CHARACTERIZING AND ENGINEERING MICROTUBULE PROPERTIES FOR USE IN HYBRID NANODEVICES

By

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To my Mom, for all the sacrifices you made to raise me up to be a respectable, responsible, and contributing member to society
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CHARACTERIZING AND ENGINEERING MICROTUBULE PROPERTIES FOR USE IN HYBRID NANODEVICES

By

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The emergence of nanotechnology in materials science research has had a major impact in biotechnology. Nature provides novel materials and structures that can be redesigned and reassembled for engineering purposes. One system in particular is the intracellular transport system consisting of the kinesin motor protein and microtubule. For synthetic devices, either the bead geometry (kinesin motors walking along a microtubule coated surface) or the gliding geometry (microtubules gliding over a kinesin-coated surface) is used. Molecular shuttles, utilizing the gliding geometry, have the potential for use in hybrid nanodevices such as biosensors.

The kinesin-powered molecular shuttle has been extensively studied. Advances have been made in controlling activation of the kinesin motors, guiding movement of kinesin motors and cargo loading onto the molecular shuttles. In this dissertation the interest in molecular shuttle development is extended with a research focus on the microtubule filament. The microtubule is a central element in the molecular shuttle. The sensing capabilities and limitations of molecular shuttles are tied to the microtubules. It would be desired to have nanodevices with molecular shuttles of predictable size, speed and lifetime.
Three materials properties of the microtubules are examined. First, the microtubule length distribution is measured and compared to the length distribution of synthetic polymers. Post polymerization processing techniques, shearing and annealing, are utilized to try to reduce the polydispersity index of the microtubule length distribution. Second, the effect of kinesin activity on the lifetime of the microtubules is observed and quantified. Degradation of microtubules is monitored as a function of kinesin activity and time. Lastly, the effect of cargo loading on microtubule gliding speed is measured to gain insight on the mechanism of cargo attachment. These property behaviors will play a role in the final development of nanodevices involving microtubules. It will also help in designing and optimizing microtubules for other synthetic uses.
CHAPTER 1
INTRODUCTION

Materials Science and Nanotechnology

Materials in Society

Materials have been and continue to play a dominant role in human experience. They are pervasive to the point that large time periods of history are often referred to by the newly discovered or most used material of that time. During the Stone Age, the stone was the most advanced material available. With discoveries of metals and alloys, came about the Copper Age, Bronze Age and Iron Age. The mid-19th century was dominated by the use of steel and is often termed the Steel Age. The 20th century will be remembered as the Polymer Age and Silicon Age as materials usage shifted. The 21st century is poised to be the Information Age. As the demand for more data storage in smaller devices increased, materials research shifted to smaller scales: micro scale to nano scale. A new field of research was coined Nanotechnology.

Emergence of Nanotechnology

Nanotechnology has been defined as the development and use of techniques to study physical phenomena and construct structures on the size scale of 1-100nm, as well as the incorporation of said structures into applications [1]. Other working definitions of nanotechnology include the deliberate manipulation of matter at size scales of less than 100nm [2], and the study of the controlling of matter on an atomic and molecular scale. The field of nanotechnology is diverse and spans research from physics and engineering to biology and chemistry [3]. Nanotechnology continues to be a driving force in materials science research, as the demand for smaller and more efficient devices remains high.
**Nanobiotechnology**

The emergence of nanotechnology has had a dramatic impact in the biotechnology and medical fields [4]. A variety of materials have been investigated and optimized for novel devices. The potential uses of nanoscale materials in imaging and diagnosis, cancer treatment, drug delivery, and gene therapy are unlimited. Nanotechnology can extend the limits of molecular diagnostics to the nanoscale, making lab-on-a-chip devices much more sensitive. Nanotechnology also has improved the development of biosensors [5-7]. Material selection for nanotechnology is abundant. Inorganic materials, metals, and nonmetals have been studied and used in conjunction with biomedical applications. Over the last 2-3 decades, advances in molecular and cell biology have brought attention to the use of biological materials, or biomolecules, in nanotechnology.

**Biomolecules and Nanotechnology**

**Bottom-up Approach**

Biomolecules make a good choice material for nanotechnology because of their size. Biomolecules provide a solution to the bottom-up approach in nanofabrication; they allow the assembly process to begin at the atomic and molecular level [8]. Several review papers have summarized the use of DNA to direct protein assembly and to serve as scaffolds for supramolecular structures [9-12]. Other bottom-up approaches include self-assembled monolayers of polymers, formation of vesicles and tubes using lipid bilayers [13, 14], and molecular self-assembly of peptides, proteins and lipids [15, 16].

**Molecular Motors**

Biomolecules can also serve as molecular motors for nanomachines [17, 18]. Molecular motors are divided into two categories [19], rotary motors and linear motors.
Rotary motors, such as the bacterial flagella and the F₀F₁-ATP synthase, take energy stored in chemical gradients and convert it to kinetic energy to generate motion. Linear motors, such as kinesin, myosin and dynein, use chemical energy stored in phosphate bonds to generate movement. Advances in nanotechnology paired with advances in molecular biology have birthed an interest in bio-nanorobots and bio-nanomachines [20, 21]. Ideally, the components of these bio-nano robots or machines, when placed in a synthetic environment, would elicit their same function in response to a stimulus as when in the body.

**Molecular Shuttles**

The kinesin motor protein has been studied extensively [22-24] for use in a molecular shuttle. A molecular shuttle, shown schematically in Figure 1-1, consists of kinesin motor proteins and the protein filament, microtubule; the microtubule will be discussed in further detail in Chapter 2. Molecular shuttles are a subset of molecular machines that have the ability to move a chosen cargo from one place to another and allow user control over cargo selectivity. Kinesin motors are quite efficient in energy conversion; they have shown promising results in being able to be manipulated for use in synthetic environments. The engineering challenges with the construction of molecular shuttles include the following: guiding movement of molecular shuttles, activation and speed of the molecular shuttles, and cargo loading and unloading. These will be discussed further in Chapter 2. Property characterization of the microtubule filament itself has been limited to its static behavior.

**Outline of Dissertation**

The work presented in this thesis begins to look at the behavior and physical properties of the microtubules in a dynamic hybrid environment. Three material
properties of microtubules, serving as the filament choice for molecular shuttles, are addressed. The length distribution of microtubules polymerized in vitro is examined and compared to length distributions of synthetic polymers. The degradation pattern of microtubules resulting from kinesin activity is assessed. Lastly the change in gliding speed of microtubules loaded with cargo will be examined.

Chapter 2 gives a detailed introduction of microtubules from both a biology perspective and an engineering perspective. The cellular and in vitro relationships between the microtubule filament and the kinesin motor protein are discussed. Further discussion about molecular shuttles, engineering challenges, and advances in nanotechnology is presented as well. The in vitro motility assay, the set-up used, is briefly explained.

In chapter 3, the length distribution of microtubules is examined. The findings are compared with other published results. An attempt is made to quantify the length distribution of microtubules given its unique polymerization process. The length of a microtubule affects its other physical properties. It has a direct relationship to the amount of cargo it can carry. Ideally, these molecular shuttles are to be like nanorobots. It would be desirable to be able to mass-produce identical constructs of the same length.

The lifetime of a device is an important factor in determining and optimizing a device for environmental use. In chapter 4, the microtubule and kinesin hybrid system is studied, with the focus being on the wear of the microtubules relative to kinesin activity. The wear pattern and mechanism will greatly affect the usefulness and efficiency of
molecular shuttles. It has to be considered along with other engineering challenges in the design of synthetic and biological nanosystems.

The gliding speed of microtubules is of interest for determining the response rate of potential sensing abilities. Cargo loading plays a role in the detection limit and capacity of a sensor. In chapter 5, the effect of cargo loading on microtubule gliding speed is examined and discussed. The response, positive or negative, has to be taken into consideration as part of the behavior and response of this device in nanotechnology applications.

The final chapter summarizes the findings and discusses future work, the potential of microtubule filaments, and their role in nanotechnology research.
Figure 1-1. Molecular shuttle – A nanoscale transport system. Adapted from [24]
CHAPTER 2
BACKGROUND

The Cellular Cytoskeleton

Eukaryotic cells have evolved and developed sophisticated systems and machineries for carrying out cellular processes such as growth, replication and information dissemination. Cells vary in shape by species, types of cells and life stages. A cell’s shape, motility and other internal processes are determined and monitored by its cytoskeleton, a dynamic proteinaceous structure. Microtubules, together with actin and intermediate filaments, make up the cytoskeleton of eukaryotic cells [25, 26].

The Microtubule

Microtubule Function

Microtubules extend from microtubule-organizing centers (MTOC) out to the periphery of the cell. The microtubules serve as tracks for the motor proteins, kinesin and dynein. The movement of these protein motors is unidirectional; kinesin travels along a microtubule towards the cell’s periphery, while dynein travels away from the periphery. Microtubules are also important during cellular division and replication. During the phases of mitosis, microtubules undergo various changes to fulfill many functions, such as lining up the chromatids, and separating the chromatids so each daughter cell gets exactly one copy of each chromosome.

Microtubule Structure

The structure of the tubulin subunit, the basic building block of the microtubule, has best been described by Nogales et al. in 1998. Tubulin is a heterodimer protein comprised of α and β tubulin monomers. Each monomer has a molecular weight of approximately 55 KDa, has dimensions of approximately 4 nm x 4 nm x 4 nm and has
three functional domains: a nucleotide binding site, a drug binding site and a motor protein binding site [27]. The α and β globular proteins are held together by noncovalent bonds. Each monomer has a guanosine triphosphate (GTP) molecule bound to it. The GTP molecule bound to the α-tubulin monomer, in the “N” site, is fixed; the GTP molecule bound to the β-tubulin monomer, in the “E” site, may be hydrolyzed to or exchanged for a guanosine diphosphate (GDP) molecule during polymerization.

Tubulin heterodimers assemble in a specific head-to-tail fashion; the top of the β-tubulin of one heterodimer binds to the bottom of the α-tubulin of another heterodimer. Many tubulins assemble to form a protofilament. Several protofilaments align themselves in parallel, with exposed α-tubulin at one end and exposed β-tubulin at the opposite end, and fold to form the final microtubule structure. Figure 2-1 illustrates the formation of a microtubule. On average, a microtubule consists of 13 protofilaments, but the actual value can vary between 11 and 17 protofilaments by changing buffer conditions during in vitro growth [28, 29]. Microtubules are approximately 25 nm in diameter and can be up to many micrometers in length.

**Microtubule Dynamics**

Microtubules exist in a state of dynamic instability, stochastically switching between a growing phase and a shrinking phase [30]. As tubulin heterodimers are assembling, the E-site GTP molecule is hydrolyzed to GDP soon after the top of the β-tubulin binds to the bottom of the α-tubulin [31]. If the rate of tubulin addition exceeds the rate of GTP hydrolysis then the β-tubulin exposed end will always have a “GTP-cap”, which stabilizes the microtubule. If GTP hydrolysis outpaces tubulin addition, there will not be a GTP-cap, which leads to a less stable microtubule and rapid depolymerization will soon follow. Depolymerization is ceased when an E-site GTP in
the microtubule lattice is encountered, then the growth phase will resume. The rapid depolymerization phenomenon is referred to as “catastrophe”, the sudden cessation of depolymerization; regrowth is termed “rescue” [32].

This dynamic behavior is vital for the reorganization of microtubules during the cell cycle and for cellular motility. The cause of microtubules switching between states is unknown. In work done by Cassimeris et al. [33], it was observed that microtubules could switch between the two states within minutes. The elongation rate of microtubules found in newt lung epithelial cells was lower than the shortening rate, but due to high frequency of rescue phase, rapid shortening did not progress to completion [33].

**Kinesin Motor Proteins**

Kinesin molecules are used in the cell for transport of vesicles to the cell periphery. Kinesin is a tetramer of two heavy (110-113 kDa) chains and two light (60-70 kDa) chains coiled around each other. The two heavy chains fold to form two globular heads, which bind specifically to the motor binding site of the β-tubulin monomer. The two light chains form the tail domain, which binds to various cargo in the cell for transportation. The motor domain, head region, also has an adenosine triphosphate (ATP) active site. Kinesin walks along the microtubule in a hand over hand fashion [34], hydrolyzing ATP molecules to adenosine diphosphate (ADP) and a phosphate ion, Figure 2-2. Kinesin preferentially walks towards the (+) end of the microtubules at an average speed of 1.5µm/s [35] in vivo and 0.8µm/s [36] in vitro.

**Engineering and Nanotechnology Applications**

Microtubules are an ideal choice material for engineering applications. They can be easily synthesized from a buffered solution containing tubulin and GTP with high integrity and negligible defects. The stability of the microtubule structure can be
enhanced for synthetic uses. Depolymerization is most commonly suppressed by keeping the microtubules in a buffered solution containing paclitaxel (Taxol) [37, 38]. The Taxol molecules form hydrogen bonds and other hydrophobic contacts with the β-tubulin dimer [39], thereby discouraging disassembly for days. Chemical crosslinking is also an option to stabilize microtubules. Microtubules cross-linked with gluteraldehyde can maintain their structure for weeks, even in extreme conditions [40, 41].

During in vitro polymerization, growth of microtubules occurs at both ends of the polymer. The α-tubulin exposed end grows at a much slower rate compared to the β-tubulin exposed end. Thus the α-tubulin exposed end is termed the minus (-) end and the β-tubulin exposed end is termed the plus (+) end. A critical concentration, Cc, of tubulin is needed for nucleation. Drechsel et al. calculated this to be 0.05 µM [42]. Rapid polymerization continues until the steady-state concentration, Cs, is reached: 3-4 µM in vivo and 14 µM in vitro [43]. Microtubule growth is promoted above the Cs. Each microtubule end has a critical concentration: Cc-plus at the plus end and Cc-minus at the minus end. When Cc-plus is below the local tubulin concentration, elongation occurs at the plus end, and the same applies at the minus end. The catastrophe frequency, the switching from growth to shrinkage at the plus end, and the rescue frequency, the switching from shrinkage to growth at the minus end, are dependent on each end’s critical concentration and local tubulin concentration.

Stabilized microtubules have been shown to have the capabilities to serve as templates for production of inorganic nanowires [44-46] and nanoparticle arrays [46, 47]. Fabrication of tubulin-carbon nanotube hybrids via microtubule encapsulation has been reported [48]. Microtubules may also be an answer to the interest in bottom-up
approaches in fabrication of microelectromechanical (MEMS) devices. Due to the polarity of microtubules, they exhibit some electrical properties. Ramalho et al. have shown that microtubules can be aligned when exposed to an electric field in the MV/m range [49].

**Methodology**

**In Vitro Assays**

Motility assays, in vitro studies of the kinesin-microtubule system, are conducted in either the bead geometry or the gliding geometry. The bead geometry has kinesin motors moving along microtubule-covered surfaces. The kinesin tail is bound to a polystyrene microsphere and its movement is observed. Optical microscopy techniques have been used to report mechanical and physical properties of kinesin motors. The gliding geometry has the tails of kinesin motors immobilized on a surface and the microtubules gliding over the kinesin heads. Gliding assays are performed in flow-cells and observed using fluorescence microscopy. In both the bead and gliding assays, kinesin motors have been reported to move a net distance of 8 nm per ATP molecule hydrolyzed. In vitro speeds can reach up to 800-1000 nm/s at saturating ATP concentrations, about 50-67% the efficiency of in vivo speeds.

For synthetic uses, kinesin can be purified from cells or expressed in recombinant bacterial systems and harvested in large quantities. Various methods for kinesin preparation can be found in references [50]. Tubulin is routinely purified from bovine brain and is readily available for purchase from such companies as Cytoskeleton Inc.
The Molecular Shuttle

Proof of Concept

The concept of a molecular shuttle system has been a driving force in nanotechnology research for over a decade. The gliding geometry has shown to be a more advantageous set-up. Control over direction of motion, activation of kinesin motors, and selectivity of cargo are easier to achieve and discriminate for.

Applications of Molecular Shuttles

The kinesin-powered molecular shuttle system has shown capabilities for surface topography analysis such as surface roughness. In surfaces that have raised areas, kinesin motors will only be attached in the valley areas and microtubules will only appear there. In fluorescent images the raised areas will appear black, indicating absence of microtubules. For surfaces that vary in roughness, the lower elevated areas will have higher concentrations of microtubules and so will appear brighter in fluorescent images.

The microtubule-kinesin system plays a role in self-assembly by providing active transport for smaller building blocks. Active transport, provided by molecular shuttles, is advantageous to microfluidic systems and lab-on-a-chip devices. Functionalized microtubules can capture various analytes and concentrate them in a collection area by active transport of kinesin motors.

Engineering Challenges

Guidance control: The path microtubules travel can be controlled by predetermining the tracks. Predesigned tracks can be achieved by physical, chemical, electrical or magnetic approaches. User-defined channels are microfabricated in devices and the bottom surface of the channels are coated with kinesin motors.
Microtubules will only glide where the kinesin motors are located. Clemmens et al. have shown microtubules, upon hitting a wall, will not climb up the wall, but rather bend and continue moving along the wall, staying attached to kinesin motors [51]. Unidirectional motion of microtubules has been demonstrated by microminiaturized circulators [52] and directional rectifiers [53] that sort and direct microtubule movement.

On a planar surface, predetermined tracks are set by assigning fouling and non-fouling regions. Kinesin proteins will only attach in the fouling regions, which have been chemically treated. Microtubules will be restricted to the regions that are high in motor density. Orientation of microtubules has also been demonstrated through applications of electric fields [54-56]. Magnetic fields have also been used to align and direct microtubules [57]. Tubulin heterodimers are functionalized with ferritic particles and controlled by passing a magnetic field [58-60].

**Activity control:** Kinesin motor activity has been reported to have a dependence on a number of factors including ATP consumption, pH, temperature, and motor density [61-63]. Start and stop motion of kinesin motors can be controlled by UV light activation of DMNPE-caged ATP [64, 65]. ATP hydrolysis rate of kinesin motors increases exponential with temperature increase [61], yielding an increase in motor activity. In the presence of an electric field, microtubules have been shown to accelerate towards the cathode region [66].

**Cargo selectivity:** Microtubule surfaces can be coated with linker molecules having binding sites for a variety of analytes. Tubulin heterodimers can be conjugated with biotin [64] prior to polymerization. In the gliding assay the biotinylated microtubules are then coated with streptavidin molecules. Various cargo or analytes can then bind to
the streptavidin molecule directly or via biotin-streptavidin linkers. Other methods include antibody attachment to the microtubule [67, 68], attachment of DNA linkers [69, 70], or binding molecules [71].

**Motivation**

In the gliding geometry, the microtubule is a very critical and essential part of the molecular shuttle. The material properties of the microtubule should be examined and exploited to optimize its engineering use. To be an effective useful material, there should be user control over the properties and behavior of such material. Three materials properties of microtubules are discussed in this work: the length distribution of microtubules polymerized in vitro, the degradation of microtubules resulting from kinesin activity, and the loading effects on microtubule gliding speed.
Figure 2-1. Hierarchal structure of a microtubule. Adapted from [26].
Figure 2-2. Processivity of kinesin motor protein. Adapted from [72].
CHAPTER 3
CHARACTERIZING THE LENGTH DISTRIBUTION OF MICROTUBULES

Introduction

In all molecular shuttle designs, the length of the microtubule or the actin filament is of interest because it determines the capacity of each shuttle for molecular or nanoscale cargo and affects the efficiency of the loading process [73]. The controlled manipulation of the length distribution would thus be very desirable for optimizing the design of molecular shuttle systems and their elements, such as loading stations [74].

Filament Length Distribution Theories

The work in this chapter builds on earlier experiments and theories regarding the length distributions of cytoskeletal filaments. Fluorescence microscopy has been previously used to determine the length distributions of microtubules [75] and actin filaments [76]. Popp et al. [77] pointed out that, while an exponential length distribution of actin filaments is generally assumed, short-range attractive forces between filaments led to bell-shaped length distributions [78]. Fygenson et al. [79] experimentally studied the dynamic assembly of microtubules and fitted the observed length distributions with a simple exponential, as suggested by Dogterom and Leibler [80]. Based on turbidity measurements and theoretical modeling, Flyvbjerg and Jobs [81] predicted a microtubule length distribution with an approximately Gaussian shape (centered at the average length and with a standard deviation equal to 0.45 times the average length). In contrast, classic experiments by Johnson and Borisy showed that the length distribution of microtubules, determined from electron microscopy as well as by turbidity measurements during depolymerization, can be approximated by a roughly symmetric curve originating at zero, rising to a maximum at the average length, and possessing a
small tail [82]. Similar length distributions have been reported in other experimental studies of that era [83-85]. Hill calculated steady-state microtubule length distributions based on the thermodynamics of the assembly process, which will be discussed later [86].

**Post-polymerization Processing of Microtubules**

Microtubules can be shortened after polymerization by exposing them to shear flow; this shearing is frequently employed to create short 'seeds' as nucleation sites for microtubule polymerization studies [30, 87]. Interestingly, the cleavage of polymers in the presence of mechanical forces, such as the viscous forces exerted under shear flow, is an established topic in the field of mechanochemistry [88]. In synthetic polymers, cleavage of the polymer backbone due to mechanical stress (e.g. exerted during milling) can reduce the polydispersity index of the molecular weight distribution, in addition to the average molecular weight [89], an effect which would be very desirable in efforts to create molecular shuttles of well-defined size. Although microtubules are composed of tubulin subunits connected by intermolecular bonds rather than covalent bonds, as in the case of synthetic polymers, it is reasonable to assume that the effects of shearing on the microtubule polymer size distribution are similar to the effects on synthetic polymers, albeit at much lower shear rates.

Microtubules can be lengthened after polymerization by end-to-end joining; this annealing process has been investigated in quite some detail [90-93]. The annealing process is strongly dependent on the length and concentration of the microtubules [94]. Deviations from a purely random coupling process, analyzed by Schulz [95], may lead to a reduction in the polydispersity index [96].
In this chapter, the length distribution of Taxol-stabilized, fluorescently-labeled microtubules polymerized for varying durations are determined experimentally. These microtubules are then processed by exposing them to shear flow and by annealing, and the resulting length distributions are analyzed. It is also demonstrated that the length distribution is described by a generalized Schulz distribution [95] for the case of a polymer growing on both ends of the chain with different growth rates.

**Experimental Methods and Materials**

**Microtubule Polymerization**

A 20-µg aliquot of rhodamine-labeled, lyophilized tubulin (TL331M, Lot 367 from Cytoskeleton Inc, Denver, CO.) was reconstituted with 6.25 µL polymerization buffer (BRB80 with 4 mM MgCl₂, 1 mM GTP, 5% dimethyl sulfoxide), polymerized at 37°C for 1, 5 and 30 minutes, and subsequently stabilized by diluting hundred-fold into a BRB80 buffer with 10 µM paclitaxel (Taxol, from Sigma, St Louis, MO). BRB80 buffer is 80 mM PIPES, 1 mM MgCl₂, 1 mM EGTA, pH 6.9 with KOH. This polymerization protocol preferentially creates microtubules with fourteen protofilaments (~70% of all microtubules) [97]. To image the microtubules, a 15-µl drop was placed on a Fisher cover glass, 35 x 50 mm and covered with a cover slip, 22 x 22 mm. To minimize shearing during pipetting, the sharp tips of the plastic pipettes were cut off.

Spectral absorption of microtubules was measured using the Nanodrop ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE). A 1-µl droplet of microtubule solution was placed on the optical pedestal of the spectrophotometer. Absorbance readings were measured at a wavelength absorbance of 340 nm on the UV-Vis setting.
Microtubule Shearing

Microtubules polymerized for thirty minutes were further processed by shearing using either a 30-gauge needle (nominal inner diameter: 140 µm) or a 25-gauge needle (nominal inner diameter: 241 µm). A 250-µL sample of the microtubule solution was passed back-and-forth once or twice through a syringe-mounted needle over a time span of 10 s, 5 s or 2 s by manual operation of a syringe. This procedure resulted in average flow rates of 22-113 µL/s (see Table 3-1).

Microtubule Annealing

Four 20-µg aliquots of rhodamine-labeled tubulin were reconstituted with 25 µL polymerization buffer (BRB80 with 10 mM MgCl₂, 1 mM GTP, and 5% dimethyl sulfoxide), including 10 µM Taxol and polymerized at 37°C for thirty minutes. The polymerized solution was then sheared by three passes through a 30-gauge needle, and incubated again at 37°C for an additional ninety minutes.

Imaging and Data Analysis

Microtubules were imaged using a Nikon TE2000-U Epi-fluorescence microscope (Nikon, Melville, NY) equipped with an X-cite 120 lamp (EXFO, Ontario, Canada) and iXON DU885LC EMCCD camera (Andor, South Windsor, CT) and a 100x oil objective (NA 1.45). The exposure time used to acquire the images was 0.2 s.

Microtubule lengths were measured using the imaging software, ImageJ (available at http://rsbweb.nih.gov/ij/). The straight-line selection and free-hand selection drawing tools were used to manually outline the straight and curved microtubules, respectively (Fig. 3-1). The software then calculates the length of the drawn outline. For each polymerization condition, shearing condition and annealing condition, several hundred microtubules were measured from 1-3 different fields of view. All microtubules enclosed
within a field of view were analyzed except for microtubules shorter than 0.5 µm, the resolution limit of the objective.

Due to the proportionality between the microtubule length and the combined molecular weight of the tubulin dimers in a microtubule, the number-average length and weight-average length of microtubules are defined in analogy to the definitions of number-average (Equation 3-1) and weight-average (Equation 3-2) molecular weight in polymer science. The polydispersity index (Equation 3-4) is the ratio of weight-average to number-average microtubule length.

\[
< L_n > = \frac{1}{n} \times \sum_{i=1}^{n} L_i 
\]

\[
\sigma_n = \frac{1}{n} \times \sum_{i=1}^{n} (L_i - < L_n >)^2 
\]

\[
< L_w > = \frac{\sum_{i=1}^{n} L_i^2}{\sum_{i=1}^{n} L_i} 
\]

\[
PDI = \frac{< L_w >}{< L_n >} 
\]

The magnitude average shear rate, \(< \gamma >\), within the needle was calculated using Equation 3-5, which applies to laminar flow in a cylindrical pipe with diameter \(D\) at a volumetric flow rate \(Q\) [98].

\[
< \gamma > = \frac{6AQ}{3\pi D^3} 
\]

**Results**

The microtubules were adsorbed to the glass surface of the coverslip and were readily imaged by wide-field epi-fluorescence microscopy. Measuring the length of several hundred microtubules enabled the accurate determination of the average length, which increased, as expected, with polymerization time. The observed number average and weight average of the length as well as the polydispersity index (PDI) -
defined by the ratio of weight average to number average [99]- are given in Table 3-2 for the different polymerization times. The turbidity (absorbance) measurements showed an increase of turbidity to a maximum value within five minutes; this was in general agreement with previous measurements for tubulin polymerization in the presence of dimethyl sulfoxide [100].

Length Distribution

The microtubule length distribution (Fig. 3-2) is clearly neither an exponential function [79] nor a Gaussian [81], but has the same general shape as the distribution observed in electron microscopy images [82]. After 30 minutes of growth, the distribution is characterized by a maximum at intermediate lengths, a slow decline in the frequency count towards higher lengths, and a polydispersity index of 1.5.

Effect of Shearing

The shearing experiments showed a shortening of the microtubules, which was more pronounced when the flow rate was higher and the needle diameter smaller (Table 3-3). The dependence on needle diameter and flow rate reflects the change in average shear rate within the needle. The shortening was mirrored in the microtubule length distributions by a shift of the peak towards shorter lengths and a decrease in the number of long microtubules (Fig. 3-3). However, the polydispersity index was unchanged within the experimental error.

Effect of Annealing

The annealing of microtubules succeeded only, as in the published experiments [90-93], when the microtubules were first sheared. Following the procedure of Williams and Rone [93], Taxol was added prior to the polymerization. The polymerized microtubules had a larger average length (8 µm), which was reduced to half its initial
value after shearing (Fig. 3-4 and Table 3-4). Allowing the microtubules to anneal for 90 minutes increased the average length of the microtubules roughly five-fold. While the shearing increased the polydispersity index from 1.5 to 1.8, after the 90-minute annealing period the polydispersity index had returned to 1.5.

**Model of Microtubule Length Distribution**

The in vitro assembly of a microtubule is initiated by a nucleation step, and proceeds by fast growth on the “plus” end of the microtubule at a rate of several micrometers per minute and three- to four-fold slower growth at the “minus” end [101, 102]. It slows as tubulin monomers are depleted and reaches a steady state during which the microtubule length fluctuates significantly due to dynamic instability [30]. Finally, the assembly process is terminated when the growth solution is highly diluted and microtubules are stabilized against disassembly with Taxol.

Significant annealing of unsheared, long microtubules was not observed within a reasonable time, six hours. Only sheared microtubules, (as used in previous studies [90-93]), at the high concentrations found in the polymerization solution exhibited annealing as described above. Since the microtubules are a hundred-fold diluted compared to the polymerization solution in our shearing experiments, annealing should not significantly impact their length distribution unless the experiments are specifically designed to achieve annealing.

The model for the microtubule length distribution illustrated in this chapter combines two theoretical insights: (1) Dogterom’s and Leibler's [80] results that a microtubule growth from one end (typical for the in vivo situation) results in an exponential steady-state distribution and (2) the classic result by Schulz [95] that
simultaneous growth from two ends results in a distribution which peaks at an intermediate length.

According to Dogterom and Leibler, the steady-state is characterized by an equilibrium between microtubule growth (polymerization) with velocity \( v_+ \) during “rescue” phases occurring with a frequency \( f_+ \) and shrinkage with velocity \( v_- \) during “catastrophe” phases occurring with a frequency \( f_- \) in the process of dynamic instability. This gives rise to an exponential distribution of microtubule lengths as described in Equation 3-6 with \( \lambda \) defined by Equation 3-7.

\[
\begin{align*}
f(L) &= \lambda e^{-\lambda L} \\
\lambda &= \frac{f_+}{v_+} - \frac{f_-}{v_-}
\end{align*}
\]  

(3-6)  
(3-7)

As Schulz described in 1939 in his classic publication regarding the molecular weight distributions in chain polymerization processes [95], simultaneous but independent growth from two ends leads to all possible combinations of lengths for each end. Therefore the length distribution \( s(L) \) of a microtubule polymerizing from the plus AND the minus end is given by Equation 3-8.

\[
s(L) = \int_0^L f_+ (L-x) f_- (x) \, dx
\]  

(3-8)

In the case of equal length distributions at each end, this reduces to the Schulz distribution (Equation 3-9) known in polymer science (where \( \ln \alpha \) is defined by Equation 3-10) with a polydispersity index of 1.5. If the two ends possess different average lengths, \( 1/\lambda_+ \) and \( 1/\lambda_- \), the integration yields a more general result (Equation 3-11).

\[
s(L) = (\ln \alpha)^2 L \propto L
\]  

(3-9)

\[
\ln \alpha = -\lambda
\]  

(3-10)
\[ s(L) = \frac{\lambda_- \lambda_+}{\lambda_- - \lambda_+} \left( e^{-\lambda_+L} - e^{-\lambda_-L} \right) \quad (3-11) \]

The polydispersity index of this generalized distribution varies between 1.5 (equal average length of both ends) and 2 (negligible average length of one end). The polydispersity index for asymmetric growth with a four-fold difference in average length of the ends is 1.68.

Assuming that the microtubule grows from individual tubulin dimers [103] of 8 nm length into a 14-protofilament structure (based on our polymerization conditions [97]), the degree of polymerization \( P \) can be readily determined from the microtubule length \( L \) as using Equation 3-12.

\[ P = \frac{14L}{(8 \, \text{nm})} \quad (3-12) \]

**Effect of Shearing**

A simple approximation to describe the effect of shearing is to assume that a fraction of microtubules will break exactly once in the center of the microtubule as a result of the applied shear. If the microtubule length distribution before shearing is given by \( s(L) \) in Equation 3-11, the length distribution \( s'(L) \) of microtubules after shearing is given by Equation 3-13, where \( L \) is the length of the microtubules, and \( b \) is the fraction of microtubules which have broken.

\[ s'(L) = (1 - b) \cdot s(L) + 2b \cdot s(2L) \quad (3-13) \]

Interestingly, if all microtubules break once in the center \( (b = 1) \), the polydispersity index of the resulting distribution is 1.5 and equal to the original polydispersity index. To reduce the polydispersity index of the microtubule ensemble, the effect of shearing likely has to be length-dependent, so that long microtubules are preferentially removed from
the ensemble. However, as will be discussed below, this simple approximation provides a good fit to the data.

**Effect of Annealing**

While the end-to-end joining of a specific number of microtubules [95] or the preferential combination of long microtubules with short microtubules [96] reduces the polydispersity of the resulting microtubule combination, a purely random process where stochastically distributed numbers of microtubules join without regard for their length, does not reduce the polydispersity. This perhaps surprising result has been obtained by Schulz, who discussed the statistical coupling of polymer chains with a (Schulz) length distribution of Equation 3-14 (corresponding to growth from two ends with equal average length), and obtained the length distribution after annealing, \( a(L) \), as Equation 3-15, where \( c \) is a normalization constant. This equation can be rewritten in the normalized shape of Equation 3-16 where \( w \) is the coupling probability at the end of a primary chain. Again, the polydispersity index varies from 1.5 (\( w = 0 \)) to 2 (\( w = 1 \)).

\[
\begin{align*}
s(L) &= \lambda^2 L e^{-\lambda L} \\
a(L) &= ce^{-\lambda L} \sinh \sqrt{w} \lambda L [95] \\
a(L) &= \frac{(1-w)\lambda}{2\sqrt{w}} \left(e^{-(1-\sqrt{w})\lambda L} - e^{-(1+\sqrt{w})\lambda L}\right)
\end{align*}
\]

Equation 3-11 and Equation 3-16 seem to suggest that it cannot be distinguished if the asymmetric length distribution of polymerizing microtubules arises from asymmetric polymerization at the ends or from symmetric polymerization with simultaneous annealing, since it is always possible to find a combination \( (w, \lambda) \) which results in the same length distribution as a combination \( (\lambda_+, \lambda_-) \). However, it has previously been demonstrated from the preparation of segmented microtubules [87] that annealing plays
no major role during microtubule polymerization, so that this question can be answered in favor of asymmetric polymerization based on experimental evidence.

Discussion

Despite its utilization in previous discussions of microtubule length distributions, [25, 79] an exponential function does not provide an accurate description of the length distribution when the microtubule polymerization reaction has run for 30 min (Fig. 3-2), nor can the observed length distribution be described by a Gaussian [81].

A fit of the length distribution proposed by Hill (eq.37 in [86]), which is of the form Equation 3-17 where \( n = 4, \ldots, 6 \), shows a significant discrepancy with the experimental data (Fig. 3-2, dashed line) even for \( n = 4 \) (the best fitting case). A possible explanation for this failure of the thermodynamic model may be that it was proposed in 1980 before the complex structure and dynamics of the microtubule was fully elucidated, so that it does not include all relevant parameters.

\[
h(L) = c_1 * L^n * C_2^L
\]

(3-17)

However, the histogram (Fig. 3-2C) is readily fit by the asymmetric growth model (Equation 3-11) if \( \lambda_+ = \lambda_-/4 \) is required (see [75, 102]) and the parameters \( \lambda_+ \) and \( \lambda_- \) are chosen as \( (3 \mu m)^{-1} \), and \( (0.75 \mu m)^{-1} \), respectively. It is thus concluded, that the length distribution of microtubules polymerized in vitro is a generalized Schulz distribution, because significant growth occurs independently from both microtubule ends.

The argument for a generalized Schulz distribution (Equation 3-11) applies for all cytoskeletal filaments growing from both ends, including actin filaments. Indeed, some observed length distributions of actin filaments have exhibited the characteristic maximum at intermediate lengths [104, 105]. However, the typically more than 10-fold
difference between association rates at the pointed and barbed end \[106\] shifts the maximum of the distribution to submicron lengths. Since only lengths larger than 0.5 \( \mu \text{m} \) are typically measured with optical microscopy, only the exponential decay of the tail of the length distribution is observed.

The second objective of this work after the determination of the length distribution of microtubules polymerized in vitro was to determine if breaking the microtubules by applying shear flow resulted in a reduction of the polydispersity index. Figure 3-3 shows that the length distributions of sheared microtubule samples are not significantly altered compared to the unsheared sample (control) if the shear rate is small \(<3 \times 10^4 \text{s}^{-1}\).

The length distribution of the unsheared sample is well-fitted by a generalized Schulz distribution (Equation 3-11), and the same fit curve describes the histograms of samples S1, S2 and S3 very well. For the intermediate shear rates \(5 \times 10^4 – 9 \times 10^4 \text{ s}^{-1}\) applied to samples S4, S5 and S6, a significant percentage of microtubules break, and the resulting length distribution can be well-fitted by Equation 3-13. For high shear rates \(5 \times 10^5 \text{s}^{-1}\) the center break model fails, since even if complete breakage \(b = 1\) is assumed, the fit underestimates the number of short microtubules and overestimates the number of longer microtubules. Under high shear rates, repeated breakage must occur.

The polydispersity of the samples is not significantly changed after shearing (Table 3-3), which is in agreement with the center break model predicting an identically shaped distribution after complete shearing but at exactly half the microtubule length. In comparison, the milling of synthetic polymers also did not reduce polydispersity when the average molecular weight decreased to half of the initial value \[89\]. Only when the
average molecular weight of the polymer was reduced by about an order of magnitude, as a result of the mechanical force exerted by the milling process, was a significant reduction of the polydispersity index observed (e.g. from 2.1 to 1.3).

Consequently, a reduction in the polydispersity index by milling/shearing is only possible if the fragmentation process preferentially removes long microtubules. Unfortunately, the shearing experiments conducted here do not reveal a pronounced increase in the fragmentation of longer microtubules.

The opposite post-processing step, annealing, was found to increase microtubule lengths several-fold, but again without decreasing the polydispersity index to below the pre-shearing value of 1.5 (Table 3-4). The length distributions of sheared microtubules can be approximated by Equation 3-14 with \( \lambda = (1.65 \, \mu m)^{-1} \) and the length distribution of annealed microtubules can be fit with Equation 3-16, again, using \( \lambda = (1.63 \, \mu m)^{-1} \) and \( w = 0.87 \). The reasonable agreement between the fit and the histogram (Fig. 3-4) suggests that there is no significant bias against the extension of longer microtubules.

This is somewhat surprising, since longer microtubules have lower rotational and translational diffusion constants and should consequently be disadvantaged relative to shorter microtubules in the annealing process [94]. It is interesting to speculate if the more recently discovered increased flexibility of the microtubule end [107-109] confers a length-independent ability on the end to associate with other microtubules. At the least, the complex architecture of the microtubule has not yet been accounted for in the classic thermodynamic treatment of the annealing process by Hill in 1983 [94].

An alternative approach to generating a population of microtubules with uniform length is sorting. A separation by length has been achieved for microtubules gliding on a
kinesin-coated surface with a kinesin density gradient, where long microtubules are preferentially retained on the surface due to their ability to bridge larger gaps between motors [110]. However, the polydispersity of the remaining (longer) population does not seem to decrease. Secondly, separation by length may potentially be achieved by exploiting the length-dependent response of microtubules to electric fields [111].

**Conclusion**

Microtubules polymerize in vitro from both ends with asymmetric growth rates. While the length distribution of each end is a simple exponential function, the length distribution of the filament is the result of the combination of these two exponential distributions. The ensuing length distribution can be described by a generalization of the Schulz distribution described in the classic literature of synthetic polymers.

The length distribution obtained after completion of the polymerization step can be engineered by post-processing methods, such as shearing and annealing. Shearing leads to fragmentation of microtubules and a concomitant decrease in the average length, however the desired reduction in polydispersity was not observed. Annealing, also referred to as end-to-end joining, of microtubules increases the average microtubule length, but again does not reduce polydispersity.

The observed polydispersity index of 1.5 after polymerization is smaller than the initially expected polydispersity index of an exponential length distribution (PDI = 2) due to the averaging effect of polymerization from both ends; however, a further reduction of the polydispersity by post-processing was unsuccessful.

The present work can be seen in the context of the larger challenge of obtaining reliable and uniform results from inherently stochastic molecular processes, which is a
classic problem in polymer science but which is now encountered by nanotechnology and synthetic biology.
Table 3-1. Shearing details.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Needle size</th>
<th>Number of passes</th>
<th>Average flow rate, &lt;Q&gt; (µL/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>n/a</td>
<td>n/a</td>
<td>0</td>
</tr>
<tr>
<td>S1</td>
<td>25G</td>
<td>1</td>
<td>22</td>
</tr>
<tr>
<td>S2</td>
<td>25G</td>
<td>2</td>
<td>22</td>
</tr>
<tr>
<td>S3</td>
<td>25G</td>
<td>1</td>
<td>46</td>
</tr>
<tr>
<td>S4</td>
<td>30G</td>
<td>1</td>
<td>21</td>
</tr>
<tr>
<td>S5</td>
<td>30G</td>
<td>2</td>
<td>21</td>
</tr>
<tr>
<td>S6</td>
<td>30G</td>
<td>1</td>
<td>36</td>
</tr>
<tr>
<td>S7</td>
<td>30G</td>
<td>1</td>
<td>113</td>
</tr>
</tbody>
</table>
Figure 3-1. Measuring microtubule lengths. Microtubule lengths were measured by tracing each microtubule in the field of view. Fairly linear microtubules, such as in upper image, were traced using the straight-line selection drawing tool in the ImageJ software. Nonlinear microtubules, such as in the lower left corner, were measured using the free-hand selection drawing tool. The lengths of the drawn outlines were calculated by the software. Caution was taken to prevent a microtubule from being measured more than once.
Table 3-2. Characterization of in vitro polymerized microtubules.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Average absorbance, ( &lt;A&gt; )</th>
<th>Number-average length, ( &lt;L_n&gt; ) (µm)</th>
<th>Standard deviation, ( \sigma_n ) (µm)</th>
<th>Weight-average length, ( &lt;L_w&gt; ) (µm)</th>
<th>Polydispersity index, PDI</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.032 ± 0.001</td>
<td>1.55 ± 0.04</td>
<td>0.65</td>
<td>1.83 ± 0.04</td>
<td>1.18 ± 0.05</td>
</tr>
<tr>
<td>5</td>
<td>0.047 ± 0.000</td>
<td>2.76 ± 0.10</td>
<td>1.45</td>
<td>3.52 ± 0.11</td>
<td>1.27 ± 0.07</td>
</tr>
<tr>
<td>30</td>
<td>0.047 ± 0.001</td>
<td>3.79 ± 0.13</td>
<td>2.73</td>
<td>5.74 ± 0.16</td>
<td>1.52 ± 0.06</td>
</tr>
</tbody>
</table>

Absorbance readings were measured at a wavelength absorbance of 340nm. Number-average and weight-average lengths, standard deviations and polydispersity indices were calculated and tabulated above for microtubules polymerized at various times.
Figure 3-2. Length histograms of in vitro polymerized microtubules. A) Polymerization time was 1 min; 283 microtubules were counted. B) Polymerization time was 5 min; 201 microtubules were counted. C) Polymerization time was 30 min; 453 microtubules were counted. Curve fits were made to the asymmetric growth model (Equation 3-11) with $\lambda_+ = \lambda_-/4$ (solid line) and the length distribution proposed by Hill [86] (dashed line).
Table 3-3. Characterization of microtubule samples after shearing.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Average shear rate, $&lt;\gamma&gt;$ (s$^{-1}$)</th>
<th>Number-average length, $&lt;L_n&gt;$ (µm)</th>
<th>Standard deviation, $\sigma_n$ (µm)</th>
<th>Weight-average length, $&lt;L_w&gt;$ (µm)</th>
<th>Polydispersity index, PDI</th>
<th>Degree of fragmentation, b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>5.03 ± 0.32</td>
<td>3.99</td>
<td>8.17 ± 0.32</td>
<td>1.63 ± 0.10</td>
<td>0</td>
</tr>
<tr>
<td>S1</td>
<td>$1.1 \times 10^4$</td>
<td>5.15 ± 0.29</td>
<td>4.14</td>
<td>8.46 ± 0.30</td>
<td>1.65 ± 0.09</td>
<td>0</td>
</tr>
<tr>
<td>S2</td>
<td>$1.1 \times 10^4$</td>
<td>4.76 ± 0.29</td>
<td>3.45</td>
<td>7.25 ± 0.25</td>
<td>1.52 ± 0.09</td>
<td>0</td>
</tr>
<tr>
<td>S3</td>
<td>$2.2 \times 10^4$</td>
<td>4.51 ± 0.27</td>
<td>3.77</td>
<td>7.66 ± 0.30</td>
<td>1.70 ± 0.10</td>
<td>0</td>
</tr>
<tr>
<td>S4</td>
<td>$5.2 \times 10^4$</td>
<td>3.63 ± 0.19</td>
<td>2.78</td>
<td>5.75 ± 0.18</td>
<td>1.59 ± 0.08</td>
<td>0.350</td>
</tr>
<tr>
<td>S5</td>
<td>$5.2 \times 10^4$</td>
<td>3.35 ± 0.19</td>
<td>2.54</td>
<td>5.26 ± 0.18</td>
<td>1.57 ± 0.09</td>
<td>0.375</td>
</tr>
<tr>
<td>S6</td>
<td>$8.9 \times 10^4$</td>
<td>2.90 ± 0.15</td>
<td>2.12</td>
<td>4.45 ± 0.14</td>
<td>1.53 ± 0.08</td>
<td>0.656</td>
</tr>
<tr>
<td>S7</td>
<td>$2.8 \times 10^5$</td>
<td>1.51 ± 0.05</td>
<td>1.00</td>
<td>2.17 ± 0.06</td>
<td>1.44 ± 0.06</td>
<td>1</td>
</tr>
</tbody>
</table>

The parameter b, degree of fragmentation, is defined in Equation 3-13.
Figure 3-3. Length histograms of the controlled and sheared microtubule samples. For the control and samples S1 to S7 the number of microtubules measured were 249, 311, 279, 272, 367, 322, 359 and 364 respectively. The black solid curve represents the fit of a Schulz distribution to the unsheared (control) population; it is replicated in all the histograms. The blue curves represent fits of the “center break” shearing model.
Table 3-4. Characterization of microtubule samples after annealing.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Number-average length, $&lt;L_n&gt;$ (µm)</th>
<th>Standard deviation, $\sigma_n$ (µm)</th>
<th>Weight-average length, $&lt;L_w&gt;$ (µm)</th>
<th>Polydispersity index, PDI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-shearing</td>
<td>7.89 ± 0.38</td>
<td>5.68</td>
<td>11.96 ± 0.47</td>
<td>1.52 ± 0.09</td>
</tr>
<tr>
<td>Post-shearing</td>
<td>3.86 ± 0.23</td>
<td>3.41</td>
<td>6.85 ± 0.30</td>
<td>1.77 ± 0.10</td>
</tr>
<tr>
<td>Annealed 90min</td>
<td>18.28 ± 0.84</td>
<td>13.39</td>
<td>28.05 ± 1.04</td>
<td>1.53 ± 0.09</td>
</tr>
</tbody>
</table>

Number-average and weight-average lengths, standard deviations and polydispersity indices were calculated and tabulated above for microtubules polymerized for 30 minutes, sheared and then annealed.
Figure 3-4. Length histogram of microtubules after annealing. A) Microtubules polymerized, in the presence of Taxol, for 30 minutes; 222 microtubules were measured. B) Microtubules were sheared with a 30G needle; 225 microtubules were measured. C) Sheared microtubules were allowed to anneal for 90 minutes; 252 microtubules were measured. The histogram was fitted with Equation 3-16 and \( w = 0.84 \).
CHAPTER 4
DEGRADATION OF KINESIN-POWERED MOLECULAR SHUTTLES

Introduction

A car engine slowly rusts when turned off but wears out quickly when running. Active nanosystems are similarly characterized by an internal flow of energy, which can be expected to cause specific types of accelerated degradation. In this chapter, the activation-dependent mechanical degradation of a reliably working nanosystem is investigated for the first time. The model system of choice is the molecular shuttle, a nanoscale transporter consisting of functionalized and stabilized microtubules propelled by surface-adhered kinesins.

Wear and Fatigue

In macroscopic machines, degradation as a result of activation is primarily the result of wear and fatigue. Wear, the sustained removal of small amounts of material from moving parts as a result of friction, causes an increasing deviation of the part dimensions from the ideal. This often leads to diminished performance, increased stresses, and further increased wear until catastrophic failure occurs. Fatigue is the result of the application of cyclic stresses, which causes an accumulation of damage to the part and eventually catastrophic failure [112]. Wear and fatigue are major considerations in machine design [113].

In molecular and nanoscale machines, wear and fatigue will occur as well, since both processes have molecular origins [114]. However, state-of-the-art active nanosystems are often operated for only a few cycles to prove their basic feasibility [115, 116]. In contrast, biological nanosystems are highly functional nanomachines, capable of sustained movements over many cycles. However, biological systems have
evolved mechanisms to minimize the effect of wear and fatigue. These unidentified mechanisms may obscure the effects of sustained activation, just as wear is difficult to observe in a well-lubricated engine.

Kinesin-powered molecular shuttles [21, 117, 118] are hybrid devices, which utilize microtubules and kinesin motor proteins [25] as biological components in a synthetic environment. These molecular shuttles combine the excellent functionality of biological nanomachines with a controlled environment, where cellular repair mechanisms are absent. As a result, they provide an outstanding test-bed to observe degradation as a result of activation.

**Motor-induced Wear**

A chain is as strong as its weakest link. Similarly the shelf life of a device is limited by the most sensitive component. Brunner et al. determined that the most sensitive component of the kinesin-powered molecular shuttle was the microtubule [119]. The lifetime of the molecular shuttles were examined on various surfaces and the outcomes indicated that the microtubules wore out long before the kinesin motors for each surface type.

In an early experiment comparing stability of microtubules in the presence of active and inactive motors, it was shown the microtubules were significantly worn out and shortened in length when exposed to active motors within a few hours. The microtubules in the presence of inactive motors remained relatively unchanged, Figure 4-1. These early results indicated a dependence of microtubule degradation on kinesin motor activity. Subsequent experiments were designed to measure the degree of degradation of microtubules as a function of kinesin motor activity.
Experimental Materials and Methods

Microtubule Preparation

Microtubules were polymerized by reconstituting a 20-µg aliquot of rhodamine-labeled, lyophilized tubulin (TL331M, Lot 367 from Cytoskeleton Inc, Denver, CO.) with 6.25 µL polymerization buffer solution (BRB80 with 4 mM MgCl₂, 1 mM GTP, 5% dimethyl sulfoxide), and grown at 37°C for approximately thirty minutes. The microtubules were then stabilized by diluting a hundred-fold into a BRB80 buffered solution with 10 µM paclitaxel (Taxol, from Sigma, St Louis, MO).

Kinesin Motor Protein Preparation

A kinesin construct consisting of the wild-type, full-length Drosophila melanogaster kinesin heavy chain and a C-terminal His-tag was expressed in Escherichia coli and purified using a Ni-NTA column [120]. The stock kinesin motors were diluted 20-fold into a BRB80 buffered solution containing 0.5 mg/mL casein and 5 µM AMP-PNP.

Motility Solution

Microtubules were diluted to a final concentration of 32 nM in buffered motility solution containing 0.5 mg/mL casein, 100 nM Taxol, 20 mM D-glucose, 20 µg/mL glucose oxidase, 8 µg/mL catalase, 10 mM dithiothreitol (DTT), 2 mM creatine phosphate (CP), 2 units/L creatine phosphokinase (CPK) and 5 µM AMP-PNP.

ATP-Antifade (AAF) Solution

The ATP-antifade solution was made by mixing 92 µL BRB80 with 0.5 mg/mL casein with 1 µL each (Taxol, D-glucose, glucose oxidase, catalase, DTT, CP, CPK) and 1 µL of the appropriate ATP concentration or AMP-PNP in the case of the control sample. The ATP concentrations tested were 10 µM, 25 µM, 80 µM and 1000 µM; for the control sample 1 mM AMP-PNP was used in place of ATP.
Motility Assay

A flow cell was constructed using two cover slips and double-sided tape; the height of the flow cell was approximately 100 µm. Initially, a solution of 0.5 mg/mL casein, diluted in BRB80, was flown into the flow cell to wet the surfaces and coat them with casein. After five minutes the solution was exchanged with the kinesin motor solution. This solution was then exchanged with the motility solution after another five minutes.

The flow cells were stored in a dark container for approximately fifteen minutes to allow the microtubules to settle and bind to kinesin motor protein heads. Lastly the solution was exchanged with the ATP-antifade solution of the appropriate fuel concentration. The openings of the flow cells were sealed with a small amount of apiezon grease to prevent evaporation of the solutions.

Motor-free Motility Assay

The microtubules were prepared as described above. A microcentrifuge tube was rinsed with a 0.5 mg/mL casein solution to prevent absorbent of proteins onto the walls. The microtubules were then diluted in a motor-free motility solution containing 100 nM Taxol, 20 mM D-glucose, 20 µg/mL glucose oxidase, 8 µg/mL catalase, 10 mM dithiothreitol (DTT), and 1 mM ATP. At various time points, a 15-µL drop of the microtubule solution was removed for imaging. The sample was placed between two Fisherfinest coverslips; no flow cells were used. Samples were discarded after imaging. Extra care was taken to not disturb the solution while removing samples.

Microscopy Imaging

Microtubules were imaged using a Nikon TE2000-U Epi-fluorescence microscope (Nikon, Melville, NY) equipped with an X-cite 120 lamp (EXFO, Ontario, Canada) and
iXON DU885LC EMCCD camera (Andor, South Windsor, CT) and a 100x oil objective (NA 1.45). Exposure time was set as 0.2 s for all samples. Acquisition time between frames was varied for each sample: 2 s was used for the 80 µM and 1000 µM, 5 s was used for the 25 µM and 10 s was used for the 10 µM and the control. Images were taken at various intervals 5 min, 10 min, 15 min, 20 min, 30 min, 45 min, 60 min, 90 min, 2 h, and hourly until movement ceases or degradation nears completion. Ten images were taken at each acquisition.

For the motor-free motility assay, the settings were as followed: 0.2 s exposure time, 2 s between acquisitions. Multiple areas were imaged; only 2-3 images were taken at each spot to minimize photodamage and photobleaching effects.

**Data Analysis**

Microtubule lengths were measured using the imaging software, ImageJ (available at http://rsbweb.nih.gov/ij/). The straight-line selection and free-hand selection drawing tools were used to manually outline the straight and curved microtubules, respectively. The software then calculates the length of the drawn outline. All microtubules in the field of view were analyzed except for microtubules shorter than 0.5 µm.

Microtubule speeds were measured using a particle analysis plug-in in Image J, Manual Tracking. The ten images were stacked; a microtubule was followed from frame to frame by manually clicking on its end. The software calculates the microtubule’s speed between each frame. An average speed is calculated for that microtubule. This is repeated for up to twenty-five microtubules chosen randomly, and then an overall average speed is calculated for that particular time acquisition.
Results

Evolution of Experimental Protocol

Standard motility assays, at varying ATP concentrations, were conducted to calculate the maximum speed and $K_m$ constant of the kinesin motors, using the Michaelis-Menten equation. ATP concentrations for future assays were intentionally selected to represent 100%, 50%, 25% and 12.5% of the maximum motor speed. In the control assays, AMP-PNP, an ATP analogue which halts motor action [121], was used in place of ATP. Early assay results were inconclusive. Two variables were convoluting the data: delayed landing and non-constant kinesin speed.

In this work, similar to work by Brunner et al., there was an initial increase in the number of microtubules on the flow cell surface that could not be explained by degradation only. This initial increase was attributed to microtubules first landing and being anchored by the kinesin motors. The microtubule landing rate appeared to have some length dependence as well. This problem was resolved by allowing time for the microtubules to land before degradation observation would begin. In all the samples, a low concentration of AMP-PNP was used in the kinesin motor solutions. In this way the starting point of kinesin activity was controlled and time was allowed for all microtubules to reach the surface before observation began.

The speed of kinesin motors decreased over time in all the samples of active motors. This decrease was not surprising and was explained by simple thermodynamics. The system was a closed system. As the assay proceeded, the speed/activity of the kinesin motors decreased as the fuel supply decreased. To overcome this obstacle an ATP regenerating system [122], CP-CPK, was introduced into the flow cell. In the presence of CP, CPK will phosphorylate ADP with a free
phosphate ion [123]. With the regeneration system, the kinesin speed remained constant, within 20%, over the assay time period (Figure 4-2).

Change in Microtubule Length and Number, in Presence of Motors

The images of gliding, rhodamine-labeled microtubules, acquired by epi-fluorescence microscopy, showed that the length of the microtubules as well as their surface density decreased over a timescale of hours (Figure 4-3A-F). This decrease was absent in the control experiment (Figure 4-3 G-I), with stationary microtubules and motors arrested by AMP-PNP. The lengths of the microtubules were measured as described in Chapter 3. An average length was calculated for each assay at each time point and plotted in Figure 4-4A. The number of microtubules per field of view was also recorded and plotted in Figure 4-4B.

Change in Microtubule Length and Number, in Absence of Motors

The average length of microtubules in a motor-free motility assay showed a slow decrease with time (Figure 4-6A). The number of microtubules per field of view remained fairly constant for the first two days (Figure 4-6B). The number count began to decrease towards the end of day three. Length histograms were constructed and are shown in Figure 4-7.

Discussion

Kinesin motor proteins were non-specifically adhered to a surface at a density of 2100 µm⁻² and supplied with their substrate ATP as a source of chemical energy [124]. The two “head” domains of the kinesin motors bind to, and walk along, the microtubules with 8 nm steps, propelling them forward with a maximum velocity of 500 nm/s at the chosen temperature of 23°C [36]. On the order of 260 motors are estimated to attach simultaneously to a microtubule. In the absence of guiding structures, the trajectory of
gliding microtubules can be described as a worm-like chain [107]. If the paths of two
gliding microtubules cross, the microtubules glide over each other with minor bending
[125]. Microtubules can glide for hours if the oxygen concentration in the buffer solution
is kept low [119].

A 5-µm long microtubule gliding for an hour with a velocity of 500 nm/s, while
interacting with 260 motors, would experience 117 million motor steps during this time.
To study the effect of active movement, the microtubules are deposited on the surface
in the presence of the AMP-PNP. Replacing the AMP-PNP solution with an ATP
solution, containing an ATP-regenerating system, results in a concurrent and constant
activation of microtubule gliding. Since oxidative reactions are known to lead to
microtubule degradation, especially during illumination for fluorescence imaging [126],
care has been taken to minimize light exposure and control for its effect.

The microtubule length distribution can initially be described with a generalized
Schulz distribution, as described in previous chapter, which peaks at roughly the
average length (Figure 4-5 and Figure 4-7). In the presence of active motors, the length
distribution shifts towards shorter lengths over a time scale of minutes to hours; the rate
of shifting varies with motor activity. There is negligible shifting in the length distribution
of the control sample with time, indicating minimal microtubule wear in the presence of
inactive motors. In the absence of kinesin motors it takes tens of hours before the shift
in length distribution towards shorter lengths is noticeable. The shape of the length
distribution transforms from a Schulz distribution to an exponential distribution.

Both microtubule number per field of view and microtubule average length
decreased exponentially over a timescale of hours with an accelerated degradation at
faster gliding speeds. In a plot of the average length as a function of the distance glided (Figure 4-8), the velocity-dependent curves collapse onto a single master curve. This master curve can be well fitted using an exponential decay function with a rate constant of 1.2 mm\(^{-1}\) and a “half distance” of 0.8mm, or \(10^5\) steps, hinting at a possible first-ordered kinetics. In a plot of microtubule number as a function of distance glided, the decrease pattern appears in two phases. The first phase is a fast degradation rate followed by a slow degradation rate. The slower degradation rate begins after the first “half distance”.

**Conclusion**

It was shown that molecular shuttles degrade by microtubule fracture when the kinesin motors were activated. The degree of degradation correlates with the degree and duration of motor activity. The motor activity itself causes only subcritical stresses, but the accumulation causes catastrophic damage to the microtubules. These results stress that the design of synthetic and biological active nanosystems has to account for engineering challenges in addition to chemical and physical considerations, such as reaction rates or maximal forces.
Figure 4-1. Microtubule degradation in the presence of active versus inactive kinesin motors. Microtubules in the presence of active motors at time A) 0 h, B) 2 h, and C) 4 h. Microtubules in the presence of inactive motors at time D) 0 h, E) 2 h, and F) 4 h.
Figure 4-2. Kinesin motor activity in presence of a regeneration system. A) Kinesin motor average speed over time. B) Microtubule gliding distance over time.
Figure 4-3. Fluorescence microscopy images of microtubules. Microtubules in the presence of active motors fueled with 1000 µM ATP at time: A) 5 min, B) 30 min, and C) 60 min. Microtubules in the presence of active motors fueled with 10 µM ATP at time: D) 5 min, E) 30 min, and F) 60 min. Microtubules in the presence of inactive motors at time: G) 10 min, H) 30 min, and I) 60 min.
Figure 4-4. Microtubule average length and number in the presence of motors. 
A) Change in microtubule average length over time. B) Change in number of microtubules per field of view, FOV, over time. Legend in A is the same for B.
Figure 4-5. Evolution of microtubule length distribution. Length distribution of microtubules in the presence of active motors fueled with 1000µM ATP at A) 5min, B) 30min, C) 60min and D) 120min. Length distribution of microtubules in the presence of active motors fueled with 10µM ATP at E) 5min, F) 30min, G) 60min and H) 120min. Length distribution of microtubules in the presence of inactive motors at I) 10min, J) 30min, K) 60min and L) 120min.
Figure 4-6. Microtubule average length and number in the absence of motors. 
A) Change in microtubule average length over time. B) Change in number of microtubules per field of view, FOV, over time.
Figure 4-7. Length histograms of microtubules in motor-free motility solution.
Figure 4-8. Microtubule average length and number as a function of gliding distance. 
CHAPTER 5
ENGINEERING THE GLIDING SPEED OF CARGO-LOADED MICROTUBULES

Introduction

As discussed in Chapter 2, each monomer of the tubulin heterodimer has three functional domains: a nucleotide binding region, a drug binding region and a motor binding region. As kinesin motors walk along the protofilament track of a microtubule, it binds to the motor binding regions of the β-tubulin. In decorated, functionalized microtubules, the additional molecules on the microtubules may pose competition for the kinesin motor heads if they are binding in the motor binding region. It is of interest to see what effect cargo loading has on the gliding speed of in vitro microtubules.

Previous works have shown that microtubule associated proteins control trafficking by reducing frequency of kinesin binding to microtubules in vivo [127] and in vitro [128]. In vitro studies showed the kinesin processivity was unaffected during single motor studies [128] and mildly decreased in multiple motor assays [129]. In a later study, cryo-electron microscopy indicated that microtubule associated proteins do not occupy the same binding region as kinesin motors [130] and thus would explain why kinesin processivity is negligibly affected. A more recent study has shown that obstacles on microtubules can reduce kinesin processivity in vitro and ex vivo [131].

The maximum dosage of streptavidin needed to fill all available biotin binding sites of biotinylated microtubules was previously determined to be 40 nM•min, or 8 nM for a 5-min incubation/exposure time, by Ramachandran et al. [68] by fluorescence measurements. However, Korten et al. [132] reported increased fluorescence intensity of streptavidin-coated biotinylated microtubules beyond the 40 nM•min maximum
dosage. Korten also reported increased reduction in microtubule gliding speed as cargo density increased.

In this chapter, the deceleration of a kinesin-powered molecular shuttles following cargo loading is determined experimentally. The molecular shuttles are varied by biotin composition and streptavidin concentration. Fluorescence measurements will be used to verify binding of streptavidin molecules to microtubules and to investigate the binding mechanism of cargo onto molecular shuttles.

**Experimental Methods and Materials**

**Microtubule Preparation**

**Zero percent, 0%, biotinylated microtubules:** A 20-µg aliquot of rhodamine-labeled tubulin was dissolved in 6.25 µL of buffered growth solution containing 4 mM MgCl₂, 1 mM GTP and 5% DMSO, and then grown at 37°C for thirty minutes. The 0% biotinylated, or rhodamine, microtubules were diluted 100-fold in a BRB80 solution containing 10 µM Taxol.

**Fifty percent, 50%, biotinylated microtubules:** One 20-µg aliquot each of biotin-unlabeled and rhodamine-labeled tubulin were combined together in 12.5 µL buffered growth solution containing 4 mM MgCl₂, 1 mM GTP and 5% DMSO, then grown at 37°C for thirty minutes. The 50% biotinylated microtubules were diluted 100-fold in a BRB80 solution containing 10µM Taxol.

**One hundred percent, 100%, biotinylated microtubules:** A 20-µg aliquot of biotin-unlabeled tubulin was dissolved in 6.25 µL of buffered growth solution containing 4 mM MgCl₂, 1 mM GTP and 5% DMSO, and then grown at 37°C for approximately thirty minutes. The biotinylated microtubules were diluted 100-fold in a BRB80 solution containing 10 µM Taxol.
Kinesin Motor Protein Preparation

Kinesin motors, prepared as described in [120] were diluted 20-fold into a BRB80 buffered solution containing 0.5 mg/mL casein and 1 mM ATP for all flow cells requiring moving microtubules. In the control assay for intensity measurements, 1 mM ATP was replaced with 1 mM AMP-PNP in the kinesin solution.

Antifade Solution

The antifade solution is a buffered solution consisting of 0.2 mg/mL casein, 10 µM Taxol, 1 mM ATP, 20 µg/mL glucose oxidase, 20 mM D-glucose, 8 µg/mL catalase and 10 mM DTT. In the control assay for intensity measurements, 1 mM ATP was replaced with 1 mM AMP-PNP in the antifade solution.

Motility Solution

The microtubules were diluted to a final concentration of 32 nM in a buffered motility solution containing 0.5 mg/mL casein, 100 nM Taxol, 20 mM D-glucose, 20 µg/mL glucose oxidase, 8 µg/mL catalase, 10 mM dithiothreitol (DTT), and 1 mM ATP. In the control assay for intensity measurements, 1 mM ATP was replaced with 1 mM AMP-PNP in the motility solution.

Streptavidin Solutions

Alexa568-labeled streptavidin (Molecular Probes) was prepared beforehand in 10 µM aliquots. The 1000 nM, 100 nM, 10 nM, 1 nM and 0.1 nM concentrations were diluted in antifade solution through serial dilution. The 9000 nM concentration was prepared by diluting the antifade components, to the appropriate concentrations, directly into the stock solution. In the control assays for intensity measurements, the antifade solution was prepared with AMP-PNP in place of ATP.
Flow Cell Washes

The flow cells were constructed with two Fisher finest cover glasses separated with double-sided tape. First a 0.5 mg/mL casein solution was flowed in the flow cells, then exchanged with a kinesin motor solution, and then again with the motility solution. A five minute wait time was executed following each wash. After the motility solution wait time, excess unbound microtubules were flushed out with two washes of the antifade solution. Then the streptavidin solution was flown in; a 10-minute incubation period is allowed for binding of streptavidin molecules to the microtubules. Following the incubation period excess streptavidin was removed with another two washes of antifade solution.

Microscopy Imaging

Microtubules were imaged under a Nikon TE2000-U Epi-fluorescence microscope (Nikon, Melville, NY) and an X-cite 120 lamp (EXFO, Ontario, Canada) and iXON DU885LC EMCCD camera (Andor, South Windsor, CT) and a 40x oil objective (NA 1.30).

Analysis

Data analysis was conducted using the imaging software, ImageJ (available at http://rsbweb.nih.gov/ij). The gliding speeds of the microtubules were measured using a particle analysis plugin in Image J. The instantaneous speed of a microtubule is measured over several frames and then averaged. This is repeated for a total of twenty microtubules and averaged.

The intensity measurements of microtubules loaded with streptavidin were measured by taking the difference between average counts within a circle, with a diameter of 3.2 µm, of both a region on a microtubule segment, and a region near the
segment but not on the microtubule, i.e. the background. This is repeated for a total of twenty-one locations and averaged.

Results

Gliding Speed

The gliding speed of microtubules (0%, 50% and 100% biotinylated) were measured before and after a 10-min incubation period with varying streptavidin concentrations and are shown graphically in Figure 5-1. The speeds of the 100% biotinylated microtubules before streptavidin binding could not be ascertained due to inability to image the biotin microtubules, as they have no fluorescent tag. There was not a significant change in the speeds of the 0% and 50% biotinylated microtubules, before streptavidin binding (data not shown); therefore, it is assumed the 0% and 100% biotinylated microtubules have identical before-speeds. The data shows there was no change in the gliding speed of the 0% biotinylated microtubules before (before-speed) and after (after-speed) streptavidin binding. There was a decrease in the after-speeds of the 50% and 100% biotinylated microtubules.

Intensity

The average intensity of the 100% biotinylated microtubules was calculated to measure the amount of streptavidin molecules adsorbed to microtubules, both in motion and stationary. The average intensity, shown in Figure 5-2, increased exponentially with an increase in streptavidin concentration. The trend in average intensity was the same for both moving and stationary microtubules.

Discussion

It cannot be proven that streptavidin molecules do not bind to rhodamine microtubule. However, the chemistry of the bonding indicates no streptavidin should
bind to the rhodamine microtubules due to the lack of biotin linkers on the tubulin heterodimers. The fact that the after-speeds of the rhodamine microtubules (referred to as 0% biotinylated microtubules) are not significantly different from the before-speeds provides support for the claim that no streptavidin molecules bonded to the rhodamine microtubules.

**Reduction in Gliding Speed**

The decrease in microtubule gliding speed showed a dependence on streptavidin concentration and percent composition of biotin. In Figure 5-3, the decrease in gliding speed is quantified and represented graphically as a function of streptavidin concentration and microtubule composition. As streptavidin concentration increased the 50% and 100% biotinylated microtubules experienced a greater decrease in gliding speed. At all streptavidin concentrations, the 100% biotinylated microtubules displayed a greater decrease in speed over the 50% biotinylated microtubules. The only exception was the 9000nM streptavidin concentration of the 50% biotinylated microtubules.

The 9000-nM-50% biotin sample is believed to be either an outlier or a high error data point. The method used to select which microtubules to measure was as followed: starting at the upper left corner of the microscopy image, the first twenty microtubules encountered were used for speed measurements. In the 9000-nM-50% biotin sample, only nine of the first twenty microtubules encountered showed any movement. The average speed of the first twenty was 49 nm/s, indicating a 92% reduction in gliding speed; the average speed of the nine moving microtubules was 357 nm/s, indicating a 44% reduction in gliding speed. Both data points are shown in Figure 5-3.
Intensity

The average intensity of the microtubules was used to show binding of streptavidin molecules to the microtubules. It is assumed, and supported by the speed measurements, that streptavidin did not bind to the 0% biotinylated microtubules, i.e. rhodamine microtubules. Rhodamine has a higher fluorescence emission than does Alexa568. The average intensity measurements of the 50% biotinylated microtubules following streptavidin incubation were inconclusive (data not shown). The increase in intensity strictly due to streptavidin binding was not distinguishable and at times not noticeable.

In the 100% biotinylated samples, fluorescence intensity was a great tool for measuring streptavidin binding. The biotinylated microtubules do not fluoresce at all, as they are not labeled or tagged. All fluorescent activity is strictly due to streptavidin binding. The data shows fluorescent intensity increased with increase in streptavidin concentration, indicating more streptavidin was binding to the microtubules. These results contradict Ramachandran's work, which predicted a 4 nM concentration for a 10 min incubation period would yield complete coverage, and supported Korten's findings. The increase in fluorescence intensity counts for the 100% biotinylated microtubules exceeded well beyond the 4 nM proposed maximum concentration.

Cargo and Microtubule Attachment

To attempt to describe the binding behavior of streptavidin, the intensity counts are plotted against streptavidin concentration on a linear scale. The data points of both the moving and stationary microtubules are used collectively. At first glance, the data points appear to follow the Langmuir curve; however, it is an improper fit, see Figure 5-4. Applying the Langmuir equation, shown as the blue curve, the parameters $I_{max}$ and $K_m$
come out to be 83.8 counts and 18.2 nM respectively; the $K_m$ parameter represents the streptavidin concentration that would yield half the maximum average intensity count.

$$I = I_{max} \times \frac{[SA]}{K_m + [SA]} \quad (5-1)$$

The Hill function (Equation 5-2) yields a slightly better curve fit, shown in red. The Hill equation is similar to the Langmuir equation, and is generally used in biochemistry to quantify the cooperative binding of substrates. In this equation:

$$I = I_{max} \times \frac{[SA]^n}{k^n + [SA]^n} \quad (5-2)$$

the Hill coefficient, $n$, describes the cooperativity of the binding between an enzyme and a substrate: $n=1$ means independent binding, i.e. Langmuir model; $n>1$ means positive cooperative binding; and $n<1$ means negative cooperative binding. Applying Equation 5-2, the parameters $I_{max}$, $k$ and $n$ come out to be 142.9 counts, 168.9 nM and 0.53 respectively. The Hill coefficient indicates negative cooperative binding between streptavidin and biotinylated microtubules. This would mean as streptavidin molecules bind to the microtubules, the affinity for other streptavidin molecules is decreased.

**Cooperative Binding of Streptavidin on Biotinylated Microtubules**

Taxol-stabilized microtubules, having an isoelectric point of 4.2 [54] at neutral pH, have a negative effective charge on their surface. Streptavidin molecules, having an isoelectric point of approximately 5, also have a negative effective charge on their surface. While the microtubule surfaces and streptavidin surfaces may repel each other, the strong affinity between streptavidin and biotin allows the binding of streptavidin onto biotinylated microtubules to occur. A streptavidin molecule has a molecular weight of approximately 50-55 kDa, about half the size of a tubulin heterodimer. A minimum separation distance of 150 angstroms is needed to overcome steric repulsion between
two streptavidin molecules [133], equal to about two heterodimers. When one streptavidin molecule binds to a biotin linker, it negatively affects the binding of another streptavidin molecule on an adjacent binding site within 150 angstroms.

In Figure 5-2, the data shows it takes about a 10-fold increase in streptavidin concentration to yield an approximate two-fold increase in the average intensity. At low concentrations of streptavidin, the streptavidin molecules are able to bind on the biotinylated microtubule surface at far enough distances that steric hindrance is not an issue. As streptavidin concentration increases, excess streptavidin molecules remain in solution. When a streptavidin molecule can get within 85Å (the maximum distance where attractive force between biotin and streptavidin is effective, [134]) of a biotin linker, the attractive force between biotin and streptavidin will pull in the streptavidin molecule and a near irreversible bond forms. The attractive force between biotin and streptavidin is greater than the repulsive force between two streptavidin molecules.

**Conclusion**

Functionalized microtubules, such as biotinylated microtubules, can be engineered to pick up cargo. The loading of the cargo onto the molecular shuttles was shown to result in a reduction of their gliding speed. The gliding speed reduction was characterized as a function of the biotin content of microtubules and streptavidin concentrations. Intensity measurements were used to indicate binding of streptavidin to biotinylated microtubules. The full profile of cargo loading onto biotinylated microtubules is yet to be achieved. One speculation presented in this chapter relates the loading of cargo onto biotinylated microtubules to that of negative cooperative binding between a substrate and an enzyme.
There still remains the question of how, mechanically, does the change in gliding speed occur. Past research has proposed several thoughts as to why loading of cargo onto molecular shuttles causes a decrease in their gliding speeds. The current accepted model states the kinesin molecule will attempt to move around the roadblock. If it succeeds, motion continues; if it does not succeed, motion ceases. The success rate decreases with increase in roadblocks [131, 132]. Would this model still hold with an increase or decrease in motor density? Is there a speed reduction dependence on motor density? Would the size of the roadblocks, cargo, affect the kinesin’s detouring ability?

Understanding the relationship between gliding speed and cargo density of molecular shuttles is paramount to its continued development for use in hybrid systems. Knowing the mechanism of cargo attachment onto biotinylated microtubules would allow the gliding speed of molecular shuttles to be predicted for all cargo densities. Potentially, molecular shuttles could be used to sort/separate analytes in a solution, similar to the polymer characterization technique, size exclusion chromatography; here, separation would be based on speed instead of size. Decrease in gliding speed can also be used to measure analyte concentration; the concentration would be derived indirectly from the reduction in gliding speed.
Figure 5-1. Average gliding speed of microtubules before and after streptavidin incubation. A) Average before- and after- speeds of the 0% and 100% biotinylated microtubules. B) Average before- and after- speeds of the 50% biotinylated microtubules.
Figure 5-2. Average intensity of 100% biotinylated microtubules. Average intensity counts were measured for the 100% biotinylated microtubules after the 10-min streptavidin incubation period. Microtubules in motion and stationary were measured.

Figure 5-3. Reduction in gliding speed of biotinylated microtubules. The percent reduction of the before-speeds of the 50% and 100% biotinylated microtubules are calculated and plotted against streptavidin concentration.
Figure 5-4. Average intensity of biotinylated microtubules as a function of streptavidin concentration. The data points for moving and stationary microtubules are used collectively to find a fit function. The blue curve was constructed using the Langmuir equation (Equation 5-1). The red curve was constructed using the Hill function (Equation 5-2).

Figure 5-5. Reduction in gliding speed as a function of average intensity count. The percent reduction of the before-speeds of the 100% biotinylated microtubules is plotted against the average intensity count.
CHAPTER 6
CONCLUSIONS AND OUTLOOK

Taking lessons from nature has offered materials scientists new sets of tools and materials for nanotechnology applications. Using biomolecules has offered the advantage of nanoscale size, chemical/biological specificity and autonomous behavior. For example, biomolecular motors are of interest to serve as actuators for nanodevices. One of the more promising biomolecular motors is the kinesin motor. For the purpose of nanobiotechnology, the kinesin and microtubule geometry is inverted in the design of molecular shuttles. Molecular shuttles can provide active transport to microfluidic devices; this can be more advantageous than pressure driven flow or simple diffusion. Advances in the engineering development of molecular shuttles have been made in activation control, guidance control, and cargo loading.

This dissertation sought to add to the working knowledge of molecular shuttles by investigating and reporting on the material properties of microtubules serving as the filament choice. The work presented here addressed three specific properties of microtubules: the length distribution of microtubules polymerized in vitro, the degradation mechanism of microtubules in active nanosystems, and the cargo loading effect on the gliding speed of molecular shuttles.

Microtubules, polymerized in vitro, grow at both ends. The growth rates are unequal and this leads to a Schulz distribution of microtubule lengths. Whereas post-polymerization processing reduces the polydispersity of synthetic polymers, such is not the case with Taxol-stabilized microtubules. Other methods besides shearing and annealing will have to be tested. Microtubule associated proteins are known to regulate microtubule length in vivo. It would be of interest to see if such proteins, when purified,
would regulate microtubule length in vitro as well. This may provide a way to reduce polydispersity and even yield uniform length microtubules.

For the first time, wear of microtubules is studied in active nanosystems. In vitro microtubules have been shown to remain intact for over a week when stabilized with Taxol in a buffered solution. This stability time is drastically reduced when in the presence of active motors. The degradation of microtubules was characterized as a function of kinesin motor activity and time. It was determined degradation was based on distance glided by kinesin motors rather than the motor speed. The complete degradation scheme is yet to be modeled and it is yet to be determined the extent of the role microtubule-breaking and microtubule-shrinking play. Other types of stabilized microtubules as well should be investigated for kinesin-activated degradation.

Previous works have shown molecular shuttles’ abilities to pick up cargo, transport cargo and drop off cargo. The mechanics of cargo loading has not previously been investigated. Here the decrease of microtubule gliding speed after cargo loading was monitored as a function of biotin composition and cargo concentration. Using fluorescence measurements, the change in intensity was used to measure cargo loading. It was shown the binding of streptavidin molecules onto biotinylated microtubules has a negative cooperative binding characteristic. A better technique is needed to measure fluorescence intensity of cargo loaded on biotinylated microtubules of other than 100% biotin content. The results can be compared with that of 100% biotin content to see if the loading mechanism follows the same scheme.

With the contributions made in this dissertation, it is anticipated that the role of microtubules in molecular shuttles has been enhanced. Further development in the
understanding of microtubules as an engineering material may lead to other potential uses and applications in nanotechnology.
APPENDIX: STANDARD MOTILITY ASSAY PROTOCOL

Buffers and Reagents

These solutions should be prepared in advance and stored in conveniently sized aliquots. An aliquot should contain sufficient solution for a typical experiment and a fresh aliquot should be used for each motility assay. The storage conditions and typical aliquot sizes are also mentioned in the following protocols.

1. BRB80 buffer, (80 mM PIPES, 1 mM MgCl₂, 1 mM EGTA in deionized distilled (dd) water, pH adjusted to 6.9 by KOH)
   a. Make up a 100 mL stock solution of 0.5 M EGTA in dd water. Adjust pH to 7.0 using 2 M NaOH solution.
   b. Make up a 100 mL stock solution of 1 M MgCl₂ in did water. Autoclave the solution.
   c. Add 24.2 g of PIPES and 3.1 g KOH pellets in approximately 800 mL of dd water and stir to dissolve. Adjust pH to 6.9 using 1 M KOH solution. Add 2 mL of 0.5 M EGTA stock solution and 1 mL of 1 M MgCl₂ stock solution. Bring up the volume to 1000 mL with dd water.
   d. Aliquot into 50 mL falcon tubes and freeze at -20°C for future use. The BRB80 tube currently being used can be stored at 4°C or at room temperature.

2. Magnesium Chloride, MgCl₂ (100 mM in dd water)
   a. Dilute in dd water to achieve a final concentration of 100 mM.
   b. Aliquot (10 µL volume) into 0.5 mL microcentrifuge tubes and store, for futures use, at -20°C.

3. Guanosine-5’-triphosphate, disodium salt, GTP (25 mM in dd water, pH adjusted to 7 by NaOH)
   a. Weigh out and dissolve in dd water and adjust the pH to 7 by 2 M NaOH solution.
   b. Verify concentration by measuring UV absorbance at 260 nm. (Use an extinction coefficient of 11.7 x 10³ M⁻¹ cm⁻¹ and the equation Aₜ =εcL).
   c. Aliquot (10 µL volume) into 0.5 mL microcentrifuge tubes and store, for futures use, at -20°C.
4. Dimethyl sulfoxide, DMSO
   a. Aliquot (10 µL volume) into 0.5 mL microcentrifuge tubes and store, for future use, at -20°C.

5. Taxol (1 mM in DMSO)
   a. Weigh out and dissolve in DMSO under fume hood to achieve a final concentration of 1 mM.
   b. Aliquot (20 µL volume) into 0.5 mL microcentrifuge tubes and store, for future use, at -20°C.

6. D-(-)-Glucose, (2 M in dd water)
   a. Weigh out and dissolve in dd water to achieve a final concentration of 2 M.
   b. Aliquot (20 µL volume) into 0.5 mL microcentrifuge tubes and store, for future use, at -20°C.

7. Glucose Oxidase, (2 mg/mL in BRB80)
   a. Dissolve in BRB80 to achieve a final concentration of 2 mg/mL.
   b. Aliquot (20 µL volume) into 0.5 mL microcentrifuge tubes and store, for future use, at -20°C.

8. Dithiothreitol, DTT (1 M in dd water)
   a. Dissolve in dd water under fume hood to achieve a 1 M final concentration.
   b. Aliquot (20 µL volume) into 0.5 mL microcentrifuge tubes and store, for future use, at -20°C.

9. Catalase, (0.8 mg/mL in BRB80)
   a. Dissolve in BRB80 in at least 2 stages to achieve a final concentration of 0.8 mg/mL. Determine the concentration at each stage by measuring UV absorbance at 276 nm and 406 nm (Use an extinction coefficient of 3.1 x 10^3 M^-1 cm^-1 at 276 nm and 2.2 x 10^5 M^-1 cm^-1 at 406 nm and the equation A_λ = εcL).
   b. Aliquot (20 µL volume) into 0.5 mL microcentrifuge tubes and store, for future use, at -20°C.

10. Adenosine-5'-triphosphate, ATP (100 mM in 100 mM MgCl2)
a. Prepare a stock solution of 100 mM MgCl₂ in dd water. Weigh out dry powder and dissolve in this stock solution to achieve a final concentration of 100 mM.

b. Verify concentration by measuring UV absorbance at 260 nm. (Use an extinction coefficient of 15.4 x 10³ M⁻¹ cm⁻¹ and the equation A₁cm = cεL).

c. Aliquot (20 µL volume) into 0.5 mL microcentrifuge tubes and store at -20°C for future use.

11. Casein solution (20 mg/mL casein in BRB80)

a. Add approximately 3 g casein to 30 mL dd water in a 50 mL falcon tube. Vortex for approximately 1 hour until solution develops thick consistency.

b. Centrifuge at approximately 15000 g for 30 minutes. Filter the supernatant through 0.5 µm and 0.2 µm syringe filters.

c. Determine the concentration of the supernatant by measuring UV absorbance at 280 nm (Use an extinction coefficient of 0.67 mL mg⁻¹ cm⁻¹ and the equation A₁cm = cεL). Dilute it to 20 mg/mL in BRB80.

d. Aliquot (20 µL volume) into 0.5 mL microcentrifuge tubes and store, for future use, at -20°C.

Standard Solutions

These solutions are prepared on the day of the experiment and should be discarded after the experiment is over. Prepare 1 mL of each.

1. BRB80CS0.5

a. Dilute casein solution in BRB80 to a final concentration of 0.5 mg/mL and store over ice. This solution is introduced into the flow cell prior to kinesin and helps retain kinesin activity after surface adsorption.

2. BRB80CA

a. Prepare 0.2 mg/mL casein and 1 mM ATP in BRB80 and store over ice. Kinesin is further diluted using this solution before introduction into the flow cell.

3. BRB80T

a. Dilute Taxol solution in BRB80 to a final concentration of 10 µM and store at room temperature. This solution is used to stabilize microtubules.
4. **BRB80CT**
   
a. Prepare 10 µM Taxol and 0.2 mg/mL casein in BRB80 and store at room temperature. This is used to further prepare the antifade and microtubule solutions.

5. **BRB80AF**
   
a. Prepare 20 mM D-glucose, 20 µg/mL glucose oxidase, 8 µg/mL catalase, 10 mM DTT, and 1 mM ATP in BRB80CT and store at room temperature. The kinesin speed can be controlled by adjusting the ATP concentration in this solution.

**Kinesin Preparation**

Express a kinesin construct consisting of the wild-type, full-length Drosophila melanogaster kinesin heavy chain and a C-terminal His-tag in Escherichia coli and purify using a Ni-NTA column as described in [50]. Make aliquots (10 µL each) in 0.5 mL microcentrifuge tubes and store at -80°C for future use. The concentration of active kinesin in these aliquots is approximately 200 nM [124].

**Microtubule Preparation**

In a 0.5 mL microcentrifuge tube, prepare 25 µL of growth solution: mix 21.75 µL BRB80, 1.25 µL DMSO and 1 µL each 100 mM MgCl₂ and 25 mM GTP. The final buffer reagent concentrations are: 5% v/v DMSO, 4 mM MgCl₂ and 1mM GTP. Add 6.25 µL of this growth buffer solution to a 20-µg aliquot of lyophilized rhodamine-labeled tubulin. Vortex, then place in a heat bath at 37°C for 30 minutes to polymerize. Note, if a water bath is used, wrap microcentrifuge tube in parafilm to prevent leakage or fluid exchange. Upon completion of polymerization, dilute the microtubules 100-fold by adding 5 µL of the grown microtubules to 495 µL BRB80T; vortex gently. Label the solution MT100 and store at room temperature. These microtubules can be used for up to one week, as long as they are stored in the dark and at room temperature.
For motility assays, the microtubules are diluted 10-fold in BRB80AF and labeled MT1000. The final concentration of microtubules in MT1000 solution is 32 nM. The MT1000 solution is good for approximately 1 hour.

**Flow Cell Construction**

Construct a flow cell using two glass coverslips separated by double-sided tape. This flow cell is approximately 2 cm long, 1 cm wide and 100 µm high, and has a volume of approximately 20 µL. Solutions are introduced into the flow cell from one side using a pipette and wicked out from the other using filter paper.

**Inverted Motility Assay Assembly**

The surfaces of the glass flow cell is first coated with a casein by flowing in 30 µL BRB80CS0.5; the casein allows kinesin to retain its functionality upon adsorption. Wait 5 min, then exchange the solution with 30 µL KIN20. (Note: During solution exchange, try to pipette in the new solution at the same rate the existing solution is being wicked out. This will prevent air pockets from forming in the flow cell and keeps flow cell from drying out.) Wait 5 min for kinesin to adsorb onto the surface. Next, exchange the solution with 30 µL MT1000.

**Microscopy**

Mount the flow cell on the microscope stage immediately after final solution exchange. In these experiments, an Eclipse TE2000-U fluorescence microscope (Nikon, Melville, NY) equipped with a 100X oil objective (N.A. 1.45), an X-cite 120 lamp (EXFO, Ontario, Canada) and an iXon EMCCD camera (ANDOR, South Windsor, CT) was used. A TRITC filter cube (#48002, Chroma Technologies, Rockingham, VT) was used to image the microtubules on the bottom surface of flow cells. The exposure time was
0.2 s, while the time between exposures was 2 s. These are typical acquisition settings and can be adjusted as needed.
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BIOGRAPHICAL SKETCH

Yolaine Jeune Smith was born in Port-au-Prince, Haiti. At the age of 6, her parents moved her and some of her siblings to live with them in the United States. She lived in Delray Beach, FL until graduation from high school. She graduated from Atlantic Community High School in the top 10% of her class; she was active in various extracurricular activities including Junior Army ROTC and Mu Alpha Theta and played sports including basketball and Track and Field.

The next phase of Yolaine's life began when she moved to Tampa, FL to pursue a higher education. Christianity has always played a large role in Yolaine's life. On April 20, 1997, Yolaine visited a church by the name of Revealing Truth Ministries (RTM), after being invited by a classmate. That same day she got saved and life has not been the same since. She attended RTM for 2 full years before officially joining; she then served in several departments including Teen/Youth Ministry, Mass Choir, Dance Ministry and Drama Ministry.

Yolaine completed a bachelor's degree in clinical laboratory sciences at the University of South Florida (USF). After a one-year hiatus, she returned and earned a master's degree in secondary science education with a concentration in biology. She then went on to teach high school chemistry in Hillsborough County for four years: one semester each at Blake High School and Tampa Bay Technical High School, and three years at Robinson Senior High School.

In her second year of teaching, Yolaine decided to pursue an engineering career; she re-enrolled at USF and began taking classes part-time. At the end of her fourth year teaching, Yolaine left the K-12 system to attend USF fulltime. One year later she transferred to the University of Florida after being recruited by Martha McDonald and
her advisor, Henry Hess. During the PhD process, Yolaine had the opportunity to do an internship at RTI Biologics, attend several research and professional development conferences where she delivered oral and poster presentations and participate in other extracurricular activities.

Yolaine was married to Andrew S.P. Smith in December 2008. They had met at USF when she re-enrolled for an engineering degree. Yolaine completed her doctorate degree in materials science and engineering from the University of Florida in May 2010. The first thing she is looking forward to doing is living with her husband fulltime and bringing an end to the commuting phase of their relationship. She also is seeking a postdoctoral appointment in medical research. Her long term career goal is to attain a tenured-track faculty position in academia. In the short term, she plans to remain very active in her community, mentoring teenagers and young adults. She also plans to be active in the rebuilding of her native country, Haiti, which suffered a tremendous loss of human lives and devastating structural damage from the 7.0-magnitude earthquake which hit the capital on January 12, 2010.