Selective attachment of F-actin with controlled length for developing an intelligent nanodevice

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1. Introduction

Protein motors are used by living organisms to convert chemical energy into mechanical energy. The human body uses such protein motors to transport materials through cells and to contract muscle [1,2]. By understanding how these biological motors work, artificial motors with improved function may be possible and may be engineered to work in complex biological and non-biological environments. Recent research efforts have focused on understanding how to harness the power of biological motors, and manipulate the functioning of such motors for integration into useful nano-scale systems. More recently, Hess’ group reported an intelligent micro-biochip that washing steps can be replaced by bio-molecular motor’s transportation [3,4]. In these protocols, antibodies were loaded in a cargo of a “smart dust” device with bio-molecular motors as wheels, and the device shuttles from “Capture” to “Tag” to “Detection” stops powered by adenosine-5’-triphosphate (ATP) in the fluidic flow. Then, the analyte was captured at the first stop, followed by a secondary dyes-tagged antibody at the second stop. Finally, the full-loaded device was stopped at the terminal of detection, in which the fluorescent signal of dyes can be read-out.

Kinesin/microtubules and myosin/actin filaments (F-actin) are two representative couples of bio-molecular motors that have been extensively studied [1,2]. Every step kinesin or myosin molecule takes involves the binding and hydrolysis of ATP converting chemical energy to mechanical work, directed by the polarity of microtubules or F-actin. In general, bio-molecular motor-based devices for nano-transportation can be classified into two categories: (1) A “slide assay” that cargos conjugated with microtubules or F-actin are moving on a kinesin or myosin-immobilized surface [3–7]; (2) A “bead assay” that micro-/nano-beads coated with kinesin or myosin are “walking” on microtubules or F-actin tracks [8–12].

Early in 1983, Sheetz’s group firstly reported myosin-coated fluorescent beads on actin cables isolated from the intermodal cell [8]. However, the development of such bead assay was hindered by the challenging, which is how to produce large-area well-oriented F-actin tracks on the surface. Several attempts have been made so far, including using a biotinylated-severin to anchor F-actin on an avidin-immobilized surface [9], performing on the actin...
bundles of Nitella axilliformis [10] and actin paracrystal surface formed by depositing F-actin on a monolayer of two-lipid-components [11]. In spite of that the movement of a single myosin-coated bead was conceived in these studies, it seems difficult to control all of the beads to move in one direction due to the random assembly of F-actin on the surface.

Compared with microtubule, actin filament is more flexible due to a smaller value in diameter (c.a. 7 nm) and is capable to conduct electrical current [13]. It is of potential to obtain a long-distance directed F-actin array (e.g. 2500 µm [3,4]) by fluidic flow [14] or electric field [15]. The success of such stable long-distance transportation is expected to shed light on the development of nanodevice in microbiochip application in vitro. One issue in consideration for such intelligent nanodevice development is the length of F-actin. It is assumed that the barbed end of one laid-down actin filament unit can be connected by the pointed end of the other unit, forming a unidirectional aligned track, on which myosin-coated beads may walk crossing one another F-actin (Fig. 1a); otherwise, the myosin-coated beads may stick to the surface after finishing moving on one unit of F-actin. Apart from severin, gelsolin is a calcium ion-dependent protein that acts to regulate control of actin filament length by binding to actin and disrupting the weak non-covalent bond between actin subunits, severing the filament [16]. The length of actin filament, therefore, can be controlled by varying the concentration of gelsolin in the mixture solution [17].

The methodology that F-actin is anchored with capped protein bridge is likely promising, in which the capped protein is attaches to the substrate surface via bioaffinity coupling, i.e. biotin/avidin bridge [15]. One reason is that such attachment is stable due to the great $K_a$ values of the bindings of two couples: capped protein to G-actin and biotin to avidin, which is $10^8$ M$^{-1}$ [18] and $10^{15}$ M$^{-1}$ respectively. The other is attributed to the strengths of biotin/avidin affinity-based immobilization [19]: (a) orientated and homogeneous protein attachment and (b) re-usability by detaching protein easily with harsh conditions, such as increase of pH or temperature. Previously, we successfully attached F-actin in a micro-channel by employing biotinylated capped protein gelsolin as an anchoring molecule [14]. The orientation of F-actin was partly controlled once applying a fluidic flow, whereas the distribution of F-actin without controlled length was found not to assemble in order. In this work, biotinylated gelsolin-capped F-actin were selectively attached on a streptavidin-patterned surface, in which homogeneous population of controlled-length F-actin was observed. The procedure in this work is demonstrated as in Fig. 1b. Glass slides were first functionalized with amino-silane aminopropyltriethoxysilane (APTES), which served as the cross-linking agent between the surface and the photo-activated biotin by establishing a covalent attachment. Roughness estimated by atomic force microscopy (AFM) was used to verify the success of APTES-modification. Prior to assembly on the patterned streptavidin surface, the length of F-actin as a function of gelsolin concentration was investigated. Fluorescent microscopy was employed to characterize the patterning of dye-tagged streptavidin and selective attachment of dye-tagged F-actin. Furthermore, non-specific binding of F-actin via electrostatic adsorption was found to be resisted by blocking the non-patterned areas. Taken together, the results presented here represent an important beginning towards achieving movement of specific cargos along patterned tracks utilizing bio-molecular motor-based nano-transportation systems.

**2. Materials and methods**

**2.1. Materials**

Aminopropyltriethoxysilane (APTES), poly-diallyldimethylammonium chloride (PDDA), bovine serum albumin (BSA), Tween-20, gelsolin, 3-[3-(4-azido-2-nitroanilino)-N-methylpropylamino]propylamide acetate salt (photobiotin) were obtained from Sigma Aldrich (St. Louis, MO). TRITC or FITC-labeled streptavidin, and NHS-PEG$_4$-Biotin were obtained from Thermo Fisher Scientific Inc. (Waltham, MA). Rabbit muscle acetone powder was acquired from Pel-freeze Arkansas, LLC (Rogers, AR). Actin and biotinylated gelsolin were obtained from Molecular Probes (Eugene, OR).

![Fig. 1.](image-url) (a) Homogeneous F-actin track for unidirectional long-distant movement of myosin-coated beads. (b) Schematic of selective attachment of F-actin in this work.
lin were prepared as described previously [14]. Summa clean solution was from Mallinckrodt Baker, Inc. (Phillipsburg, NJ). All other chemical reagents are of analytical reagent (A.R.) grade. Solutions were prepared in high-purity water (3X de-ionized water, resistivity: 18 MΩ cm) from a Millipore Milli-Q water purification system (Billerica, MA).

2.2. Methods

2.2.1. Functionalization of glass slides
A 1 mm thick, 24 mm × 50 mm glass microscope cover-slips (Fischer Scientific) were cleaned by immersed in Petri dish containing acetone and sonicated in ultrasound bath for 5 min. The same process of sonication was repeated with methanol for an additional 5 min, and slides were dried with nitrogen gas (N₂) stream. The slides were immersed in a beaker containing 300 ml of Summa clean solution, and heated to 55–60 °C, for 30 min. Afterwards the slides were rinsed with de-ionized water for 5 min and dried with N₂ stream. A solution of 3% (volume/volume) APTES in anhydrous ethanol was prepared and sonicated in ultrasound bath for 10 min to ensure proper mixing. The clean slides were then immersed in APTES solution and incubated for 12 h. The modified slides were rinsed three times with ethanol and de-ionized water and dried with N₂ stream. The slides were placed in an oven for 10 min at 125 °C to anneal APTES and form silanol groups. All of the above procedures were conducted in a Class 1000 clean-room.

2.2.2. Atomic force microscopy
Atomic force microscopy was used to characterize the APTES-functionalized slides. RMS roughness of the surface was measured after the silanization process. The AFM system (MFP3D; Asylum Research) was coupled to a PC running Igor pro 6.0.4, which served as control of the system and was used for image processing. Both tapping and contact mode imaging were used, and all experiments were conducted at room temperature. For contact mode imaging, the Veeco probe MSNL-10 with a spring constant of 0.03 N m⁻¹ and the Olympus AC240TS with a spring constant of 2 N m⁻¹ were used. The scan size was 10 × 20 μm. The same scan speed and scan size were used for tapping mode imaging using the Olympus AC160TS with a spring constant of 42 N m⁻¹ and resonant frequency of 300 K Hz. Each sample was scanned at least eight times in both imaging modes and the average roughness was determined based on the values obtained in each of the scans divided by the number of scans.

2.2.3. Streptavidin patterning
Deposition of photobiotin was achieved by pipetting 30 μl of the photobiotin solution (0.1 mg ml⁻¹) onto APTES functionalized glass slides. Samples were spun on glass slides at 400 rpm for 90 s (4000 Lauren series programmable centrifuge). Slides were then placed on a baking plate for 30 min at 40 °C. Photo-film masks with the desired features were placed on top of samples and exposed to UV light source (OAI flood exposure) with a wavelength of 365 nm and intensity of approximately 9 mw cm⁻². Next, samples were placed in Petri dishes containing de-ionized water and sonicated in an ultrasound bath for 10 min to remove unbound photobiotin. A small cover-slip 18 mm × 18 mm (Dow corning) was placed on top of each sample. Double-sided tape was used as a spacer to create a flow cell into which protein solutions were injected. After the flow cell was created, 40 μl of block agent (3% w/v BSA) solution was injected and slides were incubated at room temperature for 1 h. TRITC or FITC-labeled streptavidin in PBS buffer (0.1 M phosphate, 0.15 M NaCl, pH 7.2) was then injected and incubated with photobiotin patterned samples for 1 h (Fig. 1). The streptavidin concentrations ranging from 0.04 mg ml⁻¹ to 0.8 mg ml⁻¹ were used in intensity/concentration correlation experiments. All other experiments used streptavidin concentration of 0.08 mg ml⁻¹. Finally, the samples with streptavidin protein were washed thoroughly with PBS buffer and placed under a fluorescence inverted microscope (Nikon EclipseTE-200) coupled with an Orca-EG digital camera model C4742-80-12AG (Hamamatsu Photonic) for image acquisition. Recorded images were processed with Wasabi software (Hamamatsu Photonic).

2.2.4. Actin filament length investigation
Glass slides were cleaned using the same cleaning protocol described previously. Clean slides were placed in 1% (v/v) of PDDA dissolved in 3X de-ionized water for 1 h. Following incubation in PDDA, the slides were dried to eliminate the excess of PDDA on the slide. PDDA was used to create a sticky surface to randomly bind F-actin on the substrate. As described above, a solution of 2.47 μg ml⁻¹ for F-actin and 1.23 μg ml⁻¹ for biotinylated gelsolin and kept for at least an hour on ice. A flow cell was created by placing a glass cover-slip with a thickness of 0.16 mm on top of the PDDA coated samples. Double-sided tape was used as separators to create the space to inject solutions in the flow cell. The mixed solution was then injected onto the PDDA coated samples and allowed to incubate at room temperature for 20 min. Finally, the samples were placed under the microscope for observation and data recording. The same experimental procedures were repeated with varying concentrations of gelsolin (diluted 1:10 and 1:100).

2.2.5. Actin immobilization
Solutions of a complex containing biotinylated gelsolin at a concentration of 0.125 mg ml⁻¹ were prepared as described previously [14] and incubated with F-actin at a concentration of 0.25 mg ml⁻¹. The solution was added to 95 μl of motility assay buffer (100 mM KCl, 2 mM of MgCl₂, 1 mM EGTA and 25 mM of imidazole) followed by addition of 1 μl of 0.0356 mg ml⁻¹ of catalase, 1 μl of 4.45 mg ml⁻¹ glucose, 9.90 mM of dithiothreitol and 0.19 mg ml⁻¹ of glucose oxidase to yield a final concentration of 2.47 μg ml⁻¹ of F-actin and 1.23 μg ml⁻¹ of biotinylated gelsolin capping protein. The solution was kept on ice for at least 1 h before use. This solution was then injected into the protein patterned sample and incubated at room temperature for at least 30 min. Following incubation, samples were washed in PBS buffer (three times, 5 min each). A drop of emulsion oil was placed onto the back of the samples and they were viewed with fluorescent inverted microscope for image and video acquisition.

2.2.6. Non-specific binding of F-actin
Experiments were conducted using three types of blocking agents; Tween-20, BSA and NHS-polyethylene glycol (MS-PEG, Pierce). Control experiments were done to test the effectiveness of these blocking agents in the presence of F-actin without gelsolin (ideally all the filaments should be washed out). With an ImageJ automotive tracking procedure (see the Supplementary materials), the image was preprocessed by a thresholding function that allowed the selection of the filaments to be counted.

3. Results and discussions
The final goal of this work is to create a unidirectional F-actin track for the long-distant movement of myosin-coated beads (as shown in Fig. 1a). The most challenging requirement is to obtain a homogeneous distribution of orientated F-actin on the surface. As discussed in Section 1, the methodology that F-actin is anchored with capped protein/biotin/avidin bridge is likely promising.

In a Class 1000 clean-room, glass slides were sonically cleaned and dried in an oven. A drop of water was found to spread much
better on the cleaned glass surface than that without any treatment (see Fig. S1 in the Supplementary materials), implying that organic contaminants were washed off, leading to higher hydrophilicity of the surface. The subsequent glass slides were immersed into 3% APTES ethanol solution for a certain time, followed by characterization with AFM. As shown in Fig. 2, APTES-modified slide shows variable topography, demonstrating a non-conformal layer. Root mean square (RMS) roughness values were estimated at 0.245 nm for bare glass surface, and 0.450 nm and 0.810 nm for 30 min and 12 h modification surface, respectively, which are consistent well with that in a relevant report [20]. The significant difference in roughness values suggests that incubation time plays an important role in the density of APTES layer. In a previous study of X-ray photoelectron spectrometry, it has been pointed out that 12 h incubation in an anhydrous solution is sufficient to form a fully covered organic silane film on the surface [21]. Therefore, 12 h incubation time was chosen for APTES-modification in the following patterning section.

As described in Experimental Section 2.2.3, photobiotin was covalently binding onto the APTES modified surface under UV light via the bonding between photo-activated nitrenes and amine groups. After blocking the extra amine groups on the surface, fluorescent dye-tagged streptavidin was injected to the micro-channel and incubated to accomplish the bioaffinity reaction with biotin moieties. The concentration of dye-tagged streptavidin was optimized to be 0.4 mg ml\(^{-1}\) according to the saturation of fluorescent intensity (Fig. S2). The images recorded by a fluorescent microscopy shows correspondence to the geometrical shapes printed on the photo-film mask used during the UV irradiation of photobiotin (Fig. S3). The gray values of different patterned lanes in the intensity profile were found to range from 33 to 38, implying the formation of a homogeneous patterned streptavidin film. Photo-film masks with different types of geometrical patterns were used to assess the reproducibility of this method (Fig. 3). The edge of a lane less than 10 \(\mu\)m was clearly defined in the patterned letters. To test the stability of the patterned streptavidin film, the patterned surface was rinsed with PBS buffer (three times, 5 min each), followed by imaging with fluorescent microscope. There is marginal change of fluorescent intensity, neither the defined geometrical patterns. In comparison, a patterned film obtained from microcontact printing was found to be easily destroyed under the same rinsing conditions (Figs. S4 and S5). It needs to be pointed out that it is difficult for us to characterize the streptavidin patterning with AFM, probably because the difference in height between the streptavidin patterning and BSA-blocked surface is marginal according to their dimension sizes (streptavidin: \(45 \times 45 \times 50\) Å\(^3\); BSA: \(140 \times 40 \times 40\) Å\(^3\)).

Non-specific binding of F-actin via electrostatic adsorption needs to be in consideration, since polyelectrolyte behavior of the double-helical F-actin is analogous to ds-DNA, both of which are extremely negatively-charged [22,23]. Specifically, it is estimated to be of a nominal charge density of 11 negative fundamental charges per
monomer subunit of F-actin [24]. Therefore, it is vulnerable for F-actin to absorb on the positively charged amine functionalized surface in the non-patterned areas. To solve this problem, a secondary blocking process was conducted prior to incubation with F-actin solution. An Image J automotive tracking method (see the Supplementary materials) was employed to count the number of F-actin absorbed on the surface in a unit area, which reflects the performance of different blocking agents. To investigate the non-specific binding of F-actin, we used two different fluorescent dyes to label streptavidin and actin filament: FITC-streptavidin, emission at 521 nm; rhodamine-phallloidin–actin, emission at 580 nm. Therefore, these two types of emission can be conveniently distinguished under fluorescence microscopy. After recording the fluorescent image of F-actin, we employed image software, Image J, which could track objects in the image according to a specific brightness (fluorescence intensity), to count the number of objects of interest. In a defined area, the number of object of interest (e.g. actin) will be comparable. Obviously, much less amount of F-actin were found to absorb on BSA and PEG-NHS blocked APTES modified glass slide surface than that blocked by Tween-20 (Fig. S6, left). Electrostatic repulsion between BSA (pl = 4.7, negatively charged in neutral pH) and F-actin in neutral pH condition reduces the amount of the latter to attach, which can also be achieved by PEG that prevents the surface from non-specific binding due to its high mobility, large excluded volume, and steric hindrance effect. Served as a secondary blocking agent, BSA or PEG-NHS was introduced on the patterned streptavidin surface, followed by F-actin. Interestingly, BSA works as well as first blocker in streptavidin patterning according to that few of F-actin was found on the BSA-blocked patterned surface (Fig. 4B), whereas PEG-NHS does not (Fig. S6, right). All of measurements with fluorescent microscopy were carried out in the presence of solution at a micro-channel. As far as we known, the bright spots in Fig. 4 could probably be gas bubbles in the solution, reflecting the entrance excitation light.

As discussed in the Section 1, the length of F-actin affects the formation of a uniform track. As shown in Scheme 1, the final goal of this project is to build a bio-molecular motors-based nanotransportation system. Cargoes loaded with molecules of interest shuffles between two stations (A), which require no intervening between the couple tracks1. By controlling the distance of these two tracks (>the length of F-actin), this requirement could be meet. That is why we use micropatterning. The unidirectional track could be achieved either by motor-driving syringe pump [14] or AC electrokinetic pump. We are aiming to fabricate “small size” of such tracks (~submicron in width), although we are capable to make it around ~20 μm in this work. Instead of straight track, it is of possibility to build curve track under the elegant design of AC electrokinetic pump which may allow obtaining a circle track to accomplish repeating tasks. From our views, this task is more delightful than that in straight track form. In a curve track, the length of immobilized F-actin needs to be precisely control (B, left). Otherwise, too long F-actin will run out of the track while they are laydown. Therefore, it is in need of the optimization of the length of F-actin, especially the size of tracks are down to the value close to the length of F-actin.

To make the F-actin stretched well on the surface, a positively charged polymer PDDA was deposited on the cleaned glass slide surface via electrostatic adsorption, leading to a strong binding between PDDA and F-actin. It is believed that F-actin can be laid down completely on PDDA modified surface, which would be helpful to observe the length of F-actin. Without adding gelsolin into the mixture solution, dye-tagged F-actin were estimated to be appropriately 17 μm in length by fluorescent microscopy Image J method (Fig. S7 and Fig. 5). This value is consistent with that in several early studies [25–27]. Not surprisingly, the length of F-actin was found to decrease gradually with the gelsolin concentration increasing in the composition. To control the length be smaller than the distance of two patterning strips, a ratio of 4:1 (actin/gelsolin) was selected and consequently F-actin are appropriately of 2.5 μm in length in average. Besides, the sample of F-actin/PDDA glass slide was also observed by SEM (as Fig. S8), which

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1 For interpretation of color in Scheme 1, the reader is referred to the web version of this article.
indicates the success of immobilization of F-actin on the PDDA surface. Relying on the Image J software, it is a rapid way to calculate the length of F-actin. Most importantly, the value obtained in our method matches well with that in other reports. Therefore, this method is reliable and more simple than AFM/TEM/SEM in sample preparation.

The final part of this work was to attach biotinylated gelsolin-capped F-actin to the streptavidin-patterned areas. The procedure for elucidation was similar to previous experiments that Image J was employed to track the quantity of filaments attached to the streptavidin-patterned areas (the part under the dashed line in Fig. 6) and comparing to that attached to the non-patterned area surface (above the dashed line). Note that bright spots instead of lanes were found distributing homogenously in the patterned area, indicating that the F-actin is standing up on the surface according to the length scale of the spots. As a result, pressure driven flow was used to wash off the non-specific binding F-actin on the surface.

Fig. 4. Fluorescent microscopy image of (A) a 50 μm thick line feature of patterned FITC-labeled streptavidin film, and (B) incubated with rhodamine-labeled F-actin after blocked with 3% (w/v) BSA solution.

Scheme 1. Scheme of bio-molecular motors-based nanotransportation.

Fig. 5. Actin filament length as a function of gelsolin concentration (from three different sets of experiments). The molar ratio of actin filament/gelsolin were indicated.
surface in order to determine whether biotinylated gelsolin/actin component was immobilized on streptavidin-patterned areas. There were two ways used to characterize the attachment of F-actin: (1) direct counting of the number of filaments in both areas (Fig. S9 and Fig. 6, black column); and (2) using two distinct images one before and one after pressure driven flow was applied (Fig. 6, blank column). In method (2), the two images were then subtracted and the number of filaments from the resulting image was determined by the Image J "analyzed particles" function. Calculated in either way, the number of F-actin in patterned area is 4–6 times higher than that in the blocked areas. The density of F-actin in patterned areas was estimated as c.a. 2 µm² per actin filament. Assuming that every actin filament occupies a square area unit, the distance between each other actin filaments was calculated as in the range of c.a. 1.41 µm (side of the square) to 1.97 µm (diagonal). In this condition, it is of the possibility to build a unidirectional track by laying down F-actin that are of 2.5 µm in length with driven forces so that the F-actin would be connected head-to-tail as shown in Fig. 1a. It is not a problem to lay-down the F-actin on the surface, since F-actin was able to be laid-down by a motor-driven syringe pump at varying speeds in a previous report [14]. In this work, we were focusing on the assembly of F-actin, and we will demonstrate this part of work in the future work.

As shown in Fig. 7, we used AFM to characterize the orientation of F-actin on the surface. It is not obvious to distinguish the streptavidin patterning area and the nonpatterning area, since BSA was employed to block on the nonpatterning area. The yellow spots at high density in Fig. 7A and D with a height of c.a. 5 nm (Fig. 7B) are

Fig. 7. AFM images of F-actin/biotin–avidin bridge/APTES/glass slide surface. The dashed blue line indicates the edge of patterning. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
streptavidin molecules. Bright spots that show much bigger values in height are F-actins, which are of higher density in central part of the pattern (Fig. 7D). There might be some aggregation of F-actins, yielding the bigger bright spots in AFM images. Note that one of F-actins shows its wire-like structure at Fig. 7D. Taken together, F-actins are likely standing up on the surface. However, details of the conformation need to be revealed and it is crucial to increase the density of F-actins in future work.

As far as we known, there is only one similar report that is close to our work. Huang et al. has reported a selective assembly of F-actin, in which biotinylated gelsolin-capped F-actin is immobilized through streptavindin/biotin conjugation [15]. In their work, cysteamine patterning was achieved via microcontact printing on a thin gold film, and NHS-biotin reacted with amine minus of cysteamine for the further construction of F-actin assembly. However, the length of F-actin had not been controlled, and the gold film blocks light transmission to some extent and intervenes the observation by fluorescence microscopy. In the present work, biotinylated gelsolin-capped F-actin were selectively attached on a streptavidin-patterned surface, in which homogeneous population of controlled-length F-actin was observed. The assembly was accomplished on a glass slide surface, which is convenient for the observation by fluorescence microscopy. Moreover, the patterned film was found stable and homogenous compared to that obtained by microcontact printing method, according to the profiling with fluorescence microscopy. More efforts are needed to characterize the patterned F-actin track to reveal information in specific details to verify the feasibility of long-distant bead assay. Continued works by using AC electrokinetic pump, SEM, fluorescence microscope-AFM, and electrochemistry are undergoing, aiming to apply such nanotransportation in intelligent biosensor device development in future work.

4. Conclusions

In summary, the results presented here demonstrate an effective methodology to accomplish selective patterning of F-actin with controlled length in a homogenous distribution. A density of c.a. 2 μm² per actin filament in the patterned areas was obtained leading to a possibility to form a unidirectional F-actin track by laying down the actin filament molecules, which thus are theoretically aligning with one another. This work opens a possibility to explore the study about long-distant movement of myosin-coated beads carrying cargo.

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Appendix A. Supplementary material


References