COLLAGEN ASSEMBLY AS EXAMINED BY ATOMIC FORCE MICROSCOPY

by

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ABSTRACT

The use of atomic force microscopy (AFM) to image the assembly of Type I collagen from monomers, through the various intermediates, to mature fibrils, has been demonstrated for the first time. These stages of collagen assembly were found to proceed by a stepwise process, which commenced with axially associated oligomers (height ~1 nm, lengths 450-1200 nm) and proceeded by lateral and axial association through intermediate microfibrils of 2-3 nm in diameter (with periodicity ~67 nm). Further assembly into larger microfibrils (>10,000 nm in length) that aggregated readily was observed. The end product of assembly observed in this study was a mature fibril, which demonstrated 4D-banding with a periodicity of 67 nm.
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1 Introduction

1.1 Collagen

Collagen is a structural protein which is the principal component of many types of connective tissues. There are at least 11 different types of collagen, the most commonly occurring and well-characterized is Type I. Type I collagen is assembled into fibrils which compose the connective tissue of skin and tendons.

Fibrils are formed by interactions of collagen monomers which form a regular, periodic structure. The monomers are right-handed triple helices composed of three polypeptide chains. Since each of the peptide chains is actually a left-handed helix, the triple helix is referred to as a "coiled coil" or a supercoil. The molecular weight of a monomer is \( \sim 300,000 \). The monomers are a processed polypeptide and will spontaneously assemble into fibrils under physiological pH and temperature. The unprocessed precursor, the procollagen molecule, is represented in figure 1.1. Removal of the nonhelical extensions on both the N and the C termini of the molecule is crucial to fibril formation. From the available x-ray scattering data Rich and Crick (1961) and Ramachandran and Kartha (1967) devised two different structures for the collagen molecule. The basic difference in the models is the number of interchain hydrogen bonds. Because of the supercoiled structure of collagen monomers, the sequence constraints on collagen chains are strict. Every third amino acid must be a glycine, producing a regular pattern of triplets (Gly-X-Y) where X or Y is often proline or hydroxyproline.

Type I collagen fibril structure is well characterized, with the monomers overlapping in integral numbers of D units (1 D=67 nm) to produce the characteristic banding pattern as shown
Figure 1.1 The procollagen molecule. Arrows indicate cleavage sites of procollagen aminoprotease (N-terminus) and carboxyprotease (C-terminus). After cleavage of the non-helical ends, the 300 nm collagen molecule results.
in figure 1.2. It is not understood how this periodic pattern is built up nor the nature of the early intermediates.

Collagen in tissues is associated with a variety of other elements of the extracellular matrix. Depending on the tissue type, different diameter fibrils will be formed (this has been shown to depend partly on which other matrix components are present) and organization of the fibrils with respect to each other will vary. For example tendon, which has a strong, slightly elastic character, contains a very high percentage of collagen in large fibrils which are highly ordered bundles. In the case of softer tissues (i.e. skin) where collagen provides a structural framework, smaller fibrils are organized into a lattice.

The importance of understanding collagen assembly is underscored by the prevalence of inherited collagen disorders such as osteogenesis imperfecta (affecting bone development), Marfan's syndrome and Ehlers-Danlos syndrome (both causing vascular abnormalities).
(Yamauchi and Mecanic in Nimni, 1988, p. 158)

1.2 Fibrillogenesis in vivo

Collagen assembly in vivo takes place in specialized cells. Formation of collagen fibrils in vivo involves at least 10 distinct intracellular stages, followed by extracellular events that complete fibril formation. Inside the cell, fibrillogenesis begins with transcription of DNA into mRNA in the nucleus. The collagen gene is the most complex gene isolated thus far with many introns of 54 base pairs interspersed between exons. The mRNA then exits the nucleus and is translated into collagen protein in the endoplasmic reticulum. Translation is followed by two types of
Figure 1.2 Monomer organization in fibrils produces periodicity of 67 nm. 1-D overlap of collagen molecules (300 nm long=4.4 D) results in gap regions which contain less collagen than regions consisting solely of overlapped molecules.
post-translational modification, hydroxylation (of lysine and proline) and glycosylation.

Next the N-terminal signal peptide is removed. The purpose of the N-terminal signal peptide is postulated to be guidance of the peptide to the ribosomes. The completed peptide chains are then released from ribosomes and folding of the chains together occurs. The C-terminal extensions are critical to the correct alignment of the chains. As the chains align, there is formation of disulphide bonds between cysteine residues on adjacent chains. This is followed by formation of the triple helix, which is thought to be a spontaneous, entropy driven process catalyzed by the forming disulphide bonds. The procollagen molecules are then packed into vesicles in the Golgi apparatus. In the Golgi apparatus, the molecules are often observed to align laterally to form structures known as SLS (segment long spacing) crystallites and end overlapped SLS crystallites. The vesicles are then transported to the cell membrane where fusion occurs. Extrusion of the molecule into the extracellular matrix is then achieved.

Extrusion is followed by immediate removal of the C-terminus non-helical extensions and part of the N-terminal extensions. Initially fibrils of 20-40 nm are formed which still contain part of the N-termini. N-terminal extensions have not been observed in quarter-staggered mature fibrils (diameter >80 nm). Thus, the N-terminus is thought to modulate fibril growth. Monomeric collagen has not been observed outside the cell, but undergoes immediate assembly at the cell membrane. (Nimni and Harkness in Nimni, 1988 p. 7-15; Hulmes, 1992)

1.3 Fibrillogenesis in vitro

Turbidimetry studies show that collagen assembly proceeds in vitro in a three stage process: the
lag phase, the growth phase and the stable gel phase. In the lag phase no change in turbidity measurements is observed. The growth phase is characterized by a rapid accumulation of filaments. In the stable gel phase the rate of fibrillogenesis levels off. Collagen assembly is a complex growth process involving both fibril elongation and lateral growth. Another factor which complicates studies of collagen assembly is that the types of intermediates observed and the assembly pathway depend on experimental conditions. This was demonstrated in a study by Holmes et al. (1986) in which a collagen solution at low pH (3.5) and low temperature (4°C) was taken to assembly conditions (pH 6.8; temperature 34°C) by various pathways. If the pH was changed prior to the temperature, loose, non-banded filaments were observed as intermediates. Conversely, if the temperature was changed before the pH, banded filaments were observed as intermediates. The reasons for these differences in assembly and their dependence on pathway have still not been explained.

Characterizing collagen structure and assembly into fibrils has been a goal of researchers for over forty years. In the 1950's collagen was imaged by electron microscopy and the banding of the fibrils observed for the first time. Within the next ten years, the triple helical structure of the collagen molecule was developed and the quarter stagger array of molecules in native fibrils was proposed (a summary of early work is given in Kuhn, 1987). One question which arose from studies of collagen assembly was: is assembly a process of nucleation and growth or is it a stepwise addition of intermediate building blocks? The mode of assembly is still not well understood, partly because of contradictory findings. Type I collagen fibrillogenesis has been studied by a wide variety of techniques including: turbidimetry (MacBeath et al., 1993), dynamic light scattering (Silver et al., 1979) (Fletcher, 1976), electric birefringence (Brokaw et
al., 1985) (Bemengo et al., 1983), x-ray crystallography (Eikenberry and Brodsky, 1980), electron microscopy (EM) (Kobayashi et al., 1985) (Gelman et al., 1979), x-ray scattering (Leikin et al., 1994) and dark field light microscopy (Kadler et al., 1990) (Notbohm et al., 1993). Collagen assembly has also been studied from a theoretical perspective (Silver et al., 1992) (Wallace, 1992).

Part of the difficulty in studying collagen assembly is the isolation and characterization of intermediates, especially in the early stages. Accurate detection of the early intermediates is made difficult by their very small quantities and small size. Also, some methods are problematic for studying early intermediates. For example, dynamic light scattering and electric birefringence rely on assumptions about the geometric shape of the body being studied when, in the case of collagen, this is the information that is being sought. Another factor which complicates interpretation of collagen work is that there is no standard assembly pathway used for in vitro collagen studies. Hence, only studies using the same assembly method can truly be compared with each other. Since assembly depends on the pathway used to generate the fibrils, this may explain the conflicting results obtained in some studies.

Some studies have attempted to characterize assembly in vivo, others in vitro. In vitro methods are more commonly employed since variability of conditions allows exploration of the relative importance of various factors in determining assembly. Care must be taken in extrapolating in vitro studies to in vivo assembly since in vivo conditions are seldom created in in vitro work.
1.4 Techniques used to study collagen assembly

The methods used to study collagen fibrillogenesis are outlined below.

1.4.1 Turbidimetry

Turbidimetry is a technique which has been used to monitor collagen assembly for over forty years. Usually light (\(\lambda=313\) nm) is passed through a cuvet in which assembly is taking place and the change in absorbance is measured over time. This produces a graph for collagen assembly with the basic shape of figure 1.3a. Typically, turbidimetry curves demonstrate a lag phase, a rapid growth phase and a mature gel phase. The lag phase shows no apparent change in absorbance. In the rapid growth phase the slope of the curve becomes very steep. The mature gel phase is characterized by the absorbance reaching a plateau value. The values which characterize turbidity curves are \(\delta h\) (the total turbidity change) and \(\delta h/2\) (sometimes called \(t_{1/2}\)), the time to reach half the maximum absorbance.

Turbidity is defined as optical density (OD), since it had been shown by earlier studies (Wood and Keech, 1960; Gross and Kirk, 1958) that OD is proportional to the amount of sedimentable product. Optical density is the degree of solution opacity and is expressed by log \(I_o/I\), where \(I_o\) is the incident light intensity and \(I\) is the transmitted light intensity.

One study demonstrated that collagen, like tubulin, has a "thermal memory" if permanent aggregation resulting from cross-linking is repressed (figure 1.3b). When temperature is rapidly dropped on the formed gel, dissolution of the gel results. When the temperature is again raised to assembly conditions the lag phase is shortened; this is what is referred to as "thermal memory". (Helseth and Veis, 1981)
Figure 1.3 a) A typical turbidity curve obtained for collagen assembly (either O.D. (absorbance) vs. time or change in absorbance (in which case the curve starts at zero on the y-axis) vs. time is graphed. b) Turbidity curve illustrating thermal memory (lathyrinic collagen used). At arrows, temperature was reduced to starting temp. of 8 C (Helseth and Veis, 1981).
Some turbidity studies have examined the temperature dependence of assembly (Williams, 1978). Other studies have varied the concentrations of various extracellular components such as proteoglycans, TRAMP (tyrosine-rich acidic matrix protein) (MacBeath et al., 1993) or fibronectin (Brokaw et al., 1985). These extracellular components are known to associate with collagen fibers and are thought to determine the final diameter as well as extracellular organization of the fibrils.

A comprehensive turbidimetry study by Williams et al. (1978) demonstrated the effects of changing various parameters (ionic strength, phosphate concentration, pH, collagen concentration and temperature) on collagen assembly. Williams et al. found that if the temperature was decreased back to 4°C after assembly was complete and left overnight, the turbidity would not decrease more than 5%. By examining the slope of the growth phase curve, Williams et al. found the critical concentration for assembly to be <7μg/ml, possibly zero. From these results they concluded that turbidity measured growth by accretion rather than nucleation and growth. The growth process was suggested to be dominated by lateral association of microfibrils, whose formation in the lag phase was too small to be observed by turbidity.

Gelman et al. (1979) concluded, based on their turbidimetry results, that collagen assembly is a multistep process. As it was found that the time for fibrillogenesis to occur was inversely proportional to collagen concentration, this was interpreted to mean linear and lateral growth occur by accretion. The study tested the temperature dependence of the various steps of assembly by attempting to reverse the process at each stage by reducing the temperature. They suggested fibril formation in vitro has 3 steps: initiation, linear growth and lateral growth. The nature of the intermediates could not be determined in the initiation step. As this step appeared
to be temperature dependent, they suggested nucleation and growth as a possible mechanism. The linear growth stage showed temperature independence. The intermediates were determined to be long, thin non-banded filaments (2-4 nm in diameter). The lateral growth stage which was found to be temperature dependent. The assembly products at the lateral growth stage were native banded filaments. The critical concentration of collagen for assembly was found to be very small. Thus, it was suggested that fibrillogenesis is a non-nucleation and growth process.

In our study, turbidimetry was used to confirm each stage of assembly. This allowed correlation of AFM results with known stages of collagen assembly.

1.4.2 Light scattering and electric birefringence

Dynamic light scattering involves the measurement of the light scattered from the particles in a solution, in real time, through a range of angles. This technique permits determination of the set of translational diffusion coefficients \( D_i \) which characterize a solution. The coefficients are then coupled with hydrodynamic models to calculate the size of the macromolecule according to the model selected for the individual molecule shape. For collagen the experimental coefficients agree well with the theoretical predictions for a semiflexible rod model (Bernengo et al., 1983). The advantage of using this technique is that it does not perturb or destroy the solution (non-invasive).

Unfortunately the data from this sort of experiment are not easily interpreted. Each measurement of scattering involves signal integration which takes a certain time period. During the integration period, changes may take place in the solution and not be measured. It seems that
this could be at least partially resolved by slowing down the assembly through manipulation of
the experimental conditions (for example, lowering the temperature). Another difficulty is that a
single \( D_t \) from a solution that contains a mixture of species, does not represent a well-defined
average. Thus, in the presence of several species (sure to be the case in collagen assembly), a
deconvolution of a multiexponential correlation function is required. This deconvolution is
subject to controversy. A gradual decrease of \( D_t \) over the course of fibrillogenesis can be
interpreted as a change to less mobile, larger species. However, more specific results are
difficult to obtain because of difficulties with data analysis. (Veis and Payne in Nimni, p. 124-5)

Because of these problems, conflicting results have been obtained. Gelman et al. (1980)
found a rapid decrease in \( D_t \) during the lag phase from 7.8 to \( 1.5 \times 10^{-8} \) cm\(^2\)/sec. This correlates
well with a study by Silver et al. (1979) which found a decrease in \( D_t \) from 8.0 (monomer) to
\( 2.2 \times 10^{-8} \) cm\(^2\)/sec (interpreted as belonging to a lateral aggregate). This data yielded a molecular
weight of 930,000 at the end of the lag phase. However, scattering studies by Payne (1984) and
Payne et al. (1986) found no evidence for the existence of lag phase intermediates. They did not
detect any changes in solution parameters.

Electric birefringence is one of the most sensitive methods to determine changes in
macromolecular length since it yields rotary diffusion coefficients and relaxation times, which
are both related to the cube of the length.

A study by Bernengo et al. (1983) established that the birefringence did not decrease
until the lag phase was over. The relaxation time of molecules was found to be constant through
both the lag and growth phases. They found no evidence for intermediates even though dynamic
light scattering measurements were also performed. These measurements found no changes in
molecular weight.

1.4.3 Electron microscopy

Electron microscopy has been used to examine collagen since the 1940's. The resolution obtained with EM is far superior to that of optical microscopes and allowed observation of the banding of collagen fibrils (Hall et al., 1942) and led to the current understanding of monomer organization in fibrils. Unfortunately, EM requires extensive sample preparation and staining which can be destructive to biological structures. Recently, improved techniques for sample preparation, such as quick freezing, have been developed.

A study done by Mallein-Garin and Garrone (1990) examined in vivo collagen fibrillogenesis using quick-freezing and freeze-substitution techniques to prepare the samples for Transmission electron microscopy (TEM) study. In the chick embryo tendon samples studied, three types of intermediates were observed: 1) SLS-like aggregates surrounded by membrane containing areas with a clathrin coat, 2) fine, non cross-straited filaments connecting the cell membrane at one pole and extracellular collagen fibrils and, 3) tufts of filaments directly linked to collagen fibrils. The aggregates surrounded by membrane (1) reveal that collagen does organize to a degree within internal domains. Since fine filaments (2) were observed in Mallein-Garin and Garrone's work, this was interpreted as proof that in vivo collagen assembly proceeds by a multistep mechanism. In past work, usually only mature fibrils, with no smaller intermediates, were observed. The third category of intermediates were interpreted to be extracellular matrix components which had not been observed before by EM. Chemical fixation
of samples uses aqueous media. Thus, usually in conventional EM the associated proteoglycans and other components are washed away during sample preparation.

A Scanning transmission electron microscopy (STEM) study by Holmes et al. (1992) measured the mass present at different points along collagen fibrils, at various assembly times. The study concluded that: 1) tip shape of fibrils is not conical but paraboloid and 2) accretion, (rate of mass uptake per unit area) to maintain tip shape, cannot be the same everywhere on the tip, but must decrease as diameter increases.

1.4.4 Dark-field light microscopy

Dark-field microscopy provides better contrast than regular optical microscopy and thus facilitates the examination of structural changes in species such as collagen fibrils. When coupled with photography this technique allows observation of macroscopic fibril growth.

Kadler et al. (1990) employed dark-field light microscopy to study the growth of large fibrils in real time. They observed that growth of the fibrils was from pointed tips, usually at one, sometimes at both, ends of the fibril. Conditions were adjusted (temperature of 29°C) to produce very long, large diameter fibrils. Fibrils shown in the photographs were over 100 μm long by over 10 μm wide. The fibrils very quickly reached maximum diameter and then lengthened from the tips. This certainly does appear to be an example of nucleation and growth. However, using the technique of dark-field microscopy there was no way to ascertain how the fibril formed in the earlier stages as it was not visible until it was already very large. Also, the nature of the species adding on to the nucleus could not be ascertained due to limited resolution
of the microscope.

A theoretical model of this assembly, using computer simulation of a nucleation and growth process, was developed by Silver et al. (1992).

1.4.5 X-ray crystallography and scattering

Almost all collagen-related work in this field would actually be considered x-ray scattering since it has not been possible to crystallize collagen. In x-ray crystallography the diffraction pattern, and subsequently the molecular structure determination, is generated by passing x-rays through a single crystal. The diffraction pattern yields direct information on the repeating unit (unit cell) of the crystal. In tissue samples, only molecules which are well-ordered will diffract strongly. Disordered molecules will only produce background signal. Rat tail tendon is the best for x-ray studies as it contains ~ 90% collagen and the collagen molecules often exist in a 3-D crystalline arrangement in the tendon. It has also been found that collagen forms a liquid crystalline type arrangement where the degree of order of the molecules depends on the density of packing. Thus, Hulmes et al. (1985) found that there were regions, especially in the outer part of large fibrils, which were crystalline.

Diffraction information from collagenous tissues has been commonly used to suggest models, or refine already existing models, as opposed to direct determination of molecular structure. For example, once Cochran et al. (1952) developed a method for analyzing diffraction patterns of helical molecules, the x-ray data from collagen tissues was coupled with the
structural constraints imposed by the triplet amino acid sequence, to yield the initial models of collagen molecular structure (Rich and Crick, 1961; Ramachandran, 1967). X-ray studies are often coupled with EM, NMR or theoretical calculations in the case of collagen to yield significant results.

Data from x-ray studies of collagen are generally of three types: high angle, medium angle and low angle reflection data. High angle reflections arise from the triple helix and yield information about the rise per residue in the helix and the pitch of the helix. The triple helical part of the molecule is the only part studied as it is much more ordered than the non-helical extensions. Most x-ray work has focused on type I collagen. Medium angle reflections yield information on the molecular packing in fibrils (both axial and lateral). These studies have led to models of assembly and organization. For example, the work of Fraser et al. (1983) has led to two models: the quasihexagonal model and the distorted microfibril model. The microfibril model suggests that fibrils are composed of pentameric subunit microfibrils. Thus, the distorted microfibril model supports stepwise assembly as the mechanism for fibrillogenesis. Low angle reflections lead to information on tissue organization and fibril diameters. The binding sites of proteoglycans on collagen fibrils are periodic. This periodicity has enabled workers to discover the location, on the collagen molecule, of proteoglycan binding sites (Meck, 1985).

Bella et al. (1994) have recently performed the first single crystal x-ray diffraction study of collagen structure. A collagen-like peptide was synthesized, with a single amino acid substitution (Gly->Ala), crystallized and its diffraction pattern obtained. The Rich and Crick model II was confirmed as representing the true structure of collagen, since the x-ray structure demonstrated the pattern of interchain H-bonding predicted by the model. Also, the supercoiling
of the triple helix was confirmed. The amino acid substitution was chosen since Gly->X mutated collagens have been shown to be responsible for the inherited diseases osteogenesis imperfecta, chondrodysplasias and Ehlers-Danlos syndrome. This substitution resulted in only a local untwisting. A striking feature of the structure is that all residues in the peptide are exposed to the solvent. The triple helix is surrounded by a cylinder of hydration. This participation of the solvent in the triple helical structure is one important difference between this peptide and globular proteins. Bella et al. concluded that the 13 to 14 Å lateral spacing observed in assembled collagen fibrils is sequence independent, since it was observed in the crystal of sequence modified collagen as well. They suggested that the lateral spacing in collagen fibrils comes about as a result of the diameter of the cylinder of hydration.

1.5 Atomic Force Microscopy

Atomic force microscopy (AFM) is a technique developed by Binnig et al. (1986). A topographic image is produced by measuring sample height as a tip attached to a cantilever is raster-scanned over the sample. The tip deflection is recorded by measuring the angle of a laser beam reflected off the back of the tip. AFM is being used with increasing frequency to study biological systems because the technique has two advantages. Unlike EM, AFM requires very little sample preparation and hence the alteration of the specimen through preparative techniques is minimized. AFM also provides very high vertical resolution (< 1 Angstrom). In addition, AFM offers the option of imaging in a fluid medium in real time (Ratneshwar and John, 1994).


1.6 Atomic Force Microscopy of collagen

AFM has been used to confirm the periodicity present in mature fibrils, as well as to image monomers (E. Chernoff and D. Chernoff, 1992). A more rigorous study by AFM also confirmed two other types of periodicity inherent in mature fibrils. Forces exerted by the AFM tip on fibrils were measured both in air and in solution (Baselt et al., 1993). Recent work by Chernoff (as reported in the 1994 Denver conference of the American Vacuum Society) imaged collagen complexed with the proteoglycan decorin, which formed an ordered array on the surface of the collagen fibrils. Collagen monomers and dimers were also imaged and analyzed to determine the lengths and heights of these species. In addition, sub-bands were measured on the mature fibrils. Imaging was done using a Nanoscope III in tapping mode, under a He atmosphere. The assertion was made that collagen is too soft a substance to be imaged properly using force mode. However, the study by Baselt et al. found forces on the fibrils to produce negligible compression when imaged in air; under water the compression was found to be significant (5%).

Revenko et al. (1994) imaged mature native and self-assembled fibrils by AFM. Samples were taken from the same preparations and imaged by EM. The EM and AFM images were then compared. The periodicity of the fibrils was measured. Ridges indicative of subperiodicity were observed in the AFM images. However, ridges were not evident in the EM micrographs.

A cryogenic force microscopy (CFM) study by Shattuck et al. (1994) obtained high resolution images of collagen monomers. The mean length of the monomers by CFM was found to be 221nm (s=18 nm). This is considerably shorter than the values obtained from EM, x-ray
diffraction, and light scattering studies (290 nm) (von Hipple in Treatise on Collagen, 1967). The widths of the molecules were measured to be 4-10 nm, as opposed to the accepted value of 1.5 nm (von Hipple, 1967). Shattuck et al. observed bumps on the molecules almost always occurring within 25 nm of an end. Shattuck et al. also showed the tendency of monomers to associate with the mid-regions of other monomers. This study was done at a low temperature (143 K), under liquid isopentane, which may improve sample stability. However, it is possible that the extremely low temperature may also alter the collagen monomers. In this study, the imaging tip was produced by electron beam deposition, a technique which produces smaller, finer tips. These finer tips are capable of better resolution than the broader mass-manufactured tips, since the smaller tips are able to respond to smaller structures. Shattuck et al. found the width of a collagen monomer to be 4-10 nm, which is closer to the accepted value than the value of ~60 nm obtained in our study, using the broader tips.
2 Materials and Methods

2.1 Isolation of collagen from rat tails

Collagen was obtained from rat tail skin by acid extraction and precipitation. Initial removal of the skin and the hair from the skin was accomplished using a scalpel. This was followed by homogenization of the skin in a Waring blender (in Tris buffered saline). The homogenate was then centrifuged and the supernatant discarded. The pellet was solubilized in 0.5 M acetic acid. The collagen was then precipitated out of solution using either solid NaCl or methanol; the resulting suspension was centrifuged and the pellet collected and resuspended in water. The collagen was then stored in a freezer until needed for analysis. To facilitate AFM imaging of the collagen, a drop of the suspension was placed on freshly cleaved mica and allowed to dry at least one hour before imaging. This preparation is summarized in figure 2.1.

2.2 Collagen assembly by dialysis

Collagen assembly was initially performed by dialysis. Cow skin collagen from Sigma was dissolved in 0.5 M acetic acid to 1 mg/ml. This solution was centrifuged at 27000 g for 10 minutes. The dialysis tubing, which had been washed and soaked in distilled H₂O overnight, was filled with supernatant and suspended in a large beaker of distilled water overnight. Protein solutions can also be dialysed against buffer which would make the next step unnecessary. As the acid diffused out of the tubing, the protein solution approached physiological conditions and assembly commenced. To complete assembly, the water (now pH 4) was neutralized which drew the remainder of the acid out of the tubing. This assembly was carried out at room
Figure 2.1 Flowchart summarizing the procedure used to extract collagen from rat tail dermis.
temperature. An aliquot of the resulting suspension was taken, diluted 10x with H₂O and a drop placed on freshly cleaved mica, before imaging by AFM.

2.3 Cold start collagen assembly

Collagen assembly was done using the "cold start" procedure (Holmes et al., 1986). Type I acid-soluble collagen from calf-skin (Sigma, St. Louis, MO) was dissolved in 0.01 M acetic acid (pH 3.7), at 4°C, to a concentration of 0.36 mg/ml. The collagen solution was then mixed with an equal volume of double strength phosphate buffer (0.4 M Na₂HPO₄/KH₂PO₄, also at 4°C) to form a final solution of concentration 0.18 mg/ml, ionic strength 0.2 and pH 6.8. The temperature was raised to 34°C in a warm water bath and turbidity readings were taken at 632 nm. The incident light intensity was measured, as well as the light intensity transmitted through the 100 ml cylindrical glass cell in which assembly was performed. In Run A, the cell was kept in a 34°C bath and stirred constantly, at a very low rate. All other runs were performed with minimal stirring, only immediately before readings were to be taken. No difference was noted in turbidity readings or fibrils in the stirred vs. non-stirred runs. Approximately 0.05 ml of water-diluted (10x) collagen was deposited directly onto freshly cleaved mica substrate and allowed to air dry ~0.5 h. Dilution was used to minimize codeposition and continued assembly. Sampling was performed every 6 minutes for the first hour, every 8 minutes for the second hour and every 15 minutes for the remaining 30 minutes. In Run D sampling was every twenty five minutes for the first four samples. Sample 5 was collected 1 hour after sample 4 and sample 6 was collected 1.5 hours after sample 5.

The collagen monomers purchased from Sigma Chemical Co. were prepared from calf-
skin using a protocol modified from Gallop and Seifter (1963). This protocol involves extraction of ground skin with sodium acetate, to remove non-collagenous soluble proteins and polysaccharides. The residue is then subjected to repeated washings with water, followed by extraction with citrate buffer, resulting in a monomer solution. This solution is dialyzed against disodium hydrogen phosphate to form fibrils, which are lyophilyzed and stored. This preparation would probably contain collagen III as well as I, since skin contains both and their solubility properties are similar. The preparation is summarized in figure 2.2.

2.4 Turbidimetry

The apparatus used for collagen assembly and turbidimetry measurements in Runs B-D is shown in figure 2.3. The light source was a HeNe laser, producing light with a wavelength of 632 nm. A photodiode (PD) was used to monitor incident light intensity. Approximately 4% of the incident beam was reflected to the photodiode by a glass slide; this allowed any variations in laser intensity to be factored in when the final OD reading was calculated for each data point. For Run A a photodiode was also used to measure the intensity of the transmitted light. For all subsequent runs a photomultiplier tube (PMT) was used to measure the transmitted light intensity. As PMT's are very sensitive only a small percentage (~4%) of the actual transmitted light was reflected into the PMT by a glass slide. The output signal from the PMT was split into 2 signals, one to an oscilloscope (OSC) and the other to a chart recorder. To make the signal easier to read, a variable frequency beam chopper (BC) was inserted between the cell and
Figure 2.2 Flowchart summarizing collagen preparation used by Sigma Chemical Co.
Figure 2.3 Schematic of apparatus used for collagen assembly and turbidimetry measurements.

R represents a recording device; either a chart recorder or 386 computer, depending on the run.
reflecting glass; this produced a square wave function on the oscilloscope which made it possible to view the zero value and signal simultaneously as well as ensure the PMT was not being saturated. When the PMT is saturated, the waveform loses its square appearance since the value does not zero normally when the beam is blocked.

PMTs are much more sensitive and also more adjustable than photodiodes. The sensitivity arises from the electron cascade which multiplies the signal. The electron cascade is produced by the large applied voltage. Operating voltage setting for the power source (PS) was 600-1000 V. A black plastic tube ~ 50 cm long by 5 cm in diameter was used to isolate the light beam as it passed from the glass slide to the PMT. The tube eliminated extraneous background signal from ambient light.

Actual data collection went through an evolution as more experiments were performed. Initially, the voltage was read off the oscilloscope, which made it difficult to obtain accurate readings (data not presented). For Run A, a digital voltmeter was used to read the voltage of the photodiode. A PMT was substituted for the photodiode and initially, the readings were taken off the oscilloscope (data not shown). For Run B the signal was split between a chart recorder and an oscilloscope. The best method for data collection, in terms of ease and accuracy, was to split the signal between the oscilloscope and a box car averager. A 386 PC was then connected to the box car averager and collected data every 10 seconds (Runs C and D). The program used for data collection was Testing (Elizabeth Bishenden, Ph.D. Thesis).
2.5 Atomic Force Microscopy

AFM images were obtained using a Nanoscope II (Digital Instruments, Santa Barbara, CA) with square pyramidal silicon nitride tips (Nanoprobes, Digital Instruments), of spring constant 0.58 N/m. Typical operating forces, taken from measurements of force curves, were ~80 nN. All images were obtained in both height and force mode. However, height measurements were taken only from height mode images. Gain settings were 2 for integral gain and 3 for proportional gain. Images reported are unprocessed. A D scanner was used, with a scan range of up to 16 \( \mu \text{m} \times 16 \, \mu\text{m} \). Each sample which was quantitated, was imaged in at least 8 different, randomly selected positions on the substrate. The imaging field was usually chosen to be 8000 nm x 8000 nm.

Some scans were performed with the AFM in an isolation box to minimize vibrations. The isolation box is a plywood structure lined with fibreglass to reduce acoustic vibrations. The AFM sits on a shelf suspended by bungee cords that further reduce vibrations.

It was noted that in the summer, or at times with high ambient humidity, there were higher forces between surface and tip. Thus images taken in August and September utilized an apparatus modified to keep the samples and tip relatively dry while scanning. This apparatus consisted of a glovebag into which dried air was blown. The AFM was placed into the bag, the sample having been predried in a dessicator, and the system allowed to equilibrate for >1 h before imaging was begun. Typical humidity was 5-10% as opposed to 50-70% in the lab. (Peter Markiewicz, Ph.D. Thesis)

Scan rates were approximately 2 Hz in height mode and 8 Hz in force mode. The force mode keeps the height of the sample constant. As the sample is scanned under the cantilever the
cantilever deflection varies according to the sample topography. This mode is useful for the measurement of small objects (i.e. 1-2 nm), since the cantilever reacts more quickly than in height mode. In height mode the cantilever deflection, and thus the force, is maintained at a constant value by adjusting the height of the sample as it is scanned under the tip. The height mode is more accurate for height measurements with soft, deformable samples since the force can be kept constant at a preset low value.

2.6 Data analysis

Two different assemblies (Runs A and D) were imaged by AFM. For Run A, each sample which was quantitated, was imaged in at least 8 different positions on the substrate, which were selected randomly. The imaging field was usually chosen to be 8000 nm x 8000 nm. A minimum of 200 fibrils were counted for each sample represented by a distribution in figure 2.8. Many fibrils were counted to minimize the errors inherent in differentiating between several monomers codepositing next to each other on the mica surface and actual assembly. Only entire fibrils were counted. For fibrils which codeposited in clumps containing more than one fibril, ends were counted. For bent fibrils the straight length was taken to be the addition of the bent lengths. Height measurements were not taken where fibrils crossed each other, but only where a single fibril was deposited on the mica. Ranges were set in the distribution to allow for fluctuations in the accuracy of the measured height based on cantilever deflection: in regions where there is a higher force of attraction between the sample and cantilever, increased cantilever deflection results in a measured height which is lower than the actual height. To
calculate the amount of distortion occurring, the amount of force at any particular point on the scan is obtained from the force mode image. The spring constant of the cantilever is then used along with the force to calculate the distance the cantilever is deflected (F=-Kx) (P. Markiewicz, Ph.D. Thesis).

Run D was treated in a qualitative rather than a quantitative manner (see discussion).
3 Results and Discussion

3.1 AFM Tip effects

In imaging soft biological samples with the AFM, part of the initial study is systematizing the imaging methods in order to produce images which are representative of the sample. The AFM is prone to tip artifacts. It is necessary to distinguish correct images from faulty images that are produced by problems with the AFM tip or the sample. The following is a summary of tip effects encountered in collagen imaging.

The AFM tip is assumed, in theory, to come down to a very small point (even down to a single atom). In this case, the topographical information recorded by the scanning tip represents the interaction of a single tip atom with successive atoms in the sample. In practice this is not the case since the mass-produced Si₃N₄ tips which are commonly used are ~40 nm in diameter at the apex. Nevertheless, provided that the tip geometry is simple and symmetric the images obtained are representative of the sample, at least qualitatively. However, irregularly shaped tips produce erroneous images. An example of this is a "ghost image" (figure 3.1a), where a double of each object is produced by imaging of the object with a "double tip" (a tip with two contact points instead of one). This, and similar types of geometric distortions can be misleading. Thus, if these effects were noted while imaging, the AFM tip was changed.

In imaging soft matter, high forces can deform samples, resulting in distorted images. Care was taken to ensure images were obtained under low force (<80 nN) conditions. Generally, collagen was robust enough and high forces were not a problem except in the summer, when humidity in the lab was as high as 60%. Water from the air appeared to deposit on the sample.
Figure 3.1 AFM tip effects. a) Ghost images (small spheres) due to imperfect tip shape. b) Smearing due to high humidity (image is of mature fibrils and microfibrils).
surface. Thus, when the tip was brought into contact with the sample, there were additional forces on the tip due to its interactions with the water. The water also may have made it easier to dislodge portions of the sample which would normally remain intact. The hydrated samples resulted in images like that shown in figure 3.1b. The main feature of these images is the "smearing", which causes errors in height measurements and difficulties in counting. If smearing was observed in imaging or if the operating force rose above 80 nN, the tip was changed. As mentioned in Materials and Methods, when the ambient humidity was high, the AFM was isolated in a glovebag filled with dessicated air.

Another problem, sometimes related to humidity, was accumulated debris on the tip. The longer a tip is used, particularly with soft and/or biological samples, the more material is picked up by the tip. This often causes poorer resolution in images, and commonly structures appear wider than they normally would. As the tip is now even larger, lateral distortion is greater than usual. The lateral distortion is caused because the initial contact point of the tip with a raised object is on the side of the tip. Thus, broader tips respond to raised objects on the surface at a greater distance from the object than a narrower tip would. This results in images where the fibrils appear "smooth" (i.e. periodicity, even in mature fibrils, is difficult to find) and "fat". To avoid problems associated with debris on the tip, AFM tips were changed frequently.

A discrepancy between height and width measurements was found. A typical measured width for a fibril of height 2-4 nm in an unprocessed image was ~100 nm. It is possible that this may be due to the distortion of the fibrils because of the force of the AFM tip. However, this explanation is unlikely because Baselt et al. (1993) have shown that tip forces are quite low for air dried collagen. Collagen was demonstrated to be a robust, fibrous structure. Furthermore, if
forces were high, repeated scans of the same fibril would show displacement of the fibril or altered images on repeated scans. These high force phenomena were not observed. This distortion of the lateral dimensions is due primarily to AFM tip interactions with the sample, as the tip moves laterally across the sample, and has been discussed in detail by Markiewicz and Goh (1994). This effect was demonstrated by imaging a field of colloidal gold (diameter ~10 nm; specified by the manufacturer) deposited on mica (figure 3.2). The heights of the particles were measured to be 7-11 nm and the widths 70-100 nm. Since the size of these spherical particles is in the same range as the collagen fibrils being measured, it can be assumed that the same sort of image distortion is taking place with the fibrils. Thus, the fibril diameters are taken to correspond to the height of the fibrils above the substrate, rather than the width.

3.2 Comparison of native and assembled collagen

As can be seen from figure 3.3 there is no detectable difference between native collagen and collagen self-assembled by dialysis. All three samples show an axial periodicity of 67 nm, although the diameters vary. Native collagen was found to have larger diameter fibrils, by a factor of ~2. As mentioned in the introduction, large diameter collagen fibrils can be produced in vitro by using low temperature conditions (Williams et al., 1978). There are many factors which influence fibril diameter: the ratio of collagen III to collagen I present in the mixture, the presence of extracellular components (i.e. proteoglycans), and the intactness of collagen monomers, especially the non-helical ends. We can thus attribute the differences in diameters of native and dialysis assembled collagen to the differences in assembly conditions.
Figure 3.2 Micrographs of colloidal gold deposited on mica. a) Large scale view. b) Cross-section of area in a demonstrating width of 74 nm for a 7 nm high gold particle (also shown is 5.4 nm high gold particle).
3.3 Run A

In our study, AFM is used to quantitatively study a range of assembly products at different stages of fibrillogenesis. The results from Run A indicate that, using the cold start method, self-assembled collagen fibrils form through a series of four distinct stages of intermediates (shown in figure 3.4). The first stage is the formation of oligomers (figure 3.4a), which then laterally associate and form a network of microfibrils which show a periodicity of ~67 nm (figure 3.4b). The next stage is characterized by the presence of larger (2-10 nm height), very long, loose, non-banded microfibrils (figure 3.4c). The final stage is the mature fibrils of height 15-30 nm (figure 3.4d), which demonstrate native-type banding (~67 nm).

The turbidimetry graph for Run A (figure 3.5) indicates that this assembly only proceeded to the growth phase. The assembly was not allowed to continue through to the mature gel phase, characterized by a plateau of the absorbance measurements. Since mature fibrils are found in later samples of Run A, this run demonstrates all four stages of assembly. If assembly had been continued, it is likely that more and larger mature fibrils would have been formed.

Results from Run A demonstrate that in the starting material and in samples 1 (t=8 min) and 2 (t=13 min) only monomers and small oligomers are observed (figure 3.4a). Figure 3.6 shows the distribution of lengths for the starting material. The oligomers were all the same diameter (.7-1.5 nm). The lengths of the oligomers are mostly 250-300 nm or 500-600 nm. The spread of values in these ranges is probably due to variable deposition resulting from the semiflexible nature of collagen molecules. Collagen monomers are known to be 300 nm in length. Thus, a minimum overlap of two monomers (4D-staggered as in figure 3.7a) would produce dimers of length ~536 nm. The distribution in figure 3.6 shows a significant number of...
Figure 3.3 a) Mature collagen fibrils extracted from human brain (Markiewicz and Pollanen). b) Mature fibrils extracted from rat tail dermis. c) Collagen assembled by dialysis (source rat tail). d) Zoom of c.
Figure 3.4 Run A; micrographs showing the four level hierarchy of assembly. a) Monomers and oligomers from Starting Material. b) Small microfibrils forming network. c) Large microfibril. d) Mature fibril and microfibrils.
**Figure 3.5** Run A collagen assembly-absorbance vs. time.
Figure 3.6  Run A. Distribution of oligomer lengths in starting material.
Figure 3.7 Four possible overlap orientations of collagen monomers to form dimers. A) 4-D stagger  B) 3-D stagger  C) 2-D stagger  D) 1-D stagger (each monomer measures 300 nm long; 1-D=67 nm).
oligomers of length 500-600 nm, probably corresponding to a 4D-staggered dimer. The results from EM measurements of oligomers also support minimum axial overlap of monomers (Ward et al., 1986). A minimum overlap dimer would be expected to have the same height (measured by AFM) as a monomer since most of the dimer would consist of non-overlapped regions. As these 500-600 nm species have the same height (diameter) as monomers, it is likely that they are dimers, although this could only be determined for certain with better resolution images. If these small oligomers were composed of more than two monomers overlapping, the height would be greater than the height of a monomer.

Our results indicate that assembly of microfibrils progresses by both axial elongation and lateral aggregation. The early intermediates (small microfibrils) form a network over the mica surface. The network is observed starting in sample 3 (t=19 min), with 300-600 nm between interconnections and diameters of 1.5-2.5 nm. Some of these microfibrils show an axial periodicity of 67 nm (figure 3.4b). Further axial and lateral aggregation form the next level of assembly. Starting at time=12 min, microfibrils with diameters of 2-3 nm are seen. These fibrils are very long (>10,000 nm) and deposit on the mica in a very convoluted manner (perhaps involving self-interaction). This convoluted deposition complicates the determination of the exact number of fibrils (figure 3.4c). Once small microfibrils are formed, mature fibrils (diameter >14 nm, displaying native-type banding with periodicity ~67 nm) are observed (figure 3.4d). Later samples show increasing amounts of mature fibrils as well as larger numbers of large microfibrils (diameter 4-14 nm). Furthermore, they still demonstrate a network of microfibrils, with few or none of the small oligomers, and virtually no monomers. This would seem to indicate that at the later stages of assembly growth is taking place by the addition of
larger units, perhaps oligomers, and not addition of monomers.

Figure 3.8 shows the sequence of fibril distributions at 19, 57 and 118 minutes. These times correspond to the lag phase, the start of the growth phase and the end of the growth phase. A progression in assembly can be seen starting with sample 3, which contains only oligomers and a few small microfibrils. Sample 8 has increasing amounts of small microfibrils and a few mature fibrils while sample 14 has more large microfibrils and also a few mature fibrils.

In this study a much larger number of aggregates were observed in the lag phase than demonstrated in physical solution studies. Monomers are only visible in the starting material and samples taken within the first thirty minutes of assembly. The next largest intermediates are small oligomers (450-1000 nm long). These are present in large numbers in the starting material and the first thirty minutes of assembly. They can also be found at later assembly times but as a smaller percentage of the population. The next largest species is small microfibrils: These are present in small numbers in the starting material (even the acidic SM) and tend to clump together to form networks. These small microfibrils are present in large numbers even in samples taken much later in assembly. Our results compare favorably with results obtained in EM studies (Gelman et al., 1979; Holmes et al., 1986). Holmes et al. found aggregates in the lag phase in collagen assembled by three different pathways (cold start, warm start and simultaneous start). In the cold start lag phase long, thin (diameter 2-10 nm), non-banded filaments were observed. In warm start and simultaneous start short (1 um long), banded fibrils (just over 10 nm wide) were observed.

However, light-scattering, electric birefringence and turbidimetry studies have not observed any species larger than a pentamer in the lag phase. This apparent contradiction is
Figure 3.8 Run A. Distribution of fibril diameters at various points in fibrillogenesis. a) Sample 3 (time=19 min). b) Sample 8 (time=57 min). c) Sample 14 (time=118 min).
resolved in our study in which both turbidity and AFM techniques were performed. Although the turbidity showed no increase in absorbance in the lag phase, aggregates were found in the AFM samples. Obviously, physical solution techniques can only detect larger species if they are present in large amounts. In addition, with electric birefringence and light scattering there are the problems discussed in 1.4.2 with data interpretation.

Uncertainties in the height measurements were calculated for Run A (standard deviation/mean, using n=15, for each of the ranges shown in figure 3.8). The uncertainties are as follows: for monomers and oligomers the uncertainty is 19%, for 2-4 nm fibrils the uncertainty is 24%, for 4-6 nm fibrils the uncertainty is 10%, for 6-10 nm fibrils the uncertainty is 15% and for 20-26 nm fibrils the uncertainty is 15%. The range of monomer and oligomer lengths show a deviation of ~10% from the mean values. These uncertainties reflect the actual variations in fibril diameters within that range, as well as irregularities in fibril deposition or substrate smoothness. In comparison, the uncertainty due to the AFM, as a technique, is negligible.

As mentioned in section 3.1 there are several possible explanations involving tip effects for the noncylindrical appearance of the fibrils as indicated by height and width comparisons. However, another possibility is that the microfibrils produced by these assembly conditions are loose and thus spread out to some extent on the substrate. Holmes et al. (1986) reported that neutral start intermediate fibrils were more loosely organized. Since mica is a polar substance, some interactions with the loosely organized collagen fibrils may be sufficient to produce a flattening effect.
3.4 Runs B and C

The turbidimetry curves for Run A (figure 3.5) and Runs B and C (figure 3.9) are not the "typical curves" of figure 1.3. Only in the case of Run D was a typical curve obtained. Run A assembly was not completed as can be seen in figure 3.5. The growth phase was not yet complete when assembly was halted, thus no plateau is observed.

The time scale for Run B is longer (figure 3.9a) than run times reported in most collagen assembly studies and early experiments I performed (figure 3.5). A significant change in absorbance was not seen for at least two hours, while in other studies the growth period began ~20-30 minutes into assembly. The slower rate of assembly indicated that some factor in the experimental conditions had been changed since the previous runs. Past collagen work has been done using more stringent biochemical methods with checks on the wholeness of the collagen monomers used (for example Holmes et al., 1986). As can be seen by comparing turbidimetry curves, some assemblies took longer than others to reach the end of the lag phase and demonstrate some change in absorbance (the data for several of these runs is not shown but resemble figure 3.9a). It was found that this was due to degradation of the stored Sigma collagen. There is a noticeable trend in that the time required for assembly gets longer as the time since the opening date of the bottle gets longer. It is important that the collagen be stored at 4°C and dessicated to slow degradation. For Run C a new bottle was opened and used for assembly. The lag time for Run C was very similar to literature values (see figure 3.9b). Unfortunately, a problem occurred with the data-collecting software and the curve is incomplete in that it is missing most of growth phase data. Runs B and C were not characterized by AFM due to the problems with turbidimetry measurements.
Figure 3.9  a) Run B using PMT.  b) Run C using PMT, including final data point; data taken from oscilloscope.
3.5 Run D

One other collagen assembly was extensively characterized by AFM. The results from Run D follow the trends observed in Run A. In Run D however, assembly was allowed to go to completion, until the change in absorbance became very small and a plateau was observed (figure 3.10b). Samples were taken at all stages of assembly and imaged by AFM. The starting material (SM) was also imaged in its acidic state (as opposed to only after having been neutralized) to see if any difference occurred in the solution simply by changing the pH, while the solution was kept at 4°C. In the acidic SM occasional aggregates of small microfibrils (1000-3000 nm long) were observed, but nothing larger or more organized was found (figure 3.11ab). Upon neutralizing the SM, monomers and small oligomers were observed (figure 3.11d). However, occasional aggregates of small microfibrils, a few large microfibrils (figure 3.11c) and one small mature fibril displaying periodicity, were also observed. Thus, it appears that some assembly occurs at a neutral pH, even at 4°C. Holmes et al. (1988 in Nimni, p. 116) specifies that in cold start assembly, when the acidic solution is neutralized by addition of buffer, an immediate precipitate of a very small fraction of collagen is observed. They suggest removal of the precipitate by centrifugation since the precipitate can cause variable results by accelerating fibrillogenesis. In our experiments, the acidic SM was centrifuged to remove insoluble matter. In future studies, it is recommended that the neutralized solution be centrifuged instead.

Sample 1 was taken thirty minutes into assembly, just before the absorbance began to increase. There were many small microfibrils present, as well as some medium microfibrils and a few mature fibrils. Evidently, insufficient assembly occurred to cause a change in the intensity
Figure 3.10 Run D  a) PMT voltage vs. time (min).  b) Change in absorbance vs. time (min).
Figure 3.11 Run D-micrographs  a) Acidic starting material showing monomers and oligomers. b) Zoom of a. c) Neutralized starting material showing monomers and oligomers as well as microfibril. d) Zoom of c.
of transmitted light (figure 3.12ab).

In sample 2 (time=52 minutes) the small microfibrils observed were longer, and formed an extensive network over the mica. No mature fibrils were found. (figure 3.12cd)

In sample 3 (time=75 minutes) many medium microfibrils were observed and quite a few mature fibrils. This resulted in some mature fibrils depositing in the same frame, unlike the isolated mature fibrils found in sample 1). (figure 3.13ab)

Sample 4 (time=125 minutes, just at end of rapid growth phase) showed many mature fibrils. In some micrographs, mature fibrils were the only species that could be observed, so many of them had deposited together (figure 3.13d). In the micrographs where fewer mature fibrils were present, the network of small microfibrils and many medium microfibrils were visible (figure 3.13c).

In sample 5 (time=190 minutes, plateau region) many medium microfibrils were present. Only a few solitary mature fibrils were observed. (figure 3.14ab)

Sample 6 (time=255 minutes, plateau region) shows more mature fibrils than sample 5 but still not as many as seen in sample 4. (figure 3.14cd)

The sequence of micrographs illustrating the course of this assembly (figures 3.11-14) and the above description demonstrate an overall progression in the assembly. However, some inconsistencies do exist because of inconsistent sampling. For instance, one would expect to observe at least a couple mature fibrils in sample 2, and more mature fibrils in samples 5 and 6 than in sample 4. These inconsistencies show that there is a problem with the sampling; evidently samples are not necessarily representative of the system. A rise in absorbance should lead to a higher degree of assembly evident in the samples. The inconsistencies could be due to
Figure 3.12 Run D-micrographs a) and b) Sample 1 (time=30 min) showing mature fibrils, microfibrils and oligomers. c) and d) Sample 2 (time=52 min) showing large microfibrils and network of small microfibrils.
Figure 3.13 Run D-micrographs  a) Sample 3 (time=75 min) showing mature fibrils and microfibrils. b) Sample 3 showing densely deposited microfibrils. c) Sample 4 (time=125 min) showing mature fibrils and microfibrils. d) Sample 4 showing densely deposited mature fibrils.
Figure 3.14 Run D-micrographs  a) Sample 5 (time=190 min) showing mature fibrils and microfibrils. b) Sample 5 showing microfibrils. c) and d) Sample 6 (time=255 min) showing mature fibrils and microfibrils.
interactions between the fibrils and the glass pipet used for sampling. Another possible cause of these inconsistencies is interactions between the fibrils themselves, so that even though care was taken to ensure proper mixing both in the cell and in the dilution stage, the final samples deposited on the mica were not truly uniform. The mode of deposition of the fibrils would certainly support this last point, since fibrils usually deposit in aggregates. Sampling is discussed further in section 3.6.

3.6 Sampling

Accurate sampling of collagen assembly solutions has not been achieved by previous workers. One advantage of physical solution techniques such as light scattering and electric birefringence is that they do not require sampling. However, as mentioned in section 1.4.2, the data from these techniques do not yield useful information regarding assembly intermediates. While many studies of collagen monomers and mature fibrils have been accomplished using electron microscopy (section 1.4.3), few electron microscopy studies (Gelman et al., 1979; Holmes et al., 1986) have been performed on collagen intermediates, because of the problem of sampling. Electron microscopy samples are created by putting a drop of the assembly mixture on a carbon coated copper grid. This grid is then usually rinsed with water or buffer to rid the sample of excess assembly materials. The purpose of this rinsing is presumably to prevent further assembly from taking place. The samples are then dried and either fixed or stained. All samples are deposited and rinsed in the same manner and thus assumed to be comparable to each other. However, it is highly probable that the rinsing step renders these samples unrepresentative of the
assembly mixture. In fact, the smaller intermediates are probably washed away. Thus, EM results are presented in a qualitative rather than a quantitative manner. Although fibril distributions are discussed in a general manner, only representative micrographs rather than quantitative data are presented.

One of the goals of this study was to quantify assembly. AFM affords a better opportunity than EM for quantifying assembly because AFM requires little sample preparation. Thus, using AFM techniques, problems in sampling techniques can be discovered and corrected with more ease and efficiency than possible with EM. This evaluation forms part of my studies, which show that while AFM can be used for quantitative assembly studies, there are some problems that still need to be addressed. A summary of the sources of error and suggestions for their correction follows.

The major source of error in sampling is removing the sample from the cell using a glass pipet. Before samples were taken the contents of the cell were stirred. However, since the fibrils show a tendency to self-attract, the stirred solution is not necessarily uniform. Also the fibrils adhere to the glass pipet to a certain extent. An alternative to a glass pipet is a micropipetor with disposable polypropylene tips. Although this alternative eliminates the problem of interactions, the opening in the end of the plastic tips is very small. The small tip may cause some selectivity in sampling as well. Another alternative would be a plastic syringe with a metal tip. These metal tips are available in large bore sizes.

The drop taken only represents a very small portion of the sample which is then diluted 10-15x. This dilution is necessary to avoid over-deposition on the mica; however, it is not guaranteed that the one drop taken of the diluted mixture will be representative due to
interactions between the fibrils. One way of improving sampling at this stage would be to withdraw a larger sample (instead of 1-2 drops, 0.5 ml would be a better quantity). The probability of the sample being representative of the assembly mixture would thus be increased. The dilution could be performed in a small petri dish and then the mica could be coated by inverting it over the surface of the diluted solution. This alternative would eliminate the need for a pipet for deposition of the diluted sample onto the mica.

Another difficulty is the mode of deposition of the drop of solution on the mica. As the fibrils tend to aggregate, even when the solution is dilute, some areas of the mica are clear of aggregates while others are so thickly covered that it is difficult to count or measure fibrils exactly. The design of the AFM sample stage makes it possible to only image 30 % or so of the entire sample (the area right around the centre).

Accurate and consistent sampling is problematic using current techniques of fibril counting and data manipulation, especially at the latter stages of assembly when the number of particles have decreased substantially. In order to have truly meaningful numerical results many more samples would have to be taken during assembly. Imaging and extensive counting need to be done to provide good statistics, especially of the fibrils. Unfortunately this is not very practical using the methods presented in this work. Thus, development of an automated counting method is recommended. If a software program was written to handle counting of fibrils in micrographs and combination of data, statistically significant results, with smaller errors, could be obtained.
3.7 Reversibility of assembly

Collagen fibrils are stabilized by three different types of cross-linking. These are reducible aldol condensation links within and between monomers, reducible Schiff's base cross-links and nonreducible cross-links. The reducible cross-links allow reversibility of fibrillogenesis while the nonreducible cross-links do not. The intramolecular aldol condensation cross-links are formed early in both in vivo and in vitro assembly. They are produced when the enzyme lysyl oxidase acts on lysine residues in collagen to produce aldehydes, which then undergo condensation reactions. Lysyl oxidase is copurified with collagen (Nimni and Harkness in Nimni, p. 31). Thus, when collagen is reconstituted, neutralized and allowed to assemble, new aldehydes groups are formed on the peptide chains, allowing new cross-links to form (Deshmukh, 1971). Intermolecular Schiff’s base cross-links are formed by reaction of the aldehyde and N-amino groups of lysine in different molecules. Both of these cross-links are cleaved by weak organic acids such as acetic acid (Deshmukh, 1971). If the assembly mixture is allowed to sit for several weeks though, nonreducible cross-links form, which are heat and acid stable. This is why in any tissue extraction with acid, only a portion of collagen is recoverable as monomers.

It has been demonstrated that after in vitro fibril formation has proceeded to the mature gel phase, if the temperature is lowered to 4°C there is only a very small reduction (<5%; Williams et al., 1978) in the absorbance of the solution. Thus, most of the fibrils remain assembled. This irreversibility is known to result from spontaneous cross-linking between lysine-derived aldehydes (Tanzer et al., 1966) This result was confirmed in several of our turbidimetry studies where the cell containing assembled collagen was allowed to sit overnight at
4°C and no change was detected in absorbance. However, if the pH is lowered, along with the temperature, monomers are produced again. Thus, both pH and temperature must be changed to reverse collagen assembly. The only exception is when cross-linking is totally suppressed (as in the study by Helseth and Veis, 1981) and assembly can be reversed by decreasing the temperature alone.

3.8 Periodicity

The periodic nature of mature collagen fibrils is well documented and has been measured by EM to be 67 nm (Hall et al., 1942). As mentioned in the introduction and demonstrated in figure 1.2, this periodicity arises from the regular staggered arrangement of collagen monomers in the fibrils. Figure 3.15 shows two cross sections of fibrils from Run A demonstrating this periodicity. Figure 3.15a is a representative cross section of a mature fibril; the calculated periodicity in this case is 67.5. Figure 3.15b shows that periodicity can be found in some small microfibrils as well (66.8 nm in this case).

Periodicity in larger microfibrils was not measured; in most cases the fibrils deposited in too convoluted a manner to permit cross sections longer than several hundred nanometers. Larger microfibrils do not appear periodic, unlike mature fibrils where the periodicity is very apparent. Since periodicity has been found in small microfibrils though, larger microfibrils are expected to be periodic as well; what is necessary is a method of deposition which would enable longer cross sections to be accomplished on large microfibrils.
Figure 3.15 Crossoctions of fibrils from Run A demonstrating ~ 67 nm periodicity  a) Small microfibril (network). b) Mature fibril.
3.9 Mechanism of assembly:

There are two different models offering contrasting views of the assembly mechanism for complex biological structures. These are nucleation and growth and stepwise assembly (these mechanisms are summarized in figure 3.16). Examples of systems that have been proven to assemble by nucleation and growth include actin, tubulin and flagellin (all fibrillar biological structures). The assembly in these systems is thought to be driven by entropy considerations. Assembly by nucleation and growth requires a critical concentration of monomers in order to form stable nuclei. After nucleation, monomers are added to the nuclei one at a time to form larger structures. Nucleation and growth is a reversible process. The changing of conditions (for example, in some cases, temperature) leads to disassembly of the filaments. (Hulmes, 1992)

The biopolymers that assemble by nucleation and growth are simpler than collagen. They grow exclusively by longitudinal elongation. Collagen, in contrast, grows both laterally and longitudinally.

An example of a biological structure which assembles by stepwise growth is the tobacco mosaic virus. In stepwise assembly individual units add together to form larger structural units that are then combined to form the final structure. Growth does not occur by the addition of monomers to the larger structure.

Since evidence can be found in collagen assembly for both mechanisms, it is likely that depending on the particular stage of assembly or the conditions imposed either mechanism, or a combination of the two may be in operation. The sigmoidal shape of the turbidimetry curve found in collagen assembly has been interpreted as evidence of nucleation and growth (Nimni in Nimni, 1988, p. 15). The initial plateau region (lag phase) would represent nucleus formation.
Figure 3.16 Schematic illustrating the steps of collagen assembly by two different mechanisms: a) stepwise assembly and b) nucleation and growth.
The rapid growth phase would represent fibril formation, which would cause a rapid increase in absorbance. Fibril formation could proceed by either addition of nuclei to each other (stepwise assembly) or rapid addition of monomers to the nuclei (nucleation and growth). Either mechanism at the growth phase would result in the steep curve observed. Thus, it is possible that the initial phase of assembly could be nucleation and growth, followed by stepwise growth in the next phase.

Evidence for a stepwise mechanism is found in micrographs such as figure 3.1d, which indicate that larger fibrils are formed by the twisting together or bundling of smaller fibrils. This sort of assembly has been suggested in theoretical studies of collagen organization in formed fibrils (Smith, 1968). Collagen fibrils are observed in EM and AFM images to have tapered ends (Chernoff and Chernoff, 1992). If fibril breakage was occurring in our collagen preparations, the ends would appear blunt rather than tapered. Thus, it is unlikely that this untwisting is a result of breakage. The untwisting is observed in many micrographs at more than one level of assembly (i.e. figure 3.14b upper right portion of micrograph; microfibril level of organization).

As mentioned in the discussion, monomers are not observed after the lag phase is completed. The smallest species found in the growth phase and the mature gel phase is oligomers. This suggests stepwise assembly. If a nucleation and growth process was occurring by the addition of monomers, one would expect to observe numerous monomers in growth phase and mature gel phase samples. Our observations thus suggest the combination of oligomers to form larger fibrils through a stepwise process.

Our observations are also consistent with a combined mechanism of assembly. A
nucleation and growth process could be occurring in the earlier phases of assembly to form smaller building blocks (oligomers), which then combine in a stepwise process to form larger fibrils.

3.10 Future work:

Future studies of collagen assembly could employ an AFM liquid cell to study assembly in real time. In order for this work to be successful, several problems would have to be overcome. These include keeping the cell at a temperature suitable for assembly and finding the best substrate in order to limit the movement of the growing fibrils. However, limiting the movement of the fibrils may interfere with assembly. Early assembly events could probably not be studied using this method due to the small size and motility of monomers and small intermediates. Resolution would not be as good using the liquid cell as imaging under air, partly because the fibrils become softer when placed under solution (Baselt et al., 1993).

Another way to study collagen assembly would be to use the same general techniques used in this study with varying experimental conditions. Holmes et al. (1986) have found that the types of intermediates formed in the early growth phase depend on the order of changing the initial conditions. Depending on the order in which temperature and pH are brought to physiological levels, either short and banded or long and non-banded fibrils are observed as intermediates.

Turbidimetry studies have demonstrated that if the temperature of assembly is changed, the rate of assembly and the final diameter of the fibrils are altered (Brokaw et al., 1985). Temperature effects could also be monitored using AFM.
A more sensitive optical method than turbidimetry would be light scattering, since information can also be gained about the size of the species present in solution as opposed to just the amount of assembled material. As mentioned in the Introduction, there are difficulties associated with interpreting data from light scattering studies. The use of AFM together with light scattering would allow improved interpretation of light scattering results.

The problems with obtaining quantitative data by sampling and counting could be overcome if an automated form of counting could be introduced. If a computer program could translate the AFM images into meaningful categories based on fibril diameters and combine the data, enough frames could be quantitated to obtain statistically significant data.

Fibrils and oligomers assembled from sequence-modified collagen type proteins (i.e. Bella et al., 1994) could be imaged by AFM to see if the loops in the helix could be detected or the fibrils formed were different from regular collagen. Another variation of collagen that could be imaged is collagen from pathological tissues (i.e. ostea imperfecta; skin conditions). If the components were selected carefully, images of collagen assembled in vitro with extracellular matrix components (i.e. proteoglycans, fibrinogen) could yield information about sites of attachment. Special sharpened tips would have to be used to give the necessary resolution.

Attention should be given to the appearance of partially "untwisted" fibrils with cross sections taken in the area of the twisting to elucidate the nature of the interactions. Better resolution images of such phenomena would also yield a better understanding of the mechanism of collagen assembly. It remains to be determined which stages of assembly display twisting fibrils. If twisting fibrils were found at certain stages of assembly, this would suggest that stepwise assembly was taking place in those stages. Also, reversibility of the twisting process
could then be investigated by lowering the temperature after the twisting had taken place.
4 Conclusions

Past researchers have imaged many biological structures using AFM. The goal of this present study however, is to follow the growth of a biological structure starting from individual molecules. Collagen was the chosen system, since it has been studied extensively by a number of other techniques. Despite past studies of collagen assembly, the early stages of fibrillogenesis and the mechanism of assembly are still not well understood. Although atomic force microscopy has been employed in the last two years to image mature collagen fibrils or collagen monomers, ours is the first study of collagen assembly using AFM. We have followed all the steps of assembly from the monomer starting material through to the mature fibrils. Since past research has used turbidimetry curves to characterize different stages of collagen assembly, turbidimetry was employed in this study as a reference method. Collagen assembly was found to be a four stage, stepwise process. The initial products of fibrillogenesis were oligomers, formed by axial association of monomers. The second stage of assembly was characterized by small, 2-3 nm diameter microfibrils, formed by the lateral and axial combination of oligomers. These small microfibrils exhibited a periodicity of 67 nm and often deposited in a network. Large microfibrils (>10,000 nm long), depositing in a convoluted manner, were observed in the third stage of assembly. The fourth stage of assembly produces mature, banded fibrils with a periodicity of 67 nm.

This study has provided the groundwork for future AFM studies of collagen assembly by demonstrating that it is possible to quantify assembly using AFM techniques. However, there are some problems with the data. Due to sampling inconsistencies and the difficulty of
quantifying large numbers of fibrils, uncertainties in measurements were large. Once consistent sampling is achieved and automated methods of quantifying assembly are developed, examination of collagen assembly by AFM should yield significant quantitative results.
4 References:


Mallein-Gerin, F. and R. Garrone. 1990. Tendon collagen fibrillogenesis is a multistep


