EXPLORATIONS IN BIOLOGICAL ATOMIC FORCE MICROSCOPY: USING TAPPING MODE UNDER BIOLOGICAL BUFFERS TO EXAMINE POLYMORPHISM IN RECA PROTEIN.

By

Bernie D. Sattin

A thesis submitted in conformity with the requirements for the degree of Master of Science Graduate Department of Chemistry University of Toronto

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Abstract

RecA fibrils in physiological conditions have been successfully imaged using Tapping Mode atomic force microscopy. This represents the first time images of RecA have been obtained without drying, freezing and/or exposure to high vacuum conditions. While previously observed structures—the monomer, the hexamer, the short rod,—were seen, a new type of fibril was also observed. This mini-fibril is narrower in diameter than the standard fibril, and occurs in three distinct morphologies: aperiodic, 100-nm periodic, and 150-nm periodic. In addition, much longer rods are observed, and appear curved and even circular.
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1. Introduction

RecA Protein: The singly most important protein in the history of evolution. Why? It is the cause of evolution, the driving force in the entire process. RecA protein can be found throughout the prokaryotes, and has structural, and functional analogs in all eukaryotes. Its function in the evolutionary sense is simple. In prokaryotes, it controls two processes: SOS mutagenesis, and DNA-strand exchange (also known as Homologous Recombination). SOS mutagenesis is the process by which mutations are forcibly incorporated into the bacteria’s genome. This occurs when the cell has an urgent need to replicate, so making any functional copy of the DNA is more important than making an exact copy of the DNA (as is the norm). Since this process forces mutations into the genome, any viable cells containing this mutation have a different genetic make up than their relatives, which gives them the opportunity to excel in areas their brothers and sisters did not – this is evolution. In the case of homologous recombination, a sexual type of evolution occurs. Two cells come together to reproduce. Instead of receiving a copy of only one parents genes, the offspring contain contributions from both parents. This difference is caused when RecA brings two sections of DNA together that are similar in nature, and switches them between genomes. This difference in the offspring allows evolution to occur, much the same way the SOS mutagenesis does. Since these two processes contribute ninety percent of the genetic diversity of all living things, and RecA Protein is central to both these avenues, we can unequivocally say RecA is the most important protein in evolution.

In the past RecA has been studied by a wide variety of techniques. These range from vacuum studies by transmission electron microscopy (TEM), scanning electron microscopy (SEM), to light scattering, and small angle neutron scattering, and also include solid state X-ray crystallography, and Scanning Tunneling Microscopy (STM), to fluid studies by NMR. These techniques have been fundamental in establishing the structure and assembly of RecA with itself and to form nucleoprotein filaments. In this thesis, I present my work on establishing a better system of RecA analysis – the Atomic Force Microscope (AFM). The AFM allows us to visualize RecA structures and their assembly under conditions very similar to the conditions as they exist inside the cell.
Using Tapping Mode in fluid, I have succeeded in laying the foundation for this research, and present my results of RecA fibrils in solution, and the techniques leading up to this success.

1.1. Atomic Force Microscopy – History

Scanning tunneling microscopy is the grandfather of all scanning probe microscopies. Invented by Binning and Rohrer in 1982 (Binning et al., 1982), this microscope uses a tunneling current to position a single atom tip angstroms from the surface to determine topography. This gives it the ability to resolve single atoms (and even electron waves). Its applications include determination of crystal packing structures of metal crystals, examination of catalytic surfaces and the molecules that bind them, and the exploration of various other phenomena of this size scale (Magonov, 1993). The main difficulty lies in its inability to image non-conductive samples (e.g., biological samples), and those of larger sizes. This need to examine larger (non-conductive) surfaces led to invention of the atomic force microscope (AFM) by Binnig and Quate in 1986 (Binnig et al., 1986). Using the feedback controlled piezoelectric crystal idea, the AFM positions a bigger tip (20 to 50 nm in diameter) close to, or on the surface by holding this tip on a stiff cantilever, and accurately determining the cantilever’s position using a laser beam and a position sensitive photodetector. Around this time the human genome project (whose goal is to sequence the entire human genome) was becoming more prevalent, and with the atom sensing ability of the STM known, people conceived it to be possible to lay down a piece of DNA and read the sequence right off of it. However, while the AFM could also achieve the STM’s atomic resolution, it was soon realized that it could not see atoms on DNA in air. In addition, the high forces involved in imaging often destroyed the sample – this was not a desirable feature. The main source of these high forces was thought to be capillary forces of a water layer on the surface. So it was reasoned that examination of samples under fluids (water or alcohols) would improve resolution – thus the invention of the fluid cell (Drake et al., 1989; Marti et al., 1987). The other idea was to minimize the contact between the tip and the surface. This was done by oscillating the cantilever at its resonance frequency, and using the amplitude of resonance as the
feedback – the invention of Tapping Mode (Zhong et al., 1993). The last hurdle to overcome (and it was a big one) was to combine these two techniques, done around the same time in two groups – Tapping Mode in Fluid (Hansma et al., 1994b; Putman et al., 1994). Tapping Mode in fluid’s main drawback is its technically demanding use. This has been simplified by the invention of MAC-mode AFM (Magnetic A.C. mode) which uses a magnetized cantilever, that is oscillated by a changing magnetic field at its resonance frequency (Han et al., 1996).

1.2. Biological AFM – Aims and Goals

Clearly the AFM has a huge potential in the field of biology. It has the ability to examine structures on a wide range of scales – from just a few nanometers to around 100 microns. However, several improvements need to be made before such imaging becomes routine. These improvements can be subdivided into two areas: sample preparation, and instrumentation.

The instrumentation improvements center around finding a sharper, consistent tip, and creating a better fluid cell. Ideally, one would like an atomically sharp tip that is completely rigid and has an extremely high aspect ratio. Currently the sharpest tips commercially available are made with single-walled carbon nanotubes – having a perfect aspect ratio over many hundreds of nanometers, and a 5 nm radius. While these are expensive, improvements are being made at a rather rapid rate. The fluid cell is unfortunately making much slower progress. An ideal fluid cell would allow buffer to be flowed over the sample, without perturbation of imaging conditions. In addition, it should allow large scan sizes and Tapping Mode to be used.

The sample preparation for AFM centers around maintaining the integrity of the sample, while assuring the ease of use of the AFM. As we will see in the next sections, one of these is often compromised for the experiment to work. The major difficulty is that most biological samples do not stick to mica – the most commonly used substrate, because of its flatness on an atomic scale over many microns. If they do not stick, they cannot be imaged. The second problem is that in solutions of higher ionic strengths, procuring a nice image becomes difficult due to a loss of interaction between the tip and
the sample. The difficulty to deal with is the potential destruction of a sample from harsh imaging conditions. The solutions to these problems have been to covalently attach the sample to a modified mica (which makes it stick, for example Vinckier et al. (Vinckier et al., 1995)), to chemically fix the sample with a reagent such as glutaraldehyde (to make it stick, for example Mou et al. (Mou et al., 1996b)), to freeze or to dry the samples (which creates rigidity, for example Zhang et al. (Zhang et al., 1997)), image under alcohols instead of water (which removes the ionic strength problem, for example Hansma et al. (Hansma et al., 1995)), and/or to create a 2-D crystal of the sample (to create rigidity, and periodicity, for example Muller et al. (Muller et al., 1996)). While all of these methods introduce their own set of problems, they have allowed the field to progress at a reasonable rate.

1.3. Biological AFM — The Good, The Bad, The Ugly (history)

From its earliest days, AFM imaging of biological samples has been dogged with misinterpretations and artifacts. One of the most famous examples is the early resolution of the B-DNA helix adsorbed to graphite, which later turned out to be a section of graphite that had approximately the same period as B-DNA (Lindsay et al., 1989). However, the field has come a long way since then, providing a great deal of insight into biological systems, and allowing us to visualize processes on the nanometer size scale — a process never dreamed possible. In this section, I examine the more recent history of biological AFM. I will go over the simple air experiments, then examine the direct fluid results, the cryo-AFM, two dimensional crystal arrays, and the force (ligand-rupture) experiments.

1.3.1. Biology in Air

This large amount of data is presented chronologically. In general, the technique for imaging in air is Tapping Mode AFM. This allows minimal tip — sample interaction, so the sample is not destroyed by the tip (which can occur frequently in contact mode). In all experiments mentioned in this section, a small aliquot of the sample is adsorbed to mica, rinsed, then dried under a stream of nitrogen.
The cell has been the target many AFM experiments. One example of this is the imaging of an acetylcholine receptor in frog cells (Lal and Yu, 1993). In this experiment, the AFM could resolve the known pentameric structure of the receptor quite easily. In addition, this high resolution was seen over many microns, laying the groundwork for the study of receptors in their native state without the need for crystallization.

Tapping Mode in air has been used to determine bend-angles of DNA binding proteins. This area of research has traditionally been done by EM, but the higher resolution AFM offers makes this the more viable technique. Using this technique small organic molecules have been shown to bend DNA (Hansma et al., 1994a). This technique has been effective in providing pharmaceutical scientists with information on drug binding. SFM has also been used to determine the stoichiometry of binding of these DNA binding proteins (Wyman et al., 1995). Heat-shock Transcription factor 2 was found to require 2 trimers to associate HSF2-bound DNA sites. In addition to this, the approximate location of the binding site along the length of the DNA was found (Wyman et al., 1995). The binding site of HMG1 was also found by AFM, by a non-AFM-specializing group. This demonstrates the ease of use of the AFM (Gibb et al., 1997). The study of RNA secondary structure has also been an area of recent AFM activity in analysis of common secondary structures. A novel Stem-Loop structure for the HSP70 operon was discovered - the stem-loop-loop-stem model. This has huge implications in the mechanism of action of heat shock proteins, as the secondary structure of the RNA can determine how and when the proteins are transcribed (Ohta et al., 1996). Other transcription factors have been analyzed quantitatively, such as AP2 (Nettikadan et al., 1996). This study showed the mapping of the AP2 binding site on the DNA, and the apparent dimerization of the molecules was observed. Another DNA binding protein study studied looping under the effect of Ku and DNA-dependant protein kinase (Cary et al., 1997). These proteins are involved in the process of double strand break repair. It revealed that Ku is capable of repairing these DSB without the aid of DNA-dependant PK. However, DNA-dependant PK was found to loop the DNA in a similar manner.

Another area of DNA study done by AFM is the higher level study of nucleosome structure. This is one fundamental area of interest among biologists as it seems to hold the key to many DNA related questions. In this experiment (done before the X-ray
structure was known), the 6-8 particle substructure of the nucleosome was seen (Martin et al., 1995). These results were later confirmed in high detail by the X-ray structure (Luger et al., 1997). In addition a high resolution AFM study was done to determine the conformation of nucleosomes (Fritzsche and Henderson, 1996). This showed that AFM could achieve the same results as electron microscopy, without any averaging.

In the molecular biology world, observing DNA using AFM has led to new techniques. Revet and Fourcade (Revet and Fourcade, 1998), observed the change in ligation of DNA fragments depending on different salt concentrations. This allowed the groundwork to be laid for ligation analysis, and short length base sequence interactions.

The AFM has also been used to study the ultrastructure of biological fibers. Much insight has been gained in the area of collagen research and the study of intermediate filaments. For example, the formation and assembly of Paired Helical Filaments (PHF) and collagen have been studied (Goh et al., 1996). The AFM revealed that PHF had a right handed twisted ribbon structure previously thought to be intertwined filaments. This showed the mechanism of assembly to be nucleation elongation, instead of a stage-wise assembly. In addition, the mechanism of assembly of collagen was also been determined, and its growth visualized in stages by AFM. Another study of Amyloid-β was used to determine the ultrastructure (Stine et al., 1996). They were able to determine the molecular organization of amyloid, and see some of the stages of growth of these types of fibrils. This study lay the groundwork for the fluid mode study of amyloid growth mentioned below. The structure of FLS collagen has also been determined (Paige et al., 1998). In this case, small details of the structure were revealed that have never been seen by any other technique. This allowed the authors to speculate on the nature of assembly of this collagen. The triple-helical Schleroglucan has been imaged in air, along with Xanthan, Gellan, and κ-Carrageenan (McIntire and Brant, 1997). The main focus of this study was to determine the ability of AFM to find the sizes of these fibrils. Using polystyrene sphere standards, good agreement was found. In general, height calibration in AFM studies has been problematic – especially when the sample is dried out. While usually attributed to adsorption issues, the use of an internal standard helps greatly.

Polysialic acid, the causative agent in bacterial meningitis, was found to form filament bundle networks by AFM (Toikka et al., 1998). This unexpected result
suggested new mechanisms for the interactions of cell adhesion molecules. In addition, the degradation of these filaments was observed by sialidase. In this case the degradation of the filament was carried out outside the AFM, then periodically samples were taken and dried for AFM observation.

To date, the highest resolution air study produced by AFM showed protein secondary structure (McMaster et al., 1996). In this instance membrane proteins were collapsed onto a mica substrate, and the deflection mode image revealed a periodicity consistent with a β-Sheet. This result confirmed what was known by X-ray crystallography. It appears to be a relatively isolated event, as normally it is almost impossible to achieve this kind of resolution by AFM.

1.3.2. Biology in Fluids

The singly most appealing feature of the Atomic Force Microscope as applied towards biological systems is its ability to operate in conditions that are physiologically accurate. The invention of Tapping Mode in fluid allows minimal force to be applied to a sample, while examining it in its native state (Hansma et al., 1994b; Putman et al., 1994). While there are a wide variety of techniques for utilizing the AFM in this way, they are all quite similar. Specifically, a drop of sample is spread on freshly cleaved mica, and imaged while under this drop. Modifications to this procedure include drying the sample as in the above section, rewetting it, then imaging, flowing buffer over the sample to wash away the loosely attached molecules, chemical modifications of the mica to increase its 'stickiness', and/or the application of fixatives. In general, the simple drop, wash, and look method would seem to contain the most relevant information, with chemical fixatives decreasing resolution, and a drying step introducing some preparative artifacts. Again this section is presented in chronological order of the more recent papers in this area.

One of the first examples of the success of this technique is the imaging of microtubules (Vinckier et al., 1995). Starting a now popular trend of examining the samples both in air and under fluid, this group found a new problem with fluid imaging – the apparent decrease in fibril diameters when hydrated. In addition, they examined a
new method for immobilizing large biopolymers to a silicon wafer. This is of great necessity in fluid, as the sample is often very loosely bound to the substrate.

The ability to examine in fluid, where biological specimens are still active, allows the introduction of real time experiments. In this case, the same protein or other sample is continuously imaged over time. The results have been quite stunning. This technique was used to show the growth of a potassium channel (ROMK1) when exposed to the biological activator ATP (Henderson et al., 1996). By isolating individual protein molecules attached to the surface, then flowing an ATP containing buffer into the liquid cell, the conformation (growth) change of this protein was observed. This growth is of a single molecule! Not a measured average that would be observed using any other technique available. Another biological process in motion, under the effect of ATP is shown in (Oberleithner et al., 1996). In this case TATA-Binding protein multimers (14 subunits) where shown in real time to dissociate under the influence of an ATP containing buffer. This showed how proteins could be translocated into the nucleus – by binding the TBP, dissociating it, then entering the nucleus. One of the most exciting papers in recent memory also falls into this category. For the first time, a single molecule of RNA polymerase (RNAP) was seen in action, moving down a strand of DNA (Kasas et al., 1997). This incredible feat allowed the production of an AFM movie of RNAP in action. The rate of translocation and action of the enzyme could be seen. Finally, speculated mechanisms of action of many systems had an opportunity to be proved. The activity of a RNAP mutant was also studied (Thomson et al., 1999). In this case, the activity of the RNAP attached to a modified surface was explored, revealing the molecules orientation when transcribing DNA. In addition, the products of the reactions were then dried to reveal the large RNA transcripts produced by the RNAP activity. This unequivocally demonstrated the activity of the enzyme under in vivo conditions.

Live cell imaging has been one of the truly great advances AFM in liquid has allowed. Unfortunately, high resolution imaging has proven difficult, mostly due to the mobility of the phospholipid membrane. One higher resolution study applied the well known patch clamp technique to AFM (Lärmer et al., 1997). This is where a glass pipette is used to isolate a single ion channel on a cell’s membrane. In this case, once the channel was isolated, it was transferred to mica for imaging by AFM. This allowed the
researchers to pinpoint the size and location of many proteins within the membrane of a single cell. Again it is important to realize these results are not averages — they are actual visualizations of single molecules. The real time imaging of cells has also been done in the force mode experiments combined with height mode imaging. Using these two techniques simultaneously, the mechanical properties of living cells have been studied (A-Hassan et al., 1998). In these experiments, elasticity measurements were isolated from topography to reveal the difference between free membrane surface and that attached to an adjoining cell, among other things.

As expected, the chase for high resolution DNA images poured over into this area of AFM as well. Using the breakthrough MacMode technology, the kinking of small DNA circles was revealed (Han et al., 1997). MacMode is Tapping Mode driven by an oscillating magnet, instead of an acoustic oscillator. The authors were able to show the difference in kinking of DNA in various salt conditions. In this high resolution study, it was almost possible to envision the helical turns of the DNA, but that result is subject to a great deal of interpretation. Another area of interest is to simply explore processes of DNA molecules, without trying to take accurate height mode information. This has led to an attempt to use phase mode images to watch DNA in motion across a substrate (Argaman et al., 1997). DNA achieves high contrast in fluid phase imaging, allowing high speed high resolution of DNA molecules in motion. In addition, protein-DNA interactions have been studied in real time (van Noort et al., 1998). Using a modified fluid cell set-up that allows longer term stability, allowed them to demonstrate some intrinsic properties of DNA molecules, which helps our understanding of this fundamental molecule. In addition, the ability to find damaged DNA using photolyase, and photolyase's activity towards undamaged DNA was shown as the enzyme moved along the DNA strand.

Phase imaging has been related to the stiffness of sample among other ideas. Using this principle, the relative stiffness of Amyloid β protein was examined (Rhee et al., 1998). It was found, that the stiffness of the channel formed could be modified, depending on the buffer flowed in. This correlated well with calcium uptake studies, allowing the mechanism of action of Amyloid proteins to be examined.
Using the AFM as an analytical instrument is also being attempted. This offers a unique opportunity to measure the hydrated size of individual protein molecules. Recently, this idea has met with limited success as molecular volumes were shown by AFM to correlate to molecular weight (Schneider et al., 1998). The most difficult aspect of this area is taking into consideration tip convolution. In this case, the approach was to take the height of the protein as its diameter, and assume it was a sphere for volume calculation. This is the most accurate AFM measurement – with resolution often estimated to be on the order of 0.1 Å. While large differences can be identified, proteins around 150 KDa in size were almost indistinguishable.

Apolipoprotein(a) has also been studied by AFM (Xu, 1998). This is the so-called 'bad' cholesterol, where high levels have been linked to coronary disease. In this study, the tertiary shape of this molecule was ascertained for the first time, showing how the protein attaches Low-density lipoprotein particles to one another. AFM is the only technique that allows resolution of structures at this level. Both NMR and X-ray have limits far below the size of this molecule – whose molecular weight is on the order of 1000 KDa, and the nature of proton, neutron, X-ray, and light scattering would miss this detail as well. This has given a great deal of insight into the function of lipoprotein(a) and thus how it functions in heart disease.

The structure and motion of single proteins has also been measured. In one case the extracellular matrix protein laminin-1 was studied (Chen et al., 1998). This large protein (900 Kda) is much too large for its structure to be seen by any method other than AFM. In fluid, a cruciform structure was observed for the laminin-1, and surprisingly in fluid the motion of an arm was observed. This study laid the groundwork for the examination of the interactions between all ECM proteins, and should provide insight into how these proteins function, especially during the development of new tissue.

1.3.3. Biology by Cryo-AFM

Cryo-AFM is a specialized area of biological AFM in air. This method has produced by far the highest resolution protein images known to AFM but is limited to one research group. A good review of the instrumentation can be found in Shao and
Zhang (Shao and Zhang, 1996). In this process, the AFM is placed at ambient pressure, under liquid nitrogen vapour at a temperature close to 77 K. This freezing of the biomaterials allows for two advantages to be gained: the sample readily sticks to the mica substrate (likely held by ice), and a large stiffness is gained by the molecules, allowing more detailed probing, without damaging the specimen.

First published in 1995 (Han et al., 1995), the cryo-AFM was used to image IgG molecules in contact mode, proving their speculated ‘Y’ structure with outstanding clarity. Later monomers of IgG could be distinguished from dimers, trimers, and tetramers (Zhang et al., 1996). In addition, IgM molecules were visualized, and while pentameric as speculated, a definitive non-planar arrangement called into question the mechanism of action of the entire complement system. This microscope also has a temperature control (77 to 220 K) which allowed the probing of the Youngs Modulus of an IgG molecule. Further, the highest resolution of a red blood cell ghost is seen, with features as small as 4 nm. α2-Macroglobin domain structure was also determined, and confirmed those results achieved by cryo-EM (Zhang et al., 1996). It is often the case that AFM can get the same results and more as EM on a sample treated the same way. The most recent experiment published from this group is a study of Smooth Muscle Myosin (Zhang et al., 1997) – the protein that helps make muscles work. In this case, the head and tail could be clearly visualized, and much insight was gained into the mechanism of muscle movement.

1.3.4. Biology of two dimensional biological crystal arrays

The other solution to giving a rigidity to biomolecules was the formation of two-dimensional crystals along with some phospholipids. These are much easier to make than full-fledged crystals, and appear to only be limited by the makers ability to use a Langmuir-Blodgett trough or luck in finding the correct sample. The sample preparation is simple, either the crystals are formed and put onto mica, or the proteins are incubated for a long time on mica then washed, afterwards they are imaged in either contact or Tapping Mode under preferred buffer. In general the results fall into two categories –
detecting sub-unit structure, and/or observing a conformational change. The study of a conformation change has been limited to membrane proteins.

The first crystal examined was that of an E. coli pore protein OmpF (Schabert et al., 1995). In this case the crystal structure of the protein was known. The tight packing pores could be observed down to 10Å horizontal resolution. In addition, as the tip was rastered across the surface, enough energy is built up in the crystal to activate a conformational change. This allows the monitoring of function when exposed to various buffers, and establishes proof of actual motion within a protein. This idea was next applied to Aquaporin-1 (APQ1) (Walz et al., 1996) a protein that regulates water content inside the red blood cell. This protein's projection maps had been determined by electron crystallography, but were of relatively low resolution. The AFM experiment revealed the tetrameric shape of this protein in great detail (0.9nm resolution), and an asymmetry across the axis of the membrane. This allowed the authors to speculate on the nature of the mechanism by which osmotic pressure is controlled in the cell. This technique was further applied to Ø29 bacteriophage connectors (Muller et al., 1997). This was a well studied system, but the AFM study was able to lay to rest a number of controversies. First of all the high resolution nature of the study allowed the accurate determination of the 12 subunit structure where it was unknown to be 12 or 13. In addition, they were able to clearly show the stylus protrusion of the connector which they were able to move by increasing the force applied by the AFM tip.

The first non-membrane protein successfully imaged was the notorious GroES oligomer (Mou et al., 1996a). This is the chaperone that helps other proteins fold. This AFM study was published just after the solution of the crystal structure (Hunt et al., 1996), but at the time of the experiments, only low resolution EM data was available for comparison. In this case the crystals formed naturally on the mica surface with the help of some PEG and ammonium molybdate, and perhaps some would not consider this a 2-D crystal. It is considered in this section as the tighter packing of the molecules leads to a great deal of stability of the sample. Regardless, the 10 Å resolution achieved showed a 7 fold symmetry in the protein. In addition, a central protrusion also of 7 fold symmetry was discovered. This high resolution study was done without image averaging (as was performed in the EM study), and was later confirmed by the crystal structure. This led to
a new explorations and insight into GroES and GroEL function. Further investigations of this system led to the demonstration of the conformational changes of GroES and GroEL in the presence and absence of ATP or its analogs (Mou et al., 1996a). A second example of this mysterious formation of crystals on a mica surface was performed on the HPI layer of the purple bacteria membrane (Muller et al., 1996). This HPI layer was increased in resolution when chemically cross-linked. A six-fold symmetry was revealed as well as a conformational change. This conformational change involved the appearance and disappearance of a plug in the center of the pore repeatedly on a single molecule. This system had been examined by STM and EM previously (in a vacuum) but the AFM results correlated well with these studies.

Expanding on the idea of using a crystal lattice to hold proteins in place, some groups have turned to placing a dense layer of protein in/on a phospholipid bilayer. In one example of this, an α-hemolysin mutant was studied to determine its assembly and structure (Fang et al., 1997). This pre-pore was determined to have a heptameric structure, which was known from previous X-ray crystallographic studies. In conjunction, the assembly process could be stopped at certain points by withholding key ions thought to be involved in the process. This led to the confirmation of the current model of the αHL assembly. Further study by this group has led to the discovery of a mushroom head to the heptamer, and found the driving force of the oligomerization to involve subunit-subunit interactions in this mushroom head (Malghani et al., 1999).

1.3.5. Force experiments of Biological specimens

The final category of biological AFM experiments to be discussed is that of ligand - rupture experiments. These experiments are generally carried out in buffer conditions, and employ the AFM in force mode. Force mode is where the AFM tip is simply slowly brought into contact with the surface, then pulled away. In these experiments the surface is coated in one protein, and the tip coated in a second protein – usually known to bind the first. The researcher can then repeat thousands of force curves, and average them, attempt to deconvolute the curve, and interpret various qualitative and quantitative bits of information.
The results of this area’s first experiments started appearing in 1994. The first target was the extremely well known and well studied system of biotin and avidin (Florin et al., 1994). In this study simple binding forces were studied quantitatively. It was found that the rupture force between biotin and avidin was 160 pN and 85 pN for iminobiotin. These measurements by far exceeded the sensitivity of any previous method – however the methodology itself has been placed under great scrutiny as the interpretation of a force curve is very debatable. However the field plodded along and using the avidin-biotin, and actin monomer-monomer forces a model for the correlation between intermolecular forces and energies was devised (Moy et al., 1994). This study was also able to observe the separation of the two molecules at various pH values, and implicate various residues in binding energy. Finally this model was confirmed by a molecular mechanics simulation (Grubmüller et al., 1996). The simulation further found great details in the nature of the ligand rupture, showing a multiple-rupture pathway, and a water enhanced stability during separation. Inevitably, the actin studies led to studies between it and heavy Meromyosin (Nakajima et al., 1997). In this case a (single) HMM molecule is attached to the AFM tip via a fluorescent acrylamide nanobead (30 nm diameter), and various chemical crosslinkers. Next, this tip is lowered onto an actin filament (both can be seen by fluorescence) and thousands of force curves are recorded. Once again these force curves are interpreted so that certain individual jumps correspond to certain molecular events. These authors note that the smallest movement detectable is 0.15 nm. In this case they found long range forces pull the molecules into contact with each other, and that the binding force can be determined. Piecing these bits together, the evidence went to support the theory of a hand over hand type movement of myosin along the actin chain.

Another area of study among this area has been the innovative but controversial molecular sandwich technique (Oberleithner et al., 1997). In this case, an AFM tip coated in protein (ROMK1) is lowered into contact with a flat mica surface. After thermal equilibration, the deflection of the cantilever can be measured to determine flexibility in the protein's motion – on the order of 0.2 nm and 1 ms! Upon the addition of ATP the authors were able to see the activity of the protein as a function of change in structure. In
addition, this activity can be compared at different pH values, as a quick and easy method to determine the protein’s pKₐ value.

This technique has been combined with a light emitting tip to analyze the conformational dynamics of bacteriorhodopsin (bR; Rousso et al., 1997). By exciting the bR sample with a 532 nm laser when trapped in the Frank-Condon state (i.e. the excited stable state of bR), the authors were able to observe conformation changes on the microsecond time resolution. These results called into question the currently accepted model of the coupling mechanism of bR.

Another type of force that has been measured by AFM is that of unraveling a protein. In this case the protein tenascin was studied (Oberhauser et al., 1998). This protein is large. It contains fourteen highly elastic domains called EGF domains. These domains are all linked together by disulfide bonds, so can be considered chemically joined. Once attached to the AFM tip, this protein was stretched, and the retraction curve is recorded. Fourteen distinct 137 pN events are observed in a saw-tooth like pattern, occurring at a spacing of 25 nm. This pattern was concluded to be individual EGF domains stretching one at a time. This result was confirmed using a Monte Carlo simulation, in which the domains are assumed to behave like springs. The most controversial area of this study is that the EGF domains stretch individually in series, instead of in parallel as one intuitively suspects.

One of the most important factors to consider in the interpretation of these types of experiments is the orientation of the biomolecules as they come into contact with one another. In general, it is assumed that one of the ligands is free moving enough to bind the other in its optimal orientation. This idea has further been explored by Yip et al. (Yip et al., 1998) using the blood sugar controlling protein Insulin. Insulin can be easily modified, so it can be attached to the tip or surface in several different orientations. It is also known to form a dimer, so this was the interaction studied. In addition, the monomer-monomer contact points are known, so can be modified for the experiment. These experiments gave insight into the exact manner in which insulin monomers bind one another, giving ideas for values of interaction energies, and forces.
1.4. RecA Protein – Background and History

RecA protein was discovered in 1965 by Clark and Margulies (Roca and Cox, 1990). It was found by examining a cell line that was deficient in its ability to reproduce under stressful conditions. The protein is 38 KDa, and was first found in *E. coli*. Since then analogs have been found in all other living systems from bacteria to humans (Ogawa *et al.*, 1993). The major interest in this protein has been to examine its structure and function and their roles in relation to RecA’s strand exchange capabilities, and mutagenic activity. In this section, I outline the major contributions to this search from various fields and demonstrate the areas that require AFM to advance and prove hypotheses.

1.4.1. Electron Microscopies

Fibers of RecA were visualized by Negative Stain Electron Microscopy (Williams and Spengler, 1986). This is one of the earliest EM studies. In addition, RecA protein complexed with singlestranded X174 DNA was observed. For the first time banding was seen on the fibril. This was determined to be a RecA helical fiber. It was short and had a “open helical form”. It was found that incubation with ATPγS (an unhydrolyzable analog of the RecA activator ATP) produced the same result. With longer incubation times the fibrils became longer and bundled. The pitch of the helix was found to be 5.5 to 6 nm. The handedness of the helices could not be determined. Upon binding to the single stranded DNA, an elongation of the helix to 7.5 nm was found. Some other negative stain/no stain EM results show the fibrils to be 10 nm in diameter (Register and Griffith, 1985). While long and containing no helix, these filaments were thought to contain contaminant RNA. By late 1986, the handedness of the RecA-DNA complex was determined by EM (Egelman and Stasiak, 1986) using a three – dimensional reconstruction. The pitch of the RecA-DNA complex was determined to be 95 Angstroms.

The self-assembly of RecA was examined by EM as early as 1988 (Brenner *et al.*, 1988) in conjunction with light scattering. They found several states to RecA assembly. A small particle 4 nm in diameter, a large particle 12 nm in diameter (thought to be rings of RecA), 10 nm diameter rods 50 to 200 nm long, and large bundles of these rods. In
general, the RecA protein was postulated to go through of these stages as it self-assembled. It was also postulated that the monomeric form was not predominant at physiological conditions, so could not be the main intermediates for assembly onto DNA. An excellent resolution study of RecA protein and its DNA complexes was done by quick-freeze/deep-etch EM (Heuser and Griffith, 1989). In this method, finely ground mica flakes are mixed with the sample, then quickly frozen, and replicated in 2 nm of platinum. This platinum is then imaged by TEM. Individual particles were found (monomers) to be 5.1 nm (this includes the 2 nm of platinum) as well as rings of 14.1 nm rings of 6 nm in height. A hole in the center of the ring corresponded to 4 nm. This led them to confirm a hexamer form of RecA previously only speculated to exist (Neuendorf and Cox, 1986). In addition, the RecA protein rods of 12 nm diameter and 100 to 200 nm length are also observed with a right handed helicity of 6 nm period. Bundles of rods are observed as before. An effort is made to show the assembly process of these rods – as they are seen in conjunction with hexamers and monomers.

Now that most of the basic information that electron microscopy could reveal had been produced, these microscopists turned to the more in depth analysis EM allows. The first example of this is Yu and Egelman in 1990 (Yu and Egelman, 1990). By trapping the RecA filament in an active form, they proceed to perform a three-dimensional reconstruction and find two domains to the RecA structure. These 2 domains were equal in size. It is theorized that one domain binds the nucleotide cofactor required for activity and the other binds the target DNA. Further pursuing their analysis Yu and Egelman (Yu and Egelman, 1992) compile and compare all the known EM data of the RecA filament and the nucleofilament. Using a STEM for detailed mass analysis, they find that the elongation of the pitch of the helix leads to a different binding capacity of each form of RecA, proving that that while the two structures look the same, they are not necessarily interconvertible.

While this structural work was being carried out, function studies were also being performed. In 1993, Revet and coworkers (Revet et al., 1993), directly mapped the homologous DNA targeting ability of RecA by EM. These unstained specimens were shown alone, and then bound to a probe coated with RecA. The RecA only covered the specific section of DNA at which the homologous pairing occurred. This was the first
direct evidence that RecA could correctly position homologous DNA probes onto a larger DNA structure. It was hoped that this method could be used for sequence identification and mapping in large areas of DNA. With all this information about RecA known, it was still unknown if any of these structures formed in the cell. By producing a strain (Ruigrok et al., 1993) of E. coli that overexpressed RecA, Ruigrok was able to show the bundles of rods in inclusion bodies in these cells. This finally showed that RecA could exist in this semi-crystalline form in the cell. However, since this was inside an inclusion body, the authors could not say for sure whether this structure had any biological relevance. Another major EM landmark for RecA was the discovery of its eukaryotic analog in Saccharomyces cerevisiae (Ogawa et al., 1993). It was found to have similar activity to RecA, in its homologous recombinatorial ability. The 9.2 nm pitch of the Rad51 helix when bound to dsDNA is virtually identical to RecA. Another first for the EM was the demonstration of a triple stranded DNA inside the RecA filament (Jain et al., 1995). This result confirms a means by which RecA could orchestrate homologous recombination, which is by containing more than two strands of DNA in its core.

1.4.2. Light Scattering

Light scattering was first used in 1985 to examine the aggregation of RecA (Morrical and Cox, 1985). It was found that in the absence of DNA, RecA assembles into rigid rods of lengths on the order of 1 micron or greater, which is much longer than the EM results would predict. These results strongly favoured a the formation of rigid rods with little or no flexibility. Unfortunately, due to this rigidity, light scattering could not be used to measure filament length, but for some reason that is unclear, a length of greater than 1 micron was determined. This study found a critical RecA concentration for filament formation. In addition, this self-assembly reaction was found to compete with the DNA binding reaction. When using EM at the same time Brenner et al. (Brenner et al., 1988) multiple discrete aggregation states are found. At different concentrations exponential or sigmoidal light scattering curves were seen. This led the authors to propose the steps to be monomer to hexamer, hexamer to rod, and rod to bundle of rods. The explanation lay in the fact that if an older sample (which had already formed rods)
was diluted under the right conditions, the rods came apart quickly then slowly back together (a sigmoidal curve), whereas if they did not dilute it, a simple exponential curve occurred. They explained the large length of RecA filament previously seen as merely aggregations of rods. By 1990, light scattering was being used as a method to measure the kinetics of RecA assembly (Wilson and Benight, 1990). The assembly was found to be biphasic, the first phase being filamentation, where the rods are formed, and the second being bundling, where the bundles are formed. They found that at a certain molecular weight (or filament length) the rods began to bundle, and claimed that both states occur inside the cell. Thus light scattering has been used primarily to show the aggregation of RecA into bundles via a distinct pathway.

1.4.3. Small angle neutron Scattering

Small angle neutron scattering has limited applications to this system. Two examples will be discussed. The first shows the change in the helical pitch of the RecA filament upon binding nucleotide cofactors (Ellouze et al., 1995). It was found without any cofactors the pitch of the helix was 7.0 nm, and upon addition of ATP that increased to 9 nm, but with ADP it only increased to 8.2 nm. This led to the conclusion that more than two types of RecA rods can occur, and that the cofactor may even compete with DNA for RecA binding. This result excluded a two state model for RecA, finally the realization that this was a complex system was made. In addition, this technique has been used to determine the orientation of DNA bases inside the RecA filament (Norden et al., 1998).

1.4.4. X-ray Crystallography

The RecA crystal structure was solved in 1992 by Story et al. (Story et al., 1992). At the same time, the RecA co-crystal with ADP was solved (Story and Steitz, 1992). This was a co-crystal with ADP. To date no other RecA crystal structures are known, which is a bit odd, seeing as these bundles of rods seem to form so readily. This 2.3 Å resolution structure provided insight into the fine molecular structure of RecA, and resolved many structural ideas proposed by EM. The structure showed a central domain
rich in β-sheets, with two smaller domains nearby. An unresolved α-helix was found that separates from the structure implicating it as the DNA binding domain or a RecA binding domain. The polymer structure showed a 8.27 nm pitch and had a diameter of exactly 12.0 nm, in good agreement with the EM results. Using this crystal structure, the atomic resolution structure of the hexamer was also derived. It was found to be 6 nm high, 12 nm in diameter, and contain a central hole of 4 nm. The co-crystal allowed the exact determination of the ATP binding site, but provided little other new information.

1.4.5. Scanning Tunneling Microscopy

Although the STM studies have both been done on the RecA-DNA complex, it seems more relevant to mention them, due to the nature of the technique. This represented a major landmark in STM as it is the first time a biological sample was imaged successfully by STM. In the first effort, freeze-dried complexes were coated with a thin layer of a Pt-Ir-C film (Amrein et al., 1988). This preceded the same result found by the metal coated SEM experiments by about a year. The width of the structure corresponded to 12 nm, but the height was depressed – only 7 nm likely caused by adsorption to substrate. In addition, they claimed to be able to resolve a “tripartite structure” on the top of the structure, corresponding to the number subunits visible per turn of the helix. Indeed this metal coating experiment provided an easy high resolution method that did not require averaging to achieve the results. Unfortunately this method was not pursued for the examination of RecA. This has due do mainly with the high degree of skepticism towards the field at the time. However, one more landmark paper was published by the same group. This time they imaged the RecA-DNA complex in ambient conditions in their STM (Amrein et al., 1989). While STM should not be able to image non-conducting specimens, it was speculated that the tunneling current was achieved via the water layers coating the sample. While the result produced the same information as the previous metal coated experiment, a great deal of image processing was required to “find” the nucleofilament. However, data corresponding in size to RecA monomers, as well as the period of the nucleofilament was found.
1.4.6. Atomic Force Microscopy

In their pursuit to image DNA with the Atomic Force Microscope, the Paul Hansma Group briefly experimented with using RecA to identify DNA. This effort met with limited success, and it seems the results of these experiments were never published in detail. However, they are mentioned in passing (Hansma et al., 1995) in a review article. They found that RecA was able to elongate their X174 DNA so that they could visualize it by Tapping Mode under dry helium. A 10 nm diameter was found for the filaments – with dsDNA, and ssDNA in the core. In addition, “fused” complexes were visualized between vectors, but the resolution of this structure was impossible given the large nature of the tip. Mentioned again by a different set of authors in another review in the same year (Lyubchenko et al., 1995). Again this study was performed in air. The authors found the DNA complexes to be very circular and rigid in nature. A high order 20-30 nm periodicity is seen, caused by either the AFM tip, or higher order structure never before seen.
2. Methods
2.1. Sample Preparation

RecA protein was purchased from Sigma, or New England Biolabs. No difference was found between the two sources. The protein was spin – dialyzed using a 30000 molecular weight filter (Millipore) to change to the desired buffer. A 10000 molecular weight filter can also be used. In this process, the sample volume is increased to 400 μl then spun down to a minimal volume. This allows a dilution of one buffer to another without dilution of the sample. The solution was buffered between pH 7 and 8, with 10 to 20 mM TRIS-HCl, HEPES, or MOPS (Sigma), and contained 10 mM magnesium chloride, 0.1 mM DTT, and sometimes 0.1 mM EDTA. Once dialyzed, the samples were incubated overnight at room temperature or at four degrees Celsius. Samples were diluted to desired concentration for imaging one to six hours before a 60 microliter droplet is placed on a one cm³ piece of freshly cleaved mica (Ted Pella). The sample is allowed to reach thermal equilibrium in a high humidity environment until it is imaged up to 48 hours later. Optimal imaging conditions usually occur in three to six hours. Since imaging occurs such a long time after deposition, this allows fibril formation without incubation at 37 degrees Celsius.

2.2. Atomic Force Microscopy

All experiments were performed on a Nanoscope III (Digital Instruments) using Tapping Mode in fluid. This is where the cantilever is oscillated at a resonant frequency. The resonating tip is then brought very close to the sample, and topography is determined by local changes in the amplitude of oscillation (i.e., the piezoelectric is raised or lowered to maintain a constant amplitude of oscillation). In this case, a triangular Si₃N₄ with a nominal spring constant of 0.06 N/m was used and oscillated at a resonance peak between 16 and 36 KHz. The resulting data was collected in height mode and amplitude mode simultaneously. A height mode image displays the topography of the scanned sample – that is the amount the piezoelectric crystal has to move to maintain a constant amplitude of tip oscillation – as a function of position on the sample. An amplitude mode image can be considered an error signal. It displays the root mean square amplitude of
oscillation of the tip as a function of location on the surface. Both positive and negative values are allowed. In general, this mode of imaging can give an increased silarity of image as it is very sensitive to changes in topography, with larger changes often generating a greater change in RMS cantilever signal.

Choosing the resonance peak is not an easy task in fluid. While there are many theories on which peak should be used, it is generally necessary to perform the experiments at various peaks until one with stable desirable characteristics is found.

In general, Tapping Mode in fluid uses an O-ring to seal the fluid cell during operation. While this setup allows for flow-through experiments, it has several drawbacks, and generally makes the instrument technically difficult to use. Since my experiments do not require flowing buffers, the O-ring was not used. Instead, the edges of the mica square were surrounded by a thin layer of Teflon tape, which holds the liquid droplet in place. If desired, buffer could be flown onto the sample through the cell using a hose and syringe.
3. Results

Imaging RecA fibrils in solution is no easy task. While the AFM is capable of imaging materials under solution, it can only do so if the sample of interest remains reasonably immobilized at the substrate/solution interface. Thus, the first challenge in any solution imaging is to determine the conditions that will enable this. We observed RecA protein under various ionic strengths (10 to 20 mM) and neutral pH buffers (MOPS, HEPES, or TRIS). The results obtained were similar for experiments conducted under true physiological strength (PBS), and for those performed at lower salt and buffer concentrations. A typical image, in Figure 1 shows fibrils of 12 nm diameter and mini-fibrils of 6 nm diameter, along with a large number of monomers, hexamers and various non-specific aggregates in the background. Notice the smearing that is present in the image. This serves to emphasize one of the difficulties of AFM imaging of proteins in solution: The proteins that are loosely immobilized to the substrate can be moved or dislodged easily, while those in solution may adhere to the probe tip, effectively distorting it. The resultant smearing and other tip artefacts are typically more pronounced than occurs under ambient conditions and are subject to some interpretation. Our interpretations are discussed in the Methods section. In addition, changes in the conditions of the solution can serve to make the imaging more difficult, which we found happens at salt concentrations higher than 100 mM. This is probably attributable either to a decrease in interaction between the protein and the surface, or an increase in interaction between the protein and the probe tip.

Under the buffer conditions of the experiment, the RecA structures we observed can be classified into four categories: (i) the monomer, with cross-sectional area of 3 nm\(^2\); (ii) the hexamer, a ring-like structure which is 12 nm diameter and 6 nm high; (iii) the rod or fibril, which is 12 nm in diameter and hundreds of nm to \(\mu\)m in length; and (iv) the mini-fibril, which is of smaller diameter than the fibril, and microns in length. The first three structures – monomer, hexamer and fibril or rod – have been reported and discussed in previous literature (Roca and Cox, 1997), while the mini-fibril, and its three distinct varieties, have not been seen previously, and will be discussed in more detail below. We have seen the various mini-fibril morphologies by themselves, or accompanied by other
Figure 1. Atomic Force Micrograph of RecA under physiological conditions. Contrast is generated by topography – with brighter being higher and darker being lower. Four distinct morphologies are shown: The fibril, the mini-fibril, the hexamer and the monomer. In addition there are a large number of non-specific/specific aggregates which may be intermediates in the assembly process. The fibril is 12 nm in height. The mini-fibril is 6 nm in height. The apparent doubling of the mini-fibril is due to convolution of the image. Size bar = 500 nm.

structures, such as other types of mini-fibrils, rods and hexamers. However, there was no correlation between the various imaging conditions we used and the types of fibrils we found: All structures could be seen in all salt conditions and all buffer conditions utilized. By increasing the allotted time for deposition and assembly to occur, an increase in the
concentration of all the different types of structures is observed. Eventually, everything in
the solution ends up adsorbing to the mica surface, and if samples are allowed to sit for a
prolonged period of time (but still kept moist) complete surface coverage occurs. Under
these conditions, one would expect the next level of assembly, the formation of bundles
of rods, to occur. We do not observe such structures presumably because no details can
be resolved with the AFM at this point.

3.1. Monomers, Hexamers, and Short Rods

The hexamer is easily identifiable by matching the dimensions with those
reported from X-ray crystallography (Story et al., 1992). Figure 2 shows a close-up view
of three different hexamers, which are also seen in the background of almost all of the
other figures. Due to tip convolution effects, which effectively broaden an image, it is
difficult to ascertain the lateral dimensions of the sample. However, the AFM has an
excellent vertical resolution and we can determine the height of a sample above the
substrate quite routinely. In this case, the observed height of 6 nm corresponds well with
the thickness of the hexamer from X-ray studies. However, the individual sub-units of the
hexamer are not clearly defined, nor is a hole in the middle observed, as would be
expected from the postulated hexamer structure (Story et al., 1992). This is likely a
consequence of imaging conditions. The force applied by the probe tip in TM-AFM is
very slight, and may be inadequate to break through strongly-bound water layers.

Figure 3 shows an example of the RecA fibril. This versatile polymer begins to
wrap itself up – as two fibrils are seen split apart, then rejoining at both ends. This is
probably a step towards the formation of the well documented bundle of rods seen in EM.
Figure 4 is an example of the same process. This time, the fibrils come together into a
large non-specific mass. If one could resolve such a structure, it is likely a 8 nm
periodicity would appear. Figure 5 further extends this idea. In this figure we see two
additional elements: a much higher non-specific, and specific background, and some
Figure 2. Three typical examples of TM-AFM images of hexamers. In the left column we see the hexamers without any resolved subunit structure or even a clear donut shape. However, the sections in the right column reveal these shapes to all be 6 nm in height, which is exactly as expected. As discussed in the text, tip broadening effects make a lateral width measurement highly inaccurate.
Figure 3. Solution TM-AFM image of RecA fibrils. The fibrils are joined at both ends, but split in the middle. The fibrils have a diameter of 12 nm apart, and 16 nm when joined together. This may be an instance of fibrils bundling to their storage form. In the background we can see a few large non-specific aggregates.
Figure 4. RecA fibrils in the process of bundling. This image, captured under buffer conditions, shows many fibrils of 12 nm diameter joining or aggregating into a single large mass. In the background a large number of hexamers and RecA monomers are seen.
AFM image of RecA under buffer solution. In this case we see two distinct sets of bundles of fibrils, and the associated fibrils moving to form them. In addition, we see some mini-fibrils attached to the fibrils, and a background of hexamers and non-specific aggregates.

Figure 6 shows a slightly different type of bent fibril. These fibrils are 10 nm high above the substrate, and are curvilinear, but do not appear to form closed rings. While this height is lower than the expected 12 nm, we believe this is an artefact of the height measurement of the AFM (Vinckier et al., 1995), and we can classify these as among the...
Figure 6. Solution AFM image of curvilinear RecA fibrils. These fibrils are seen amongst a background of monomers and hexamers. The length of the fibrils extends up to 4 μm. Fibrils with these characteristics have not been observed by other methods. Size bar = 500 nm.

This curvilinear fibril is also seen in Figure 7, but this time in a more locally concentrated way. Mini-fibrils (discussed below) are also present. Seen again in a different variety in Figure 8, the fibril can also make short tight curves. This curve can be seen over and over by itself and in conjunction with other fibril types. These curved fibrils have a uniform diameter, and can continue consistently for over 4 μm. In fact, fibrils shorter than 1 μm, whether straight or bent, are rarely seen, in complete contrast with the EM results.
Figure 7. Curvilinear and SP-fibrils co-exist in solution. Here we see a high local concentration of curved RecA fibrils with a cross-sectional diameter of 14 nm. As we examine the area with increasing resolution, we are able to see that two very long SP-fibrils crisscross through the area. Even in the extreme close up with a scan size of 750 nm it is difficult to determine if these SP-fibrils actually join the fibrils or simply pass by.
Figure 8. A particularly clear AFM image of one of the many forms RecA fibrils takes. We see a 12 nm cross-sectional diameter fibril which also curves gently. In the background we see a bed of monomers, around 3 nm in height. Near the top of the scan, the fibril is cut by the AFM tip – it just goes to show that one cannot be too careful with these sensitive biological samples.

3.2. Mini-fibrils

In addition to the 12 nm fibrils (or rods), we observe many fibrils that are significantly smaller in diameter. For the purposes of this thesis, we will consider all these narrower fibrillar structures under the category “mini-fibril”, although there are a variety of morphologies within this class. The most common mini-fibrils are of 6 nm
diameter, which is exactly half that of the fibrils, examples of which are seen in Figures 1, 4, and 9 - 14. Like the rods, these mini-fibrils can extend for quite a distance (over 3 μm). Some of them, like those in Figures 1, and 5, are generally straight with a few bends. However, they can also be strongly curved, as illustrated best in Figures 6, and 13. While these are primarily aperiodic mini-fibrils, others are periodic, with either 100 nm (Figures 15 - 18) or 150 nm period (Figure 19).

3.2.1. Mini-fibrils with no apparent periodicity

Shown in Figures 7, and 10-13, are ring-shaped structures, containing fibrils of which are 6 nm in diameter (corresponding to mini-fibrils), some which are 12 nm (corresponding to fibrils), and some which are both. Notice the different forms present in these figures. Completely circular closed rings are seen side-by-side with open full-size rings, and some incomplete semi-circles and arcs. Let us examine each of these forms one by one. In Figure 10 we see a series of increasing resolutions scans of the same rings. As we can see from the cross-section in 10(D), the mini-fibril is exactly half the height of the fibril. 10(A) shows a large scan area: we see that these rings occur amongst a background of monomers and hexamers, and often contain mini-fibrils and fibrils on the same ring. In addition, complete rings of mini-fibrils are seen. If we examine this area a little more closely (as in 10(B)), we see the rings are not necessarily complete circles. We also see their ability to overlap without forming fibrils. Finally, 10(C) shows us in greater detail the joint between the fibril and the mini-fibril, showing them clearly joined, but not revealing how. These rings can be seen again in Figure 11. This time the rings are all over each other, but amongst a very low background. The amplitude image shown on the right shows some intense details, and allows you to trace the mini-fibrils along there length to reveal fibrils and mini-fibrils existing on the same strand. The cross-section of the image also reveals that the mini-fibrils are exactly half the height of the fibrils. In addition these rings exist in several distinct groupings. The AFM image shown in Figure 12 presents a closer look at these low background ring shaped fibrils and mini-fibrils. Using the amplitude image on the right makes it easier to trace out the details of this overlapping structure. Once again the section shows that the mini-fibrils (6 nm) are
Figure 9. TM-AFM image of RecA polymer in solution. This scan has three distinct features: A stiff fibril is extended at both ends by curved short mini-fibrils, semicircular mini-fibrils of 6 nm height, and at the bottom some more straight fibrils. In the background we see hexamers and non-specific aggregates.
Figure 10. A, B, C, scans of continually increasing resolution of rings of RecA. In A-C we see the fibrils and mini-fibrils that make up the rings, amongst a high background of hexamers, monomers, and non-specific aggregates. A shows how the rings are clustered. B allows to see the overlap of the rings of mini-fibrils, and the closed ring made up of a fibril and a mini-fibril. As we move to C this fully closed ring is seen and we can see that the mini-fibril and fibril are definitely attached together. The cross-section in D reveals that the mini-fibrils (6 nm) are exactly half the height of the fibrils (12 nm).
Figure 11. A 1.8 micron scan of RecA fibrils in buffer. The image on the left represents topography (grayscale = 25 nm), and the image on the right represents amplitude (grayscale = 5 nm). The section displayed below the AFM images is a cross-section of the topographic image. In this section, we see two different heights – 6 nm (mini fibril), and 12 nm (fibril). The topographic image shows these groups of ring shaped structures, while the amplitude image allows us to more clearly trace the path of the RecA polymers.
Figure 12. A 1 micron scan of RecA fibrils in buffer. The image on the left represents topography (grayscale = 25 nm), and the image on the right represents Amplitude (grayscale = 5 nm). The section displayed below the AFM images is a cross-section of the topographic image. In this section, we see two different heights – 6 nm (mini fibril), and 12 nm (fibril). The topographic image shows these groups of ring shaped structures, while the amplitude image allows us to more clearly trace the path of the RecA polymers. In contrast to Figure 11 we are able to trace the RecA rings a lot more closely. Note that while complete rings of mini-fibrils are observed, complete rings of fibrils are rarely seen.
Figure 13. Stereo images of RecA ring shaped fibrils, and mini-fibrils. Under similar conditions, these two morphologies of RecA fibrils in solution have co-deposited with (A), and without (B) a background of hexamers, monomers, and other non-specific aggregates. Complete strands of the mini-fibril (6 nm height) are observed, along with strands of both fibril (12 nm height) and mini-fibril. Size bar =500 nm in (A) and the scan size is 1.0 μm in (B).
Figure 14. RecA mini-fibril performing an extremely tight hairpin turn observed using TM-AFM under buffer conditions. This 6 nm diameter mini-fibril is slightly shorter than a micron, and is able to turn with a radius of curvature on the order of 30 nm. In the background a large mass of hexamers gather to witness this outstanding event.
Figure 15. In this solution AFM image we can see primarily short period mini-fibrils in the area. These fibrils have a periodicity of 100 nm and a cross-sectional diameter of less than 6 nm. In addition, this area is rampant with hexamers, mini-fibrils, and fibrils.
Figure 16. An SP-fibril combines into a LP-fibril in this TM-AFM image. This lasso type structure has two components: The circle is complete, has a periodicity of 100 nm, and a height above the substrate of around 5 nm, the rope is several microns long, has a periodicity of 150 nm, and extends 7 nm above the substrate.
Figure 17. A 100 nm period mini-fibril observed in buffer by AFM. The overlain section emphasizes the periodicity of the mini-fibril. This fibril is 6 nm in cross-sectional diameter, and extends for many microns. Note the high background of non-specific aggregates.
Figure 18. A 100nm period mini-fibril observed in buffer by atomic force microscopy. The overlaid section emphasizes the periodicity of the fibril. This mini-fibril is approximately 6 nm in height. Size bar = 250 nm.
Figure 19. A 150 nm period mini-fibril observed in buffer by AFM. The overlay emphasizes the periodicity. This striking fibril with high rigidity extended for over 10 μm in length. The height of the nodules is 12 nm while the rest of the mini-fibril is only 4 nm tall. In the background, a number of aggregates can be seen. The surface plot view of three of the nodules seen in B shows that the fibril may be turning over itself at a regular interval. Size bar = 150 nm for A (the image has been rotated for clarity of presentation).
exactly half the height of the fibrils (12 nm). These ring-like forms exhibit a wide range of diameters, from 300 nm to 1.3 μm, which correspond to fibril lengths ranging from 1 to 4 μm, respectively. Even if stretched out, these values are much longer than expected from other reports (Roca and Cox, 1997). The most stunning bend I have ever observed is depicted in Figure 14. This extremely tight hairpin turn on an otherwise straight fibril as a radius of curvature of approximately 30 nm.

The most fascinating detail about these rings of fibrils is visible in stereo in Figure 13. This allows us to see the joints between fibril and mini-fibril. Here we see several rings which are 6 nm in diameter on one side (corresponding to mini-fibril), and 12 nm along the other (corresponding to fibril). This region would thus appear to be the nucleation point for conversion from mini-fibril to fibril. This type of structure, wherein a ring doubles in diameter, was observed in many different samples. We also noted that while complete circles of 6-nm cross-sectional height were observed regularly, a complete ring with 12 nm cross-sectional height is rarely seen. These observations lead us to infer that the doubling in cross-sectional height corresponds to the build up of fibrils, and not their disassembly.

3.2.2. Mini-fibrils with a short period

RecA mini-fibrils that show distinct periodicities are also observed regularly. In this section we examine the many variations of the short period mini-fibril. This mini-fibril, depicted in Figures 15-18 is quite commonly observed in our experiments. The cross-sectional height of these fibrils is slightly less than 6 nm, and the periodicity is approximately 100 nm. We see this mini-fibril in many distinct environments. Figure 15 shows the short period mini-fibril (SP-fibril) in a low background area in conjunction with fibrils and mini-fibrils. Figure 16 shows a very unique SP-fibril – it is a lasso. In this case the SP-fibril extends from a single nucleation point into a circle forming the loop of the lasso. The rope end of this SP-fibril also contains a slight twist corresponding to a period of 150 nm. Figures 17 and 18 contain very similar looking SP-fibrils. They both occur in a high noise background containing many non-specific aggregates. The SP-fibril in Figure 17 is very straight, while the SP-fibril in Figure 18 exhibits a large bend. Thus
we see this highly diverse mini-fibril type that can be seen in the presence of all other fibril types.

3.2.3. Mini-fibrils with a long period

The final type of fibril that was observed is the long period mini-fibril (LP-fibril). This rarely observed fibril seen in Figures 1, and 19 has a periodicity of approximately 150 nm. The LP-fibril seen in Figure 16 is connected to a SP-fibril. This is a slow gradual twisting form with a cross-sectional diameter of 4 nm, except when it crosses itself. In this case the diameter is 10 nm. The LP-fibril in Figure 19 has very similar characteristics: it is also 4 nm in diameter except when it crosses over itself. However this time the cross-overs appear as nodules of sorts, and rise to the height of 12 nm above the substrate. The image shown in Figure 19(B) displays a close-up view in three dimensions of three of the nodules revealing that the extreme height of the cross-over could be from coiling of the LP-fibril around over itself.

3.2.4. Summary – all types of fibrils

We have determined that RecA forms a wide diversity of structural types using TM-AFM under buffer conditions. These types can be grouped as hexamers of 6 nm height (Figure 2), fibrils of 12 nm height and microns in length (Figure 6), and mini-fibrils of around 6 nm in cross-sectional diameter, which come in three distinct morphologies: Aperiodic (e.g., Figure 14), 100 nm periodic (e.g., Figure 18), and 150 nm periodic (e.g., Figure 19). All fibril shapes are in conjunction with each other, and take on a variety of turns and bends.

Both types of periodic mini-fibrils extend for several microns in length, and have been observed repeatedly in a number of samples. However, as one can see by comparing Figures 18 and 19, the environment of the long period mini-fibril differs from that of the short period one. That is to say that the SP-fibril (Figure 18) occurs in a situation where there is a great deal of non-specific debris in the background, while the LP-fibril (Figure 19) occurs with a relatively clean background of a few monomers and hexamers.
4. Discussion

The AFM of RecA fibrils in solution has confirmed some previously known facts, and gone on to demonstrate a wider polymorphism of the fibril. The classical picture of the RecA fibrils derived from EM shows them to have a diameter of 12 nm, less than a micron in length –typically only two or three hundred nanometers (Heuser and Griffith, 1989; Roca and Cox, 1990)– and possessing a periodicity of 8 nm. Our AFM results indicate that these rods can actually extend many microns (for example, Figures 1 and 6), with a constant height above the substrate of 12 nm over such range. It is possible that this is due to a bias in the sampling, that is, only long fibrils adsorb to the mica substrate under the conditions of the experiment. However, we do not believe this to be the case, since in all cases, we see the fibrils in conjunction with smaller aggregates such as hexamers in the background: If hexamers and longer fibrils can adsorb under these conditions, then so should shorter fibrils. It may also be the case that with such a long incubation time, the polymers have grown to much longer sizes. In addition, the 8 nm periodicity is not observed. This is beyond a doubt a resolution issue. We believe that in buffer solution, the RecA fibrils are surrounded by a tightly bound water layer. This water layer is impenetrable to the mild forces of a TM-AFM tip (on the order of 100 pN) obscuring the 8 nm periodicity. Unfortunately, any increase in the tapping force would result in loss of image quality, by dislocation of the RecA off the surface, or destruction of the sample.

Similarly, the RecA hexamer is also observed that correspond to dimensions known by X-ray crystallography and EM. We observe a height of 6nm corresponding to the height of the hexamer (Figure 2), but all other information is lost. The lateral dimensions cannot be observed due to tip broadening effects, and the subunit structure remains obscured by tightly bound water molecules as in the RecA fibril.

The mini-fibril has been observed for the first time. As seen in Figures 10-19 It comes in three distinct varieties. The aperiodic mini-fibril (Figures 10-14), and short period mini-fibril (Figures 15-18), and the long period mini-fibril (Figure 19). The mini-fibril is always exactly half the diameter of any fibril it appears in the same image as – usually 6 nm. It appears to have all the features of the fibril. We have shown it to be
straight or curved, even forming circles, much like the fibril. The fact that in Figures 10-13 the fibrils and mini-fibrils are joined in circles and curves must indicate something about the nature of these mini-fibrils, and likely means they exist in an equilibrium of some sort. These mini-fibrils may be beyond the resolution of TEM experiments, especially if it is actually aperiodic.

The most puzzling aspect of the periodic mini-fibrils is the scale at which periodicity occurs. The monomer and hexamer units, which are postulated to be important intermediates to the fibril assembly are only are few nanometers in any dimension. The observed periodicity of 100 or 150 nm are an order of magnitude larger by comparison. How can the sub-units maintain correlations these far? The simplest way is by a very slow twist propagating along the axial direction, as is observed in a number of examples (Pollanen et al., 1994). However, we find no evidence for such twist. In particular, examination of a longitudinal section along the long-period mini-fibril in Figure 19 shows that the mini-fibril may actually fold over itself at regular intervals for an apparently unknown reason. Closer examination (shown in Figure 19(B)) shows the coiling to indeed exist. Whether this is an artifact of imaging conditions is difficult to say.

The wide variety of polymer structure seen in all varieties of mini-fibril leads us to propose an additional step in the mechanism of RecA nucleofilamentation. We propose that these mini-fibrils are a more versatile form of RecA polymer. Their reduced height likely means a longer pitch than other fibrils, conferring an added element of flexibility to the fibril. It is possible that all fibrils are actually the same, and just at various states of a pitch extending/relaxing equilibrium, waiting to encounter a nucleic acid molecule and begin the nucleofilament assembly process. This process is outlined in figure 20.

One observation common among the various EM micrographs of RecA fibrils is their apparent rigidity. In previously published studies, only images of RecA-DNA complexes were curvy in nature, presumably dictated by the morphology of the plasmid DNA around which the RecA protein wraps. Contrary to this, our AFM studies of RecA without DNA show a variety of bent, curved and circular fibrils. However, it should be pointed out that while we see many curved fibrils, the features manifested in the images do not necessarily imply a higher flexibility of the fibril. That is, unlike a flexible coil,
the curved fibrils are mostly in rings or semi-circles, which are determinate structures, and in fact may be reasonably rigid, only in a different way. Furthermore, if one was to simply examine a short (200-300 nm) section of any of these curved fibrils, they appear the same as the short rods seen in the previous literature (see Brenner et al., 1988; Egelman and Stasiak, 1986). The short period mini-fibril, which has a larger diameter, appears to be slightly flexible, while the long period mini-fibril is highly rigid. This is exactly opposite to what one would expect: the rigidity of a material increases with diameter if the microstructure is preserved. Thus, while we cannot determine ultrastructural details, their trend in rigidity implies that there are fundamental differences between the short- and long-period mini-fibrils.

Figure 20. Proposed model for RecA/DNA nucleofilament assembly. The monomers and hexamers are in equilibrium with one another, as are the fibrils and mini-fibrils. All of these groups of RecA polymer and monomer may combine with DNA in some way to form the nucleofilament.
5. Summary and Conclusions

For the first time polymers of RecA have been examined in cell-like conditions by Atomic Force Microscopy. While many similarities to previous experiments were found, a novel polymorphism in the fibril type and variety was found. This similarity included the observation of the monomer, hexamer, and rod, with characteristics seen in the previous literature (Roca and Cox, 1997). These polymers are seen in figures 1 through 8. The hexamer (Figure 2) was found to have the same height as reported by the crystal structure – 6 nm. The fibril (also referred to as the rod) was found at its characteristic cross-sectional diameter of 12 nm. In addition, the AFM revealed the fibrils to be much longer than expected and often non-linear in nature. Upon careful examination, one could conclude that the rods in the EM and AFM images are the same, as the fibrils appear to be short rods when examined on the length of 200 to 300 nm.

Along with the fibril, a never before seen morphology of RecA polymer was observed. This mini-fibril (Figures 9 through 19) was found to occur in three distinct varieties: aperiodic, 150 nm periodic, and 100 nm periodic. The mini-fibril was found to have much the same characteristics as the fibril, with the exception that it is half the diameter. The SP-fibril has a long range periodicity of 100 nm, and the LP-fibril has a long range periodicity of 150 nm, which are also not observed amongst the fibrils.

The observation of all of these polymer types along with a large number of monomers and non-specific aggregates has led us to believe that the entire RecA system is in an equilibrium. This equilibrium is outlined in Figure 20, and introduces the mini-fibril as a more versatile form of the RecA polymer, that may have the ability to bind or enhance the binding of various strands of DNA in order for RecA to perform its function.

One question that remains is the significance of these observations inside the cell. Since RecA protein is known to exist inside the cell in large numbers (~10000 copies) it has long been thought that the possibility of aggregates occurring inside the cell existed. The function of these aggregates would mainly be as a storage form of RecA, but the hexamer and short rods have been shown to be the predominant forms under strand exchange conditions (Brenner et al., 1988). As we postulate the mini-fibril to be the intermediate in the formation of these 2 species, we conclude it too is a highly relevant
species during the strand exchange reaction. In addition, previous studies show that mutations in RecA monomer-monomer contact points lead to cell death (Roca and Cox, 1997). In the broader picture, RecA interacts with a large number of other proteins in the various pathways with which it is involved. In this case, the aggregates of RecA in the cell may serve as collection points for maintaining these supporting proteins nearby.

In the future, technical improvements in the operation of the AFM should allow the detection of the well characterized short range periodicity of the fibril. Indeed one would expect a shorter form periodicity may be found to exist in the mini-fibril as well. Additionally, future experiments will include exploration of the nucleofilaments RecA has the capability to form. Once this ability is found, one should be able to watch processes (such as homologous recombination) conducted by RecA in real time.
6. Bibliography


Goh, M. C., M. F. Paige, P. Markiewicz, I. Yadegari, and M. Edirisinghe. 1996. The Formation of Fibrillar Structures from Biopolymers. *In Scanning Probe*


Register, J. C. d., and J. Griffith. 1985. 10 nm RecA protein filaments formed in the presence of Mg2+ and ATP gamma S may contain RNA. *Molecular & General Genetics.* 199:415-20.


