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Control of Myosin motor activity and Actin filament translation by alteration of Assay Reducing Potential

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TITLE

Control of Myosin motor activity and Actin filament translation by alteration of Assay Reducing Potential

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ABSTRACT

The use of biomolecular motors for nanotechnological applications has been impaired by an inability to control biomolecular motor activity in a temporal fashion. Previous data has suggested that myosin activity can be modulated by inducing changes in the degree of myosin oxidation. The hypothesis of this study is that myosin motor activity can be regulated by altering the amount of antioxidants and reducing agents present in assay solution. To test this hypothesis we systematically altered the concentration of reducing agents and examined the effects of these changes on myosin-dependent actin filament translation. Our findings indicate that myosin activity can be controlled over several on-off cycles in a controlled fashion by varying the amount of antioxidants and reducing agents in the assay medium. This strategy if further developed may lead to new ways to control myosin motor activity in bionanotechnological devices.

(Total words 140)

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LIST OF ABBREVIATIONS AND SYMBOLS

ADP	Adenosine diphosphate
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
$Cacl_2$	Calcium chloride
CCD	Charge-coupled device
DNA	Deoxy ribonucleic acid
DTT	Dithiothretol
F-actin	Filamentous actin
G-actin	Glomerular actin
GODCAT	Glucose oxidase and catalase in the medium of glucose
HMM	Heavy meromyosin
K	Potassium
KCl	Potassium chloride
KDa	Kilodalton
LMM	Light-meromyosin
$MgCl_2$	Magnesium Chloride
MRI	Magnetic Resonance imaging
mRNA	Messenger Ribonucleic acid
PDMS	Polydimethylsiloxane
Pi	Inorganic phosphate
SEM	Standard error of the mean
-SH	Sulphydral
-SS-	Thiol
TMCS	Trimethylchlorosilane
UV	Ultraviolet
Zn^{2+}	Zinc

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CHAPTER 1

INTRODUCTION

Biomolecular motors are cellular machines that are involved in the contraction of muscles, cell movement and the transport of cargo molecules within cells [1]. Nanobiotechnology is a young and rapidly evolving field of research at the cross roads of biotechnology and nano science that is considered one of the key technologies of the 21st century. A long-term goal of nanobiotechnology is the utilization of biomolecular motors for controlled cargo carriers such as liposomes to be carried on a chip, with applications in sorting, separation, purification or assembly of materials.

Biomolecular motors use the hydrolysis of adenosine triphosphate (ATP) to perform mechanical work with a remarkable degree of efficiency [2]. In the cell, biomolecular motors perform a wide variety of transport functions suggesting that they may someday be useful for the development of novel biomedical devices. There are two classes of biomolecular motors: linear and rotary. Linear motors include the myosin and kinesin motors while the rotary motors include the F0-F1 ATP synthase [3]. Among the linear motor type, myosin plays an important role in the contraction of muscles and in the transport of molecular cargo within cells. The transport of cargo by myosin occurs along actin filaments. Actin filaments are made up of glomerular actin (G-actin) subunits that are arranged in a head-to-tail fashion [4]. Recent work has demonstrated the potential use of biomolecular motors for the construction of actuators and

transporters capable of transporting micrometer and nanometer-sized objects like streptavidin coated beads and DNA molecules [3]. Other research in this field has been centered on developing suitable biocompatible substrates capable of supporting myosin activity [5]. While progress in these areas has been exciting, the application of biomolecular motors for nanotechnological applications has been hampered by an inability to accurately control motor activity.

In an effort to control the activity of biomolecular motors, several different approaches have been utilized over the past few years including (1) the engineering of Zn^{2+} binding sites into kinesin motors and using the modulation of Zn^{2+} availability to govern the on / off state of the motor [6] , (2) the use of photo-activated ATP in an effort to modulate motor activity by altering the amount of ATP available for hydrolysis [7] and (3) varying the temperature of the external environment surrounding the biomolecular motor to take advantage of the relationship between temperature and enzyme activity [8]. Although each approach has shown promise to control protein motor activity, these methods have yet to clearly demonstrate the ability to reversibly modulate motor activity over several cycles of activation.

Purpose

The purpose of this research is to develop the means to temporally control actin filament motility across a motor patterned surface. To fulfill this purpose we will examine how the presence and absence of reducing agents affects the ability of the myosin motor to translate actin filaments. The overall hypothesis of this study is that myosin motor activity can be regulated by altering the amount of antioxidants and reducing agents present in assay solution.

Specific Aims

Protein molecular motors such as the actin-myosin system are natural nano-machines that convert chemical energy directly into mechanical work. Our long-range goal is to use molecular motors for new biomedical applications. The ability to control the activity of molecular motors in an external environment is currently lacking. Without better mechanisms of motor control the use of these machines for applied purposes is significantly hampered. The objective of this study is to investigate the possibility of controlling molecular motor activity by altering the chemical composition of the buffer that the motor resides in. The working hypothesis of this study is that myosin motor activity can be regulated by altering the amount of antioxidants and reducing agents present in assay solution. To test this hypothesis two specific aims will be pursued:

Specific aim #1

To determine if absence of reducing agents has an effect on myosin motor activity in the *in vitro* motility assay.

Hypothesis

The absence of reducing agents will have a significant effect on myosin motor activity.

Specific aim #2

To investigate if myosin motor activity can be cyclically controlled by altering the concentration of reducing agents in the assay medium

Hypothesis

Manipulation of reducing agent concentration can be used to cyclically control myosin motor activity.

CHAPTER 2

REVIEW OF LITERATURE

Introduction

A review of the pertinent literature concerning the present study will be presented in the following chapter. The following areas will be addressed: 1) nano technology and nanobiotechnology: potential applications to mankind, 2) Structure and function of motor proteins, 3) Structure of the actomyosin motor system and, 4) Use of protein biomotors for nanotechnological applications.

Nanotechnology and nanobiotechnology: potential applications to mankind

Richard Feynman is widely credited for establishing the field of nanotechnology. Much of this credit arises from his famous talk “There’s plenty of room at the bottom” (American Physical Society: 1959) [9]. Initial progress in the field of nanotechnology was slow as it not until the 1980s that the scanning tunneling microscope and atomic force microscope were developed which allowed the manipulation and observation of single molecules. Since then, progress in the nanosciences has been rapid and has resulted in the ability of scientists to manipulate matter at its most fundamental level. For example, the development of the atomic force microscope has allowed researchers to individually pattern 35 xenon atoms to spell out the word “IBM” [10]. Similarly, other devices such as the atomic force microscopy now make it possible for scientists to probe the atomic structure of DNA [11]. Key goals of nanotechnology are to develop

new molecular treatments for disease, new types of manufacturing processes and new materials that would increase productivity while diminishing the effects of man on the environment.

Nanobiotechnology is defined as a field that applies nanoscale principles and techniques to understand and transform biosystems or use biological principles and materials to create new devices and systems integrated from the nanoscale [12]. Many of the most basic processes of life occur at the nanoscale suggesting that understanding the design of biological systems can be useful for the development of novel nanodevices and systems. For example, developing a better understanding of how a cell is designed and interacts with its environment may help researchers to develop new types of biological or chemical sensors. Such devices could have use in many fields including diagnostics, DNA separation and in the development of new nanomaterials that could be used imaging [13]. Recent advances in the field of nanobiotechnology have enabled higher sensitivity in detecting cancer cells and proteins, new methods of tumor imaging, and new materials e.g. liposomes, that could be used to deliver medicine or other types of molecules for the treatment of cancer [14].

Structure and function of motor proteins

Motor proteins convert chemical energy derived from the hydrolysis of ATP into mechanical energy [2]. Bionanomotors are used in muscle contraction and for the transport of cargo molecules within cells [15]. There are two types of motor systems: linear and rotary motor systems. Linear systems operate in a linear fashion while rotary

motors produce rotary motion [3]. Most of the current research in the field of these motor proteins has centered on linear motor proteins. There are three linear motor proteins in the cell: myosin motors which move on actin filaments and the kinesin and dynein motors which use microtubules [3].

Compared to kinesin and dynein, the actomyosin motility system may be advantageous for applications that involve the transport of materials. For example, the actomyosin system enables faster transportation (up to 10 times faster with muscle myosin II) [16] and the capacity to move heavier loads given its ability to generate higher amount of force [3].

Actin-myosin motor system

Muscle fibers are actually composed of large single cells that had been formed from many separate cells that have fused together. The bulk of the muscle fiber cytoplasm is made up of myofibrils. These myofibrils contract in the presence of ATP and calcium and in doing so generate the force used to produce movement. The structural unit of the myofibril is the sarcomere. Each sarcomere contains an anisotropic band that is bound by two isotropic bands. The anisotropic band is called the A-band; the isotropic band is called the I-band [17]. In the center of the A band, there is a lighter region known as the H-zone or H-band. The myofibrils in turn are composed of two proteinaceous structures called myofilaments. One filament is termed the thick filament and is about 11 nm in diameter[18] whereas the other filament is called the thin filament

and is 5 nm in diameter [19]. Thick filaments are made up of several hundred myosin molecules.

Within the cell there are many types of myosin such as myosin I, myosin II, myosin V and others[20]. Among these myosin types, myosin II is the most well known. Myosin II is known as a conventional myosin. It is present in all non-plant eukaryotic cells[4]. In the cells, myosin II plays an important role in muscular contraction, cytokinesis, and vesicle transport[21].

Structure of myosin filament

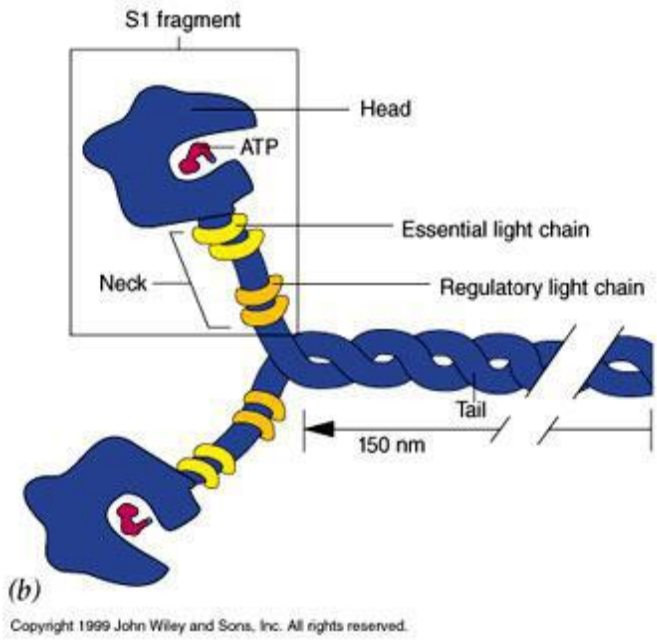

Figure 1. Structure of the myosin filament

Myosin is an actin-based molecular motor (molecular weight ~ 520 kDa). It consists of two large pear-shaped domains (S1) attached by their stems to a long tail (Figure 1). It is made of six subunits. There are two heavy chains of about 220 kDa in the head region. There is a regulatory domain in the region of the myosin head immediately behind the globular catalytic domain consisting of four light chains. The light chains are arranged in the form of pairs and one pair is called as essential light chain while the other pair is known as the regulatory light chain. These light chain pairs also used to support the neck portion of the myosin molecule and are useful to form the core of the thick filament [19].

Heavy meromyosin (HMM) is obtained by the chymotrypsin digestion of the myosin molecule. HMM contains two globular structures termed the S1 domain. The structure of the S1 domain has been determined using x-ray crystallographic techniques [18]. Within the S1 domain are two clearly defined morphological domains, which have been termed the “motor domain” and the “regulatory domain”. The large cleft at the front in the motor domain is the actin binding site. The smaller cleft on the side of the motor domain is the ATP-binding site or the nucleotide binding site. The ATP binding site contains ATPase activity and is responsible for the hydrolysis of the ATP into adenosine di-phosphate (ADP) and inorganic phosphate (Pi). The energy released from the hydrolysis of ATP causes the power stroke of myosin which in turn is responsible for the translation of the actin molecule [19]. Because the S1 domain (myosin head) contains the ATP and actin-binding sites, the myosin head can generate force (Figure 2). The head joins with another heavy chain to form a long α-helical coiled-coil tail (Figure 1).

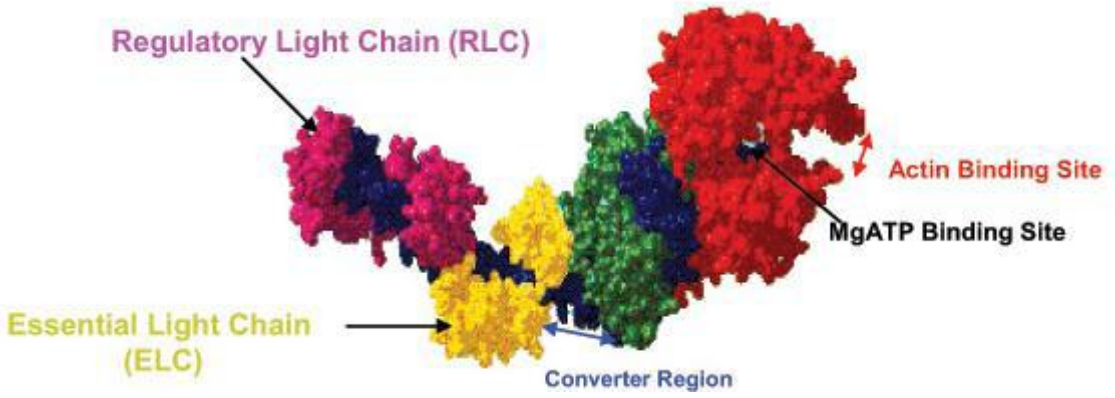

Figure 2. Myosin showing the actin and ATP binding sites

Adopted from (http://www.biomed.miami.edu/pharm/faculty_Szczesna.html)

Structure of actin filament

Actin is a very common structural component of the eukaryotic cell [22]. Actin is also an important component of the cytoskeleton. Actin filaments are found in the sarcomere where they play an important role in muscle contraction by binding with the myosin filaments. Actin typically exists in two different states: globular actin (G-actin) and filamentous actin (F-actin) [19]. G-actins can polymerize and form polar F-actin. F-actin is a two-start right-handed helix, with a crossover every 36 nm consisting of 13-14 actin monomers and has a fast growing (plus) and slow growing (minus) end [19].

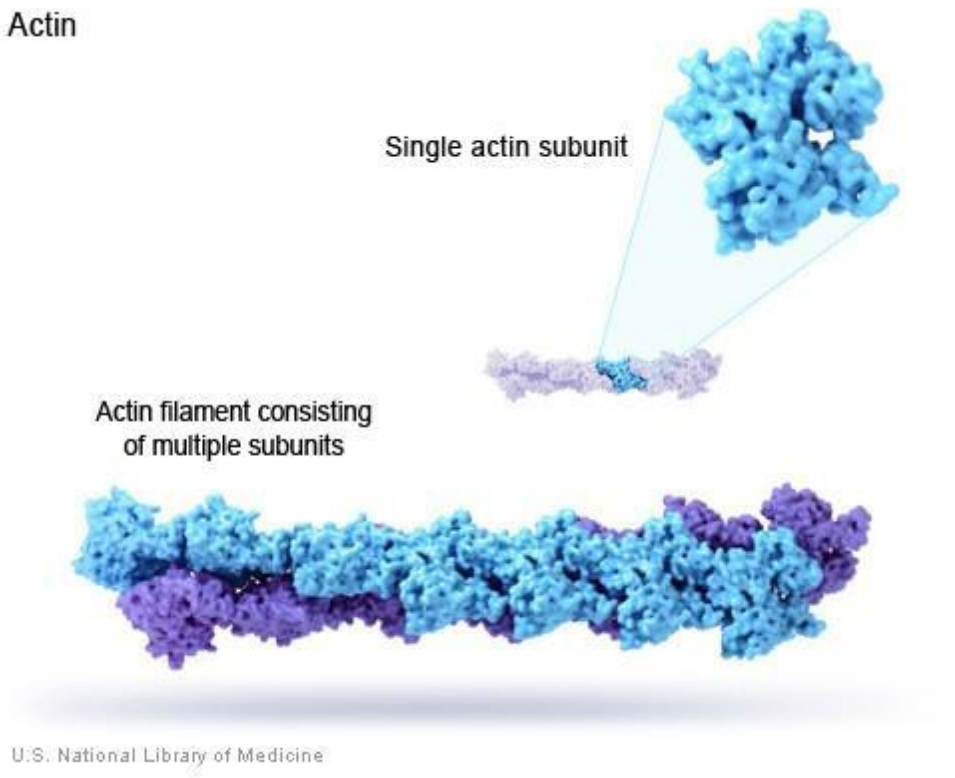

Figure 3. Three dimensional structure of actin

Use of protein biomotors for nanotechnological applications

Currently research activity concerning the use of protein biomotors is primarily centered on the utilization of myosin motors which move on actin filaments and kinesin and dynein motors which move on microtubules. As the force producing capacity of myosin motors is high (10 times that of the kinesin motors) the utilization of myosin motors may provide a mechanical advantage over that realized with the kinesin and dynein motors. The development of nanodevices that could be powered by protein

biomotors has been hampered due to a current failure to overcome three important problems:

(1.) The immobilization and patterning of protein biomotors onto a surface in such a fashion that still allows enzyme function,

(2.) An inability to develop a reliable and reversible means of coupling molecular cargo in such a way that it could be transported by protein biomotor systems,

(3.) The ability to temporally controlled protein biomotor activity.

Efforts to develop biomotor immobilization and patterning

Although very useful for the study of biomotor function, the application of biomolecular motors for nanotechnological applications has been hindered by limitations in the ability to accurately control the motion of the moving filaments as they travel across the substrate surface. Given this restraint, several approaches have been developed and utilized over the past few years to create well defined patterns of motor proteins across a micro patterned surface. For example, motor protein binding tracks have been produced by aligned poly (tetrafluoroethylene) films on glass, micrometer scale grooves, and nanoimprinting lithography [23] [24]. However, in most cases, the motility is not entirely confined to the tracks. To achieve confinement of the motility of F-actin filaments on myosin motors, trimethylchlorosilane (TMCS) has been investigated. An advantage of this surface type is its ability to be patterned by electron beam lithography or by exposure to ultraviolet (UV)/ ozone treatment [25].

Methods to load molecular cargo for transport using protein biomotor systems

Limited work has been done in the area of cargo loading and transport in biological nano systems since it is often viewed as the next step after directional control as discussed above. Efforts in this area to date have focused on binding synthetic nano elements to specific proteins, and the interaction of specific biological materials, such as biotin and streptavidin. This strategy has been quite successful as it has been demonstrated that microtubules and actin filaments functionalized with biotin can be used for the transport of streptavidin coated polystyrene beads, DNA molecules and quantum dots [24, 26]. A disadvantage of these methods is that the cargo has to be pre-functionalized and the “cargo loading” is largely irreversible in nature.

A promising and versatile method is the use of filaments that are coated with antibodies or single-stranded DNA oligonucleotides or any other type of biochemical affinity capture molecule specific for the cargo to be transported [27, 28]. Several research groups have demonstrated the transportation of materials, including DNA [29], viruses[26], and myoglobin and BSA [28], through specific bioaffinity capture materials. However, unloading these cargoes is still limiting the application of this technology.

Methods to modulate protein biomotor activity

Currently there are no efficient methods to control biomotor activity that are reversible in nature. In an effort to overcome this limitation, several approaches have been developed and utilized over the past few years include (1) the engineering of Zn^{2+}

binding sites into the kinesin motors and using the modulation of Zn^{2+} availability to govern the on / off state of the motor [6], (2) the use of photo-activated ATP in an effort to modulate motor activity by altering the amount of ATP available for hydrolysis [7] and (3) varying the temperature of the external environment surrounding the biomolecular motor to take advantage of the relationship between temperature and enzyme activity [8].

Initial efforts to control motor activity have been centered on the genetically engineering new regulatory domains e.g. a Zn^{+2} binding site, into the native biomotor structure [6]. Although this type of approach has demonstrated the cyclical activation and inactivation of microtubule motility by kinesin it appears that this type of control system exhibits a high degree of variability as the microtubule velocity is not constant after one period of cycling. Why the velocity and percentage of moving microtubules might vary between cycles is not known but may be related to alterations in the way the kinesin motor is structured after the incorporation of the new regulatory domain. Current efforts in this field are now investigating how other modifications of the kinesin protein may affect motor activity.

In addition to genetic engineering approaches, the use of photo-activated ATP for controlling kinesin activity has also been investigated [7]. In this approach, caged ATP which can be activated and inactivated by exposure to ultra violet (UV) radiation is used. It is based on the premise that exposure of the caged ATP to UV radiation will result in the controlled liberation of ATP from the caged compound. The released ATP in turn

can be used by the kinesin motors to induce microtubule movement. The efficiency of this approach and its effects on microtubule movement is dependent upon the concentration of ATP, caged ATP, ADP, kinesin activity, the quantum yield of the uncaging reaction, temperature, light intensity, and the mutual dependencies between these parameters. Similar to research using engineered constructs that are responsive to divalent metal concentration, this approach has yet to demonstrate the ability to cycle motor activity over time.

It is well known that temperature can affect enzyme activity. Recent efforts have demonstrated that temperature can be used to modulate myosin activity [8]. In these experiments, thermal heating and cooling pads have been incorporated into a flow cell that is used to house the motility assay. By controlling the flow of electricity to these pads, the temperature of the flow cell can be varied. Although this study showed that changes in temperature can be used to modulate the motility of F-actin filaments the temperature extremes needed to inhibit myosin activity are likely to be incompatible over time with currently used motility substrates. In addition, this procedure also requires extensive modification of the flow cell.

In summary, a review of the current literature demonstrates progress in the area of motor activity control has been made but that limitations still exist. The major limitation in this area of inquiry is the inability to cyclically control motor activation and inactivation. This thesis project has been designed to specifically address this gap.

Summary

Nanotechnology is the study of molecules or atoms at the nanoscale while nanobiotechnology integrates the investigation of biological systems with the nanosciences. A long term goal of nanobiotechnology is the utilization of biomolecular motors for controlled cargo manipulation on a chip, with applications in sorting, separation, and the purification or assembly of materials. Biomolecular motors exhibit the ability to convert chemical energy into mechanical energy with high efficiency. For nanotechnological purposes, most research investigating biomolecular motors has used either myosin or kinesin. The incorporation of biomolecular motors into nanodevices will require the ability to surmount three challenges: the development of appropriate surfaces capable of maintaining motor activity, the reversible coupling of cargo to motor driven structures and the ability to modulate motor activity over time. Progress in this latter area has been steady however the ability to cycle biomolecular motors on and off over several cycles has yet to be achieved. This thesis will address this gap in our understanding.

CHAPTER 3

Control of myosin motor activity and actin filament translation by alteration of assay reducing potential*

*The results of this thesis have been formatted for publication purposes.

Abstract

The study of biomolecular motors represents a rapidly and progressing field of nanobiotechnology. Here, we present a simple method for controlling the activity of myosin and movement of actin filaments on a myosin coated surface. Our findings indicate varying the amount of anti oxidants in the assay buffer can be used to alter filament motility. This data suggest that antioxidants may be useful for the selective control of myosin II activity.

Introduction

Bionanomotors such as myosin and kinesin use the hydrolysis of adenosine triphosphate (ATP) to perform mechanical work with a remarkable degree of efficiency. In the cell, these motors perform a wide variety of transport functions suggesting that they may someday be useful for the development of novel new biomedical devices [15, 30]. Recent work has demonstrated the potential use of bionanomotors for the construction of actuators and transporters capable of transporting micrometer and nanometer-sized objects [3]. While this progress have been exciting, the application of biomolecular motors for nanotechnological applications has been hampered by an inability to accurately control motor activity.

In an effort to overcome this limitation, several approaches have been developed and utilized over the past few years including Zn^{2+} binding to genetically engineered kinesin motors[6], photo-activated ATP[7], local anesthetics [31] and varying the temperature of the motility chamber[8]. Although promising, these control systems have yet to demonstrate the ability to reversibly modulate motor activity over several cycles of activation. Here we investigate the potential of altering the concentration of reducing agents as a means of controlling myosin activity. Our results demonstrate that myosin motor activity can be accurately and reversibly controlled by varying amounts of reducing agents within the assay buffer.

Materials and methods

Surface preparation

Glass cover slips (Cat. No. 12-540A, Fisher) were functionalized with nitrocellulose (2% colloidin in isoamyl acetate, Okenshoji Co, Japan) using solution deposition and soft-baking at 40ºC for 1 hr as detailed previously [32].

Motility assays

The procedures of the motility assay have been described previously [33]. Briefly, HMM was extracted from the back and leg muscle of a rabbit and purified. HMM was prepared by limited digestion of myosin with α-chymotrypsin [32]. Actin was extracted in the monomeric form from an actin acetone powder of chicken breast muscle [34] and then the polymerized actin filaments were labeled with tetramethylrhodamine-phalloidin (Molecular Probes, Inc., OR, USA). Flow cells were constructed from a No. 2 cover slip using strips of double sided 90 µm thick tape (Nichiban Co, Japan). The flow cell was filled with 120 µg/ml HMM diluted in the assay buffer which contained 25 mM KCl, 2 mM $MgCl_2$, 0.2 mM $CaCl_2$ and 25 mM imidazole at pH 7.0 and incubates for 5 min. Bovine serum albumin (0.1 % BSA in water) was used to prevent the filaments from nonspecifically binding to the surface. After a 5 min incubation, the flow cell was washed with the assay buffer, and 0.25 µg/ml labeled filaments in assay buffer supplemented with 10 mM dithiothreitol (DTT), 4.5 mg/ml D(+)-glucose, 0.22 mg/ml glucose oxidase and 0.036 mg/ml catalase was introduced into the flow cell. Motility was activated by exchange of actin loading solution with assay buffer containing 1.5mM adenosine-5`-triphosate (ATP). Motility assays were performed at

room temperature (20-23°C) and observed under fluorescence microscope (Olympus BH-2, Japan) with a 100X objective (1.3 N.A., oil-immersion; UVFL100, Olympus, Japan) through a CCD camera (Hamamatsu Photonics C2400, Japan) and a monitor.

Cycling experiments

To determine the effect of varying the concentration of reducing agents on filament movement *in vitro* motility experiments were performed in the absence and presence of reducing agents (10 mM DTT, 4.5 mg/ml D (+)-glucose, 0.22 mg/ml glucose oxidase and 0.036 mg/ml catalase). To examine the potential of using reducing agents to reversibly alter filament movement, filament motility was assayed before and after the addition of motility buffer with and without antioxidants. Up to three cycles of buffer exchange were tested. To investigate the contribution that each of the different reducing agents may have on myosin activity, motility assays were performed using standard conditions after the omission of an individual compound.

Determination of filament velocity

Images of the moving actin filaments were digitally recorded onto a computer (Dell, Dimension 4300). The velocity of actin filament movement was analyzed using software (Image J) as outlined by the manufacturer (http://rsb.info.nih.gov/ij/). Image frames were collected at 0.5 Hz for 30 s and used for the determination of sliding velocity. The sliding of all filaments in the images was obtained by tracking the leading end of each filament in each frame.

Dot blot analysis for nitro tyrosine and nitro cysteine

The procedure for the dot blot analysis to identify the oxidative modification of nitro cysteine and nitro tyrosine residues of myosin has been described previously [35] For these experiments, HMM was exposed to fluorescent light for one minute to mimic the conditions of the actual experiment. In a parallel set of experiments, GODCAT, DTT, or GODCAT+DTT were excluded from the assay medium prior to the exposure of the HMM to fluorescent light. Once exposed, 5 µL of protein [120 µg/mL] from each tube was spotted onto a Hybond nitrocellulose membrane (Piscataway, NJ). Membranes were air-dried for 20 min and blocked in 5 % BSA prepared in Tris-buffered saline containing 0.5% Tween 20 (TBS-T) for 1 h at room temperature. Membranes were incubated in primary antibody for 1 hour at room temperature and washed three times in TBS-T. The following primary antibodies and dilutions were used: Nitro-Tyrosine (# 9691, Cell signaling technology) and Anti-S-Nitroso-Cysteine (SNO-Cys) (# N5411, Sigma Aldrich) which are added at the dilutions of 1:1000 in 5% BSA prepared in TBS-T. Immuonoreactivity was detected following incubation with a horseradish peroxidase-labeled (HRP) IgG secondary antibody and reaction with enhanced chemiluminescence (ECL) Western blotting detection reagent (# RPN 2106V, GE Health care). The exposure time was adjusted to keep the integrated optical densities within a linear and nonsaturated range. Band signal intensity was quantified by densitometry using a flatbed scanner (Epson Pefection 3200 PHOTO) and Imaging software (AlphaEaseFC). At least four independent experiments were performed for each condition.

Oxidized Protein Analysis

Oxidized myosin protein was evaluated using the OxyBlot™ Oxidized Protein Detection Kit (Chemicon International, Monterrey, Nuevo León, México) according to manufacture specifications. Band signal intensity was quantified by densitometry using a flatbed scanner (Epson Perfection 3200 PHOTO) and Imaging software (AlphaEaseFC). At least four independent experiments were performed for each condition.

Statistical analysis

Results are presented as mean ± SEM. All comparisons were performed by *Students t-tests* or a two-way analysis of variance (ANOVA) with post hoc analysis where appropriate. For all comparisons the alpha level was set *a priori* at $P \leq 0.05$.

Results

Effect of reducing agent concentration on actin filament motility

Once introduced into the flow cell, the F-actin bound rigidly to the myosin immobilized on the cover slip. Similar to previous work by our laboratory and others [36] the addition of assay buffer containing 1.5mM ATP, 10 mM dithiothreitol, 4.5 mg/ml D (+)-glucose, 0.22 mg/ml glucose oxidase and 0.036 mg/ml catalase stimulated the continuous movement of the actin filaments with an average velocity in the range of 2.62 ± 0.07 μm/s at 20 – 23 °C (Figure 4 a). The average percentage of moving filaments under these conditions was 84 ± 2% (Figure 4 b).

When the buffer was exchanged with an assay medium which contained 1.5mM ATP in the absence of reducing agents, the actin filament movement quickly came to a stop (Figures 4a, 4b). After observation for 5 minutes under these conditions, the assay buffer was exchanged once again to one which contained antioxidants. Upon addition of assay buffer with antioxidants the filaments began to move. Within a few seconds the average velocity of the actin filaments was 2.64±0.06 μm/s while the percentage of moving filaments was 74±2%. This cycle was repeated two additional times. During the third cycle the average velocity of filament movement was 2.76±0.06 μm/s and percentage of moving filaments was 73±2%. Taken together, these experiments demonstrate that actin filament motility can be controlled by altering the presence or absence of reducing agents in the motility buffer (Figures 5a, 5b).

To determine the individual effect that DTT and the oxygen scavengers contribute to the on/off cycle, motility assays were performed using standard conditions after omission of individual compound. Compared to that observed using the "complete" motility buffer, the average velocity and percentage of moving actin filaments in assay medium lacking in DTT was unchanged (2.62 ± 0.07 µm/s vs. 2.27 ± 0.05 µm/s; 84 ± 2% vs. 85 ± 2 % (Figures 3a, 3b). Conversely, the average velocity and percentage of moving actin filaments in assay medium lacking glucose oxidase and catalase was significantly reduced (2.62 ± 0.07 µm/s vs. 1.25 ± 0.08 µm/s, ($P < 0.05$); 84 ± 2% vs. 25±4 %, ($P < 0.05$) (Figures 3a, 3b). Taken together, these data suggest that the omission of DTT has less of an effect on actin filament motility than the oxygen scavengers, glucose oxidase and catalase.

To better understand the effects of the antioxidants on myosin motors, we next analyzed the effects of fluorescent light on myosin modification using dot blotting and oxyblot analysis (Figures 8, 9, 10). Compared to control conditions, the omission of GODCAT or GODCAT plus DTT resulted in a significant decrease (50- 60%; $P < 0.05$) in the oxidative modification of myosin. The data suggest that myosin needs to exhibit a basal amount of oxidative modification to attain optimal activity.

Discussion

A current limitation for the use of bionanomotors in the development of nanodevices is an inability to control motor activity. Here we examine if altering the amount of reducing equivalents can be used as a means of controlling actin filament movement. For these experiments we varied the concentration of DTT, glucose oxidase and catalase in the motility buffer and determined the effect of these alterations on actin filament movement. Our data demonstrate the actin motility can be halted by the omission of antioxidants from the motility buffer (Figs 4a, 4b) and then restored again by the addition of antioxidants to the motility chamber (Figures 5a, 5b). Importantly, the restoration of filament velocity and the percentage of motile filaments did not change between three cycles of activation and inhibition. To our knowledge this finding has not been reported before. Indeed, although previous work using either genetically engineered motor proteins or temperature modulation have failed to demonstrate an ability to maintain or restore motor activity following inhibition [6, 8, 37].

The mechanism(s) by which alterations in the amount of DTT, glucose oxidase or catalase may affect myosin activity are currently unknown. Previous research has demonstrated that myosin ATPase activity can be activated and inactivated by chemical oxidation and reduction. In these studies ATPase activity could be inhibited by the addition of hydrogen peroxide and then restored later by the addition of the antioxidants like cysteine or glutathione [38]. It is thought that the inactivation of myosin by hydrogen peroxide may arise from the oxidation of sulphydral [(-SH) thiol] groups to form disulphide linkages (-SS-). The addition of cysteine or glutathione, in turn act as

reducing agents to restore the thiol groups and enzyme function. In another study, it was shown that the modification of myosin sulphydral groups causes ATPase activity to follow a bell shaped curve [39]. Our data demonstrate a reduced myosin activity with decreased myosin modification. Whether further increases in myosin activity with modification beyond that observed in the present study is currently unclear. Nonetheless, our data clearly show that oxidative modifications in the myosin molecule can be used to regulate myosin activity in an on- and off-fashion.

Conclusion

We demonstrate that the inclusion or absence of glucose oxidase, catalase and DTT in the myosin motility buffer can be used to control myosin activity during the *in vitro* motility assay. Unlike other methods that involve metal chelation [37], the use of local anesthetics [31] or caged-ATP [7], this technique appears to be a simple alternative that does not require expensive equipment. These findings may be useful to develop new means of controlling myosin motility.

Figure 4

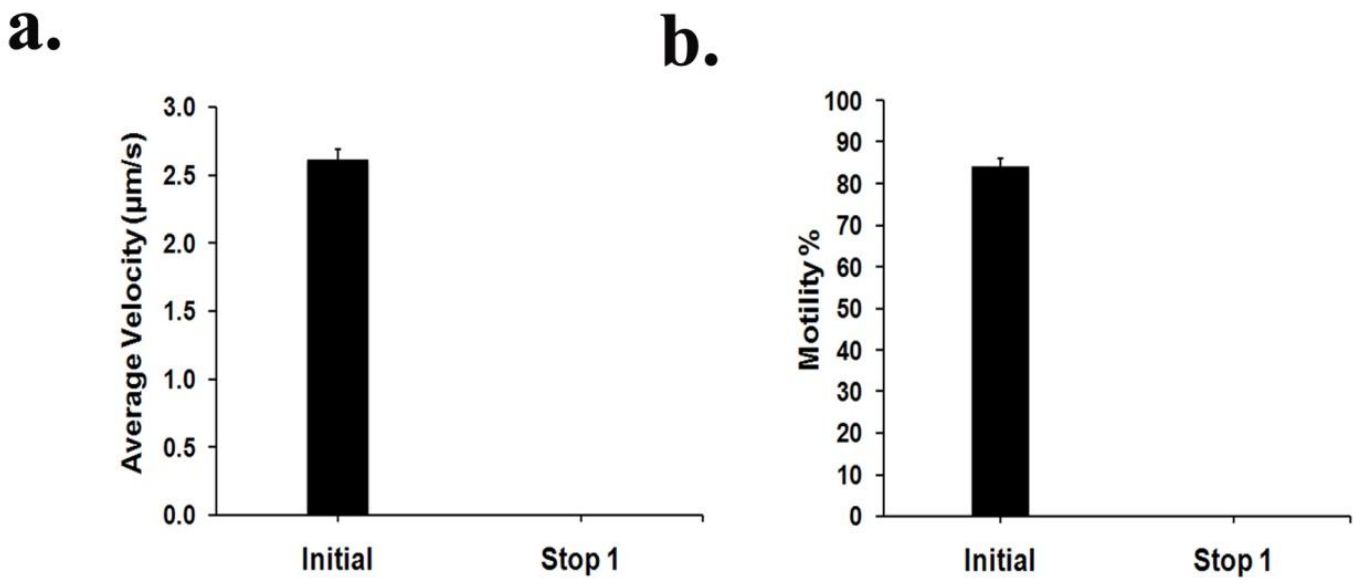

Figure 4. a). Velocity of actin filaments in assay buffer with and without antioxidants

b). Percentage of moving actin filaments in assay buffer with and without antioxidants

Figure 5

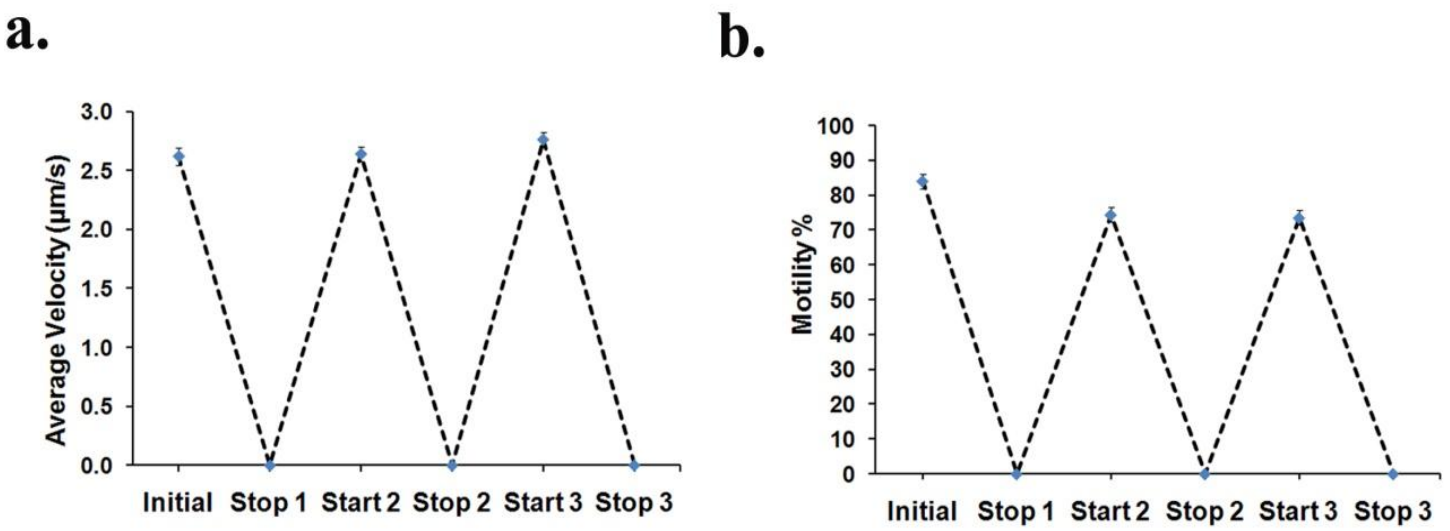

Figure 5 a). Cyclical variation in the average velocity of filaments with and without antioxidants in the assay medium. **b).** Cyclical Variation in the percentage of motile filaments with and without antioxidants in the assay medium

Figure 6

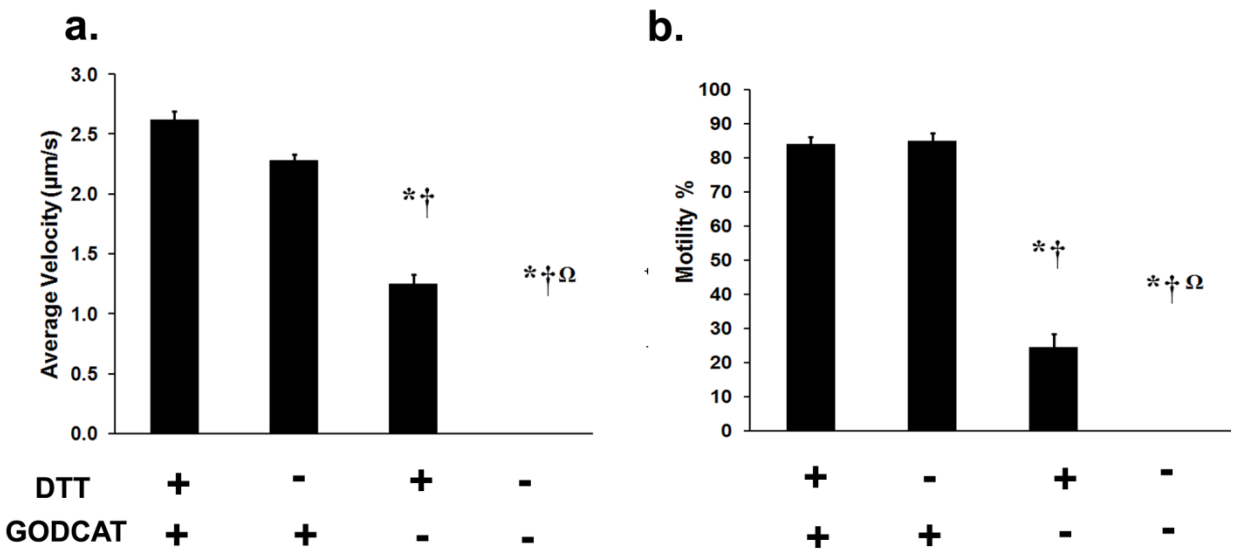

Figure 6 a). Average velocity of actin filaments at different levels of oxidation by removing the reducing agents in the assay medium. b). Percentage of motile actin filaments at different levels of oxidation by removing the reducing agents in the assay medium. * Significantly different from amount present in assay medium containing antioxidants. † Significantly different from amount present in assay medium lacking DTT. Ω Significantly different from assay medium lacking GODCAT.

Figure 7 a

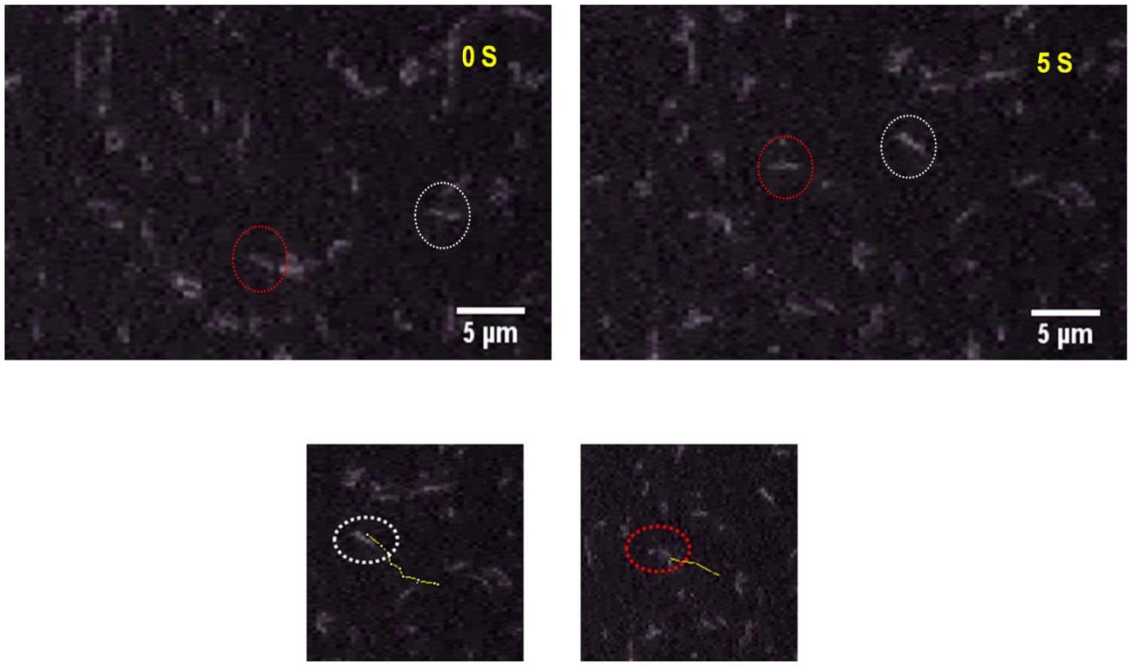

Figure 7 a). Displacement of actin filaments during an interval of 5 s after the initial movement. The paths of two filaments measured with Image-J software are shown. Cropped images with yellow tracks show the displacement of the filaments with an interval of 5 sec.

Figure 7 b

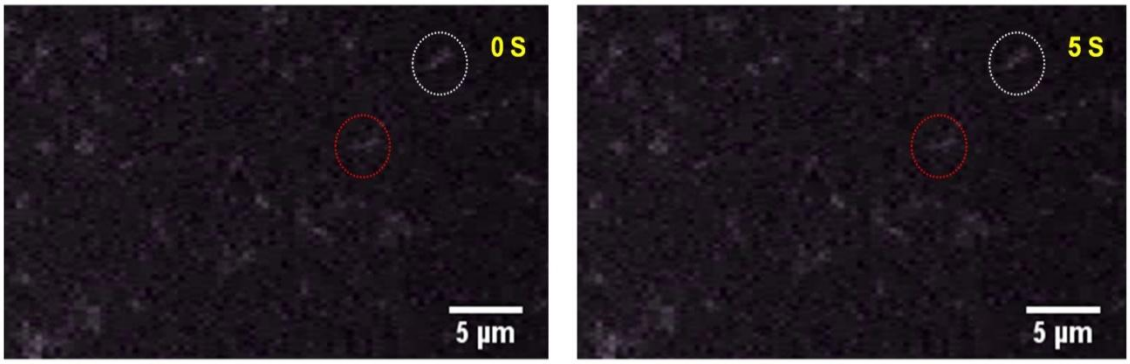

Figure 7 b). Complete stop of the actin filaments after exchanging the buffer without antioxidants.

Figure 7 c

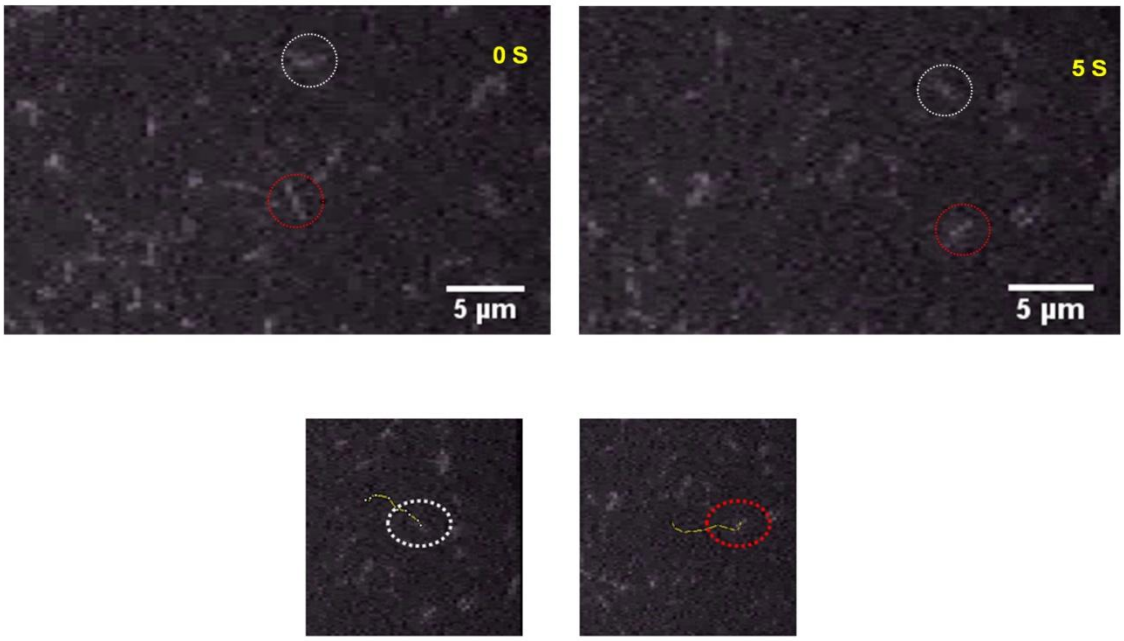

Figure 7 c). Restart of the actin filaments after exchanging the buffer with reducing agents. The paths of two filaments measured with Image- J software are shown. Cropped images with yellow tracks show the displacement of the filaments with an interval of 5 sec

Figure 8

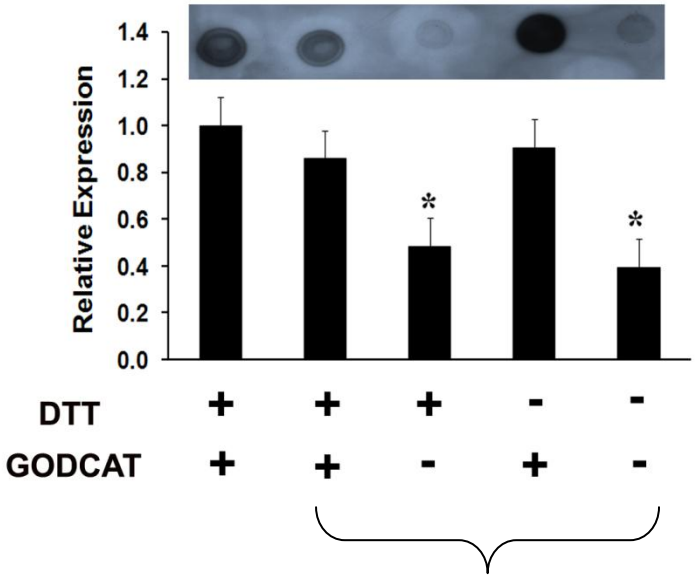

Figure 8. Effect of solution reducing potential on protein nitrosylation. * Significantly different from positive control.

Figure 9

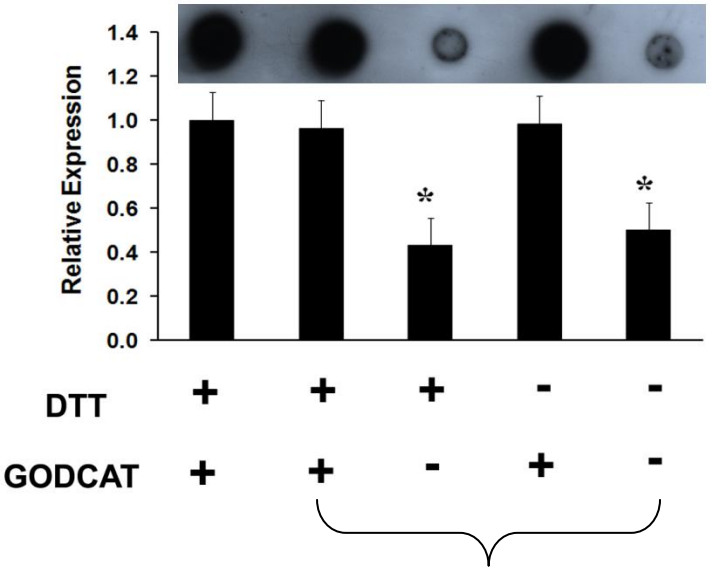

Figure 9. Effect of solution reducing potential on the degree of nitrocysteine residues present in the assay medium. * Significantly different from positive control.

Figure 10

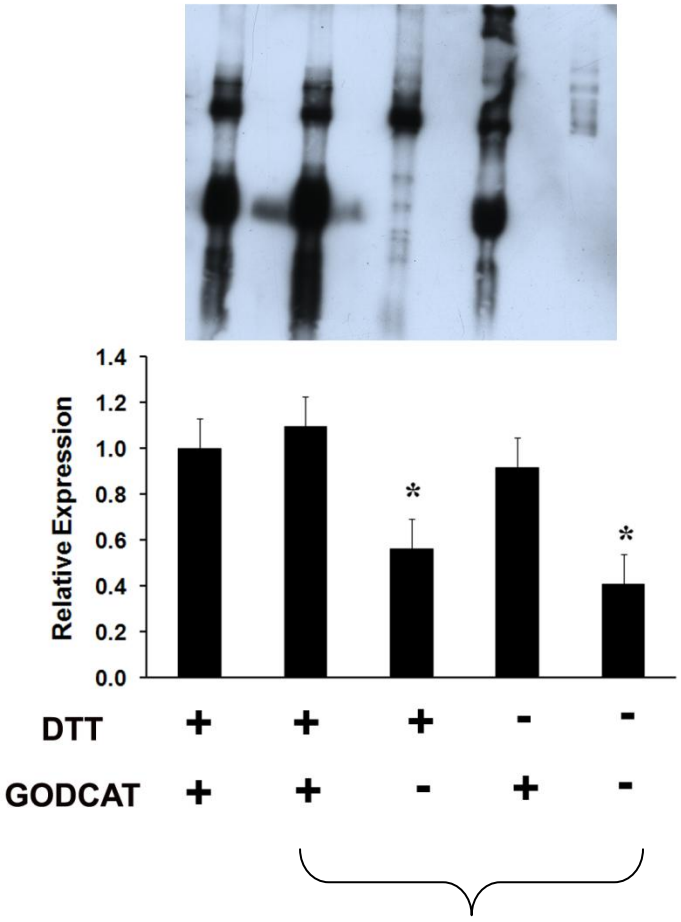

Figure 10. Effect of solution reducing potential on the degree of protein carbonylation.

* Significantly different from positive control.

CHAPTER 4

Conclusions

In this thesis document our recent research concerning the use of reducing agents to control the activity of myosin ATPase and actin filament movement during the *in vitro* motility assay is presented. Here we present a simple way to control the motor activity of myosin motors in a switch on/off fashion. To accomplish this, we modified the internal environment of the motility assay medium without any change in the average velocity of actin filaments, by introducing and removing the antioxidants from the assay medium with the aid of motility assay buffer. We demonstrate that alterations in the concentration of reducing agents can be used to control actin filament movement across a myosin coated substrate. Unlike other control methods such as use of genetically engineered motor constructs, photo activated ATP or modulating enzyme activity by altering environmental temperature, this technique appears to be a simple alternative that does not require expensive equipment and obviates the use of metal chelation or genetical engineering. Our findings may be useful in future nanodevices that use myosin bionanomotors. These devices are imagined to load the bionanomotors with the cargo carriers like liposomes at one place and unload the molecules from these liposomes at a different location. To realize this, we need the motors to be controlled temporally which is possible if they are controlled in a switch on/off fashion. So we conclude that the development of nanodevices can be realized with the results we got, if they are incorporated into a suitable nanodevice.

Future Directions

To expand upon the present research future research efforts could be undertaken to:

1. To develop a means for the transport of cargo molecules in a directed and controlled fashion. For example, it is evident that the actin filaments can be loaded and unloaded with the cargo carriers like liposomes (unpublished observation). To enable this transport in a controlled fashion, the motility of the actin filaments should be controlled precisely so that the loading and unloading the cargo at the specific location of interest could be achieved.

2. To incorporate the use of bionanomotors into a device that could be used to sort different molecules from one another. To achieve this goal, polydimethylsiloxane (PDMS) molded channels could be integrated with biomolecular motors such as myosin. Coupling a motor control system into this device may allow the ability to stop and start motility at pre-designated areas where molecular cargo could be picked up or dropped off.

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APPENDIX

Standard *in vitro* motility assay

Nitrocellulose coated cover glass: 0.2% Collodion in Isoamyl acetate

Assay buffer: 25mM KCl, 2mM MgCl2, 0.2mM CaCl2, and 25mM Imidazole, pH 7.0

BSA: 1mg/ml

HMM: 120µg/ml

Actin solution(X100):

95µL Assay buffer

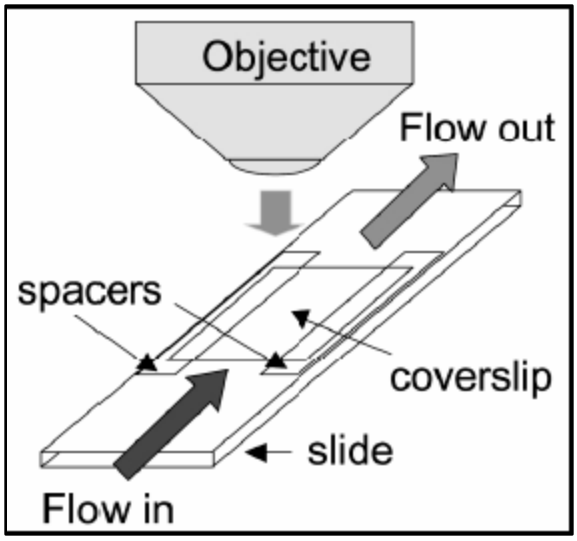
1µL 1mM DTT

1µL 0.216mg/ml Glucose oxidase,

1µL 0.036mg/ml Catalase

1µL 4.5mg/ml Glucose

1µL 0.25ug/ml RP-labeled F-actin



ATP solution(X100):

95µL Assay buffer

1µL 1mM DTT

1µL 0.216mg/ml Glucose oxidase,

1µL 0.036mg/ml Catalase

1µL 4.5mg/ml Glucose

1µL 1.5mMATP

Procedure

Assemble a flow cell with slide glass, nitrocellulose coated cover glass, and double adhesive tape.

1. Add 8μL of HMM to the flow cell and incubate for 5min
2. Block with 25μL of BSA and incubate for 5min. (capillary action and using kimwipe to introduce the solution)
3. Wash with 25μL of Assay buffer.
4. Add 25μL of F-actin labeled with Rhodamine phalloidin solution.
5. Observe under the fluorescent microscope. Add 25μL of ATP solution. Non-binding Actin will be washed off, and Binding actin will start to move.
6. Adjust the sensitivity of CCD camera and the intensity of fluorescent. (Rhodamine will be fluoresced under the green light)

Results

Table 1.Cyclical variation of the average velocity (μm/s) of the actin filaments

	Start 1	Stop 1	Start 2	Stop 2	Start 3	Stop 3
	2.94	0.00	2.91	0.00	3.61	0.00
	2.73	0.00	3.12	0.00	3.64	0.00
	2.79	0.00	3.51	0.00	3.09	0.00
	2.32	0.00	2.86	0.00	2.80	0.00
	3.41	0.00	2.80	0.00	2.61	0.00
	3.39	0.00	2.65	0.00	3.62	0.00
	3.64	0.00	2.22	0.00	2.35	0.00
	3.64	0.00	3.49	0.00	3.41	0.00
	3.33	0.00	3.48	0.00	2.97	0.00
	3.72	0.00	2.70	0.00	2.99	0.00
	2.83	0.00	2.60	0.00	2.53	0.00
	3.89	0.00	3.07	0.00	3.06	0.00
	2.92	0.00	2.70	0.00	3.49	0.00
	3.06	0.00	3.76	0.00	2.75	0.00
	2.51	0.00	3.85	0.00	3.16	0.00
	2.91	0.00	2.83	0.00	2.42	0.00
	4.11	0.00	2.37	0.00	3.87	0.00
	3.11	0.00	3.11	0.00	4.07	0.00
	3.66	0.00	3.25	0.00	1.88	0.00
	3.16	0.00	2.98	0.00	3.66	0.00
	3.88	0.00	3.59	0.00	3.19	0.00
	3.52	0.00	2.89	0.00	3.26	0.00

	3.42	0.00	2.72	0.00	3.15	0.00
	4.03	0.00	3.18	0.00	3.45	0.00
	4.22	0.00	2.65	0.00	2.95	0.00
	1.79	0.00	2.61	0.00	2.40	0.00
	1.97	0.00	2.32	0.00	2.42	0.00
	1.83	0.00	2.14	0.00	1.75	0.00
	2.36	0.00	1.51	0.00	2.16	0.00
	1.64	0.00	2.40	0.00	2.29	0.00
	2.53	0.00	2.11	0.00	2.25	0.00
	1.74	0.00	2.57	0.00	2.25	0.00
	1.48	0.00	2.05	0.00	2.38	0.00
	2.03	0.00	2.55	0.00	2.72	0.00
	2.53	0.00	2.18	0.00	2.94	0.00
	1.98	0.00	1.67	0.00	2.47	0.00
	2.09	0.00	1.49	0.00	2.27	0.00
	2.79	0.00	2.95	0.00	2.42	0.00
	1.76	0.00	1.94	0.00	1.83	0.00
	1.93	0.00	2.08	0.00	1.83	0.00
	2.11	0.00	2.79	0.00	2.83	0.00
	2.01	0.00	1.82	0.00	1.36	0.00
	2.62	0.00	2.44	0.00	2.12	0.00
	2.50	0.00	2.35	0.00	1.74	0.00
	2.40	0.00	2.51	0.00	2.56	0.00
	2.21	0.00	2.39	0.00	2.77	0.00
	1.97	0.00	2.21	0.00	2.39	0.00
	2.27	0.00	2.68	0.00	2.89	0.00

	2.46	0.00	1.65	0.00	2.47	0.00
	1.87	0.00	2.33	0.00	2.52	0.00
	2.32	0.00	2.02	0.00	2.74	0.00
	2.02	0.00	2.32	0.00	2.53	0.00
	2.48	0.00	1.73	0.00	2.38	0.00
	2.57	0.00	2.27	0.00	2.94	0.00
	2.27	0.00	2.31	0.00	3.96	0.00
	2.22	0.00	2.97	0.00	2.76	0.00
	2.66	0.00	2.60	0.00	2.44	0.00
	2.02	0.00	2.77	0.00	2.81	0.00
	2.66	0.00	3.03	0.00	2.17	0.00
	2.28	0.00	2.39	0.00	2.66	0.00
	1.67	0.00	2.45	0.00	2.97	0.00
	2.85	0.00	2.16	0.00	2.60	0.00
	2.88	0.00	3.08	0.00	2.46	0.00
	2.55	0.00	2.37	0.00	2.91	0.00
	2.98	0.00	2.70	0.00	3.35	0.00
	2.75	0.00	2.55	0.00	3.03	0.00
	2.94	0.00	2.88	0.00	3.53	0.00
	2.10	0.00	2.80	0.00	2.84	0.00
	2.60	0.00	3.41	0.00	3.29	0.00
	2.43	0.00	2.50	0.00	2.42	0.00
	2.43	0.00	2.70	0.00	2.69	0.00
	1.83	0.00	2.87	0.00	2.86	0.00
	2.10	0.00	4.41	0.00	2.76	0.00
	2.08	0.00	2.81	0.00	2.85	0.00

	2.63	0.00			2.99	0.00
N	75.00	75.00	74.00	74.00	75.00	75.00
Mean	2.62	0.00	2.64	0.00	2.76	0.00
STDEV	0.66	0.00	0.55	0.00	0.55	0.00
SEM	0.08	0.00	0.06	0.00	0.06	0.00
Maximum	4.22	0.00	4.41	0.00	4.07	0.00
Minimum	1.48	0.00	1.49	0.00	1.36	0.00
Total	196.26	0.00	195.10	0.00	206.88	0.00

Table 2.Cyclical variation of the motility percentage of the actin filaments

	Start 1	Stop 1	Start 2	Stop 2	Start 3	Stop 3
	90.00	0.00	81.58	0.00	75.00	0.00
	96.23	0.00	83.33	0.00	78.57	0.00
	88.10	0.00	83.33	0.00	73.91	0.00
	82.86	0.00	73.33	0.00	78.05	0.00
	88.89	0.00	77.78	0.00	82.86	0.00
	73.33	0.00	80.00	0.00	72.73	0.00
	94.74	0.00	69.23	0.00	88.24	0.00
	71.43	0.00	80.00	0.00	57.14	0.00
	78.95	0.00	54.55	0.00	60.00	0.00
	70.59	0.00	66.67	0.00	75.00	0.00
	84.52	0.00	69.33	0.00	68.66	0.00
	85.71	0.00	77.27	0.00	77.63	0.00
	84.71	0.00	75.34	0.00	69.32	0.00
	86.25	0.00	68.89	0.00	71.79	0.00
	84.00	0.00	74.73	0.00	73.24	0.00
N	15.00	15.00	15.00	15.00	15.00	15.00
Average	84.02	0.00	74.36	0.00	73.48	0.00
STDEV	7.69	0.00	7.71	0.00	7.87	0.00
SEM	2.06	0.00	2.06	0.00	2.10	0.00

Table 3.Variation in the average velocity of the filaments at various levels of the oxidation

	Reduced	Without DTT	Without GODCAT	Oxidized
	2.94	2.23	1.08	0.00
	2.73	2.18	1.31	0.00
	2.79	2.09	0.87	0.00
	2.32	2.09	0.79	0.00
	3.41	2.72	0.95	0.00
	3.39	2.72	1.15	0.00
	3.64	2.32	1.57	0.00
	3.64	1.73	1.40	0.00
	3.33	1.92	1.31	0.00
	3.72	1.88	1.32	0.00
	2.83	2.70	1.21	0.00
	3.89	2.30	1.19	0.00
	2.92	3.03	1.41	0.00
	3.06	2.61	1.85	0.00
	2.51	2.29	1.52	0.00
	2.91	2.49	1.49	0.00
	4.11	2.03	1.07	0.00
	3.11	2.34	1.59	0.00
	3.66	2.79	0.95	0.00
	3.16	4.01	1.50	0.00
	3.88	2.70	1.58	0.00
	3.52	2.48	1.55	0.00

	3.42	1.98	1.12	0.00
	4.03	2.18	1.64	0.00
	4.22	2.02	0.00	0.00
	1.79	2.19	0.00	0.00
	1.97	2.19	0.00	0.00
	1.83	1.73	0.00	0.00
	2.36	2.60	0.00	0.00
	1.64	2.03	1.60	0.00
	2.53	2.03	1.53	0.00
	1.74	2.06	1.18	0.00
	1.48	2.45	1.09	0.00
	2.03	2.15	1.90	0.00
	2.53	2.65	1.84	0.00
	1.98	1.90	1.41	0.00
	2.09	1.57	2.24	0.00
	2.79	2.78	1.71	0.00
	1.76	1.62	2.10	0.00
	1.93	2.21	1.34	0.00
	2.11	1.88	1.43	0.00
	2.01	2.03	1.85	0.00
	2.62	3.16	1.81	0.00
	2.50	1.80	1.22	0.00
	2.40	1.63	1.56	0.00
	2.21	1.94	0.00	0.00
	1.97	2.53	0.00	0.00
	2.27	1.78	0.00	0.00

	2.46	2.16	0.00	0.00
	1.87	1.79	1.75	0.00
	2.32	2.08	1.49	0.00
	2.02	2.19	1.82	0.00
	2.47	2.03	2.26	0.00
	2.57	2.32	1.83	0.00
	2.27	2.70	1.71	0.00
	2.22	2.38	1.75	0.00
	2.66	2.84	1.24	0.00
	2.02	2.43	1.58	0.00
	2.66	2.00	1.35	0.00
	2.28	2.36	1.85	0.00
	1.67	2.15	1.67	0.00
	2.85	2.98	1.38	0.00
	2.88	2.65	1.33	0.00
	2.55	2.61	1.44	0.00
	2.98	2.07	1.55	0.00
	2.75	2.93	1.53	0.00
	2.94	1.92	1.34	0.00
	2.10	3.05	0.00	0.00
	2.60	2.67	0.00	0.00
	2.43	2.95	1.48	0.00
	2.43	2.05	1.78	0.00
	1.83	1.46	1.77	0.00
	2.10	1.87	0.00	0.00
	2.08	1.95	0.00	0.00

	2.63	1.38	2.63	0.00
				0.00
N	75.00	75.00	75.00	75.00
Mean	2.62	2.28	1.25	0.00
STDEV	0.66	0.45	0.65	0.00
SEM	0.08	0.05	0.08	0.00
Max	4.22	4.01	2.63	0.00
Min	1.48	1.38	0.00	0.00
Total	196.26	170.71	93.75	0.00

Table 4.Variation in the motility percentage of the filaments at various levels of the oxidation

	Reduced	Without DTT	Without GODCAT	Oxidized
	90.00	80.00	17.65	0.00
	96.23	70.00	25.58	0.00
	88.10	82.35	33.33	0.00
	82.86	85.00	19.05	0.00
	88.89	80.56	22.22	0.00
	73.33	92.31	0.00	0.00
	94.74	100.00	31.71	0.00
	71.43	100.00	32.43	0.00
	78.95	71.43	35.00	0.00
	70.59	84.62	3.23	0.00
	84.52	86.49	33.33	0.00
	85.71	85.71	42.86	0.00
	84.71	88.00	48.48	0.00
	86.25	85.19	10.34	0.00
	84.00	83.33	13.64	0.00
N	15.00	15.00	15.00	15.00
Average	84.02	85.00	24.59	0.00
STD DEV	7.69	8.36	14.01	0.00
SEM	2.06	2.23	3.74	0.00

STATISTICAL ANALYSIS

One Way Analysis of Variance Wednesday, February 25, 2009, 3:44:58 PM

Data source: Average velocities of the actin filaments in different cycles.

Normality Test: Passed (P = 0.064)

Equal Variance Test: Passed (P = 0.170)

Group Name	N	Missing	Mean	STD Dev	SEM
Start-1	75	0	2.617	0.657	0.0758
Start-2	74	0	2.636	0.549	0.0638
Start-3	75	0	2.758	0.545	0.0629

Source of Variation	DF	SS	MS	F	P
Between Groups	2	0.881	0.440	1.283	0.279
Residual	221	75.837	0.343		
Total	223	76.718			

The differences in the mean values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0.279).

Power of performed test with alpha = 0.050: 0.091

The power of the performed test (0.091) is below the desired power of 0.800.

Less than desired power indicates you are less likely to detect a difference when one actually exists. Negative results should be interpreted cautiously.

One Way Analysis of Variance Wednesday, February 25, 2009, 3:49:42 PM

Data source: Motility percentage of the actin filaments in different cycles.

Normality Test: Passed (P = 0.057)

Equal Variance Test: Passed (P = 0.989)

Group Name	N	Missing	Mean	STD Dev	SEM
Start-1	15	0	84.020	7.694	1.987
Start-2	15	0	74.357	7.711	1.991
Start-3	15	0	73.476	7.870	2.032

Source of Variation	DF	SS	MS	F	P
Between Groups	2	1026.558	513.279	8.527	<0.001
Residual	42	2528.290	60.197		
Total	44	3554.848			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 0.940

All Pair wise Multiple Comparison Procedures (Tukey Test):

Comparisons for factor:

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.050**
Start-1 vs. start-3	10.544	3	5.263	0.002	Yes
Start-1 vs. start-2	9.662	3	4.823	0.004	Yes
Start-2 vs. start-3	0.882	3	0.440	0.948	No

One Way Analysis of Variance Friday, April 10, 2009, 9:54:47 AM

Data source: Variability in the velocity of the actin filaments with the variability of the level of the antioxidants.

Normality Test: Failed (P < 0.050)

Test execution ended by user request, ANOVA on Ranks begun

Kruskal-Wallis One Way Analysis of Variance on Ranks Friday, April 10, 2009, 9:54:47 AM

Data source: Variability in the velocity of the actin filaments with the variability of the level of the antioxidants.

Group	N	Missing	Median	25%	75%
Reduced	75	0	2.525	2.093	2.939
Without DTT	75	0	2.190	1.981	2.614
Without oxygen scavengers	75	0	1.406	1.084	1.662
Oxidized	75	0	0.000	0.000	0.000

H = 235.314 with 3 degrees of freedom. (P = <0.001)

The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001)

To isolate the group or groups that differ from the others use a multiple comparison procedure.

All Pair wise Multiple Comparison Procedures (Tukey Test):

Comparison	Diff of Ranks	q	P<0.05
Reduced vs. Oxidized	14197.000	18.898	Yes
Reduced vs. without OSS	8985.500	11.961	Yes
Reduced vs. Without DTT	1805.500	2.403	No
Without DTT vs. Oxidized	12391.500	16.495	Yes

Without DTT vs. Without OSS	7180.000	9.557	Yes
Without OSS vs. Oxidized	5211.500	6.937	Yes

Note: The multiple comparisons on ranks do not include an adjustment for ties.

Abbreviations: OSS (Oxygen Scavenging System- Glucose oxidase, catalase in the medium of glucose)

One Way Analysis of Variance Friday, April 10, 2009, 10:01:57 AM

Data source: Variability in the percentage of motile actin filaments with the variability of the level of the antioxidants.

Normality Test: Failed (P < 0.050)

Test execution ended by user request, ANOVA on Ranks begun

Kruskal-Wallis One Way Analysis of Variance on Ranks Friday, April 10, 2009, 10:01:57 AM

Data source: Variability in the percentage of motile actin filaments with the variability of the level of the antioxidants.

Group	N	Missing	Median	25%	75%
Reduced	15	0	84.706	79.925	88.690
Without DTT	15	0	85.000	81.005	87.622
Without OSS	15	0	25.581	14.639	33.333
Oxidized	15	0	0.000	0.000	0.000

H = 50.033 with 3 degrees of freedom. (P = <0.001)

The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001)

To isolate the group or groups that differ from the others use a multiple comparison procedure.

All Pair wise Multiple Comparison Procedures (Tukey Test):

Comparison	Diff of Ranks	q	P<0.05
Without DTT vs. Oxidized	555.500	8.213	Yes
Without DTT vs. without OSS	345.500	5.108	Yes
Without DTT vs. Reduced	1.000	0.0148	No
Reduced vs. Oxidized	554.500	8.198	Yes
Reduced vs. without OSS	344.500	5.093	Yes
Without oxygen vs. Oxidized	210.000	3.105	No

Note: The multiple comparisons on ranks do not include an adjustment for ties.

Abbreviations: OSS (Oxygen Scavenging System- Glucose oxidase, catalase in the medium of glucose).

One Way Analysis of Variance Thursday, June 18, 2009, 9:29:47 AM

Data source: Nitro tyrosine

Normality Test: Passed (P = 0.470)

Equal Variance Test: Passed (P = 0.149)

Group Name	N	Missing	Mean	Std Dev	SEM
HMM	6	0	27.433	3.448	1.408
HMM+F W/O	6	0	23.533	3.564	1.455
GODCAT	6	0	13.283	2.581	1.054

W/O DTT	6	0	24.850	4.400	1.796
W/O AO	6	0	10.850	0.565	0.231

Source of Variation	**DF**	**SS**	**MS**	**F**	**P**
Between Groups	4	1320.582	330.146	32.407	<0.001
Residual	25	254.685	10.187		
Total	29	1575.267			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

All Pairwise Multiple Comparison Procedures (Student-Newman-Keuls Method) :

Comparisons for factor:

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.050**
HMM vs. W/O AO	16.583	5	12.727	<0.001	Yes
HMM vs. W/O GODCAT	14.150	4	10.859	<0.001	Yes
HMM vs. HMM+F	3.900	3	2.993	0.107	No
HMM vs. W/O DTT	2.583	2	1.983	0.173	Do Not Test
W/O DTT vs. W/O AO	14.000	4	10.744	<0.001	Yes
W/O DTT vs. W/O GODCAT	11.567	3	8.877	<0.001	Yes
W/O DTT vs. HMM+F	1.317	2	1.010	0.482	Do Not Test
HMM+F vs. W/O AO	12.683	3	9.734	<0.001	Yes
HMM+F vs. W/O GODCAT	10.250	2	7.866	<0.001	Yes
W/O GODCAT vs. W/O AO	2.433	2	1.867	0.199	No

A result of "Do Not Test" occurs for a comparison when no significant difference is found between two means that enclose that comparison. For example, if you had four means sorted in order, and found no difference between means 4 vs. 2, then you would not test 4 vs. 3 and 3 vs. 2, but still test 4 vs. 1 and 3 vs. 1 (4 vs. 3 and 3 vs. 2 are enclosed by 4 vs. 2: 4 3 2 1). Note that not testing the enclosed means is a procedural rule, and a result of Do Not Test should be treated as if there is no significant difference between the means, even though one may appear to exist.

One Way Analysis of Variance Thursday, June 18, 2009, 10:41:48 AM

Data source: Nitro cysteine

Normality Test: Passed (P = 0.056)

Equal Variance Test: Failed (P < 0.050)

Test execution ended by user request, ANOVA on Ranks begun

Kruskal-Wallis One Way Analysis of Variance on Ranks Thursday, June 18, 2009, 10:41:48 AM

Data source: Nitro cysteine

Group	**N**	**Missing**	**Median**	**25%**	**75%**
HMM	6	0	24.700	21.800	31.000
HMM+F W/o	6	0	24.850	21.600	28.200
GODCAT	6	0	11.150	10.900	11.300
W/o DTT	6	0	26.450	23.100	26.600
W/o AO	6	0	12.900	12.700	13.200

H = 22.368 with 4 degrees of freedom. (P = <0.001)

The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001)

To isolate the group or groups that differ from the others use a multiple comparison procedure.

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparison	Diff of Ranks	q	P<0.05
W/o DTT vs W/o GODCAT	112.000	5.194	Yes
W/o DTT vs W/o AO	76.000	3.524	No
W/o DTT vs HMM+F	7.500	0.348	Do Not Test
W/o DTT vs HMM	4.500	0.209	Do Not Test
HMM vs W/o GODCAT	107.500	4.985	Yes
HMM vs W/o AO	71.500	3.316	Do Not Test
HMM vs HMM+F	3.000	0.139	Do Not Test
HMM+F vs W/o GODCAT	104.500	4.846	Yes
HMM+F vs W/o AO	68.500	3.177	Do Not Test
W/o AO vs W/o GODCAT	36.000	1.669	No

Note: The multiple comparisons on ranks do not include an adjustment for ties.

A result of "Do Not Test" occurs for a comparison when no significant difference is found between the two rank sums that enclose that comparison. For example, if you had four rank sums sorted in order, and found no significant difference between rank sums 4 vs. 2, then you would not test 4 vs. 3 and 3 vs. 2, but still test 4 vs. 1 and 3 vs. 1 (4 vs. 3 and 3 vs. 2 are enclosed by 4 vs. 2: 4 3 2 1). Note that not testing the enclosed rank sums is a procedural rule, and a result of Do Not Test should be treated as if there is no significant difference between the rank sums, even though one may appear to exist.

One Way Analysis of Variance Thursday, June 18, 2009, 10:44:20 AM

Data source: Oxyblot

Normality Test: Passed (P = 0.863)

Equal Variance Test: Passed (P = 0.563)

Group Name	N	Missing	Mean	Std Dev	SEM
HMM	6	0	25.133	2.448	1.000
HMM+F	6	0	27.483	2.079	0.849
W/o GODCAT	6	0	14.100	1.103	0.450
W/o DTT	6	0	23.017	2.388	0.975
W/o AO	6	0	10.233	2.433	0.993

Source of Variation	DF	SS	MS	F	P
Between Groups	4	1329.895	332.474	71.800	<0.001
Residual	25	115.763	4.631		
Total	29	1445.659			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

All Pairwise Multiple Comparison Procedures (Student-Newman-Keuls Method) :

Comparisons for factor:

Comparison	Diff of Means	p	q	P	P<0.050
HMM+F vs. W/o AO	17.250	5	19.636	<0.001	Yes
HMM+F vs. W/o GODCAT	13.383	4	15.234	<0.001	Yes
HMM+F vs. W/o DTT	4.467	3	5.084	0.004	Yes
HMM+F vs. HMM	2.350	2	2.675	0.070	No
HMM vs. W/o AO	14.900	4	16.961	<0.001	Yes
HMM vs. W/o GODCAT	11.033	3	12.559	<0.001	Yes
HMM vs. W/o DTT	2.117	2	2.409	0.101	No
W/o DTT vs. W/o AO	12.783	3	14.551	<0.001	Yes
W/o DTT vs. W/o GODCAT	8.917	2	10.150	<0.001	Yes
W/o GODCAT vs. W/o AO	3.867	2	4.401	0.005	Yes

CURRICULUM VITAE

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OBJECTIVES

- Explore the field of science to quench my thirst for knowledge.
- To work efficiently and effectively as well as grow with a prestigious organization.

WORK EXPERIENCE

- Jan 2008 – Present: Research Assistant / Assistant lab manager, Marshall University, Huntington, WV.
    - Duties: Thesis research, responsible for laboratory ordering, inventory upkeep, P-card management, laboratory record keeping, tracking of project resources, preparation of stock solutions, involved in overseeing laboratory safety and compliance.

- Oct 2007 - Dec 2007: Poultry pathologist, Srinivasa hatcheries, Vijayawada, India.
    - Duties: Disease diagnosis, treatment and prevention in poultry farms. Identification of cause of disease by performing postmortem. Educating the people about prevention and control of diseases in the farms.

- April 2007 – Oct 2007: Student Internship, State institute of animal health, Ongole, Andhra Pradesh, India.
    - Duties: Performing various surgical, medical and gynecological procedures on animals for curing the disease process, diagnosis, treatment and prevention of diseases in various livestock, educating the

people about how to improve the productivity of live stock, control of diseases by vaccination, attending various vetero legal cases like toxicities and performing postmortem to identify the cause of death, making people aware of various zoonotic diseases and community outreach programs.

EDUCATION

- Jan 2008- Present **(Grade:3.56/4.0)**
    - Pursuing Master of Science Department of Biological Sciences, Marshall University, Huntington, WV.

- Aug 2002-Oct 2007 **(Grade 4.0/4.0)**
    - Bachelor of Veterinary Science and Animal Husbandry (Equivalent to **DVM**), College of Veterinary Science, Sri Venkateswara veterinary university Tirupati, India.

LICENSE EXAMS CLEARED- For ECFVG certification

- BCSE (Basic and Clinical Sciences Examination)
- NAVLE (North American Veterinary Licensing Examination)

TESTS

- GRE : Dec 2006, Verbal-640, Quantitative-700, Analytical writing-2.5
- TOEFL: Aug 2007, Total-82.

PUBLICATIONS

1. Application of PAMAM dendrimers for use in bionanomotor systems Madhukar Kolli, B Scott Day, Kevin M. Rice, Hideyo Takatsuki, Kazahiro Kohama, Murali K Gadde, **Siva K Nalabotu**, Sunil K Kakarla and Eric R. Blough (In preparation)

2. Control of myosin motor activity and actin filament translation by alteration of assay reducing potential **Siva K Nalabotu**, Eric R. Blough (In preparation)

3. Application of liposomes for cargo transport and unloading through actin bundle-myosin motor system Hideyo Takatsuki, Hideyuki Tanaka, Madhukar Kolli, **Siva K. Nalabotu**, Kazuhiro Kohama, and Eric R. Blough (In preparation)

PROFESSIONAL MEMBERSHIPS

- Association of Young Scientists Jan 2008-present
- Cell Differentiation and Development centre Jan 2008-present
- Veterinary Council of India Oct 2007-life time
- Andhra Pradesh Veterinary Council Oct 2007-life time
- AAAS (American Association for the advancements in Science).

NANO TECHNOLOGY TECHNIQUES

- Purification of actin from chicken skeletal muscle.
- Purification of myosin from scallop (a mollusk) and skeletal muscle of rabbit.
- *In-vitro* motility assays.
- Epi fluorescent microscopy.
- Analysis of data using win DVD creator project software.
- Making PDMS molds and silicon masters.
- Photo lithographical patterning of proteins.
- Photo patterning of proteins using UV light.

MOLECULAR BIOLOGY TECHNIQUES

- SDS–PAGE
- Western Blot Analysis

- Immunohistochemistry
- Protein extraction & Purification from muscle samples

ANIMAL MODELS

- Trained to handle animals in research as per IACUC guidelines.
- Experienced with F344XBN F1 Aging rat model, Gerbils and Obese Syndrome X Zucker rat model.
- Rat Hind limb Suspension model of Muscle Atrophy; Skeletal Muscle Stretch.
- Echocardiography, electrocardiography, physiological and pathological aspects of rats.

MICROSCOPIC TECHNIQUES

- Light microscopy
- Fluorescence microscopy
- Confocal microscopy

CURRENT PROJECTS

- Master's Thesis Project: Control of myosin motor activity and actin filament translation by alteration of assay reducing potential, PI: Eric R Blough, Ph. D (Fall 2008). Marshall University, Huntington, WV.

- Application of PAMAM dendrimers for use in bionanomotor systems, Primary Investigator: Eric R Blough, PhD Marshall University, Huntington, WV (Spring 2008).

PREVIOUS RESEARCH EXPERIENCE

- June 2005-Oct 2005: "Earn while you learn" poultry project in conjunction with the department of poultry science. Primary Investigator: Dr. Ch. Srilatha, Ph.D. College of Veterinary Science, Tirupati.

PRESENTATIONS

- Spring 2008: 1. “Use of erythrocyte ghost cells as cargo carriers”.
  2. “Metabolic syndrome - pros and cons”
  Marshall University, College of Science.

- Fall 2008: “Use of liposomes for controlled delivery of nano cargo in in-vitro motility assays”, Marshall University, College of Science.

AWARDS

- Jan 2008 onwards: Marshall University, NSFEPSCoR #9871948 graduate assistantship with stipend.
- Oct 2007: Best volunteer for National Social Service program during veterinary internship program.

COMPUTING AND STATISTICAL SOFTWARE

- Well-acquainted with MS Office,
- Adobe® Photoshop,
- Adobe® Acrobat,
- Sigma Stat®,
- Endnote®,
- BIOPAC® Systems.
- Analysis with Win DVD creator project software.
- Alpha ease software,
- Well acquainted with IMAGE J analysis.

COMMUNITY SERVICE AND OTHER ACTIVITIES

- July 2007: Participated in National Social Service (NSS) animal health camps and gynecology camps to treat infertility in animals.
- Aug 2007 - Sep 2007: Participated in free animal health camp to treat ailments of animals.

- Participated in various free vaccination camps, infertility camps, spaying, castration of stray dogs, making awareness camps for the ethical treatment and preservation of animals and ecosystem.

REFERENCES

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3) Dr. Bin Wang,
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