acidified collagen monomers. Note the presence of multiple ridges, suggesting that the aggregate consists of several SLS units connected in an end-on manner.
Figure 2.15: Structures of polyanions used to induce SLS aggregation.
a) Amaranth  b) Cibacron Blue 3GA  c) ATP  d) ATP-γ-s  e) GTP  f) UTP
**Figure 2.16:** UV/Vis spectrum of ATP in acidic solution (pH 3.5) at room temperature. Absorbance maxima occurs at approximately 270 nm.

**Figure 2.17:** Plot showing absorbance of supernatant as a function of washing. Excess ATP was removed by washing cycles until the total absorbance was \( \sim 0.1 \) units. Collagen aggregates were then re-dissolved and yielded the absorbance peak labeled 'Sample 1', indicating amount of ATP liberated from aggregates.
Figure 2.18: Schematic description of process used to determine ATP content of SLS aggregates. Excess ATP was removed from solution by repeated washing / vortexing / sonicating cycles, with the concentration being determined by UV-Vis spectroscopy. After removing all excess ATP from solution, the SLS aggregates were re-dissolved and the amount of ATP liberated into solution was quantitatively determined through Beer's Law.
Figure 2.19: AFM images of SLS aggregates frozen in the process of aggregating.
a) Height mode image (11 x 11 μm)  b) Deflection mode image (11 x 11 μm)
Figure 2.20: Labeled AFM image of SLS collagen assembly intermediates. Certain labeled regions have been expanded and are shown in the following set of figures.
Table 2.2: Tabulation and description of labeled regions in Figure 2.19

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<th>Object Diameter (nm)</th>
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</tbody>
</table>
| 7      | Seven discrete objects: highly entangled: numerate from right to left | Object 1 = 9.3  
|        |                                 | Object 2 = 10.2      
|        |                                 | Object 3 = 12.4      
|        |                                 | Object 4 = 14.9      
|        |                                 | Object 5 = 12.2      
|        |                                 | Object 6 = 8.7       
|        |                                 | Object 7 = 10.6      |
| 8      | Four objects, three of which are discrete, one a fused product of several objects: all are highly entangled: numerate from right to left | Object 1 = 11.9  
|        |                                 | Object 2 = 12.2      
|        |                                 | Object 3 = 11.4      
<p>|        |                                 | Object 4 = 16.3      |</p>
<table>
<thead>
<tr>
<th>Page</th>
<th>Description</th>
<th>Measurements</th>
</tr>
</thead>
</table>
| 9    | Five discrete objects plus one fused object: numerate from top to bottom | Object 1 = 10.7  
|      |             | Object 2 = 10.8  
|      |             | Object 3 = 10.6  
|      |             | Object 4 = 12.5  
|      |             | Object 5 = 10.2  
|      |             | Object 6 = 11.4  |
| 10   | Nine highly entangled objects: numerate from top to bottom | Object 1 = 5.9  
|      |             | Object 2 = 7.2  
|      |             | Object 3 = 8.3  
|      |             | Object 4 = 10.7  
|      |             | Object 5 = 3.6  
|      |             | Object 6 = 5.4  
|      |             | Object 7 = 8.6  
|      |             | Object 8 = 8.8  
|      |             | Object 9 = 9.1  |
| 11   | Two partially fused objects: numerate from left to right | Object 1 = 15.8  
|      |             | Object 2 = 15.4  |
| 12   | One large object: one associated fibril | Object 1 = 25.0  
|      |             | Object 2 = 15.4  |
| 13   | Two discrete objects | Object 1 = 11.8  
|      |             | Object 2 = 18.5  |
| 14   | Three discrete objects: one large fused one, two minor ones: numerate from right to left | Object 1 = 9.3  
|      |             | Object 2 = 10.2  
|      |             | Object 3 = 12.4  |
| 15   | Three discrete objects: numerate from right to left | Object 1 = 15.6  
|      |             | Object 2 = 15.4  
<p>|      |             | Object 3 = 16.0  |
| 16   | Three discrete objects: numerate from right to left | Object 1 = 16.9  |</p>
<table>
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<th></th>
<th>Description</th>
<th>Object 1</th>
<th>Object 2</th>
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<tbody>
<tr>
<td>17</td>
<td>Five filamentous objects: highly entangled; numerate from top to bottom</td>
<td></td>
<td>Object 2 = 18.4</td>
<td>Object 3 = 15.9</td>
<td></td>
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<tr>
<td>18</td>
<td>Seven entangled objects in group; numerate from top to bottom</td>
<td></td>
<td>Object 1 = 17.8</td>
<td>Object 2 = 15.3</td>
<td>Object 3 = 15.9</td>
<td>Object 4 = 15.2</td>
<td>Object 5 = 10.2</td>
<td>Object 6 = 9.0</td>
</tr>
</tbody>
</table>
**Figure 2.21:** 2.25 x 2.25 μm image of region 7

**Figure 2.22:** 2.25 x 2.25 mm image of region 8
**Figure 2.23:** 2 x 2 μm image of region 9

**Figure 2.24:** 3x3 μm image of region 10
Figure 2.25: 2.25 x 2.25μm image of region 11

Figure 2.26: 2.25 x 2.25 μm image of region 12
Figure 2.27: 2.25 x 2.25 μm image of region 13

Figure 2.28: 2.25 x 2.25 μm image of region 15
Figure 2.29: 1.5 x 1.5 μm image of region 17

Figure 2.30: 2.25 x 2.25 μm image of region 18
**Figure 2.31:** Cross-sectional analysis of SLS intermediate in region 1.

a) Longitudinal section  

b) Cross section
Figure 2.32: Cross sectional analysis of SLS intermediates in region 7
Figure 2.33: Cross sectional analysis of SLS intermediates in region 5.
a) Longitudinal section  b) Cross section
Figure 2.34: Cross sectional analysis of SLS intermediates in region 11.
**Figure 2.35:** Schematic depiction of proposed mechanism for SLS crystallite formation. Process involves formation of a stable intermediate, likely an oligomer containing multiple collagen molecules. These stable intermediates then fuse together to form the final structure.
Chapter 3: Kinetics and Thermodynamics of SLS Aggregation

3.1 Introduction

3.1.1 Preliminary remarks

Results from the AFM imaging experiments discussed in Chapter 2 strongly suggest that the formation of segmental long spacing collagen aggregates proceeds in a hierarchical fashion, through the lateral grouping and fusion of collagen oligomers. In this chapter, this model is tested through an exploration of both the kinetics and thermodynamics of SLS aggregation.

In terms of kinetic experiments, the growth rate of the aggregates has been monitored by means of laser light scattering, a technique commonly used to measure kinetic properties of many dynamic, biological systems, including type I collagen fibrils (1). Experiments of this kind can provide useful insight into the mechanism of aggregate growth, as demonstrated in Chapter 1.

While kinetic data can be used to extract thermodynamic information about the system under study (e.g. see (2)), a more direct approach to measuring thermodynamic properties shall be taken. The technique of Isothermal Titration Calorimetry (ITC) has been applied to the SLS system, and has provided both fundamental calorimetric data, as well as low-resolution kinetic data which will prove to be of importance in this work. A brief introduction to ITC is provided in this introductory section.

In the following chapter, results of these kinetic and thermodynamic measurements are correlated with the data obtained through AFM imaging in the previous section, with a view towards determining a clearly defined mechanism for SLS formation.
3.1.2 Thermodynamic measurements: Isothermal titration calorimetry

ITC is a technique that measures the heat evolved from a chemical reaction or process which occurs at constant temperature. The method involves titrating a sample with a series of fixed-volume injections of reactant and measuring the heat associated with the resulting reaction. This technique is particularly useful for biological applications, such as measuring enthalpies of protein binding reactions, as many of these processes are active under isothermal conditions. ITC is also quite sensitive, capable of measuring the heat evolved from reactions involving nanomole amounts of reagents, and as such it is useful when dealing with in vitro biological samples which can only be prepared in very small amounts. This method has been well reviewed by Brandts and Freire et al. (3.4)

A schematic diagram of an isothermal titration calorimeter, in this case one produced by the company MicroCal™, is shown in Figure 3.1 (5). The instrument essentially consists of a sample holding container, a feedback-controlled water bath and a syringe of injectable reactant. Samples are equilibrated at the temperature of choice, and then fixed volumes of the reactant (typically μL quantities) are injected into the sample at regular intervals. Reaction between the sample and reactant gives off or takes in heat, the amount of which is quantified through the feedback loop of the temperature control system. The reaction mixture is stirred continuously, often by rotating the injection syringe which ends in a paddle-shape.

Results of an ITC experiment are commonly expressed as a plot showing thermal power as a function of time. Some typical results for the isothermal titration of an antibody – antigen system are shown in Figure 3.2. Plots typically consist of a series of
sharp spikes, corresponding to heat evolution / uptake during the course of the binding event. Using the notation of Freire et al. (4), the heat evolution/uptake for each individual injection is given as:

\[ q = V \Delta H \Delta[L_B] \] \[ [1] \]

where \( V \) is the reaction volume
\( \Delta H \) is the binding enthalpy
\( \Delta[L_B] \) is the change in concentration of the bound ligand

The peaks corresponding to the heat uptake or evolution decrease as the saturation of the sample is titrated to completion. In turn, the total heat evolved, determined through integration of the total area under peaks in an ITC experiment, is correlated with the total amount of bound ligand:

\[ Q = V \Delta H \sum \Delta[L_B] \] \[ [2] \]

where \( Q \) is the total heat evolved for all additions.

Upon collecting the raw data, one typically attempts to fit it to a molecule-ligand binding model. This is generally an empirical procedure, carried out by iterative, computer-based calculations. A number of fitting models and procedures exist, the most common being the so-called "multiple sets of independent binding sites". Readers are referred to the review by Freire (4) and the textbook by Cantor (6) for further details. By fitting the experimentally determined value of \( Q \) to models of this sort, one can extract data such as binding enthalpies and equilibrium binding constants, and distinguish different types of binding sites on the macromolecule of interest. Note, this is very much an empirical method, and great care must be taken in interpretation of results obtained using this sort of approach.
3.2 Materials and Methods

3.2.1 Kinetic measurements: Laser light scattering

Light scattering experiments were carried out using the 'cold-start' procedure devised by Holmes during his investigations of native-type collagen fibril aggregation (7). Initial stock solutions of collagen monomer, prepared as described previously, were made at concentrations of 1 mg/mL and used within 2 weeks of preparation. Stock solutions of nucleotide triphosphates (NTPs) were also made at concentrations of 2 mg/mL. Immediately prior to carrying out an experiment, the collagen solutions were filtered through an 0.45 µm syringe filter (Sigma). All volume measurements were carried out using volumetric autopipettes.

Once solutions of the appropriate concentration had been made, 1 mL aliquots of NTP and collagen monomer solutions were autopipetted into a plastic cuvette, which was immediately sealed with parafilm and placed into the light scattering apparatus. All solutions were kept at 5-10°C until their introduction into the light-scattering apparatus.

A schematic diagram of the light scattering apparatus is shown in Figure 3.3. The system essentially consisted of an unpolarised He-Ne laser beam directed at the sample cuvette, which was immersed in a continuously-stirred water bath. A fibre-optic light guide was placed at a fixed angle of 90° to the cuvette, with the scattered light signal being collected by a photomultiplier tube. The incident beam was split prior to its interaction with the sample, with a fraction of the beam being directed to a photodiode to monitor fluctuations in the beam power. Signals from both the PMT and photodiode were collected and averaged by a Tektronix 210 digital oscilloscope. The resulting signal was sent to a computer, displayed to screen and saved to file using the program...
"MODIF.BAS" (Appendix A). Time resolution of either 10 seconds or 1 minute were typically employed and were sufficient for these experiments. Temperature of the system was controlled by a Neslab water bath and was accurate to within 0.2°C. After their initial introduction into the apparatus, samples typically required ~ 4 minutes to reach the same temperature as the bath. A calibration curve illustrating this is shown in Figure 3.4.

3.2.2 Thermodynamic measurements: Isothermal titration calorimetry

Measurements were carried out using a MicroCal™ isothermal titration calorimeter. Collagen monomer solution of 2 mg/mL was prepared as previously discussed, filtered with 0.45 μm filters, and allowed to equilibrate at 22.1°C in the ITC sample cell. ATP solutions of 3 mg/mL were prepared and allowed to equilibrate in the injection syringe. Solution concentrations were chosen such that the SLS aggregation process would occur very rapidly upon addition and that the reaction between collagen and the entire aliquot of injected ATP would be complete before subsequent injections took place. Several different injection volumes were tried for the titration experiments and are described in subsequent sections. Data was fitted and analysed using the software ‘Origin’ and the fitting routines included with the calorimeter package.

3.3 Results & Discussion:

3.3.1 Kinetic measurements:

Measurement of scattered light intensity as a function of time for SLS aggregation yielded sigmoidal or s-shaped curves, as illustrated in Figure 3.5. The curves consisted of a so-called ‘lag-phase’ (a period in which there is minimal change in the intensity of scattered light), followed by a steeply rising portion, and finally a plateau region in which the scattered light intensity level is constant. These are the same general shape of curves
that are obtained when studying light-scattering of native-type collagen (1). One can characterize the rate of the process by determining the reaction half-time, \( t_{1/2} \), which shall be defined as the time required for the scattered intensity to reach one half of its final plateau value. In the experiments carried out, the intensity of scattered light was expressed as an arbitrary number generated by the D/A converters of the oscilloscope. As the absolute values of these numbers are arbitrary, one is free to alter their magnitudes as long as their relative values remain intact. In this case, the intensity values were shifted to an initial intensity of zero, and were normalized to yield a final value of unity. Reaction half-times were determined by empirically fitting the experimental curve with the function:

\[
y = \frac{A_1 - A_2}{1 + e^{(x-x_0)/d \tau}} + A_2
\]

where \( A_1 \) = the initial intensity value
\( A_2 \) = final intensity value
\( x_0 \) = center point (taken to be \( t_{1/2} \))
\( d \tau \) = time increment

3.3.2 Aggregation rate dependence on collagen and ATP concentration

In the following series of experiments, the dependence of the aggregation rate upon collagen concentration, ATP concentration, ionic strength and temperature were monitored in a systematic fashion, by varying one parameter and holding all others constant. When attempting to determine a generalised rate law for a reaction, one typically uses an approach known as the ‘isolation method’, in which the concentration of one parameter is varied while the others are held in large excess (e.g. see (8)). One may
then assume that the concentration of the excess reactant remains essentially constant throughout the process. and one can establish the order of reaction for a particular reactant by monitoring the net effect on the reaction rate of changing its concentration. However, such an approach was not strictly possible for the case of SLS aggregation. for reasons which will soon become apparent. Nonetheless, the kinetic experiments do provide valuable insight into the aggregation mechanism.

For determining the effect of collagen concentration on the reaction rate, the collagen concentration was varied systematically while holding the concentration of ATP, temperature and ionic strength constant. It is possible to determine the order of the aggregation rate law in terms of collagen concentration. because, for the concentrations used here, ATP was in excess - typically on an order of 1000:1 mole ratio of ATP to collagen. Some typical light-scattering data is shown in Figure 3.6. for a set of experiments carried out at [ATP]=2.0 mg/mL and 25.1°C. Also included is a chart showing a summary of the dependence of t 1/2 upon collagen concentration (Figure 3.7). In general, there was a linear (first-order) dependence of the reaction half-time with collagen concentration over the range of temperatures and ATP concentrations investigated.

Determination of the kinetic dependence of the aggregation on ATP concentration was somewhat more complicated. In the same manner as described previously. the ATP concentration was varied at constant collagen concentration and temperature. However, an unusual phenomena was encountered: a 'critical' concentration of ATP was required to induce a change in the intensity of scattered light. That is.

at initial ATP concentrations below a certain value, no change in scattered intensity could be observed.
This critical concentration dependence is shown in Figure 3.8, in which raw light scattering data for experiments carried out over a range of ATP concentrations is shown. The critical concentration was at approximately 1.8 mg/mL: the reaction mixtures prepared at higher concentrations showed the characteristic sigmoidal scattering curves while those at lower concentrations showed no measurable change in scattered intensity. These latter solutions prepared showed no change in scattered intensity even after several days, and indeed remained transparent to the eye for an indefinite period. There was no apparent temperature dependence for this critical concentration within the precision of these experiments: it seemed to lie at the value of 1.8 mg/mL over a range of temperatures from \(-20-40 \ ^\circ\text{C}\). Because of this lack of change in scattering signal at low ATP-to-collagen ratios, an isolation method type approach to determining the generalized rate law could not be used.

The existence of a critical salt concentration required to induce aggregation is not unusual in colloid science. As noted in section 1.3, salts in solution tend to screen electrostatic interactions between similarly charged particles, and at sufficient concentration, allow particles to be drawn together through van der Waal's interactions. Below this salt concentration, one would expect kinetic stability of the colloid, with no aggregation occurring. AFM images of the non-turbid solutions, however, revealed that aggregation was occurring at least to some extent. Figure 3.9 a,b,c shows AFM images of an ATP-collagen mixture produced by the addition of a sub-critical concentration of ATP. As is apparent from these images, aggregate structures are being formed, even while the intensity of scattered light remains unchanged. In Figure 3.9b, a number of different structures are present, including fully mature SLS aggregates as well as those
with much smaller diameters. On average, however, the structures formed are much smaller in diameter than a fully-formed aggregate, as indicated by the diameter distribution in Figure 3.10.

Inspection of the basic equations governing light scattering (Chapter 2, equations [12], [13]) can help to provide some insight into this situation. Because aggregation is occurring to at least some extent, the average molecular weight of all species in solution must be increasing. If the signal depended only upon this factor, one should expect some increase in scattered intensity. One of the other parameters which affects the scattering signal, the optical constant, $K$, should simply act as a scaling factor which is not affected by the size and shape distribution of the aggregates. The most likely explanation for this lack of change in signal is the role played by the average particle structure factor, $P(0)$. This factor, as shall be explored in greater depth in section 3.3.5, acts to attenuate the total scattering signal because of intraparticle scattering interference effects. The attenuation will be more significant at early stages in the assembly because at these times, the small, rod-like collagen molecules cause a greater amount of destructive interference, and hence signal decrease, than the final collagen aggregates. What is likely occurring in the case of the minimum ATP concentration, is that the effect on the scattering signal by the increase in average molecular weight of the solution is entirely offset by the attenuation of the signal by the particle scattering function. The ATP concentration is sufficiently low such that the majority of collagen remains as a free monomer – that is, the concentration is too low to result in large polymer to monomer ratios. In effect, the assembly appears to be caught in a perpetual ‘lag phase’.
By selecting ATP concentrations well above this critical concentration, the reaction rate dependence on ATP concentration could be determined. As one might expect, aggregation proceeded more quickly with increasing concentration of ATP. Some typical light scattering results (collagen=1 mg/mL, T=19.0°C), along with summarized t_{1/2} data showing the dependence of the reaction rate on ATP concentration is shown in Figure 3.11 and 3.12. Again, reaction half-times were determined using the empirical fitting equation [4]. As noted, because of the inability to carry out measurements at conditions where collagen was in great excess, the overall order of the rate law on ATP concentration could not be determined.

### 3.3.3 Temperature dependence

The effect of reaction temperature on the kinetics of the process was investigated using the same basic approach described previously. Both collagen and ATP concentration were held constant, while the temperature was varied systematically. Some typical light scattering data, along with summarized reaction half-times, are shown in Figure 3.13 a,b. The data was fit with an Arrhenius-type temperature dependence:

\[ y = A \exp\left(-\frac{E_A}{kT}\right) \]  

[4]

with the parameters:

\[ E_A = -720 \text{ Jmol}^{-1} \]

\[ A = 1.14 \times 10^{12} \text{ min}^{-1} \]

A rather unusual feature of SLS aggregation is that the rate of the process shows an inverse temperature dependence. That is, at higher temperatures, the aggregation proceeds at a markedly slower rate. This can be interpreted as the existence of a negative activation energy, as shall be discussed momentarily. While obviously different from a
typical Arrhenius-type dependence. Such behaviour has been known to occur in certain types of gas-phase reactions - typically systems which possess a moderately complex reaction mechanism. One of the more well-known systems which shows such kinetic behaviour is the oxidation of nitrogen (II) oxide to nitrogen(IV) oxide (8):

\[2\text{NO}_{(g)} + \text{O}_2(g) = 2\text{NO}_2(g) \quad [5]\]

It was observed, experimentally, that this reaction had an overall rate law which was third-order:

\[\frac{d[\text{NO}_2]}{dt} = k[\text{NO}]^2[\text{O}_2] \quad [6]\]

The rate law and the inverse temperature dependence of this process can be accounted for, in kinetic terms, as follows:

Define an initial pre-equilibrium step:

\[2\text{NO}(g) = \text{N}_2\text{O}_2(g) \quad [7a] \quad K = \frac{[\text{N}_2\text{O}_2]}{[\text{NO}]^2} \quad [7b]\]

which, in turn, is followed by a bimolecular process:

\[\text{N}_2\text{O}_2(g) + \text{O}_2(g) = 2\text{NO}_2(g) \quad [8a] \quad \frac{d[\text{NO}_2]}{dt} = k_h[\text{N}_2\text{O}_2][\text{O}_2] \quad [8b]\]

The final, third-order, rate equation is obtained by combining equations [7b] and [8b] into:

\[\frac{d[\text{NO}_2]}{dt} = k_hK[\text{NO}]^2[\text{O}_2] \quad [9]\]

Now from the van't Hoff equation, we know that for an exothermic reaction, the equilibrium constant \(K\) decreases with increasing temperature:

\[\ln\left(\frac{K}{K_1}\right) = \frac{\Delta H_m^\circ}{R} \left(\frac{T_2 - T_1}{T_1T_2}\right) \quad [10]\]
The dimerization of NO is a known exothermic process. Under the assumption that the rate constant $k_b$ does not increase more with temperature than $K$, then the net product $k_bK$ will decrease with an increased temperature.

In effect, an inverse temperature dependence, and consequently a negative activation energy, implies the existence of a stable intermediate in the course of the reaction. In the case of the NO reaction, the intermediate species is generally thought to be the stable $N_2O_2$ dimer. Rice (9) has approached the problem of inverse temperature dependence in the NO reaction from the point of view of transition state theory, under the assumption that the process involves a trimolecular collision event. Using this alternate approach, the conclusion reached was that the process necessarily require the formation of a stable intermediate transition state complex. In Rice’s own words, “A negative activation energy must mean the formation of a more or less stable intermediate compound...”. Despite approaching the problem from a completely different point of view, Rice’s conclusion is that the inverse temperature dependence is indicative of a stable intermediate forming in the process. Now, while a generalized rate law like equation [9] could not be determined from the light scattering data, the AFM data shown in Chapter 2 strongly indicates that the assembly involves the formation of stable intermediate oligomers. The two measurements both suggest the same general type of growth mechanism.

3.3.4 Ionic strength dependence

To investigate the role played by ionic strength on the aggregation, ATP – NaCl solutions were combined with collagen monomer and studied by light scattering. As with previous isolation method experiments, the concentration of collagen, ATP and the
reaction temperature were kept constant (collagen = 1 mg/mL. ATP = 1.7 mg/mL. temperature = 19.2°C) as the total concentration of NaCl was varied in a systematic manner. In general, the reaction rate decreased with increasing concentration of sodium chloride, with the most rapid aggregation occurring when there was no salt present in solution. Typical light scattering curves are shown in Figure 3.14. Above a salt concentration of ~2.4 mM, the reaction mixture was found to be indefinitely stable, with no measurable change in scattered light intensity.

The effect of ionic strength on the aggregation rate seems counterintuitive. As discussed in section 1.3, the general stability of colloids decreases with increasing salt concentration, primarily because of electrostatic screening of electrical double layers on the similarly charged particles. In an attempt to explore the peculiar ionic strength dependent behaviour exhibited by SLS aggregation, the role of specific cation type was quantified by light scattering experiments. A series of solutions of ATP and salt were prepared in which the ionic strength of all solutions was equal but established by a different type of cation. Solutions of ATP with NaCl, KCl and LiCl were made, in which the net concentration of ATP was 2.0 mg/mL and salt of 5x10⁻⁴ M. Light scattering measurements at 24.8°C were carried out using these solutions, as well as a reference ATP solution to which no salt had been added. The raw light scattering data, along with summarized t₁/₂ data, is shown in Figure 3.15. a,b. Similar experiments were carried out with the divalent cations Mg²⁺ and Ca²⁺, with relevant data shown in Figure 3.16.

When dealing with biological molecules and related systems, many unusual effects which depend upon salt type can arise. In fact, a large number of phenomenological observations dealing with the effect of charged ions on biological
molecules has been observed, giving rise to the so-called 'Hofmeister series'. The Hofmeister series is essentially a ranking scheme for ionic species based upon their ability to precipitate out or stabilize colloidal molecules – typically ones of a biological nature. The topic has been recently reviewed by Cacace et al. (10). The series has been built up on the basis of a large amount of phenomenological data. While some insight has been gained into understanding the driving forces underlying the series, it remains for the most part, largely elusive. This is generally thought to be so, because so-called Hofmeister effects, that is, increased or decreased stability to aggregation with ion type, likely occurs because of a combination of many smaller effects such as solvent structure and specific salt-biomolecule interactions. For cations, the Hofmeister series is generally written as:

\[(\text{CH}_3)_4N^+ (\text{CH}_3)_2\text{NH}_2^+ \text{NH}_4^+ \text{K}^+ \text{Na}^+ \text{Cs}^- \text{Li}^- \text{Mg}^{2+} \text{Ca}^{2-}\] [11]

As one proceeds from left to right across the series, the more the destabilizing (salting-in) the nature of the salt; i.e., proceeding across the series, the ability of the salt to induce aggregation of particles increases. Even in terms of the generalized Hofmeister phenomenology, however, the effect of salt on SLS aggregation is peculiar. In the light scattering experiments, it was observed that the ability of the salts to destabilize colloidal solutions and to induce aggregation was \(\text{K}^+>\text{Na}^+>\text{Li}^+\), the opposite of the Hofmeister series.

The solution to this puzzle lies in the nature of interactions between salts and ATP. It is well known that in many biological systems, ATP is found in close association with divalent cations, particularly \(\text{Mg}^{2+}\). In fact, the functional form of ATP is, at least in living systems, generally believed to be a complex between ATP and \(\text{Mg}^{2+}\) (11).
However, the biologically functional ATP-Mg complex will have quite different solution properties to ATP alone, which is obviously of critical importance for SLS aggregation. The likely explanation for the ionic strength dependence of SLS aggregation is one of binding of ATP with salt ions. In addition to binding with divalent cations, ATP can also bind to monovalent cations such as K+, Na+, and Li+. Wilson and Chin (12) have characterized the strength of binding of ATP with various cationic species by means of isothermal titration microcalorimetry. In these experiments, it was found that the strength of binding of monovalent cations to ATP followed the general trend Li⁺>Na⁺>K⁺, and for divalents, Mg²⁺>Ca²⁺. These trends are in excellent agreement with the observed light scattering data. From the point of view of inducing SLS aggregation, ATP can be 'quenched' by binding to cations. The bound ATP cannot be taken up into an SLS aggregate and as such aggregation is impeded. One can envision a competitive process for unbound ATP, between binding with a cation and with the reactive sites on the collagen monomer. When the concentration of salt is too high, the monomer is unable to compete for ATP and remains in its unaggregated form, hence the observation that excessively high salt concentrations completely inhibit SLS formation.

3.3.5 Computational analysis of light scattering curves

As noted in section 1.7, a characteristic property of processes which occur via a nucleation and growth type mechanism is a sigmoidal growth curve. For example, if one defines a simple nucleation and growth process as consisting of the following series of mechanistic steps:

\[ A + A \xrightleftharpoons{K_1} A_2 \]  

[12]
\[ A_2 + A_{k'} = A_3 \]
\[ A_{i-1} + A_{k'} = A_i \]

One can write the following differential rate equations:

\[
\frac{d[A_3]}{dt} = k_\chi [A]^2 - k_\chi [A_2] \quad [13]
\]
\[
\frac{d[A_i]}{dt} = k[A_{i-1}][A] - k'[A_i] - k[A_i][A] + k'[A_{i-1}] \quad [14]
\]

By solving these equations numerically, and by defining a mass of polymerized material:

\[
\text{Polymer mass} = \sum_{i=2}^{n} [A_i] \quad [15]
\]

one can calculate a polymer mass evolution as a function of time for the nucleation and growth process. Calculations of this type were carried out by means of the program ‘Kin.cpp’, shown in Appendix B. Some typical sigmoidal growth curves calculated in this fashion are shown in Figure 3.17.

As noted, our proposed formation mechanism for SLS aggregates is not nucleation and growth, rather a stepwise process. In the remainder of this section, we shall attempt to show that a stepwise model of growth is also consistent with the generation of a sigmoidal light scattering curve. Consider, now, a simple model for a
stepwise growth process. We shall first define an initiation step in which a stable intermediate is formed via the addition of two basic monomer units, followed by a second series of steps in which the intermediates merge to form the final aggregate:

\[ B + B \xrightarrow{k_Y} A \]  \hspace{1cm} \text{(Formation of stable intermediate)} \hspace{1cm} [16]

\[ A \xrightarrow{k'} A_i A \]  \hspace{1cm} \text{(Aggregation of intermediates to form final product)}

\[ A_{i-1} + A \xrightarrow{k'} A_i \]

Hence, one can define:

\[ \frac{d[B]}{dt} = -2k_Y[B]^2 + 2k_Y[A] \]  \hspace{1cm} [17]

\[ \frac{d[A_i]}{dt} = k[A_i][A] - k'[A_i] - k[A_i][A] + k'[A_{i-1}] \]  \hspace{1cm} [18]

Following the same approach as with the nucleation and growth-type experiments, one can numerically integrate these equations and calculate the time evolution of polymer mass. This is shown for the stepwise mechanism in Figure 3.18 a.
While the generated curve is an increasing, monotonic function, it is clearly not sigmoidal. This would appear to represent a significant flaw in the analysis of the light scattering data, as the experimental results obtained are sigmoidal in shape. One possible interpretation is that the assembly simply does not proceed in a stepwise manner. However, a point which must be considered, and one which is quite often overlooked in the literature when analysing similar light scattering curves, is that the scattering signal does not track the mass of polymerized material alone, but rather surveys a combination of both particle mass and shape. Consider, now, from section 2.2.3, the basic equation which dictates the intensity of the scattered light signal:

\[
R(\theta) = \frac{2\pi^2 n^2}{\lambda^4 N_A} \left( \frac{dn}{dc} \right)^2 \left( \sum_i \frac{c_i M_i P_i(\theta)}{c M_w} \right) \left( \frac{\sum_i c_i M_i}{\sum_i c_i} \right) c
\]  

[19]

From equation [19], one notices that while the scattered light intensity will increase in proportion to the mass of polymerized material, as evinced by the fourth term, it also has a strong dependence upon the averaged particle structure factor, the third term in this expression. In effect, the light scattering measurement is tracking the time evolution of both average particle mass and shape. Solving the differential rate equations from a simple reaction mechanism is useful to predict the former, but generally ignores the latter.

In an attempt to quantify the effect of the averaged particle structure factor upon the light scattering signal, the following strategy was adopted: The program ‘Kin.cpp’ was modified so as to track the total number of polymers present in solution. All ‘monomers’ in solution were considered to behave as thin, rigid rods. This is one of the few shapes for which the general particle structure factor equation has been solved:
\[ P(\theta) = \frac{1}{\sigma^2} \sum_{i=1}^{\sigma} \sum_{j=1}^{\sigma} \frac{\sin \mu h_{ij}}{\mu h_{ij}} \]  

[20]

where \( h_{ij} \) is the distance between the \( i \)-th and \( j \)-th scattering centre. \( \mu = (4\pi/\lambda)\sin(\theta/2) \)

(scattering vector) and \( \theta \) is the angle between the incident and scattered beam.

For the thin, long rod (13):

\[ P(\theta) = \frac{1}{x} \int_{0}^{x} \frac{\sin u}{u} du - \left( \frac{\sin x}{x} \right)^2 \]  

[21]

where \( x = \frac{2\pi L}{\lambda} \sin \left( \frac{\theta}{2} \right) \)

The basic dimensions of the collagen monomer and the experimental details of the scattering setup were substituted into this equation (monomer length=290 nm, \( \lambda = 632 \) nm, \( \theta = 90^\circ \)), which was then solved numerically using the program "Maple". The calculated particle structure factor for the collagen monomer was approximately 0.68.

Determining a reasonable value for the structure factor of the polymerized material was somewhat more difficult. As the calculations carried out could track the total number of polymers but not the exact number of monomer units which made up each polymer. In addition, the exact shape of the scattering units was difficult to account for:

While the AFM data suggests that a good approximation for the polymer shapes might be cylinders whose diameters vary from that of an individual monomer up to \( \sim 50 \) nm for a final aggregate, a solution to equation [20] for cylinders could neither be found, nor could one be readily determined. Instead, it was decided to approximate all polymers as acting like spheres with a volume equal to a mature SLS aggregate. This is obviously not correct, but if one acknowledges the limitations of this model and does not attempt to extend it too far, it does provide some rather useful insight.
The solution to equation [20] for a homogeneous sphere of diameter D is given by Kratochvil (13):

\[ P(\theta) = \left( \frac{3}{x^2} \right) (\sin x - x \cos x) \]  \hspace{1cm} [22]

where \( x = \left( \frac{2\pi D}{\lambda} \right) \sin \left( \frac{\theta}{2} \right) \)

Substituting the dimensions of SLS and instrumental parameters into equation [22] and solving, yields a particle structure factor of 0.91.

Now, the effect of particle on the scattered light signal shall be accounted for by multiplying the polymer mass evolution value (Figure 3.18 a) by a weighted average structure factor defined by:

\[ \overline{P(\theta)_{\text{weighted}}} = [ P(\theta)_{\text{sphere}} \cdot (1-N) + P(\theta)_{\text{rod}}(N) ] \quad \text{where } N = \# \text{ of polymers} \]  \hspace{1cm} [23]

\[ \overline{P(\theta)_{\text{weighted}}} = [0.91 \cdot (1-N) + 0.68N] \]

In these particular calculations, the number of polymers formed was quite small relative to the initial number of monomers, and as such, the effect of the particle structure factor is difficult to show in a graphical nature. However, the effect can be amplified by scaling the number of polymers formed by a factor of 1000. Presumably, the same amplification could be produced by a more judicious selection of rate constants. However, the time required to complete these calculations on the computing facilities available was prohibitive, and a more detailed survey of rate constants was impractical.

The result of multiplying the polymer mass evolution values by the structure factor is shown in Figure 3.18 b. As is readily apparent, the most significant effect of the structure factor is to decrease the magnitude of the scattering signal at early times in the
assembly, producing a lag phase. This seems quite reasonable in regards to the relative sizes of the particle structure factors for the rods and the polymer 'spheres'. At early stages in the assembly, collagen will exist primarily as individual molecules, which strongly attenuate the scattering signal. However, as more and more polymer molecule 'spheres' begin to form, the higher scattering factor becomes weighted more heavily, and the scattered light is not attenuated so strongly. This leads to the pronounced lag phase observed in the calculated curve.

Of course, this argument is not intended to be a rigorous attempt at modeling the light scattering data, but rather a way of describing how sigmoidal curves can arise from stepwise growth. Caution must be exercised before applying this model to the measured kinetic data. Clearly, the final SLS aggregates in solution are not spheres, and the intermediates discussed in Chapter 2 are even less so. As such, it would not be appropriate to use this model to fit and extract experimental rate constants. A more rigorous attempt at modeling, preferably one which takes into account the detailed shape of the aggregation intermediates, must be carried out before rate constants can be extracted.

3.3.6 Thermodynamic Measurements:

3.3.7 Baseline experiments

Prior to carrying out an SLS forming experiment, the effect of ATP addition to the acetic acid buffer in which collagen monomers were typically solubilised was examined. ATP solutions of 3 mg/mL in acetic acid was injected into a solution of 0.05% acetic acid in a series of 5 additions of 10 μL volume. All solutions were maintained at a temperature of 21.1°C. The raw ITC results, shown in Figure 3.19 a.
consisted of a series of endothermic peaks. which, after integration, yielded an average
$\Delta H$ value of $30.1 \pm 2.7$ kcal mol$^{-1}$. This baseline effect can be attributed to the heat of
dilution of ATP with possible contributions from ATP hydrolysis.

3.3.8 SLS aggregation

In these experiments, collagen monomer solutions were titrated with a series of
ATP solution injections. Injections into the 2 mg/mL collagen solution were carried out
with 3 mg/mL ATP in acetic acid. All solutions were maintained at 21.2°C throughout.
Raw ITC data is shown in Figure 3.19 a.b.c along with the same plot at a higher time
resolution. The best empirical fit of the data was obtained using a model of three
independent binding sites, with each binding site having an associated equilibrium
constant and enthalpy of binding as follows:

- $K_1 = 5.4 \times 10^4$ $\Delta H_1 = 1.48 \times 10^3$ kcal/mole
- $K_2 = 1.8 \times 10^5$ $\Delta H_2 = -1.04 \times 10^3$ kcal/mole
- $K_3 = 7.07 \times 10^4$ $\Delta H_3 = 3.21 \times 10^3$ kcal/mole

Note, no particular credence should be placed on the fact that the data was best fit by a
model of three independent binding sites; this should not be viewed as there being three
different types of binding processes occurring between collagen and ATP. rather a simple
statistical result from the fitting procedure.

As is evident from Figure 3.19 b, each injection of titrant gave rise to a pair of
peaks, the first a small but rapid exothermic peak, followed by a large but slower
endothermic peak. The existence of two peaks can be interpreted in terms of two distinct
processes which are initiated by the addition of ATP to collagen. The calorimeter used
had a response half-time on the order of ten seconds (3), and as such, can be used to give
an estimate of the rates of these processes. Defining a growth/decay constant for the
peaks as being the reciprocal of the time required for the signal to change from its
baseline value to its maxima / minima, yields, for the first exothermic peak, a time
constant of ~8 s and for the endothermic peak, a time constant of ~130 s. Note, the rate
of the former is approaching the rate at which the calorimeter can respond and should be
taken to be correct to an order of magnitude, only.

The existence of multiple peaks and hence, multiple processes, in the ITC data is
further evidence in favour of the postulated model of multi-step aggregation. As
discussed in Section 3.3.5, the aggregation of SLS shows an inverse temperature
dependence, which can be rationalized if the overall mechanism of growth occurs in a
manner similar to:

\[ 2A = A_2 \quad [24a] \quad K = \frac{[A_2]}{[A]^2} \quad [24b] \]

which, in turn, is followed by a bimolecular process:

\[ (A_2)_{i-1} + A_2 = (A_2)_i \quad \text{with forward rate constant } k_b \quad [25a] \]

\[ \frac{d[(A_2)_i]}{dt} = k_b K[(A_2)_{i-1}][A]^2 \quad [25b] \]

It shall be assumed that the first peak in the ITC data corresponds to step [24a],
the formation of a stable intermediate. This first, rapid peak in the ITC data is
exothermic, and as described in section 3.3.5, an initial exothermic step is required to
explain the negative activation energy of the aggregation. That is, for an exothermic step
[24a], the term \( k_b K \) will decrease with increasing temperature. Obviously, there is strong
agreement between the kinetic and thermodynamic data, as analysis of both types of data
suggest the same type of aggregation mechanism.
3.4 Summary and Conclusions:

The aggregation of Type I collagen into segmental long spacing aggregates by the addition of NTPs to collagen monomers has been explored using a variety of techniques. All of these techniques have generated data which suggest aggregation occurs through the formation of stable, intermediate particles which then merge together to yield the final aggregate structure. Atomic force microscopy has been used to characterize aggregate shapes and sizes, and has allowed direct visualization of the intermediate particles in the process of merging. Laser light scattering has been used to track the kinetics of the aggregate growth, with one important feature of these kinetics being an inverse temperature dependence of the aggregation rate, a hallmark of a process occurring in a stepwise fashion. Finally, Isothermal Titration Calorimetry has been used to explore the thermodynamics of the ATP-collagen interaction, and again, in excellent agreement with the other techniques, this data indicates that aggregation proceeds in a hierarchical manner.
3.5 References


**Figure 3.1:** Schematic illustration of MicroCal™ Isothermal titration calorimeter (5).
Figure 3.2: Typical ITC results for titration of an antibody with an antigen (5).
**Figure 3.3**: Schematic diagram of light scattering apparatus used for kinetic experiments
Figure 3.4: Calibration curve showing change in temperature of sample in cuvette upon immersion into water bath. The initial sample temperature was 10°C, and the water bath temperature was 34.2°C as indicated by the dotted line.
Figure 3.5: Typical sigmoidal light scattering curve obtained from the aggregation of SLS collagen. Aggregation conditions: collagen concentration = 1 mg/mL, ATP concentration = 1.6 mg/mL, temperature = 14.6°C.
Figure 3.6: Raw light scattering data showing collagen concentration dependence of SLS aggregation. Aggregation conditions: ATP concentration = 2mg/mL, temperature = 19.0°C. Note that while a data point was collected every ten seconds, we have displayed only a limited number of these points in order to increase the visual clarity of the plot. The fitting calculations are carried out with all data points in the set.
Figure 3.7: Plot showing light scattering reaction half-time ($t_{1/2}$) as a function of collagen concentration for SLS collagen aggregation. Aggregation conditions: ATP concentration = 2mg/mL, temperature = 25.1°C. The half-time dependence on collagen concentration was fitted with a linear dependence, yielding a slope of $-2.51 \times 10^4$ s mL mg$^{-1}$ and an intercept of $2.91 \times 10^4$ s.
Figure 3.8: Raw light scattering data showing dependence of aggregation rate on concentration of ATP. Aggregation conditions: collagen concentration = 1 mg/mL, temperature = 20.9°C. Below a critical concentration of ~1.8 mg/mL, addition of ATP to collagen results in no change in scattered intensity.
Figure 3.9 a: AFM image of collagen aggregates formed by addition of ATP to collagen at ATP concentrations below the ‘critical’ 1.6 mg/mL. Image size is 30 μm x 30 μm
**Figure 3.9 b:** Higher resolution image of aggregates shown in Figure 3.7 a. Note presence of mature SLS aggregates as well as those which are much smaller in diameter. Image size is 10 μm x 10 μm.
**Figure 3.9 c**: High resolution AFM image of collagen aggregates shown in Figures 3.7a,b. Image size is 5 μm x 5 μm.
The aggregate diameter distribution for aggregates formed below 'critical' ATP concentration. The aggregates were generally smaller than mature SLS, with a mean diameter of 17.2 nm as compared with 53 nm as shown in Figure 2.8.
Figure 3.11: Raw light scattering data showing ATP concentration dependence of SLS aggregation. Aggregation conditions: collagen concentration = 2mg/mL, temperature = 25.1°C. Again, data was collected at ten second intervals but not all points are plotted in order to increase the visual clarity of the plots; all relevant fitting calculations are carried out with the complete data set.
Figure 3.12: Plot showing light scattering reaction half-time ($t_{1/2}$) as a function of ATP concentration for SLS collagen aggregation. Aggregation conditions: collagen concentration = 2mg/mL, temperature = 19.0°C. The half-time dependence on ATP concentration was linearized by taking the logarithm of the half-time. The resulting data was fitted with a linear dependence, yielding a slope of 2.6 s mL mg$^{-1}$ and an intercept of 8.6 s.

**Linear Fit for Data on linearized scales.**  
$y = A + B \cdot (X)$

<table>
<thead>
<tr>
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<th>Value</th>
<th>Error</th>
</tr>
</thead>
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<tr>
<td>B</td>
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<td>0.67494</td>
</tr>
</tbody>
</table>

**R**

-0.89334  0.11401  6  0.01646
Figure 3.13: Plots showing temperature dependence of SLS aggregation. Aggregation conditions: collagen concentration = 1 mg/mL, ATP = 1.6 mg/mL.

a) Raw data
b) Reaction half-time ($t_{1/2}$) as a function of temperature.
Figure 3.14: Raw light scattering data showing role of ionic strength on aggregation rate. Ionic strength was adjusted by addition of an amount of NaCl as indicated in the captions. Rate decreased with increasing salt concentration. Upon sufficiently high salt concentration, the aggregation remained infinitely stable over the course of several days.
Figure 3.15 a, b: Light scattering data showing monovalent ion type dependence of SLS aggregation.
Aggregation conditions:
Collagen concentration = 1 mg/mL. ATP concentration = 2mg/mL. Temperature = 24.8°C.
Ionic strength for all runs except the control (labeled ATP Only in the plot) was 5x10^{-4}M.
a) Raw data
b) Summarized $t_{1/2}$ results.
Figure 3.16: Raw light scattering data showing effect of divalent cations upon reaction rate.
Reaction conditions: collagen concentration = 1 mg/mL. ATP concentration = 2 mg/mL.
ion concentration = 5 \times 10^{-5} M. temperature = 24.8^\circ C
Figure 3.17: Sigmoidal growth curves calculated by numerical integration of eqns [15], [16]. Kinetic parameters are shown on inset.
Figure 3.18 a, b: Growth curve generated by numerical integration of ‘intermediates model’, shown in equation [16].

a) curve is obtained by solutions to eqns [17]. [18] without accounting for the particle structure factor
b) same curve as in a) but an averaged structure factor was accounted for. resulting in the formation of an initial lag phase.
ITC Analysis: Injection of ATP into HOAc
(ATP = 3 mg/mL)

ITC Analysis of SLS Formation
(2 mg/mL collagen, 3 mg/mL ATP @ 21.1 C)

Figure 3.19 a,b,c: Results of ITC experiments of SLS formation.
a) endothermic peaks resulting from multiple injections of ATP into acetic acid
b) peaks resulting from titration of collagen sample with ATP; peaks contain an initial
exothermic peak followed by a slower endothermic peak
c) expanded view of a "doublet" peak in figure b)
ITC Analysis of SLS Formation
(2 mg/mL collagen, 3 mg/mL ATP @ 21.1 °C)
Chapter 4: Fibrous Long Spacing Collagen Structure and Assembly

4.1 Abstract

Fibrous long spacing collagen (FLS) fibrils are collagen fibrils that display a banding with periodicity greater than the 67 nm periodicity of native-type collagen fibrils. FLS fibrils can be formed in vitro by addition of α1-acid glycoprotein to an acidified solution of monomeric collagen, followed by dialysis of the resulting mixture. Investigations of the ultrastructure and assembly mechanism of FLS fibrils formed in vitro using the atomic force microscope have been carried out. The majority of the fibrils imaged showed typical diameters of ~150 nm and had a distinct banding pattern with a ~250 nm periodicity. However, an additional type of FLS fibril, which is typified by a secondary banding pattern surrounding the primary bands, was also observed. These results are compared with those obtained in past investigations of FLS ultrastructure carried out using the transmission electron microscope (TEM). The importance of the fibril's surface topography in TEM staining patterns is also discussed in relation to previous models of collagen fibril structure, and an alternate interpretation of previously reported TEM experiments is suggested. In addition, images of FLS fibrils in various stages of assembly have also been collected, and the implications of these images for the mechanism of assembly of FLS collagen is discussed. From these results, a model for FLS collagen assembly is proposed for the first time.

4.2 Introduction

4.2.1 Fibrous Long Spacing Collagen

Type I collagen fibrils in their native form typically display a banding pattern with a 67 nm periodic spacing when visualized with electron microscopy (EM) (1,2) or
atomic force microscopy (3.4). As has been discussed in previous chapters, however, collagen is a system which shows a large degree of polymorphism. Depending upon the *in vitro* conditions of ordered aggregation, collagen molecules may form a variety of different structures. One important variant of the fibrous forms of Type I collagen are those which are classified as "fibrous long spacing" fibrils (FLS). By general definition, any collagen fibril with a banding periodicity greater than 67 nm can be classified as FLS collagen (1).

The first observation of FLS collagen was made in 1950 by Highberger *et al.* during the examination of fibrous structures formed by the dialysis of an acidified connective tissue extract (5). The fibrils produced by dialysis displayed a banding periodicity of ~200-300 nm by transmission electron microscopy (TEM). Because the collagen monomer is ~280 nm in length (2), this observation led to the hypothesis that this new collagen variant was formed by collagen monomers lined up in register, taking on a random head-to-tail, head-to-head or tail-to-tail alignment (Figure 4.1) (6). As with the initial Hodge-Petruska model used to describe native-type collagen fibril structures, this model was intended to help describe banding patterns observed in the TEM (7), rather than be a full, three-dimensional description of the fibril structure. Despite its rather preliminary and incomplete nature, however, this has become the accepted model for FLS collagen structure (8.1). FLS collagen formed *in vitro* can exhibit polymorphism, even within the same fibril, and similar models have been employed to help describe these variants. As depicted schematically in Figure 4.2, Chapman and Armitage (9) have classified FLS collagen into four different categories - Types I-IV - depending upon the
banding pattern observed by TEM. Based on the observed patterns, various monomer stagger patterns were proposed for the structures of each of the types I-IV FLS collagen.

During subsequent investigations by Hightberger et al., it was found that careful purification of collagen solutions inhibited the formation of FLS fibrils. This suggested that a second component, removed by the more careful purification scheme, was necessary for inducing FLS formation. Further work suggested this component may be $\alpha_1$-acid glycoprotein, a species commonly found in mammalian plasma (10). A comparison of FLS and native collagen fibril amino acid compositions using radioactively labelled $\alpha_1$-acid glycoprotein suggested that $\alpha_1$-acid glycoprotein is incorporated directly into the FLS fibrils (8). However, the exact role of the $\alpha_1$-acid glycoprotein in both fibril formation and morphology is yet to be determined. A number of researchers have also carried out experiments wherein the glycosaminoglycan chondroitin sulfate was used instead of $\alpha_1$-acid glycoprotein; this substitution resulted in the formation of similar FLS structures (9,11).

Soon after the first in vitro detection of FLS collagen, Jakus detected similar fibrils in vivo, located in ocular tissue (12). FLS collagen has since been detected in a variety of normal and pathological tissues (1). It has regularly been observed in nervous tissue, in both normal (13) and cancerous tissue (14,15). Other pathological conditions in which FLS collagen has been observed are atherosclerotic plaques (16), Hodgkin’s disease (17), myeloproliferative disorder (18,19) and silicosis (20).

The banding periodicity that is most frequently observed in vivo is approximately half that of FLS collagen formed in vitro (~100-150 nm as compared with ~200-300 nm) (1). In terms of the Hodge-Petruska type models, this has lead to the hypothesis that FLS
collagen formed in vivo is simply made up of collagen monomers aligned in a half-stagger. One might be inclined to dismiss FLS collagen formed in living systems as being completely different structures from those formed in vitro. In at least one case, however, FLS collagen from a hemorrhagic and necrotic tissue specimen was observed to have a banding periodicity of ~250 nm (21). The FLS collagen found in vivo also contained glycoproteins (1.22), making the general discrepancy in banding periods between the in vitro and in vivo samples puzzling. This is especially so when compared with the behaviour of normal collagen fibrils, where the 67 nm periodicity is observed in both the in vivo and those assembled in vitro (23).

Because of its possible linkage with disease pathology, considerable effort has been made towards determining a mechanism of FLS formation in vivo. There are two current hypotheses accounting for the driving force for formation of FLS fibrils in vivo. In the first, proposed by Kajikawa et al., the degradation of reticular collagen by collagenase is proposed to be an essential factor (24). This was shown by the promotion and inhibition of collagenase activity in tissue cell culture, with a large amount of FLS collagen being produced upon elevation of collagenase activity. In the case of EDTA inhibition of collagenase, no FLS collagen was observed. An independent study by Kobayasi confirmed increased FLS formation promoted by collagenase activity (25). In the second proposed mechanism, from Park and Ohno, glycosaminoglycans from basal laminae of neoplastic cells are thought to interact with immature collagen fibrils and promote FLS collagen formation (26). This was postulated after histochemical examination of neural tumors, in which FLS collagen appeared to be contiguous with the basal laminae. Additional studies from the same research group showed further cases in
which FLS collagen was associated with basal laminae (27). Of course, it is possible that FLS collagen *in vivo* may be formed through either mechanism, depending upon the physiological conditions in a given tissue. It is also quite possible that there are other, as of yet undetermined, mechanisms of *in vivo* FLS collagen formation. Unfortunately, the applicability of the first pathway for *in vitro* systems is minimal; the fact that fibril formation can be induced without the presence of collagenases indicates that collagenases, if relevant to FLS formation, are only so for living biological systems.

The fundamental goal of these investigations has been to determine mechanisms of assembly for collagen aggregates. In the past, the majority of research on *in vitro* formation of FLS fibrils has been dedicated towards the characterization of the fibrillar structure, with scant effort being directed towards elucidating its assembly mechanism. The reasons for paucity of work in this area are likely twofold: First, the structure of the final fibril is still poorly understood, and without a clearer understanding of the nature of the final product, determining how it forms has been relegated to being of secondary importance; and second, the nature of the experimental preparation, namely dialysis, makes vital kinetic measurements such as light-scattering, which have been applied with moderate success to other collagen systems, problematic. In the following sections, experiments directed towards further elucidation of both the structure of FLS fibrils and their mechanism of formation shall be described. The primary tool used in these studies is the AFM, which is eminently suited for structural characterization of features on collagen's length scale.

In terms of structural characterization, attempts shall be made to correlate AFM images with results obtained by electron microscopy, and in particular with the structural
variants of FLS collagen proposed by Chapman and Armitage. A new interpretation of the EM staining patterns used in studying collagen fibrils is suggested, which may well have profound implications for the field of collagen research as a whole. In terms of an overall fibril aggregation mechanism, the AFM measurements carried out will be used to provide insight by characterizing various intermediates in the course of the assembly. On the basis of these results, the first ever model for the assembly of these complex structures will be postulated.

4.3 Materials and Methods

4.3.1 In vitro FLS collagen assembly

Type I calf-skin collagen (~ 95% purity, Sigma, St. Louis, MO) was dissolved over ice in 0.05% acetic acid, with occasional sonication to facilitate the breakdown of collagen aggregates. The mixture was centrifuged at 10000 rpm for 60 minutes at 4°C. After centrifugation, the supernatant was filtered through 0.45 μm Millipore filters (Sigma) and mixed with the α1-acid glycoprotein solution. Solutions of various α1-acid glycoprotein concentrations were prepared by dissolving the solid bovine serum derived protein (99% purity, Sigma) in 0.05% acetic acid. Collagen and glycoprotein solutions were combined to yield a mixture with a final collagen concentration of ~ 0.5mg/mL, an α1-acid glycoprotein concentration ranging from 0-0.75 mg/mL and a final pH of 3.5. The mixture was transferred into dialysis tubing (molecular weight cutoff 12-14 kDa) and dialyzed at 21°C against Millipore filtered water overnight (~ 17 hrs). Dialysis produced a white, turbid solution with a final pH of 7. A series of dilutions ranging from 10-1000 fold were prepared by mixing the dialysis product with an appropriate volume of Millipore water. The diluted samples were deposited onto freshly cleaved sheets of mica
in 20μL aliquots and dried. either under a stream of filtered nitrogen or at ambient temperature and pressure under air, prior to imaging with the AFM. A similar procedure was followed for the experiments involving chondroitin sulfate A and B (70% and 90% purity, respectively, Sigma).

4.3.2 Atomic force microscopy

Samples were imaged with a Nanoscope III instrument (Digital Instruments, Santa Barbara, CA), typically using square pyramidal silicon nitride tips of spring constant 0.58 N/m. Occasionally, oxide sharpened tips were used for imaging. Images were obtained using contact mode in air. Electron microscopy locator grids were fixed underneath the mica prior to cleavage in order to facilitate the repeated examination of the same area of a given sample (28). Tips were changed frequently in order to check for possible tip artifacts. Both height and force mode images were taken simultaneously, with height measurements being taken from the height mode images only.

4.3.3 Fluorescent labeling and confocal microscopy

Samples of acidified α1-acid glycoprotein were fluorescently labeled using a procedure similar to Busby (29). A stock fluoresceine isothiocyanate solution was made by dissolving the isothiocyanate salt in 75% methanol to yield a final concentration of 6 mg/mL. A 6mL aliquot of 4 mg/mL α1-acid glycoprotein in 0.05M NaHCO3 (pH 9.0) was combined with 180μL of the fluoresceine isothiocyanate. This mixture was allowed to incubate at 37°C for ~2 hours. Excess free dye was removed by running the products through a Sephadex G-100 gel chromatograph column, which had previously been equilibrated with a neutral pH phosphate buffer solution. The final, labeled protein was re-acidified by addition of HCl to pH 3.5, and used to prepare FLS collagen in the
manner described previously. Fluorescently labeled samples were deposited onto a mica substrate and imaged with a BioRad confocal microscope using a 488 nm excitation line.

4.4 Results and Discussion

4.4.1 Native type versus FLS collagen ultrastructure

The preparation described above yielded white, turbid solutions after dialysis. AFM imaging of the resultant solutions revealed an abundance of fibrous long spacing collagen fibrils, which were identifiable because of their distinct banding pattern (~245 nm periodicity). The measured periodicity of 245 nm agrees with the results of Highberger et al. (10), which were obtained using electron microscopy (EM). However, the AFM measurements show differences in morphology between normal and FLS fibrils that are not readily apparent in the EM results. In addition, the high vertical resolution of the AFM allows for examination of minute ultrastructural details found on the surface of the fibrils. Figures 4.3 (a)-(c) show some typical examples of FLS fibrils.

Longitudinal and cross-sectional measurements of different mature fibrils were taken, showing them to be typically ~100-200 nm in diameter and ~ ten microns in length. A typical fibril and its sections are shown in Figures 4.4 (a)-(c). For comparison, a native-type collagen fibril, assembled as described in (30), is shown in Figures 4.4 (d)-(f). Aside from their markedly different periodicities, FLS fibrils are much thicker in diameter than native type collagen fibrils, while their lengths are roughly the same.

As can be seen in Figure 4.4 (b), the banding pattern in FLS is very periodic, much more so than the native type collagen fibril (Fig. 4.4 (e)). In addition, it is clear that the banding pattern in Figures 4.4 (a) and (b) consists of a series of evenly spaced ridges. These ridges are ~30 nm high and ~100 nm wide, and are separated by a relatively flat
region that spans about 150 nm. This is in stark contrast with the observations for normal collagen fibrils, where the fibril surface consists of a continuous series of bumps. This can be seen in the section (Fig. 4.4 (e)). The banding pattern seen in Figure 4.4 (d) is due to relatively sharp depressions or grooves instead of protrusions.

Because the bands in FLS are well-separated, the AFM tip can access both the interband regions and the bands themselves for closer inspection. Within these regions, fine ultrastructural features can be resolved. These features, shown in Figure 4.5, appear as small ridges and grooves oriented roughly along the main axis of the fibril. The grooves were typically 2 nm deep, 20 nm in width and were continuous along the bands and the interband regions for all mature fibrils examined. It is important to note that the finite tip width means that the depth of these ridges may be an underestimate, while tip convolution effects mean that the width is an overestimate (31,32).

Several different AFM tips, including standard silicon nitride tips, sharpened oxide tips and tips produced by electron beam deposition were used to confirm that the observed features were not simply due to tip effects. The same ultrastructural features could be observed using all of the different tips. A better measurement of the dimensions of these features can be obtained by processing the tip geometry out of the AFM image (33,34). However, it is simpler to estimate their dimensions by noticing that their apparent widths are about the same as those of the filamentous structures that are on the mica substrate. The real diameters (free of tip convolution effects) of these filamentous structures can then be obtained by measuring their height above the mica surface (31): this was found to be \( \approx 5-7 \) nm.
In their EM study of *in vitro* FLS fibrils, Hhighberger et al. (10) also mentioned the presence of some interband region ultrastructure in FLS. but because of the inability of electron microscopy to characterize depths, they were unable to obtain much information about these features. AFM has provided information about this ultrastructure, and has also revealed the presence of ultrastructure on the bands themselves. Other studies of *in vitro* FLS fibrils note the presence of striations or minor bands that were oriented parallel to the main bands (9). In these investigations, there was no indication of the presence of minor bands that are parallel to the main bands. However, on a longitudinal section of a mature fibril, one can sometimes discern the presence of small lumps, which may possibly appear as minor bands when stained for EM.

This ultrastructure does not remain the same throughout the mature fibril. Consider the fibril shown in Figure 4.4 (a), which is narrower and compact at one section and grows wider in the other direction. On the narrower end, as highlighted in Figure 4.6 (a), the corrugations are less visible and appear more orderly and parallel to the main fibril axis. As the fibril becomes wider, shown in Figure 4.6 (b), these corrugations become more clearly defined and less ordered, showing mostly as lumps instead of clear filaments. Using the AFM, it is possible to examine the full length of a fibril and locate its tip. Examination of several such FLS fibrils, some of which are shown in Figure 4.7 (a), indicates that their tips do not have the same parabolic shape found in native type collagen (35). Instead, they are wide and somewhat blunted, as can be seen in Figures 4.7 (a) and (b).

In these images and in other similar samples, the wide tip is always surrounded by a large number of the ~5-7 nm-diameter filamentous structures, similar to those that were
mentioned previously. The fine, disordered ultrastructure discussed previously is also evident and is much more pronounced. Based on these results, one may postulate that the mature FLS fibril is composed of these finer filamentous structures, which shall henceforth call protofibrils. With this assumption, the ultrastructural pattern in Figure 4.6 (a) shall be ascribed to the existence of a tighter packing of the protofibrils at the narrower section of the fibril, and a looser packing at the wider section. As one nears the wide tip of the mature fibril, the protofibrils become even more loosely packed as can be seen in Figure 4.6 (b). This implies that the tip is the region of growth, and that growth occurs by addition of protofibrils to the mature fibril. This view is further supported by a closer inspection of the tip in the AFM deflection mode, which is more sensitive to changes in topography. In Figure 4.7 (b) one observes a region of protofibrils (indicated by an arrow) that appears to be giving rise to a partially formed band, which is axially located about 245 nm from a complete band. This is further evidence suggesting the importance of protofibrils in FLS structure.

While it is difficult to get a precise value for the length of the protofibrils because of their high degree of entanglement, a reasonable estimate is that they are about 1-2 microns. These protofibrils are much larger than collagen monomers, which are roughly 280 nm in length and 1 nm in diameter. Their relative uniformity in size suggests that they are stable intermediates in the assembly of FLS fibrils.
4.4.2 Novel FLS fibril ultrastructure

As stated, the majority of FLS fibrils observed showed the characteristic features described above. For sake of convenience, fibrils with the previously described ultrastructure shall temporarily be referred to as 'Type A' fibrils. Occasionally, fibrils with an ultrastructure which is different from Type A are observed, as shown along with appropriate cross-sections in Figure 4.8 a-c. The second type of FLS fibril, which shall be referred to as 'Type B', again possesses a series of periodic bands running along the length of the fibril at ~250 nm periodicity. In Type B collagen, however, each band consists of two minor ridges surrounding a larger, central peak. A narrow interband region separates adjacent bands. The central peak is typically on the order of ~18 nm above the lowest point on the interband region, while the smaller ridges are ~6 nm high. The width of the central peak, measured as in Fig 5 a, is ~90-100 nm, while the width of the minor ridges are ~70-90 nm. Diameters of the Type B fibrils are generally smaller than Type A, being typically around 50 nm, while their lengths are, again, on the order of tens of microns.

A range of $\alpha_1$-acid glycoprotein concentrations (0–0.75 mg/mL) have been explored to see if this parameter plays any apparent role in the formation of one FLS polymorph over another. Over the range of $\alpha_1$-acid glycoprotein concentrations explored, there was no particular tendency to form one variant of FLS over another. However, it was observed that at total glycoprotein concentrations between 0.1-0.2 mg/mL, a mixture of native-type (~67nm periodicity) fibrils and FLS fibrils are formed. One occasionally observes hybrid fibrils, showing both FLS and native-type periodicity on the same structure (Figure 4.9). Above this glycoprotein concentration range, FLS
only is observed: below, only native-type is observed. Unlike Franzblau et al., who observed the complete inhibition of fibril formation at very high collagen to glycoprotein ratios. these experiments indicated that native-type fibrils form at all glycoprotein concentrations below 0.1 mg/mL. The most obvious explanation for this is that the glycoprotein is incorporated directly into the growing fibril. If the glycoprotein is not present in sufficient quantity, the fibril assembly ‘defaults’ into forming native fibrils. Presumably, at concentrations in the range of 0.1-0.2 mg/mL there are regions in the reaction mixture which contain sufficiently high local concentrations of glycoprotein to promote FLS formation.

4.4.3 Fluorescent labeling and confocal microscopy

Experiments with fluorescently labeled glycoprotein and confocal microscopy have been carried out in an attempt to determine the role of the glycoprotein in the assembly. Following the procedure above, the glycoprotein was fluorescently labeled with a fluoresceine functionality. The exact nature of the labeling process has been well studied and is discussed elsewhere (29).

The fluorescently labeled glycoprotein is capable of inducing the formation of FLS. In Figure 4.10 (a)-(c), AFM images of FLS collagen produced by the dialysis of a collagen monomer / labeled glycoprotein mixture is shown. The labeled FLS had a strong tendency to form large clumps of fibrils, which were difficult to disperse into individual fibrils. Some of these clumps are shown in Figure 4.10 (a). However, even in these clumps, the periodicity of FLS can readily be discerned. In Figure 4.10 (c), several individual FLS fibrils can be seen, despite the obvious presence of the clumped fibrils. The FLS fibrils had dimensions similar to those prepared without the fluorescently
labeled glycoprotein, suggesting that the modification does not alter the final product's structure in any significant way.

The FLS formed with the labeled glycoprotein was deposited onto mica and imaged with a confocal microscope. Confocal microscope images, shown in Figure 4.11 (a)-(c), reveal that the clumps of FLS collagen observed in the AFM are strongly fluorescent. This suggests that the glycoproteins are incorporated directly into the FLS fibrils, as previously postulated by Franzblau et al. (8). However, locating individual FLS fibrils was problematic, even when the position of fibrils had been determined previously by AFM imaging and use of locator grids, as suggested by Markiewicz and Goh (28). Objects with a fibrous appearance were occasionally found, as shown in Figure 4.11 (b), and had an approximately uniform distribution of fluorescence intensity along their length. This would suggest that the glycoprotein is distributed uniformly throughout the fibrils, instead of being localized in a particular region such as in the fibril bands. However, these objects could not be definitively identified as FLS, and such statements can only be taken as speculation. Unfortunately, it seems unlikely that confocal microscopy can provide the spatial resolution required to pinpoint their exact location (e.g. in the bands, interband region etc.) of glycoprotein in the fibrils. With the recent development of the near-field scanning optical microscope (NSOM) (for recent reviews see (36)) the required resolution might now be attained to answer this question.

4.4.4 Investigation of chondroitin sulfate activity in FLS fibril formation

As noted in the introduction, it has been observed that FLS formation can be induced by substituting the glycosaminoglycan chondroitin sulfate for $\alpha_1$-acid glycoprotein in the fibril preparation scheme (e.g. see (9.11)). Glycosaminoglycans such
as chondroitin sulfate are important components of interstitial tissues and are commonly found in close association with collagen (37). The structures of several common isomers of chondroitin sulfate are shown in Figure 4.12.

Attempts were made to reproduce these results by using both chondroitin sulfate A and B in place of \(\alpha_1\)-glycoprotein. In all of these experiments, FLS collagen failed to form, although fibrils which had no discernible banding pattern were produced. Some typical fibrils of this type are shown in Figure 4.13 a,b. An explanation for this failure is not immediately apparent, though a distinct possibility lies in the nature of the preparation used to obtain the chondroitin sulfate. The past works typically made use of a crude and poorly characterized isomeric mixture of chondroitin sulfate (9). In fact, all of collagen, \(\alpha_1\)-glycoprotein, chondroitin sulfate and the closely associated class of molecules referred to as proteoglycans (the nomenclature of glycosaminoglycans, glycoproteins and proteoglycans is at best unclear and at times remarkably confusing: readers are referred to (38) for further clarification) are often prepared from the same connective tissues. What has likely occurred, is that the original, crude mixtures of chondroitin sulfate used by Chapman and others were contaminated with substantial quantities of \(\alpha_1\)-glycoprotein, which were responsible for the FLS formation. The more highly-purified (70 & 90% for chondroitin A and B, respectively) samples used in these studies failed to induce FLS formation because they did not contain any significant amounts of glycoprotein. Regardless of the explanation, FLS formation could not be induced with chondroitin sulfate.
4.4.5 Comparison of fibril banding patterns observed by AFM and TEM

The transmission electron microscope (TEM) has provided much of the current insight on the structure of collagen fibrils. The generally accepted Hodge-Petruska model, which describes the axial arrangement of collagen molecules in the native fibril, has its basis in TEM micrographs of negatively stained samples (39). As a tool for visualization, the AFM can serve as an excellent complementary approach to EM, and in a number of polymer studies, a combination of both AFM and TEM have been used to successfully obtain structural information (40). The first question to consider is the correlation of information from both techniques, which may not be a simple task because of their intrinsically different contrast mechanisms. In the mode of operation employed in these studies, the AFM attains contrast primarily from surface topography, whereas in negatively stained TEM images, contrast is generated by preferential exclusion, or by uptake of heavy metal ions into the sample. In the following section, an attempt will be made to correlate AFM and TEM images for the case of FLS fibrils.

As noted in section 4.2, FLS fibrils refer to a class of collagen fibrils with axial periodicity greater than the 60-70 nm of native type collagen. Chapman and Armitage (9) have classified these forms into four types based on the differences in their TEM banding patterns. These variants are shown schematically in Figure 4.2 and in electron micrograph form in Figure 4.14 a-d (reprinted with permission of the publisher). The micrographs were generated by negative contrast staining with a sodium phosphotungstate solution, and thus the observed periodicity of FLS is due to a pattern of light-coloured bands, which are rich in electron-transmitting material, separated by broad dark bands, which are localized electron-opaque material. The dark regions in TEM
micrographs are generally believed to correspond to stain-penetrable. less densely packed regions of the fibril: in normal collagen, these are the so-called 'gap-zones' of the Hodge-Petruska model. A more careful consideration also takes into account an enhanced uptake of ions into polar regions of molecules, as well as stain exclusion by bulky amino acid groups (41).

In the AFM studies of FLS fibrils carried out here, two types that can be differentiated by their topographic banding patterns were observed, which were labeled Type A and Type B. It is not immediately obvious which of the various classifications assigned by Chapman and Armitage (e.g., FLS I – FLS IV), if any, correspond to these. It shall be proposed that Type A corresponds to FLS I and Type B to FLS IV based on the arguments below.

In the TEM images of collagen, one typically observes broad gap / overlap zones with small scale variations in the stain patterns in these regions. The nature of these small-scale variations have been studied in detail and are well understood (41). Consider however, the collagen fibril staining process itself. In this process, a solution of sodium phosphotungstate is directly applied to the sample, and allowed to interact for a few seconds. Note that the approximate size of a phosphotungstate polyanion is on the order of 1 nm (41,42). In order to settle into crevices buried within the fibrils, this anion will have to penetrate the surface of the fibril. The surface topography of the fibril is easily visualized with the AFM. and hence, the AFM images can be used to provide guidance in estimating possible stain penetration into the fibril. In the mature FLS fibrils, such as those shown in Figure 4.6 a, one observes a tight packing of protofibrils. Given the spacing between protofibrils, it is highly unlikely that the stain can penetrate into the
fibril to much more than a small fraction of its total diameter. This would mean that if there are gap zones in the fibril’s interior, they cannot be easily reached by stain molecules, because the stain is too large to penetrate into the fibril to any significant degree. Instead, it shall be proposed that the contrast is mostly due to the behaviour of the stain at the surface of the fibrils.

Consider, instead, the deposition pattern of the stain on the surface of the fibril itself. Notice that the topographical section along an FLS fibril (in Figure 4.6 b) show that the banding pattern is due to alternating peaks (‘bands’) and valleys (‘interbands’). The valleys are thus crevices between the peaks, and as the solution deposits the phosphotungstate stain, the crevices will be filled preferentially over the peaks. The net density of high electron scattering material in these interband regions will thus be much greater than on the bands themselves, making these regions appear dark in TEM. Thus, in this view, the dark bands in TEM, which indicate a high amount of deposited metal stain, will correspond to topographical crevices and valleys observed in the AFM, while the light bands in TEM will correspond to higher regions in the AFM images. This is illustrated, schematically, in Figure 4.15.

Further evidence in favour of this interpretation comes from features in the original TEM results of Chapman and Armitage which were overlooked. Closer inspection of Figure 4.14 a.b.c reveals long grooves running parallel to the main axis of the fibrils. These grooves can be attributed to the presence of closely packed protofibrils, as observed with the AFM. The contrast in these micrographs is probably caused by the metal stain settling preferentially into the gaps between protofibrils, giving rise to dark
grooves. Clearly, the topography of the fibrils strongly affects the nature of the staining process, an effect which is too often ignored in interpreting EM results.

With these arguments in mind, one can attempt to correlate the different structural variants of collagen measured with the AFM to the corresponding TEM classifications. It shall generally be assumed that the light bands in TEM micrographs correspond to elevated topographical regions, and the dark bands correspond to ‘low lying’ regions in which the metal stain can collect. The Type A FLS observed corresponds most closely to Chapman and Armitage’s FLS I. From the TEM micrographs, the periodicity is caused by single bands separated by approximately 260 nm, with a band width of ~60 nm. The AFM images yield a comparable periodicity, and band widths of ~90-100 nm, with this latter value greater than the width measured by TEM because of the aforementioned AFM tip convolution effect. An examination of FLS IV shows that the periodicity in the TEM micrographs is caused by broad, light-coloured bands, separated by 260 nm, with the bands having an estimated width of ~150 nm. This is comparable with the Type B fibrils observed in the AFM measurements: the periodicity is the same and the band widths are ~200 nm. Again, accounting for tip convolution effects in the measured band widths, these are values which compare well with those for FLS IV.

4.4.6 Insights into assembly mechanism of FLS collagen

Numerous images have been collected which provide insight, albeit incomplete, into the mechanism by which FLS fibrils form. In the previous section the presence of protofibrils, stable intermediates in the assembly of FLS, with lengths of several microns and diameters of ~5-7 nm, were noted. As mentioned above, the grooves observed along the length of the fibril are actually due to the spaces between adjacent protofibrils. These
protofibrils bulge outwards at each band, but appear as continuous structures along the length of the fibril. These protofibrils were also observed adding to the end of a mature FLS fibril, suggesting that, at least in the later stages of assembly, FLS fibril growth occurs via the interweaving of protofibrils at the ends of fibrils. In order to elucidate the assembly mechanism, it would be most desirable if one can observe fibrillogenesis as it occurs. However, technical difficulties have precluded such studies. Instead, a larger range of samples have been examined to provide information about the mechanism by which FLS forms. Figure 4.16 a-f shows a series of AFM images that were taken from samples prepared in the manner described above. The structures observed in these images coexist with one-another as well as with mature FLS fibrils within the same sample; however, the mature fibrils are the dominant forms, which is why they were more readily observed.

In Figure 4.16 a, one observes a periodic framework of collagenous material, which appears to be aligned in a lateral fashion. The spacing between these structures is ~300 nm, somewhat larger but close to the expected periodicity of FLS. These structures are stable in the absence of any resolvable fibrillar structure. In Figure 4.16 b, one observes a similar pattern of laterally aligned collagenous material, but with the beginning of an internal structure forming between them, something that hints at the subsequent appearance of a fibril. The banding pattern in this structure is easily discerned, and corresponds to the characteristic 260 nm of FLS. Figure 4.16 c is a zoom in on the aligned, fibrous collagen material in one of these structures. This material has smaller dimensions than the protofibrils which have been characterized previously: The diameters are on the order of ~1-2 nm, whereas the previously observed protofibrils had
diameters from 5-7 nm. Whether this fibrous material is purely oligomeric or contains monomers is unclear, as their high degree of entanglement and their tendency to pile on top of each other makes any exact classification problematic. Figure 4.16 d shows a further development of this stage, again with the aligned collagenous material still being plainly obvious but with a much larger, banded internal structure connecting the periodic bands together. At this stage, the internal structure has a diameter of 20-30 nm, indicating that a great deal more lateral growth is required before it reaches the size of a typical mature FLS fibril (~150 nm diameter). Finally, in Figure 4.16 e, one observes a blunted fibril end, with one end of the fibril showing the typical FLS banding pattern and the other showing protofibrils merging with the growing tip.

Because of the recurrence of images such as these, it is suggested that FLS may grow in the following manner, which is schematically illustrated in Figure 4.17. An initial framework consisting of entangled collagen oligomers aligns in solution, with the periodic arrangement being determined either by long range interparticle forces, or by a thin network of collagen linkers that are smaller than the resolution attained in these experiments (e.g. Figure 4.16 a). Concurrent to this interweaving of monomers and oligomers is the growth of protofibrils, a process likely competitive with the formation of the interwoven collagen framework. While maintaining the collagenous framework, more oligomers and monomers add laterally, resulting in the gradual thickening of the FLS fibril (e.g. Figure 4.16 b). Again, occurring simultaneously, is the addition of protofibrils to the end of the fibrils. This results in structures that have the banding pattern of mature FLS, but not the thickness of the final fibrils. Simultaneous lateral growth, via addition of oligomers and monomers, as well as growth through the fibril tip.
end proceeds until, at some point in the assembly, lateral growth ceases and growth of the fibril becomes dominated by addition of protofibrils to its tip end (e.g. Figure 4.16 e). This may occur because the supply of free monomers and oligomers in solution becomes exhausted; the only species able to add to the growing fibril are protofibrils. As protofibrillar addition progresses, the fibril is drawn together more tightly until the final, banded structure is formed. At some point in the assembly, addition of collagen between protofibrils ceases and the growth of the fibril becomes dominated by addition of protofibrils to its tip end. As addition progresses, the fibril is drawn together increasingly tightly, until the final structure is formed.

This model is, of course, speculative, and there are several issues that need to be addressed. The first is one of sample preparation. In the methods used here, samples are dried onto the mica substrate. Drying samples can induce unusual patterns in the material being deposited. In many cases, these drying patterns can be distinguished by an experienced microscopist. A common example of this would be the formation of circular patches, caused by the gradual evaporation of droplets on the surface. Other patterns can be less easy to recognize, raising the possibility that some of the results observed here may be a consequence of sample drying. However, this is likely not the case. If the patterns observed were caused by drying, one would expect that the samples would orient themselves along the drying front: that is, all the fibrils, protofibrils and partially formed fibrils with their associated collagenous material would all be aligned in the same direction. This was certainly not the case in these experiments: For example, in Figure 4.16, the partially formed fibrils have a random orientation with respect to one another.
Another issue that should be addressed is one of kinetics. It has been assumed that the diverse structures imaged represent different stages of the assembly process. Ideally, one would like to carry out a kinetic investigation, in which the appearance and development of these different structures is tracked as a function of time. However, this has not been possible so far. When samples were taken prior to completion of the dialysis, the collagenous material coagulates and cannot be dispersed on the mica substrate. Thus, they generally appear as amorphous clumps in the AFM images, preventing one from obtaining a better resolution. Obviously, further work on sample preparation is necessary.

4.5 Summary

The ultrastructure of fibrous long spacing collagen has been examined by the atomic force microscope. Measurements yield fibril diameters and periodicities of ~100-200 nm and ~245 nm, respectively, which are in good agreement with electron microscopy results. The banding pattern consists of evenly spaced ridges separated by large, relatively flat interband regions. Probing the surface of mature FLS fibrils at higher resolution has revealed details about their ultrastructure. A fine structure consisting of side-by-side protofibrils aligned roughly along the axis of the fibril was observed in both the interband spaces and on the bands. The tips of FLS fibrils were found to be blunt and flattened, in contrast to the parabolic shape found in native type I collagen fibrils. The existence of two structural variants of FLS fibrils has also been noted, the major difference between structures being the presence of additional ridges adjacent to the central banding peak. The structures observed have been compared with those obtained from electron microscopy, and, taking into account the role of AFM tip convolution on
the structure. the fibrils have tentatively been classified as FLS I and IV. as assigned by Chapman and Armitage in earlier TEM experiments.

In addition, a series of images which show different stages of assembly of FLS suggest possible mechanisms of fibril formation. Growth of the fibrils appears to be hierarchical, with an important stable intermediate in the process being a protofibril. a fibrous collagen species of approximate dimensions ~ 5-7 nm diameter and ~ 1-2 μm length. The AFM images obtained suggest that the formation of FLS in vitro is related to entanglement of protofibrils at the blunted tip, followed by the eventual tightening of this entanglement, and a final close packing of the protofibrils. Our proposed model involves the establishment of a preliminary framework of interwoven oligomers and monomers, followed by growth of the fibril around this framework and a final addition of protofibrils to the fibril tip ends. Furthermore, the periodicity of FLS fibril banding may in fact be attributable to the incorporation of this primary collagenous framework into the fibril during assembly.
4.6 References


Figure 4.1:
a) Hypothesized structure of in vitro FLS collagen fibril with schematic representation of collagen monomers (~280 nm in length). The observed periodicity in electron micrographs of FLS collagen is accredited to the spacing between rows of monomers. Note that no directionality is implied for the monomers.

Figure 4.2: Schematic representation of the four proposed morphologies of in vitro FLS collagen fibrils classified by transmission electron microscope analysis. The dashed arrow represents one period of an FLS collagen monomer (i.e. ~230-260 nm).
Figure 4.3: Deflection mode AFM images of some typical FLS fibrils
a) 25 x 25 μm
b) 9 x 9 μm
Figure 4.3:  c) Height mode AFM image of FLS fibril (top view)
3.5 x 3.5 μm
Figure 4.1: AFM images and sectional analyses of FLS and native collagen fibrils.

a) Deflection mode image of FLS collagen fibril (3.5 x 3.5 μm)
b) Longitudinal section of FLS collagen fibril; note ~250 nm periodicity
c) Cross section of FLS collagen fibril
Figure 4.4: Native-type collagen fibril with appropriate cross-sectional analysis

d) Deflection mode image (2.5 x 2.5 μm)
e) Longitudinal section showing ~67 nm periodic banding pattern
f) Cross section
Figure 4.5: Deflection mode image of ultrastructural features in interband region and on the bands themselves (1.5 x 1.5 μm).
Figure 4.6: Deflection mode images of the same FLS fibril at different regions along fibril
a) Tightly packed region of fibril (1.75 x 1.75 μm)
b) Loosely packed region of fibril (2 x 2 μm)
Figure 4.7: Deflection mode images of FLS fibril tip ends.

a) 12 x 12 μm
b) 5 x5 μm
**Figure 4.8:** AFM image and sectional analyses of new FLS collagen variant

a) Deflection mode image of FLS collagen variant (2 x 2 µm)
b) Longitudinal section of FLS collagen variant
(A = 86 nm – width of central peak; B = 250 nm – banding periodicity; C = 220 nm – width of entire band)
c) Cross section of FLS collagen variant
Figure 4.9: Deflection mode image of hybrid FLS / native collagen fibril. Periodicity on the left hand side of the fibril is the $\sim 67 \text{ nm}$ of native-type collagen, while that on the right is the $\sim 250 \text{ nm}$ of FLS (10 x 10 $\mu$m).
Figure 4.10: Deflection mode images of FLS collagen fibrils prepared using fluorescently labeled $\alpha_1$-acid glycoprotein.

a) $15 \times 15 \mu m$
b) $20 \times 20 \mu m$
c) $10 \times 10 \mu m$
Figure 4.11: Confocal microscope images of collagen deposits produced by fluorescently labeled \( \alpha_1 \)-acid glycoprotein. Excitation wavelength is 488 nm. Scale bars at bottom are approximately 100 \( \mu \text{m} \).
Figure 4.12: Structures of chondroitin sulfate isomers.

a) chondroitin sulfate A

b) chondroitin sulfate B
**Figure 4.13:** Height mode AFM images showing collagen aggregates formed by the dialysis of a collagen-chondroitin sulfate A mixture. Unlike native-type and FLS fibrils, structures which formed had no discernible banding pattern.

a) 10x10 μm image

b) 2 x 2 μm image
Figure 4.14: TEM micrographs of FLS collagen variants reproduced from Chapman and Armitage (9) with permission of the publisher. The scale in these images is indicated by the solid line, which represents the length of an individual collagen monomer.

a) FLS I (one narrow overlap band per monomer length)
b) FLS II (two narrow overlap bands per monomer length)
c) FLS IV (one broad overlap band per monomer length)
d) FLS III (four narrow overlap bands per monomer length)
Figure 4.15: Schematic illustration showing proposed effect of negative contrast staining on FLS collagen fibril. The metal stain preferentially fills the cracks and crevices on the fibril’s surface; these regions of accumulated stain will scatter electrons more efficiently than the bands themselves, giving rise to dark regions on TEM micrographs. Note, this diagram is schematic only and is not intended to represent the actual morphology of the stain deposits.
Figure 4.16: a)-f) Deflection mode images showing various stages of FLS fibril assembly.

a) Network of interwoven collagenous material. Sectional analysis shows this material consists of collagen monomers or oligomers but not protofibrils. The network has a periodicity approaching the 250 nm of FLS collagen, yet no fibrillar structure has formed (25 x 25 μm).
Figure 4.16: b) Interwoven network of collagenous material showing an internal structure connecting it together (10 x 10 μm).
Figure 4.16: c) Zoom in of aligned collagenous material (2 x 2 μm).
Figure 4.16: d) FLS fibril with surrounding collagenous network (25 x 25 μm).
Figure 4.16: e) FLS fibril with protofibrils adding to its tip end (8 x 8 μm).
Figure 4.17: Schematic illustration of postulated FLS assembly mechanism. Collagen monomers aggregate (step A) to form a framework of collagenous scaffolding material at the periodicity of banding (~250 nm) for the mature fibril. Protofibril structures begin interweaving (step B) with the framework, beginning to form bulk along the long axis of the fibril. As the fibril matures (step C), the scaffolding materials are incorporated into the fibril as it tightens and the protofibrils pack in a more orderly fashion. Growth continues via protofibrillar addition at the tips of the fibril.
Chapter 5: In Situ AFM imaging of collagenase action

5.1 Abstract

In this chapter, the ability of the AFM to perform imaging of dynamic, biological processes in a liquid environment as they occur in real time is examined. A generalized overview of the literature, as well as the experimental requirements needed to perform such imaging, is provided. To demonstrate the capabilities of the instrument, a test case involving the enzymatic digestion of collagen fibrils by a commercial Clostridium histolyticum collagenase mixture was investigated using Tapping Mode® atomic force microscopy under liquid. Using a commercial AFM with fluid cell attachment, solution conditions compatible with stable imaging of mature collagen fibrils while simultaneously maintaining activity of the collagenase were found. Upon exposure to collagenase, collagen fibrils became both shorter and thinner with time, suggesting that collagenase degrades the entire fibrillar structure in a non-specific manner. The results are discussed in relation to the postulated mechanism of C. histolyticum collagenase activity. These experiments suggest the possibility of performing kinetic measurements of collagenolysis using the AFM. However, the quality of the images and their detailed quantification were somewhat impaired by the tendency of collagen degradation products to adsorb to both the substrate surface and the AFM tip. Experimental advantages and shortcomings of this technique are compared with other methods of following enzymatic degradation and biological processes, and suggestions are made in order to overcome some of the technical difficulties encountered.
5.2 Introduction

5.2.1 Atomic force microscopy under fluid

One of the fundamental assets of the atomic force microscope is its ability to obtain high resolution images of objects which are immersed in a liquid environment. This capability has filled an important niche in the field of microscopy: Traditional high resolution microscopy methods such as TEM and SEM often require extensive sample treatment such as staining or embedding and operate under vacuum conditions, which generally preclude studying samples in a liquid. In light of these limitations, the versatility of the atomic force microscope becomes apparent: In principle, any surface or surface bound species can be immersed in a liquid and imaged.

There are a number of advantages to performing AFM experiments in a liquid environment. One of the most significant is that the adhesive forces exerted between tip and sample are decreased when imaging in fluid. When imaging objects in air, the surface of the substrate is usually covered with a thin layer of adsorbed water. This water layer can cause the tip to adhere strongly to the surface, resulting in a reduction in image resolution and in some cases, potential damage to the sample and the tip. However, this limitation of the AFM can be overcome. It was noted by Weisenhorn and Hansma (1) that the typical adhesive force between an AFM tip and a mica substrate in air is \( \sim 10^{-7} \text{ N} \), but by immersing the sample in water, this adhesive force can be decreased by approximately two orders of magnitude. Smaller imaging forces often allows higher resolution imaging of soft samples, though some samples may become more compliant upon hydration. In general, AFM imaging under fluid has proven to be
very useful in a wide range of systems and has been applied to problems such as corrosion, adsorption and a host of others (eg.(2.3)).

Another advantage to operating the instrument in a liquid environment is that because the dominant adhesive forces between tip and sample are eliminated, the AFM can be used to measure smaller forces which would otherwise be overwhelmed by the adhesive interaction. The AFM has a force sensitivity on the order of $10^{-12}$ N (4) and as such, it is amenable to probe interactions with magnitudes on the order of electrostatic and Van der Waal's forces. Furthermore, interactions between the AFM probe tip and the substrate surface can be tailored by adjusting the conditions of the liquid environment, such as pH and ionic strength. Much progress has been made in this field and has been well reviewed by Ducker (5,6).

While of general utility in many fields, AFM measurements under liquid have been applied most extensively to biological systems in their native, hydrated states. For a recent review, see the paper by Hansma (7). As stated previously, other types of microscopy often require extensive sample preparation and dehydration via exposure to vacuum. As noted in Chapter 4, procedures such as staining may lead to erroneous conclusions about structures of biomolecules. Such procedures are generally not required for the AFM though, as shall be discussed in subsequent sections. Preparation of samples is not necessarily trivial. It seems preferable to image samples in states as close to their native environments as possible in order to obtain realistic measurements of their true structures. There are many examples of biological molecules and assemblies that have been structurally characterized by means of high-resolution AFM imaging under fluid, a recent review of which is given by Colton (8). Among these include a photosystem I
complex (9), microtubules (10), amyloid fibrils (11) and myosin (12). By far the most active field of liquid-phase AFM research is currently centred on the imaging of DNA in liquid (e.g. see (13,14)). The ultimate goal of these studies is the sequencing of DNA with the AFM, although this goal seems unlikely to be realized in the comparatively near future. Another actively growing area of research in liquid-phase AFM is cell biology. Recent advances in this field include the imaging of surface and submembrane structures (15), a survey of the mitotic cycle (16) as well as cell motility and apoptosis (17).

Aside from the advantage of maintaining sample hydration in structural studies, imaging under liquid also offers the unique possibility of imaging biological processes as they occur in real-time and under near-physiological conditions. Some examples of processes which have been investigated in this manner include the transcription of DNA (18), the dynamics of actin filaments in living cells (19) and membrane spreading during the activation of human platelets (20). Furthermore, the AFM can offer true molecular scale resolution (e.g. see (7,12)), suggesting that the instrument might be used to observe individual molecular events. In order to perform imaging of biological processes in near-physiological conditions some basic experimental considerations must be taken. These will be discussed in the following section.

5.2.2 Experimental considerations

While the AFM has been in existence for almost fifteen years, it has only been successfully applied to liquid-phase imaging of biological molecules in the past ~ five years. The primary reasons for both the delay and the recent successes have, for the most part, been purely technological. The greatest improvement in AFM imaging technology has been the advent of Tapping Mode® imaging in liquid (see Chapter 2).
necessity of biological AFM imaging is, of course, that the sample of interest be bound firmly to the substrate – typically mica because of its atomically flat nature. The first type of AFM imaging to be implemented, the so-called contact mode, generally exerts significant lateral forces upon a sample, even when imaging under liquid. While the adhesive forces between tip and sample are lowered because of the liquid surroundings, biological molecules tend to adsorb to surfaces quite weakly and the lateral forces exerted by the probe tip are often large enough to dislodge the sample from the substrate. While in certain cases, this may be overcome (e.g. (21)), more often than not, contact mode imaging can not be used to image biomolecules under fluid. Tapping Mode® imaging, described in Chapter 2, exerts much smaller lateral forces on adsorbed molecules, and in many cases, the forces are sufficiently small to prevent dislodging materials. For this reason, almost all imaging of biological materials carried out in fluid is performed using Tapping Mode®.

While important, application of Tapping Mode® imaging alone is not sufficient to ensure that a biomolecule can be successfully imaged. By far and away the most important factor for imaging is the general conditions of the imaging solvent, including buffer concentration, pH and ionic strength. Optimizing these imaging conditions, unfortunately, is a process which currently still belongs in the realm of experimental trial and error. The conditions required appear to depend very specifically upon the nature of the biomolecule itself. For example, Kasas found that the conditions for successfully imaging DNA was a solution of 20 mM Tris, 5 mM KCl, 5 mM MgCl₂, 1 mM β-mercaptoethanol and 1-2 mM ZnCl₂, all at pH 7.5 (18). Goldsbury et al. (11) found that the conditions required for stable imaging of amylin fibrils on mica was 10 mM Tris
buffer (pH 7.3), and that the images remained stable in both the presence or absence of 50-200 mM KCl. In general, an a priori approach to predicting the conditions under which a molecule will stick to a substrate has not yet been developed. As such, carrying out the imaging of a dynamic process generally requires very careful selection of solvent conditions. Of course, one is not only obliged to select conditions under which the biomolecule of interest will adsorb strongly to the surface: to image a process one must also maintain the functionality of the biomolecule. These two conditions must be met simultaneously, and are, unfortunately, not always compatible with one another.

In this chapter, these principles shall be demonstrated on a test case, that of a collagenolytic enzyme, collagenase, acting upon mature collagen fibrils.

5.2.3 Collagenase

A noteworthy property of collagen is its inertness to cleavage by a variety of common, nonspecific proteinases such as trypsin, chymotrypsin and pepsin (22). It is, however, actively digested by a class of metalloproteinases known as collagenases, which are broadly defined as enzymes that can catalyze the hydrolytic cleavage of undenatured collagen (23). A host of collagenases have been recognized and, to varying degrees, characterized, including human-fibroblast collagenase, tadpole collagenase and several others from bacterial sources (24,25). The mechanism of collagenase action is very much dependent upon the nature of the enzyme itself. Collagenase from fibroblasts and neutrophils cut the triple-helix of the collagen monomer at a single site, splitting it into two discrete fragments (26,27). Collagenase extracted from the bacterium Clostridium histolyticum cleaves monomers at multiple sites, resulting in a wide range of degradation
endproducts (28). Collagenases are thought to play vital roles in biological functions such as the remodeling of animal tissues during growth and in tissue repair (29).

One of the more well-studied of the collagenases are those extracted from the bacterium *C. histolyticum* (22). Crude preparations from this source are known to contain at least seven different subtypes of collagenases, as well as associated proteases such as clostripain and an aminopeptidase (30-32). As noted, collagenase from *C. histolyticum* cuts collagen molecules in multiple sites, and as such produces multiple fragments of degraded material. However, until now, investigations of collagenase action typically rely upon indirect methods, such as SDS-PAGE analysis of endproducts, for characterizing the digestion (e.g. see (33)). Application of a high-resolution imaging technique would allow for a direct survey of collagenase activity and perhaps provide insight into the mechanisms of collagenase activity at a molecular level.

As an initial step towards imaging the degradation of individual collagen molecules, the AFM has been used to investigate the digestion of FLS collagen fibrils by a crude collagenase extract from *C. histolyticum*. Careful selection of solution conditions have allowed collagen to be adsorbed stably to a substrate and simultaneously maintain activity of the collagenase such that direct AFM imaging of the digestion process occurring in solution can be performed.
5.3 Materials and Methods

5.3.1 In vitro FLS Collagen Assembly

FLS collagen was prepared as described in Chapter 4. Samples were deposited onto freshly cleaved sheets of mica in 30 μL aliquots and dried with a stream of filtered nitrogen. Collagenase solutions were prepared by dissolving Type H Sigma Blend Collagenase (Sigma) in 10 mM Tris-HCl (pH 7.5) buffer and adding CaCl₂ to a final collagenase concentration of 1 mg/mL, calcium concentration of 20 mM and pH of 7.5.

5.3.2 Atomic Force Microscopy

Samples were imaged with a Nanoscope III instrument (Digital Instruments, Santa Barbara, CA), using thin-legged NP-type silicon nitride tips (same supplier) of nominal spring constant 0.06 N/m. The previously dried samples were placed in the AFM Tapping Mode® fluid cell and allowed to equilibrate for ~2 hrs under 10 mM Tris-HCl buffer (pH 7.5). Resonance frequencies in the range 10-25 kHz were used for imaging, with the peak at ~16 kHz most commonly being employed. Once a stable image of collagen fibrils had been obtained, collagenase solution was flowed into the cell. In some cases, the sample was imaged continuously upon collagenase addition, in others, the tip was withdrawn for a measured period of time and then re-engaged to collect an image. Both height and amplitude mode images were taken simultaneously. Amplitude images often highlighted features not easily seen in the height mode. However, quantitative measurements were taken from the height mode images only.
5.4 Results and Discussion

5.4.1 Determination of imaging conditions

As noted in the introduction, determining appropriate solvent conditions for stable imaging of biomolecules is very often a case of trial and error, and collagen fibrils are no exception. Given that the isoelectronic point of the collagen monomer is \( \sim 8.7 \) (34), one might expect that at pHs lower than this value, collagen aggregates would be positively charged and adsorb strongly to the negatively charged mica substrate. This was not the case: a variety of neutral and acidic phosphate buffers failed in this regard, as did distilled water and several different types of alcohols, including ethanol, methanol and n-propanol which have been used successfully in the past for imaging other biomolecules (e.g. see (35)). Success was finally achieved with use of a Tris-HCl buffer, at a pH of 7.4. It should also be noted that the ionic strength of this buffer solution was also very important. An 100 mM solution allowed only the smallest fibrous species to remain adsorbed to the surface. By decreasing the buffer concentration ten-fold, conditions were obtained in which mature collagen fibrils could be imaged stably on mica. Shortly after these results were obtained, another research group described an alternative set of conditions which allow successful imaging of collagen fibrils under fluid. Taatjes et al. (36) used a sodium carbonate-bicarbonate buffer (16 mM Na\(_2\)CO\(_3\) / 34 mM NaHCO\(_3\)) of pH 9.53 and apparently also obtained stable images of fibrils. That images can be obtained with these two markedly different set of conditions, while a wide range of others are completely unsuccessful, makes the difficulty in selecting imaging conditions all the more apparent.
A different approach to solving this problem may lie in tailoring the substrate surface. A number of studies have made use of chemically modified surfaces to stably adsorb biomolecules, including surfaces which have been silanized or covered with self-assembled monolayers (37.38). However, these modifications are rarely simple, and again, obtaining stable adsorption depends strongly upon the properties of the biomolecule being imaged. That is, one may be obliged to carry out a different type of surface modification for each type of biomolecule one wishes to image. A more useful and generally applicable approach would be to produce a "tunable" surface, one which has surface properties which could be perhaps altered by an applied voltage, or one which has a large gradient in properties such as surface charge. A surface which might fill the latter description has been produced by Cremer et al., in which a stable charge gradient in a solid-supported lipid bilayer was generated via an electrophoretic approach (39.40).

5.4.2 Collagen fibril characterization under fluid

The preparation described above produced both FLS and native-type collagen fibrils in abundance. Both types of fibrils adsorbed with sufficient strength to the mica substrate to allow imaging under the Tris-HCl buffer. Amplitude mode images of some typical fibrils are shown in Figure 5.1 a-e. The FLS fibrils observed had diameters of ~20 nm, which were markedly smaller than the ~150 nm which is typically observed in air (e.g. see Chapter 4). Lengths of the fibrils were on the order of tens of microns, again, with measurements of this value being hindered by the entangled nature of the fibrils. A cross-sectional analysis of a characteristic fibril is shown in Figure 5.2 a,b. The longitudinal section clearly shows the ~250 nm periodicity expected from FLS. Why the diameters of the fibrils measured in fluid is much smaller than those in air is unclear.
One might expect that the hydrated fibrils would be more compressible than those which have been dessicated. However, the difference in fibril diameters is well over 100 nm, and a compression of that magnitude would lead to horribly distorted images in the AFM. A more likely explanation is that the solution conditions are such that only the thinnest of FLS fibrils formed adsorb strongly to the surface. This is what was observed when imaging under 100 mM Tris-HCl buffer – only small strands of fibrous material remained bound to the surface. Why this is so remains uncertain.

Images of FLS collagen fibrils obtained under fluid lacked the resolution which could be obtained when imaging under ambient conditions. For FLS, the basic banding pattern could be resolved (Fig. 5.2 a). However, the fine intraband ultrastructure, which consists of small ridges and grooves oriented along the main axis of the fibril and is readily observable in air, could not be resolved under liquid. This inability to distinguish small features suggests that there is an increased compliance of the fibrils upon hydration. For the case of native-type fibrils, this increased compliance made it very difficult to resolve the ~ 67 nm periodicity. Figure 5.3 a,b shows an image of a native-type fibril taken in amplitude mode, along with the corresponding longitudinal section. While the amplitude mode image has enough contrast to show the repeat unit of the fibril, the longitudinal section from the height mode image fails to resolve these features. More detailed quantification of collagen fibril structure carried out in fluid will have to overcome this difficulty in order to be useful; at present, imaging in air offers far superior resolution of features.
5.4.3 Collagenase addition

Addition of collagenase solution into the fluid cell resulted in the digestion of fibrils adsorbed to the substrate. By adjusting the concentration of the collagenase solution used, the rate at which the digestion proceeded could be controlled and optimized such that the process occurred on a time scale amenable to analysis by AFM imaging. With the imaging conditions found suitable for these experiments, each image took approximately 2 minutes to collect. Scanning at substantially greater speeds results in a drastic decrease in resolution and occasionally results in material being dislodged from the surface. Technological improvements which may greatly improve the speed with which high-quality images can be obtained are now under development. For example, Hansma (41) has recently developed smaller AFM cantilevers which have high resonance frequencies in liquid. These cantilevers can collect a complete image in liquid in approximately 1 second. While obviously a great improvement, these cantilevers are not yet commercially available and their use requires significant modification of the existing AFM.

Two series of images showing the time evolution of collagenase digesting several fibrils over the course of approximately half-an-hour are shown in Figure 5.4 a-f and in Figure 5.5 a-j. In Figure 5.4, the AFM tip was withdrawn after each image was obtained, and re-engaged only after a time period of five minutes had elapsed. In the second series of images, Figure 5.5 a-j, the AFM tip was scanned continuously across the sample.

Consider, first, the series of images in Figure 5.4. After the initial addition of collagenase, some fibrils disappeared completely; it is likely that at least in some cases.
the fibrils are being lifted entirely off the substrate and are carried off into the surrounding medium. However, several fibrils remain stably adsorbed to the substrate throughout the experiment, allowing one to monitor their dimensions as they change with time. From a mature fibril all the way down to the limits of the instrument's ability to resolve sizes. As is apparent in Figure 5.4, both the collagen fibril length and diameter decrease as a function of time after addition of the collagenase solution. The decrease in diameter appears to be uniform throughout the fibril: there was no preferential digestion of fibrils at their tip ends, and there was no tendency to cleave the fibrils into multiple fragments. These results would imply that the enzyme molecules attack the monomers exposed along the surface of the fibril with equal probability, and at the same time does not penetrate to the core of the fibrils. This view is schematically depicted in Figure 5.6, and is consistent with the proposed mechanism of *C. histolyticum* collagenase activity (22).

In this scheme, the rate of decrease of fibril diameter and length is directly related to the enzyme activity, and one can estimate the rate of breakdown of collagen monomers from the change of volume of fibril with time. To show this, consider one particular fibril, highlighted by an arrow in Figure 5.4 a-e. The cross-section of this fibril has been measured both prior to and during the course of the collagenase digestion: the corresponding fibril diameters are included in the figure captions.

From these measurements, one estimates an approximate rate of change in fibril diameter of 1.3 nm/minute, and in volume of $2.0 \times 10^5$ nm$^3$/minute. It has been postulated that the FLS fibrils consist of monomers arranged in register (42). With this model, and using the known dimensions of collagen monomers, the observed rate of
change corresponds to a breaking down of about 2100 molecules/minute or 1.26x10^5 molecules/hour for this one fibril. Under near-physiological buffer conditions, the rate constant for formation of collagen hydrolysis products by \textit{C. histolyticum} collagenases, $k_{\text{cat}}$, is 250-2100 hr$^{-1}$ over a range of temperatures from 15-30°C (43-45). Of course, to make a viable comparison between the two rates, one must be able to measure every fibril which is digested in the AFM experiment, a requirement which is impractical at best. A parameter which also requires more careful control is that of sample temperature. This is particularly difficult to control in an AFM experiment because of heating of the sample by the diode laser used in the feedback system, heating by the overhead camera used to align the diode laser on the cantilever and because of heating of the electronic components of the instrument itself. Another significant issue is that the results shown in Chapter 4 suggest that the model proposed by Gross is incorrect. Nevertheless, the estimate should not be off by much if one assumes that the fibril is reasonably close-packed and thus the fibril density will not be too model dependent.

The collagen samples investigated contained both FLS and native-type fibrils. Collagenase attacked both types of fibrils indiscriminately, to within the resolution of the instrument. There was no evidence for the transformation of native-type ~ 67 nm period fibrils into FLS fibrils. This is in agreement with the results of studies carried out on rat-skin (46). Kajikawa has reported the formation of FLS fibrils only in regions of rat-skin which were known to contain poorly-characterized 'reticular fibres'. Presumably, the conversion of reticular fibres to FLS observed with the \textit{in vivo} system is strongly dependent upon the presence of mucopolysaccharides and other supporting substances found in the living system.
5.4.4 Imaging difficulties and artifacts

While it is readily apparent that collagenase digestion can be visualized in real time, there are several issues which currently limit the success of these experiments. The first issue that must be resolved in order to improve resolution and quantification is that of tip contamination. Collagen degradation products slough off from the fibrils during the course of digestion, and float into the solution, occasionally finding their way to the AFM probe tip. Their adsorption to the tip has the undesirable result of diminishing image quality. This problem is most clearly illustrated by Figure 5.4 f. and Figure 5.5 j which show a larger view of a region imaged previously, which is now visible as a square pattern. This pattern is a result of the probe tip sweeping the collagenolysis products out of the previously imaged (square) area. While large structures can still be resolved, the added material on the tip further reduces the resolution which could be achieved. In these experiments, for example, the characteristic banding patterns of collagen become very difficult to resolve after the initial addition of collagenase, but it is unclear whether this disappearance of the banding is an essential part of the collagenase digestion process and thus provides a clue to the mechanism of enzyme action, or that the decreased resolution is simply because of a contaminated tip. Possible approaches to alleviate this problem are chemical modifications of the tip, either with a gold coating followed by functionalization with a hydrophobic thiol (47), or by using electron beam deposited tips which tend to be naturally hydrophobic (48).

A second issue which must be addressed is the proclivity of Tapping Mode® imaging in fluid to yield imaging artifacts. Discussions in previous chapters have touched upon some imaging artifacts which occur with the AFM, such as tip convolution
effects. Unfortunately, Tapping Mode® imaging under fluid is particularly prone to imaging artifacts, and great care must be taken with both setting instrumental parameters throughout the imaging process and also during data analysis so as not to make erroneous conclusions based upon a faulty image. An image artifact which appeared on several occasions during these experiments is made readily apparent by closer inspection of Figure 5.5.

Careful scrutiny of the images in Figure 5.5 a-j. and a closer view shown in Figure 5.7 reveals an unusual distortion of the collagen fibrils. The edges of the fibrils appear much darker than the mica substrate. This indicates that the edges of the fibrils apparently lie beneath the solid substrate itself – clearly a nonsensical situation. This edge-inversion is an artificial effect, likely brought on by a failure of the tip to come into contact with the fibril, and represents a significant problem in a data set. The inverted edges render all height data which might be collected from an experiment practically useless. Further illustration of this effect is shown in cross-section form, in Figure 5.8 a,b. Figure 5.8 a shows a sectional-analysis of fibrils which show an edge-inversion artifact. Fibrils appear as sharp spikes which penetrate below the apparent substrate surface. Clearly, making measurements such as fibril diameters from these images is problematic at best. Because of this reason, the data in Figure 5.5 a-j were not used to extract collagenolysis rate information. These results are compared with an image in which no edge-inversion effect was present, shown in cross-section in Figure 5.8 (taken from Figure 5.4). In this case, the fibrils appear as elevated regions above a flat mica substrate, as they should, allowing for accurate measurement of fibril diameters.
Avoiding this edge inversion effect is not always trivial. Careful selection of the cantilever oscillation amplitude and the applied force between tip and sample can eliminate the effect, but for these experiments, one does not have a lot of range in these parameters. For example, exceeding a certain tip oscillation amplitude causes the tip to disengage from the surface, but not having sufficiently large value can give rise to edge inversion. Furthermore, clogging of the tip with degradation products can make the problem worse.

The investigations described here are initial explorations into the possibility of using the AFM to extract kinetic data on collagenase activity by quantification of the real-time images. The initial results are promising, but in order to obtain rate constants, the experiments need to be refined in several ways. One needs to perform studies at different concentrations and temperatures, which would require improvements of the apparatus for these measurements. The use of a higher purity enzyme would make for a better defined system. Finally, the resolution should be improved by addressing the issue of tip contamination, and fibril immobilization.

5.5 Summary

Type I FLS collagen fibrils with ~250nm banding were prepared, and subsequently exposed to a *C. histolyticum* collagenase mixture while being observed in real time with atomic force microscopy. Imaging conditions that allowed successful imaging of fibrils loosely held to the substrate, while simultaneously maintaining the activity of the collagenase were determined. Through careful selection of collagenase concentrations, the rate of fibril digestion was selected such that the process could be monitored in real time with the AFM. Upon exposure to collagenase, it was observed that the collagen
fibrils decrease both in diameter and length as a function of time, with no obvious preferential degradation occurring at the tip ends. There appeared to be no general tendency for the fibrils to cleave into multiple segments. These observations are consistent with proposed mechanisms of collagenase action. Measurement of changes in the fibril dimensions enabled an estimate of the rate of collagen breakdown, showing the possibility of application of the AFM for kinetic studies. However, the image resolution and quantification has been limited by the tendency of collagenolysis degradation products to adhere to the substrate surface and the probe tip. Modified probe tips would help to control this non-specific binding to ultimately allow the investigation of collagen digestion mechanisms at a molecular level.
5.5 References


Figure 5.1: AFM amplitude mode images showing mixed FLS and native-type collagen fibrils imaged using 'Tapping Mode' imaging under buffer (10 mM Tris-HCl, pH 7.4).

a) Image size: 7 x 7 μm
Figure 5.1: AFM amplitude mode image of mixed FLS and native-type collagen fibrils.

b) Image size: 12.5 x 12.5 μm

c) Image size: 3 x 3 μm
Figure 5.1: AFM amplitude mode images of mixed FLS/native-type collagen fibrils imaged under fluid.
d) 12 x 12 μm
e) 2.5 x 2.5 μm
Figure 5.2: Sectional analysis of an FLS fibril imaged under fluid
a) longitudinal section (periodicity of fibrils as indicated on figure)
b) cross section (diameter indicated on figure)
Figure 5.3:
a) AFM amplitude mode image of native-type collagen fibril imaged under fluid (Image size 5 x 5 µm). Note, the banding can be easily distinguished, visually, but the longitudinal section (b), does not appear particularly periodic. Fibril compliance may be playing a significant role in this, giving rise to partially distorted measurements.
Figure 5.4: AFM amplitude mode images showing affect of collagenase upon collagen fibrils. The AFM tip was withdrawn between images in order to minimize possible contamination by degradation products. Arrow points to a reference fibril upon which diameter measurements were made. All images are 15 x 15 μm in size.

a) Time = 0 minutes (prior to addition of collagenase) (Ref. Fib. Diameter = 27.3 ± 2.2 nm)
b) Time = 5 minutes of digestion with collagenase (Ref. Fib. Diameter = 26.3 ± 1.3 nm)
Figure 5.4:

c) Time = 10 minutes (Ref. Fib. Diameter = 12.0 ± 1.0 nm)

d) Time = 15 minutes (Ref. Fib. Diameter = 8.3 ± 0.5 nm)
Figure 5.4:

e) $T = 20$ minutes (Ref. Fib. Diameter = $2.4 \pm 0.5$ nm)

f) AFM amplitude mode images of collagen fibrils after partial digestion with collagenase. Earlier imaging has swept degradation products into a square pattern, as is apparent at the centre of the image. (Image size: $50 \times 50 \mu m$)
Figure 5.5: Height mode images of collagen fibrils being digested by collagenase. The AFM tip was scanned continuously over the fibrils throughout the digestion period. Image size is 20 x 20 μm.

a) Prior to addition of collagenase
b) Initial addition of collagenase (T = 0 - 4 mins)
c) $T = 4 - 7$ minutes

d) $T = 7 - 10$ minutes
e) \( T = 10 - 13 \) minutes

f) \( T = 14 - 17 \) minutes
g) $T = 17 - 20$ minutes
h) $T = 20 - 23$ minutes
Figure 5.5:
i) $T = 23 - 25$ minutes
j) Height mode image showing the effect of imaging on collagen degradation products. The square region in the centre of this $50 \times 50 \, \mu m$ was the region in images a)-i) which was continuously scanned. The square is $\sim 20 \times 20 \, \mu m$ in size, corresponding to the scan size of the previous images.
**Figure 5.6:** Schematic illustration of proposed mechanism of action of collagenase on collagen fibrils. Collagenase appears to attack collagen fibril in a non-specific manner, degrading the fibril along its entire length, with no particular tendency to cut the fibril into multiple fragments. Degradation products slough off into solution, where they can interact with the surface, and potentially impede the imaging process.
Figure 5.7: Height mode image of collagen fibrils imaged prior to addition of collagenase. Note that the edges of the fibrils appear dark in comparison with the substrate, indicating that the edges are lower than the substrate itself. This is an example of an edge inversion imaging artifact. Image size: 7.5 x 7.5 μm
Figure 5.8: Cross-sectional analyses of collagen fibrils imaged under fluid. Section a) shows a common imaging artifact encountered in these experiments, that of edge inversion. Fibrils showing this effect appear to have edges which are lower in height than the substrate itself, making measurements of their diameters impossible. Careful adjustment of parameters such as the amplitude of oscillation and the applied force can remove this effect, however, one only has a limited ability to control these parameters: drastic changes will tend to cause the tip to disengage the surface entirely. Section b) shows a partially digested fibril in which the imaging parameters could be adjusted successfully. Note, the fibril appears as an elevated object, with no evidence of edge inversion.
Chapter 6: Summary of Thesis

Research into understanding the nature of soft, biological materials is a challenging endeavor that truly lies at the frontier of modern research in physical chemistry. One of the most exciting aspects of some of these materials is their ability to undergo ordered aggregation, which has been defined as being the formation of a highly ordered entities from an initially random mixture of precursor units. From a fundamental point of view, systems of this nature are complicated because of the sheer size of the molecules involved and, consequently, because of the huge number of interactions taking place within and between these molecules. Despite the complex nature of these soft materials, however, the works presented here show that these systems can be studied and understood by applying standard methods of physical chemistry.

In this thesis, the Type I collagen system has been investigated with a view towards achieving a better understanding of collagen's tendency to form highly organized aggregates. The ultimate goal of these studies has been to gain a better understanding of the mechanisms by which collagen aggregates form. That collagen is a complex system to study is beyond reproach; however, insight into the problem of collagen aggregate formation mechanisms can and has been attained through application of physical chemical approaches such as characterizing aggregate structures and by investigating chemical kinetics as well as the thermodynamics of the processes.

In an effort to further understand the mechanisms of ordered aggregation in collagen, two variants of Type I collagen aggregates, the so-called segmental long spacing (SLS) aggregates and the fibrous long spacing fibrils (FLS), have been studied.
In the former case, SLS collagen aggregates are produced via a reaction between collagen monomers and nucleotide triphosphates, typically adenosine triphosphate (ATP). The reaction between these two components results in the formation of a block-like aggregate, which consists of laterally aligned collagen molecules. It has been shown here that SLS aggregates incorporate the ATP into their structure, with the ATP likely acting as a bridging unit between aligned collagen molecules. A combination of techniques, including atomic force microscopy, light scattering and isothermal titration calorimetry have been applied towards understanding the mechanism of formation of these aggregates. Each of these methods has indicated that SLS forms via a step-wise or hierarchical growth mechanism, through the formation of stable intermediates. AFM measurements show that growth occurs by lateral merging of oligomers to form the final structures; light scattering measurements show an inverse temperature dependence in the kinetics of the process, a hallmark of step-wise growth; and ITC also indicates a multiple-step process upon addition of ATP to collagen, which is in excellent agreement with the light scattering results.

The case of FLS collagen fibril assembly appears more complicated than that of SLS, mainly because the structure of the final aggregate is less well understood. Efforts have been made to further characterize the fibril structure using the AFM, and much progress has been made. The previously postulated model for FLS fibril structure has been brought into question by the results obtained in this thesis. Instead of monomers aligned in a staggered pattern, FLS is now believed to consist of aligned protofibrils, entities that are stable intermediates in the assembly of the fibril. Additionally, the results collected have cast doubt upon earlier interpretations of electron microscopy data.
prompting suggestions to re-visit the previously accepted model of native-type collagen fibril structure. On the strength of AFM evidence, it was also proposed that the fibrils grow in a hierarchical fashion, with the protofibril playing a crucial role in the assembly. The first ever model for the postulated growth mechanism of FLS was presented on the basis of these results.

In the final part of this thesis, the capabilities of the AFM, an increasingly important tool in soft material research, to perform imaging of dynamic, biological processes in real-time was explored. The basic requirements for applying the AFM to real-time imaging have been discussed, with particular emphasis being placed upon the importance of solvent conditions for successful experimentation. As an illustration of these capabilities, and to demonstrate the current limitations of the technique, the digestion of collagen fibrils by the collagenase of *Clostridium histolyticum* was investigated. It was observed that the digestion of fibrils occurred non-preferentially along their entire length, in keeping with the proposed mechanism of *C. histolyticum* collagenase activity. The potential of the technique for performing real-time kinetic measurements was discussed, along with limitations and possible improvements to the experimental method devised.

In conclusion, these investigations have provided much insight into the nature of collagen assembly, and the use of the AFM to survey properties of such systems in a dynamic fashion. A number of future works suggest themselves from these studies. For the case of SLS, further modeling and fitting of the kinetic data might prove useful if a more accurate expression for the average particle structure factor can be attained. For FLS structure characterization, further attention should be paid to the comparison of EM
and AFM data, as the AFM results have called into question the accepted FLS structure, as well as bringing to light some new interpretations of EM staining patterns. These results may have ramifications for the structure of native-type fibrils and should be carefully explored by the collagen community at large. For the assembly of FLS, the fundamental question of the role of $\alpha_1$-glycoprotein remains unanswered. One hopes that application of near-field techniques might resolve this question in the near future. Finally, a method which holds much promise, AFM imaging in fluid, has proven to be extremely valuable and will continue to do so for the foreseeable future. An important consideration in this field, which should be studied in more detail, is that of making biomolecules adsorb stably to a surface. If a method to either predict a priori the required conditions for sticking molecules to a substrate, or a surface with “tunable” properties can be developed, then this field would benefit greatly.
Appendix A: Light-scattering apparatus control software

DECLARE SUB graphset (xmin!, xmax!, ymin!, ymax!)
REM *****************************************************************************
REM * GPIB CONTROLLER SOFTWARE FOR INTERFACE BETWEEN *
REM * TEKTRONIX TDS-210 OSCILLOSCOPE AND IBM-PC *
REM *-----------------------------------------------------------------------------*
REM *
REM * USED TO OPERATE LIGHT-SCATTERING APPARATUS *
REM *-----------------------------------------------------------------------------*
REM *
REM *
REM *
REM *
REM *
REM *
REM *
REM *
REM *
REM *
REM *
REM *
REM *
REM *
REM *****************************************************************************
REM IN QBASIC HIT <F2> TO SELECT WHICH SUBROUTINE TO VIEW

DECLARE SUB gpibOut (device%. output$)
DECLARE SUB sampleData (now%, pointsToAverage%, scope%, filename$)
DECLARE SUB extractSeconds (secondsSinceMidnight%)

COMMON SHARED ByteCount%, pointsToAverage%

OPTION BASE 1

CLS

' Set some universal variables

' This is the number of data points
pointsToAverage% = 2500

' Calculate the number of bytes to be read in
ByteCount% = pointsToAverage% * 2

' Set the number of time units we want to observe
maxtime = 32000
REM << ACTIVATE TDS 210 TEKTRONIX OSCILLOSCOPE >>
CALL ibfind("TDS210", scope%)

REM << SET DESIRED DATA FORMAT >>

PRINT TAB(16): "<<< INITIALISING SCOPE - JUST A MOMENT >>>"

CALL ibwrn(scope%, "*cls")

REM TO PRINT THINGS FROM SCOPE TO A STRING VARIABLE IT IS
REM NECESSARY TO ZERO THE STRING VARIABLE WITH A SET OF BLANK
REM CHARACTERS - I DON'T KNOW WHY. YOU JUST HAVE TO DO IT

preamble$ = " "

FOR count = 1 TO 550
    preamble$ = preamble$ + " "
NEXT count

REM << SET DATA TYPE TO BE SIGNED BINARY >>

dcommand$ = "DAT:ENC RIB"

CALL gpibOut(scope%, dcommand$)

REM << SET DATA TO BE TWO BYTES IN LENGTH >>
REM << VALUES RANGE FROM +/- 32767 >>

dcommand$ = "DAT:WID 2"

CALL gpibOut(scope%, dcommand$)

REM << SAMPLE DATA FROM STORED WAVEFORMS STARTING AT 500TH POINT >>
REM THIS WAS LARGELY AN ARBITRARY DECISION
REM IT CAN BE ALTERED TO FIT YOUR CONVENIENCE. HOWEVER
REM THE TEKTRONIX DIVIDES EACH WAVEFORM INTO 2500 POINTS
REM SO THE MINIMUM START VALUE IS 1 AND THE MAX STOP IS 2500

dcommand$ = "DAT:STAR 0"

CALL gpibOut(scope%, dcommand$)
REM << SAMPLE DATA ENDS AT 2500TH POINT >>
REM   SEE PREVIOUS STATEMENT

dcommand$ = "DAT:STOP 2500"
CALL gpibOut(scope%, dcommand$)

REM << CONFIRM CHANGES BY CHECKING WAVEFORM PREAMBLE >>

REM AGAIN, NOT NEEDED BUT NOT A BAD IDEA TO
REM CONFIRM THAT APPROPRIATE CHANGES HAVE BEEN
REM MADE - CURRENTLY UNACTIVE - REMOVE THE '
REM IN FRONT OF PRINT STATEMENTS TO DISPLAY RESULTS

dcommand$ = "WFMPR?"
CALL gpibOut(scope%, dcommand$)

CALL ibrd(scope%, preamble$)
PRINT
PRINT TAB(24); "<< INITIALISATION COMPLETE >>"

PRINT
' PRINT TAB(20); "WAVEFORM PREAMBLE:"
' PRINT
' PRINT preamble$
' PRINT LEN(preamble$)
preamble$ = " "

'DO: LOOP WHILE INKEY$ = ""

REM << INITIALISATION STAGES COMPLETE - READY TO COLLECT DATA >>

REM << MAIN PROGRAM BODY >>

PRINT
INPUT "ENTER FILENAME ": filename$

'PRINT

'5 INPUT "ENTER DURATION OF RUN IN HOURS (Max. 24h) ": duration%

'IF duration% > 24 THEN

' PRINT : PRINT "INVALID TIME ENTERED 

'PRINT

'FOR PAUSE = 1 TO 30000

' FOR PAUSE2 = 1 TO 15

' NEXT PAUSE2

' NEXT PAUSE

'CLS

'GOTO 5

'END IF

'maxtime = duration% * 360

INPUT "HOW MANY SECONDS BETWEEN DATA POINTS ": period%

PRINT

PRINT TAB(20): "<< Hit Any Key to Continue >>"

DO: LOOP WHILE INKEY$ = ""

CLS

' REM << PRINT SCREEN HEADERS >>

' PRINT TAB(20): "TIME". "PMT SIGNAL". "PHOTODIODE"
'PRINT TAB(20): "-----". "-------------". "--------------".

'Set up the graphics screen

xmin = 0: xmax = maxtime
ymin = -32768: ymax = 32768
CALL graphset(xmin, xmax, ymin, ymax)

REM << SET UP TIMING MECHANISM >>

'set time to midnight'
TIMES$ = "00:00:00"

'make sure to sample first time through loop
previousSampleTime% = -period%

'main loop
WHILE continue$ <> "s"
   continue$ = INKEY$
   CALL extractSeconds(currentTime%)
   IF currentTime% - previousSampleTime% >= period% THEN
      previousSampleTime% = currentTime%
      CALL sampleData(currentTime%, pointsToAverage%, scope%, filename$)
   END IF
WEND

REM << SUBROUTINE EXTRACT SECONDS >>
REM
REM THE PURPOSE OF THIS SUBROUTINE IS TO DETERMINE
REM THE NUMBER OF MINUTES WHICH HAVE PASSED SINCE THE
REM DATA WAS LAST SAMPLED: THIS IS DONE BY CHECKING
REM THE COMPUTER'S INTERNAL CLOCK
REM
SUB extractSeconds (secondsSinceMidnight%)
   now$ = TIMES$
   hours% = VAL(LEFT$(now$, 2))
   minutes% = VAL(MID$(now$, 4, 2))
   seconds% = VAL(MID$(now$, 7, 2))
   secondsSinceMidnight% = 3600 * hours% + 60 * minutes% + seconds%
END SUB

REM << SUBROUTINE gpibOut >>
REM
REM THE PURPOSE OF THIS SUBROUTINE IS TO WRITE COMMANDS
REM TO THE GPIB CONTROLLER: THIS IS DONE THROUGH THE
REM IBWRIT COMMAND AND WRITING THE APPROPRIATE COMMAND
REM THROUGH THE VARIABLE OUTPUTS. VALID COMMANDS AND
REM THEIR SYNTAX ARE EXPLAINED IN THE MANUAL
REM 'TDS200 SERIES PROGRAMMER MANUAL'
REM
SUB gpibOut (device%, output$)

    CALL ibwrt(device%, output$)

END SUB

SUB graphset (xmin, xmax, ymin, ymax)

' This subroutine is intended to provide the initial settings for
' the subsequent graphical display of data.

SCREEN 12

' Place the name of the program in a noticable spot.

LOCATE 3. 5

PRINT " << LIGHT SCATTERING DATA COLLECTION PROGRAM : V1.1 >>" 

PRINT " ( Modified for SLS Data Collection )"

' VIEW (55. 50)-(585. 430). 5. 8
VIEW (100. 100)-(585. 400). 1

' Set the chart recorder's view-window.

WINDOW (xmin, ymin)-(xmax, ymax)

' Draw a box around the window screen

LINE (xmin, ymax)-(xmax, ymax). 8. B
' Set tick marks

xrange = xmax - xmin
yrange = ymax - ymin

xtickstep = xrange / 4
ytickstep = yrange / 4

ystep = yrange / 50
xstep = xrange / 50

ypos = ymin + ystep
xpos = xmin + xstep

xtickpos = xmin
ytickpos = ymin

FOR i% = 0 TO 4
    xtickpos = xtickpos + xtickstep
    PSET (xtickpos. ymin)
    LINE -(xtickpos. ypos)

    ytickpos = ytickpos + ytickstep
    PSET (xmin. ytickpos)
    LINE -(xpos. ytickpos)
NEXT i%

END SUB

REM << SUBROUTINE sampleData >>
REM
REM THE HEART OF THE PROGRAM - TAKES DATA FROM THE
REM OSCILLOSCOPE AND SENDS IT TO THE COMPUTER.
REM THE COMPUTER STORES THE DATA IN THE ARRAY CALLED
REM SPECTRUM%() AND PERFORMS AN AVERAGING CALCULATION
REM UPON IT.
REM
SUB sampleData (now%. pointsToAverage%. scope%. filename$)

    DIM spectrum%(32767). spectrum2%(32767)

FOR x = 1 TO 32767
    spectrum%(x) = 0
spectrum2%(x) = 0
NEXT x
sumone = 0
sumtwo = 0

REM << SET DATA SOURCE TO BE CHANNEL ONE >>
dcommand$ = "DAT:SOURCE CH1"
CALL gpibOut(scope%, dcommand$)

REM << SEND DATA FROM SCOPE TO COMPUTER >>
dcommand$ = "CURV?"
CALL gpibOut(scope%, dcommand$)

REM << READ PMT DATA INTO ARRAY CALLED SPECTRUM% >>
' This is where we read in what we assume is a 12 byte header
' which the CURVE? query adds to the beginning of the data.
CALL ibrdi(scope%, spectrum%(). 12)

' Read in the data
CALL ibrdi(scope%, spectrum%(). ByteCount%)
average! = 0

REM << SET DATA SOURCE TO BE CHANNEL TWO >>
dcommand$ = "DAT:SOURCE CH2"
CALL gpibOut(scope%, dcommand$)
REM << SEND DATA FROM SCOPE TO COMPUTER >>

dcommand$ = "CURV?"

CALL gpibOut(scope%. dcommand$)

REM << READ PHOTODIODE DATA INTO ARRAY CALLED SPECTRUM2% >>

' This is where we read in what we assume is a 12 byte header
' which the CURVE? query adds to the beginning of the data.

CALL ibrdi(scope%. spectrum2%(). 12)

' Read in the data

CALL ibrdi(scope%. spectrum2%(). ByteCount%)

average2! = 0

REM << CALCULATE AVERAGE VALUE >>

FOR counter = 1 TO pointsToAverage%

    sumone = sumone + spectrum%(counter)

    sumtwo = sumtwo + spectrum2%(counter)

NEXT

average! = -(sumone / pointsToAverage%)

average2! = -(sumtwo / pointsToAverage%)

' Display the data point.

IF now% = 0 THEN

    PSET (now%. average!)

ELSE

    LINE -(now%. average!)

END IF
REM << SAVE DATA TO FILE AND DISPLAY RESULTS >>

OPEN filename$ FOR APPEND AS #1
    PRINT #1. now%. average!.. average2!
CLOSE
LOCATE 27. 32
PRINT "Time(s)". " PMT". " PD"

LOCATE 28. 32
PRINT now%. average!.. average2!

END SUB
Appendix B: Program "Kin.cpp"

/*Jacek Strzelczyk
July 9, 1998
Simulation of assembly kinetics through iterated function system*
/*output is of the form:

[time] [total polymer mass] [total polymer number] [number of monomers] [number of dimers] [number of tetramers] [number hexamers]...

#include <stdio.h>
#include <conio.h>
#include <math.h>
#include <stdlib.h>
#include <io.h>

#define DELTAT 0.001 //time increment
#define kn 0.000001 //backward nucleation rate constant
#define KN 0.000001 //forward nucleation rate constant
#define K 0.1 //forward growth rate constant
#define k 0.01 //backward growth rate constant
#define IMAX 200 //half the maximum polymer size
#define SKIP 1000 //number of data points to skip
#define NOCHANGE 0.0000001
*====================================================================

void main(void)
{
  double b[2], a[IMAX][2], t=0, perdiff, pmass=0, pnum=0, trial;
  int i, counter=0;
  FILE *fptr;

c1rscr();
printf("Program Running....");
/*opens output file*/
fptr=fopen("output.txt", "w");
/*initializes array, and writes to file*/
b[0]=1;
fprintf(fptr, "%f %f %f ", t, pmass, pnum);

for(i=0; i<IMAX; i++)
{
  a[i][0]=0;
  //fprintf(fptr, " %f", a[i][0]);
}
do
{
  counter++;  
  /*calculates the new concentrations*/
  b[1]=b[0]+DELTAT*(2*kn*a[0][0]-2*KN*b[0]*b[0]);
  for(i=1; i<IMAX-1; i++)
    a[i][1]=a[i][0]+DELTAT*(k*a[i-1][0]*a[0][0]-
  k*a[i][0]-K*a[i][0]*a[0][0]+k*a[i+1][0]);
  a[IMAX-1][1]=a[IMAX-1][0]+DELTAT*(K*a[IMAX-1-1][0]*a[0][0]-k*a[IMAX-1][0]);
  a[0][1]=(double)1/2-b[1]/2;
  for(i=1; i<IMAX; i++)
    a[0][1]=a[0][1]-a[i][1]*(double)(i+1);
  /*calculates the total mass and number of polymers*/
  pmass=0;
  pnum=0;
  for(i=0; i<IMAX; i++)
  {
    pmass=pmass+(double)2*(i+1)*a[i][1];
    pnum=pnum+a[i][1];
  }
  /*increases time*/
  t=t+DELTAT;
  gotoxy(1, 3);
  printf("%2.3f %2.4f %2.4f", t, pmass, pnum);
  if(fabs(1-pmass-b[1])>0.01)
  {
    printf("Mass not conserved");
    getch();
  }
  /*writes time and polymer mass and number to file*/
  if(counter===SKIP)
  {
    fprintf(fptr, "\n%f %f %f", t, pmass, pnum);
    counter=0;
  }
  /*writes the species distribution to file, and
calculates the largest percentage change*/
  //fprintf(fptr, "%f", b[1]);
  if(b[0]!=0)
    perdiff=fabs(b[1]-b[0])/b[0];
  else if(b[1]==0)
    perdiff=0;
  else
    perdiff=NOCHANGE*2;
  if(b[1]<0)
printf("ALARM! Negative concentration on B.");
getche();
exit(EXIT_FAILURE);
}

b[0]=b[1];
for(i=0; i<IMAX; i++)
{
    if(a[i][0]!=0)
        trial=fabs(a[i][1]-a[i][0])/a[i][0];
    else if(a[i][1]==0)
        trial=0;
    else
        trial=NOCHANGE+2;
    if(trial>perdiff)
        perdiff=trial;
    //fprintf(fptr, "$ %f", a[i][1]);
    if(a[i][1]<0)
    {
        printf("ALARM! Negative concentration on A%d.", i+1);              
        getche();
        exit(EXIT_FAILURE);
    }
    a[i][0]=a[i][1];
}

while(perdiff>NOCHANGE);
printf("\nDONE.");
fclose(fptr);
getche();