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Covalent immobilization of myosin for *in-vitro* motility of actin

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Abstract. The present study reports the covalent immobilization of myosin on glass surface and *in-vitro* motility of actin-myosin biomolecular motor. Myosin was immobilized on poly-L-lysine coated glass using heterobifunctional cross linker EDC and characterized by AFM. The *in-vitro* motility of actin was carried out on the immobilized myosin. It was observed that velocity of actin over myosin increases with increasing actin concentration (0.4–1.0 mg/ml) and was found in the range of 0.40–3.25 μm/s. The motility of actin-myosin motor on artificial surfaces is of immense importance for developing nanodevices for healthcare and engineering applications.

Keywords. Molecular motors; atomic force microscope (AFM); immobilization; biomolecular electronics and nanotechnology.

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1. Introduction

Proteins in the body perform very sophisticated functions which cannot be achieved with man-made creations. Hence, they are sometimes called biological 'nanomachines. Developing hybrid devices by taking advantage of functional biological molecules such as DNA, biomolecular motors, and other proteins has been actively considered as future technology [1–3]. *In-vivo* function of motor proteins is well established but the challenge lies in creating controlled function of these biomolecules *in-vitro*. At present, three major classes of molecular motors have been identified (myosin, dynein and kinesin) and all are important in cellular movement [4].

Skeletal muscle contraction is obtained by the cyclic interaction between heads of the myosin motor protein, extending from the thick filaments, and specific sites on the thin actin filaments [5]. Myosin molecules are elongated molecules, which run parallel in the thick filament backbone in muscle cells. Each myosin protein has one head with two motor domains that bind the actin filament; the conformational changes of the motor domain due to the ATP hydrolysis determine the swinging out of the head and consequently the movement of actin filament sliding [6]. Actin

is a major constituent of the cytoskeleton of almost all eukaryotic cells. Actin exists either in a monomeric form (G-Actin) or in a filamentous form (F-Actin) and each actin subunit binds to either ATP or ADP [7]. Actin consists of a series of monomers that can be described in terms of a double helix with a half periodicity of 36 nm. Myosin binding is governed by two structural aspects of the actin filament; the 5.5 nm monomer–monomer repeat distance and the 36 nm half repeat of the actin double helix.

The *in-vitro* motility assay is a powerful tool for studying the function and properties of myosin [8–12]. Several systems for observing movement of purified actin on myosin have been reported [13–18]. The first quantitative measurements of rates of purified myosin along actin *in-vitro* were made by using the Nitella-based movement assay of Sheetz and Spudich [19]. In that assay, myosin filaments are attached to polystyrene beads, and these myosin beads are deposited on the cytoplasmic face of a dissected Nitella axillaries cell. Yanagida *et al* [16] observed single fluorescent actin filaments in solution by using a video light microscope. They found that the amplitude and frequency of bending of the filaments increases in the presence of soluble myosin filaments and ATP. Spudich *et al* [20] reported that in the presence of ATP myosin filaments attached to glass are indeed capable of supporting the movement of single actin filaments labeled with rhodamine phalloidin. Previous studies used native myosin [8,12,21] or its enzymatic derivatives, heavy meromyosin (HMM) and S1 [9–11], all of which support actin filament motion, though at markedly different velocities. It has been shown that the velocity of actin filaments on an HMM-coated nitrocellulose surface is higher than that on native myosin, while the actin filaments move slower on S1 than on myosin [9,11].

In the present study we report immobilization of myosin on lysine-coated glass slide using zero length cross linker EDC (1-ethyl-3(3-dimethylaminopropyl)-carbodiimide) and actin motility assay on myosin under different conditions.

2. Material and methods

2.1 *Materials*

Rabbit skeletal myosin and actin, rhodamine phalloidin, and poly-L-lysine-coated glass slides and other biochemicals were procured from Sigma Aldrich and EDC (1-ethyl-3(3-dimethylaminopropyl)-carbodiimide) was procured from Pierce, USA. In all the experiments, microbes- and pyrogen-free deionized water having 18.2 MΩ resistivity obtained from PureLab, Elga, UK, water purification system was used.

2.2 *Immobilization of myosin*

Myosin was immobilized on poly-L-lysine coated glass slide. Poly-L-lysine-coated glass slides (1 cm × 1 cm) were washed with 500 μl of conjugation buffer (0.1 M MES (2-[N-morpholino]ethane sulfonic acid), pH 4.5–5), then EDC (10 mg/ml) was dispersed on lysine-coated glass wafers and incubated for 15 min. Myosin was immobilized using the following procedure: 100 μl of EDC (10 mg/ml) was poured on the glass slide, after 15 min 200 μl of myosin (1 mg/ml) was added into the reaction mixture on the glass slide and then incubated at room temperature for

Covalent immobilization of myosin

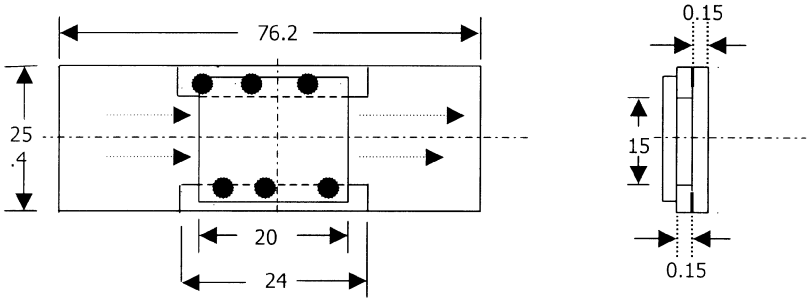

Figure 1. Flow cell prepared from coverslip and glass slide. The arrows show the inflow and outflow of the buffer solution during motility assay. (Note: All dimensions are in mm.)

2 h. After 2 h, the slide was washed with MES buffer and then air dried. Then immobilization was confirmed using atomic force microscope (Nanoscope II).

2.3 *Fluorescence labeling of actin*

Rhodamine phalloidin specifically binds to F-actin without any influence on physiological functions of F-actin and its fluorescence intensity is enhanced 4.2-fold by the binding [22]. RhPh has an excitation maximum of 550 nm and an emission maximum at 575 nm. RhPh was dissolved in methanol and 10 μl of the dissolved RhPh was dried by passing dry nitrogen. The resulting pellet was dissolved in 2 μl of ethanol. Then 290 μl of AB buffer (25 mM imidazole hydrochloride, 25 mM KCl, 4 mM $MgCl_2$, 1 mM EGTA and 1 mM DTT, pH 7.4) was added and the pellet was dissolved by vortexing. Then finally 2 μl of 1 mg/ml of actin was added and the mixture was incubated overnight on ice in dark.

2.4 *Flow cell preparation*

A flow cell of 20 mm $\times$ 15 mm $\times$ 0.15 mm corresponding to a volume of 50 μl was prepared. After coating EDC on lysine-coated glass slide, two slivers of cover slip were placed onto two parallel beads of grease (filled circles in figure 1). Another cover slip was placed onto the grease and pressed down with the help of forceps until it rests on the coverslip slivers. The resulting volume of the flow cell was 50 μl (figure 1).

2.5 *In-vitro motility assay*

For *in-vitro* assay the flow cell was inclined at an angle of 30°. Then 100 μl of 40 μg/ml myosin was infused from one side of the flow cell and kept for 2 h incubation on ice. After incubation 100 μl of AB/BSA [(25 mM imidazole hydrochloride, 25 mM KCl, 4 mM $MgCl_2$, 1 mM EGTA and 1 mM DTT, pH 7.4)/Bovine Serum

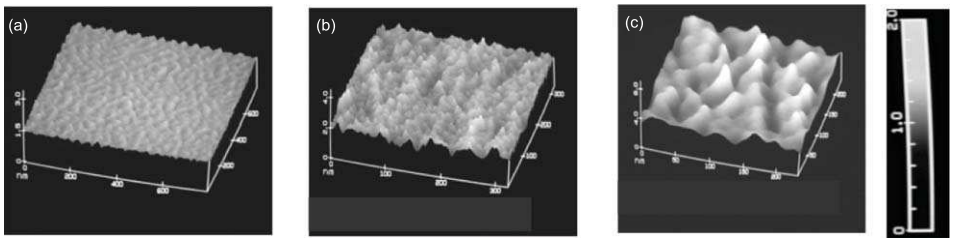

Figure 2. Contact mode AFM images of myosin cross-linked to lysine-coated glass slide. (**a**) Glass slide coated with lysine (roughness 0.5 nm), (**b**) EDC–coated on lysine-coated glass slide (roughness 1.2–1.4 nm), (**c**) myosin molecules immobilized with EDC (roughness 2.4–4.5 nm).

Albumin)] buffer was infused to prevent further nonspecific protein adsorption. This was kept for 1 min incubation at 25°C. RhPh-labeled actin (100 μl) was infused and incubated for 2 min. Then 100 μl of AB/BSA/GOC buffer (3 mg/ml glucose, 0.1 mg/ml glucose oxidase and 0.018 mg/ml catalase) was added. The slide was kept on the microscope stage and the last buffer for motility, i.e. AB/BSA/GOC/ATP (Adenosine Triphosphate) was infused to induce motility which was observed and recorded with inverted fluorescence microscope (Axiovert 200, Carl Zeiss).

The average velocities of moving filaments (scalar velocity equivalent to speed) were determined using custom-built program written in MATLAB. Pictures of the *in-vitro* motility assay were recorded at 15 frames per second. The programs seek the filaments in different frames to locate its centroid position. The velocity is calculated from the changing positions of the centroid.

3. Results and discussion

The immobilization of functional proteins on flat surfaces is of crucial importance for studying their interaction with ligands and examining their structure by means of electron and scanning probe microscopy and other biophysical techniques requiring a solid interface. We have used heterobifunctional cross linker EDC to immobilize myosin on poly-L-lysine-coated glass slide. The surface characterization of myosin on lysine-coated glass slide was done by taking AFM images (figure 2) in contact mode in air and calculated the roughness of the surface.

In-vitro motility of actin filaments on myosin tracks on glass surface (figure 3) was conducted at 25°C. The energy for mobility of actin–myosin was driven by ATP. In the absence of myosin, actin floated freely in solution and did not bind to the glass surface. Actin binds rigidly with myosin immobilized on the cover slip using EDC. On addition of 1 mM ATP, the actin moved in a continuous fashion with velocity in the range of 0–4 μm/s (figure 3). Results of this study shows mobility of actin over myosin in the presence of ATP and the velocity increases with increasing actin concentration in the range 0.40–3.25 μm/s. The velocity of the actin filament was recorded in 1 s intervals during the course of reaction up to 6 s. Beyond 6 s, the velocity could not be recorded due to quenching of the dye. All the three concentrations are studied and the velocity initially increases with time and then decreases after attaining a maximum velocity. This initial increase

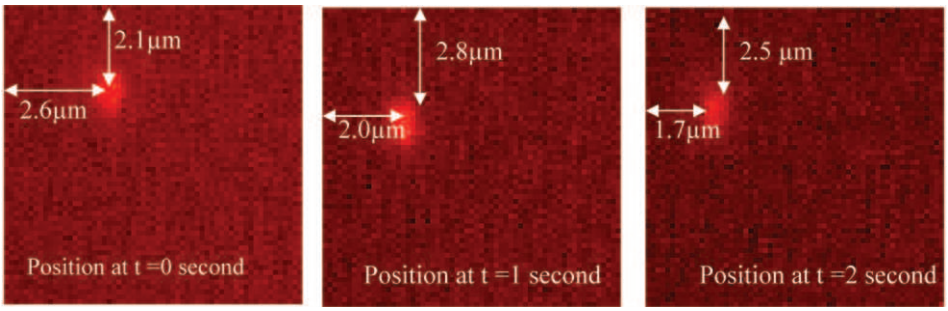

Figure 3. Sequential images (1000 × magnification) of *in-vitro* assay at 1 s interval. The fluorescent spot is the actin filament moving on myosin track. The figure indicates the changing position of the actin filament at different time intervals.

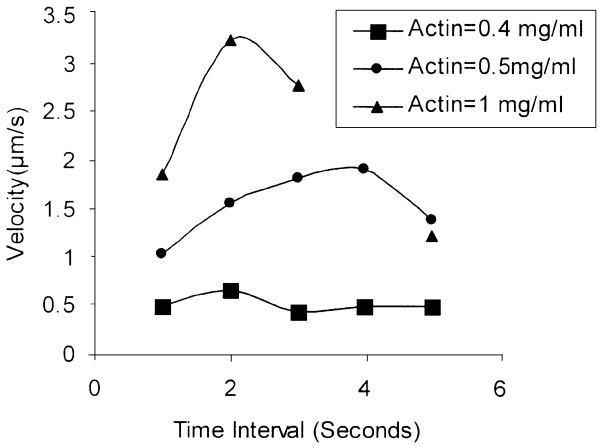

Figure 4. Velocities of actin filament at different actin concentrations.

in velocity can be attributed to slow interaction of ATP with myosin and decrease in velocity to depletion of ATP concentration (figure 4).

We have demonstrated an *in-vitro* movement assay system for actin filaments on myosin covalently bound on lysine-coated slide using zero length EDC cross linker. The covalent binding based actin–myosin motor mobility will find application in the domain of biomolecular electronics (nanoswitching), nanorobotics, targeted drug delivery etc.

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