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Translocation of cationic polystyrene microspheres associated with actin filaments on a surface coated with myosin motors

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Abstract

Muscular actin filaments can glide on a myosin-fixed glass surface by mechanochemical energy transduction. Here, we examined whether artificial objects bound to actin filaments can be translocated actively by myosin motors. Actin molecules may have a monomeric (G-actin) or fibrous form (F-actin), depending on the ionic conditions. Each form of actin molecules was mixed with a suspension of polystyrene microspheres carrying amine groups. After myosin motors were fixed on the collodion-coated glass surface, the mixture of actin molecules and microspheres was applied in the presence of adenosine triphosphate. The motion of the microspheres was monitored under a phase-contrast optical microscope. The microspheres mixed with G-actin exhibited thermal fluctuations due to insufficient binding of G-actin. In contrast, F-actin could bind to the microspheres and they were translocated in linear manner at approximately 1.5 μm/s on the myosin-coated surface. These results demonstrate that the actin filaments bound to polystyrene microspheres are capable of translocation between the actin and myosin.

Keywords: Motor protein; Delivery system; Brownian motion; *In vitro* motility assay

1. Introduction

Actin is a major muscular protein that interacts with myosin motor proteins, resulting in the sliding movement underlying muscle contractions[1]. A combination of purified actin and myosin possesses the ability to be motile in the presence of adenosine triphosphate (ATP). This motile system can be reconstituted on either a myosin-fixed or an actin-fixed glass surface[2]. The movement of actin filaments or myosin motors can be observed under a fluorescence

microscope by labeling them with fluorescent dyes, using a technique referred to as "*in vitro* motility assay"[2]. This method revealed that, in myosin motors from a class II skeletal muscle, a plural number of motors coordinately produce faster movement of actin filaments than that the non-muscular myosin motors such as myosin V[3, 4].

These motor proteins have the potential for targeted transport and accumulation of certain objects for analysis in Lab-on-a-chip devices[5]. In addition, the actin-myosin system would allow attached objects to diffuse extensively beyond thermal diffusion. Various methods have been developed to attach actin or myosin molecules to targets by using the biotin-avidin complex, antibodies, or actin-binding proteins[6, 7]. The following simpler concept is proposed for the binding of actin molecules to objects. At neutral pH, actin molecules carry a cluster of negative charge on their subdomain-I[8]. When actin molecules form double helical filaments by polymerization, the clusters of negative charge concentrate in the outer surface, along the filaments[9, 10], meaning that actin filaments could associate with cationic matter. We have already demonstrated that actin filaments bind to positively charged liposomes consisting of cationic lipids and are transported by myosin motors via ATP hydrolysis[11]. However, it is unknown whether this motility is confined to liposomes or applicable to other materials as well.

Here, we employed a polystyrene microsphere containing amino groups as a model for the translocation of artificial matter. The microspheres bound to actin filaments were translocated through actin-myosin interactions, except for microspheres with G-actin. KCl-dependence of the translocation was also examined.

2. Materials and Methods

2.1 Microsphere, proteins, and chemicals

Actin and myosin were prepared from rabbit skeletal muscles[12]. Purified actin was stored at 3 mg/mL in G-buffer (2 mM Tris-HCl (pH 7.5), 0.2 mM ATP, 0.1 mM $CaCl_2$, and 1 mM DTT) on ice. Myosin was digested with α-chymotrypsin to obtain the HMM (soluble motor fragment)[13], and the product was stored in liquid nitrogen. Microspheres carrying amino groups (polystyrene, 2.5%w/v, diameter 1.0 μm, 4.55×10^{10} particles per mL) were purchased from Polysciences, Inc. (Pennsylvania, USA). Before use, this suspension was sonicated for 30 min. Bovine serum albumin (BSA), phalloidin, catalase, and glucose oxidase of biochemical grade were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Other chemicals were of special grade and were purchased from Nacalai Tesque (Kyoto, Japan). Collodion (2%) was purchased from Okensyouji Co., Ltd. (Tokyo, Japan).

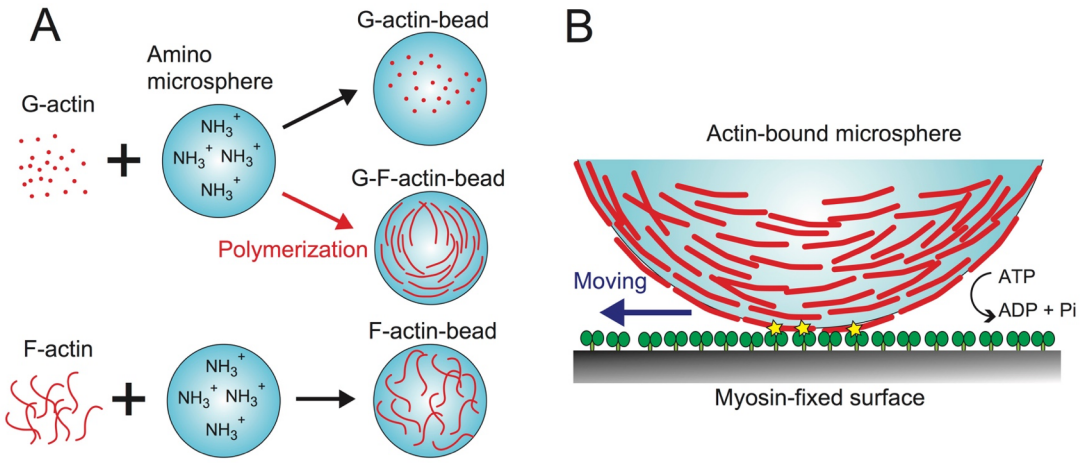

Figure 1: (A) A schematic diagram of the mixture of actin and microspheres carrying amino groups. Three preparations of actin-bound microspheres were tested: G-actin-bead, F-actin-bead, and G-F-actin-bead (See Sec. 2.2). (B) An illustration of the motility assay for the actin-bound microsphere interacting with myosin motors.

2.2 Preparation of the mixture of microspheres and actin

We prepared three kinds of actin and cationic microsphere mixtures. For the first one, 10 µL of microsphere suspension (2.5%) was added to 290 µL of G-actin (200 µg/mL) in G-buffer. After incubating for 60 min at 25°C, 300 µL of G-buffer was added (final content: 0.042% microspheres, 0.1 mg/mL actin in 600 µL). This sample is referred to as "G-actin-bead." For the second one, 10 µL of microsphere suspension (2.5%) was added to 290 µL of pre-formed F-actin (200 µg/mL) with phalloidin (2 µg/mL) in F-buffer (25 mM imidazole-HCl (pH 7.4), 4 mM $MgCl_2$, 0.2 mM ATP, 1 mM DTT, and variable concentrations of KCl). After incubating for 60 min at 25°C, 300 µL of F-buffer was added. This sample is referred to as "F-actin-bead." For the final one, 10 µL of microsphere suspension (2.5%) was added to 290 µL of G-actin (200 µg/mL) in G-buffer, following which 300 µL of F-buffer (2-fold concentrations) with phalloidin (2 µg/mL) was added. This mixture was incubated for 60 min at 25°C for polymerization. This sample is referred to as "G-F-actin-bead." Figure 1A summarizes the three kinds of preparations.

2.3 Co-sedimentation assay and sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis

To determine the binding amount of actin to the microsphere, the bound fraction was separated from the mixture (600 µL) by centrifugation. The mixture was centrifuged at 15,000 rpm for 20 min, precipitate the microspheres with bound actin, whereas the

unbound actin filaments remain in the supernatant. After removing the supernatant, the precipitate was dissolved in 60 μL of 4% SDS solution and centrifugation was carried out again. The dissolved actin, which was bound to microspheres, was obtained in the supernatant. This fraction was analyzed by SDS-polyacrylamide gel electrophoresis at a gel density of 12.5%. After electrophoresis, the gel was stained with Coomassie Brilliant Blue, and the amount of bound actin was estimated from the image of the stained gel by densitometry, performed with an image scanner (GT-X800, Seiko Epson Corp., Nagano, Japan) and ImageJ software (Rasband, W.S., ImageJ; National Institutes of Health, Bethesda, MD, USA).

2.4 Motility assay

The surface of a glass slide (S1111, No. 1, 24×76 mm; Matsunami Glass Industries, Osaka, Japan) was treated with 0.2% collodion in isoamyl acetate and thoroughly air-dried. The HMM molecules were fixed onto the collodion-coated glass slide by perfusing 0.2 mg/mL HMM in standard solution (25 mM KCl, 25 mM imidazole-HCl (pH 7.4), 4 mM $MgCl_2$, 1 mM DTT) between the slide and the cover glass (C218181, No. 1, 18×18 mm; Matsunami Glass Industries), separated by 0.1 mm. Sixty seconds after perfusion, the solution was replaced with 10 mg/mL BSA in standard solution to remove unbound HMM molecules. The slide was then perfused with an actin-bead suspension, to which an ATP solution (1 mM ATP in the standard solution, with various concentrations of KCl) was immediately added. Figure 1B shows a schematic illustration of the motility assay. The assay was performed at room temperature (25°C ± 2°C). The motion of microspheres was observed under a phase-contrast microscope (objective ph3 40DL 0.55LWD 40×, Diaphoto-TMD; Nikon, Tokyo, Japan). Images were acquired using a CMOS camera and a recording system (SV9M001M; Epix Inc., Buffalo Grove, IL, USA), and were recorded on a personal computer (Vistro 220; Dell, Austin, Texas, USA) with a XCAP software (Epix Inc., Buffalo Grove, IL, USA). The center position of the microspheres was traced at intervals of 0.5 s by ImageJ software. We only measured the moving microspheres and excluded the stationary ones from the sampling due to irreversible binding to the glass surface. Additionally, sudden changes in the direction of movement were observed, and these were also excluded from sampling. The overall distance and the displacement were defined as the successive distance covered in 10 s at intervals of 0.5 s and as the distance between the start point and the point travelled at each time interval, respectively. The linearity of the movement was defined as the ratio of displacement to the overall distance.

3. Results and Discussion

3.1 The amount of actin bound to microspheres

Because actin molecules have a net negative charge (isoelectric point, 5.4) at neutral pH[14], it was expected that they would bind to positively charged matter. For each of the preparations, G-actin-bead, F-actin-bead, and G-F-actin-bead, the amount of actin bound to microspheres was estimated by a combination of centrifugation and electrophoresis. Figure 2 shows a stained polyacrylamide gel with actin bands. Each amount of bound actin to microspheres for G-actin, F-actin and G-F-actin was calculated to be 1.1%, 3.8%, and 4.4% of the overall actin content (60 μg), respectively. This result indicates that, compared to G-actin, F-actin and G-F-actin had a stronger affinity to microspheres. When KCl concentration of the G-F-actin-bead was increased from 25 mM to 100 mM, the percentage of bound actin slightly decreased from 4.4% to 4.2% of the overall actin content.

Geometrically, the maximum percentage of possible bound actin in this experimental concentration was roughly calculated to be 13% by comparing the surface area (3.14×10^6 nm^2) of a microsphere with the contact area (13 nm^2) of an actin molecule. The number of amino groups on microspheres we used was estimated through the spectroscopy of bound amino-reactive fluorescent dyes (Cy3-NHS, data not shown). After Cy3-NHS

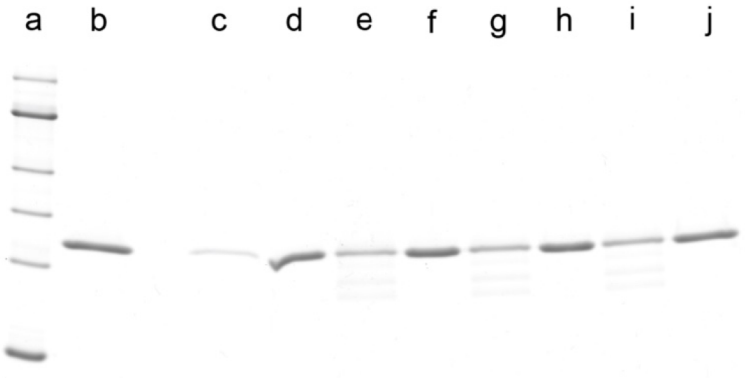

Figure 2: Use of sodium dodecyl sulfate (SDS)-polyacrylamide gel (12.5%) electrophoresis to determine the amount of actin bound to cationic microspheres. The molecular weight of actin molecules is approximately 42000. Lane symbols: (a) Ladder marker, 100 kDa, 80 kDa, 60 kDa, 50 kDa, 40 kDa, and 30 kDa from top to bottom; (b) Actin standard (0.1 mg/mL); (c) precipitate (ppt) in G-actin-bead; (d) supernatant (sup) in G-actin-bead; (e) ppt in F-actin-bead; (f) sup in F-actin-bead; (g) ppt in G-F-actin-bead at 25 mM KCl; (h) sup in G-F-actin-bead at 25 mM KCl; (i) ppt in G-F-actin-bead at 100 mM KCl; (j) sup in G-F-actin-bead at 100 mM KCl.

and the amino-microsphere were thoroughly reacted, the fraction of unbound Cy3-NHS (supernatant) was collected using a centrifugation (Cy3-bound microspheres were removed). Absorbance of the supernatant was measured to determine the concentration of Cy3-NHS. The surface density of amino groups was calculated by considering the difference between total and unbound Cy3 amounts, resulting in approximately 5.7×10^{-2} amino groups per nm^2 of surface.

In order to determine non-specific (or hydrophobic) interactions between actin and microsphere, the binding of actin molecules to polystyrene microspheres without amino groups was examined. It was found that actin filaments also bind to these microspheres though this amount was 1.5 times smaller than those carrying amino groups. This result indicates that polystyrene microspheres possess within themselves an ability to absorb actin filaments, and the addition of amino groups to microspheres improves the binding amount due to electrostatic interactions.

3.2 Translocation of the actin-microsphere mixture on HMM-fixed surface

Various mixtures of actin and microspheres were applied on the HMM-fixed glass surface in the presence of ATP. G-actin-bead did not bind to the HMM-fixed surface and thermally fluctuated in solution. The fluctuating motion was similar to the motion of microspheres without actin. Figure 3 shows typical images of translocated microspheres in the case of F-actin-bead. There were both translocated microspheres and stationary ones just above

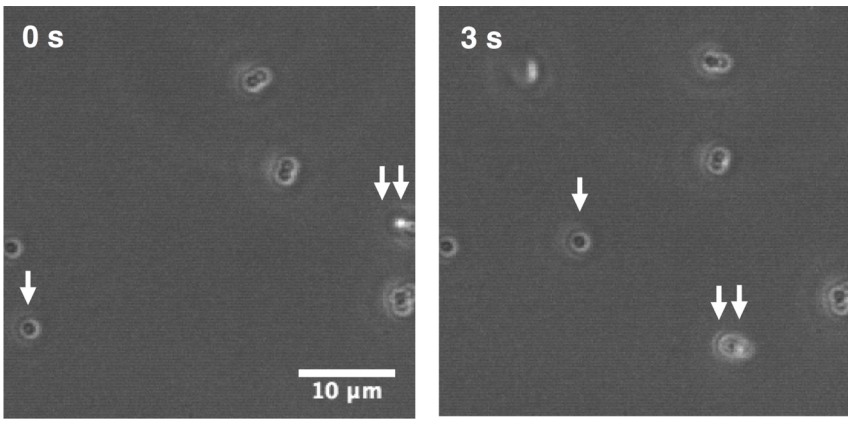

Figure 3: Phase-contrast microscopic images of the microspheres in case of F-actin-bead at start time (left) and 3 s later (right). A single arrow indicates a microsphere translocated from left to center. Double arrow indicates a microsphere translocated from right to center. Scale bar at the right bottom (left) is 10 µm.

the HMM-fixed glass surface. The ratio of translocated microspheres relative to the total was at most 50%. In the case of polystyrene microspheres without amino groups, the ratio was even lower (data not shown). The cause of immotility cannot be identified at present. Some F-actin-bead and G-F-actin-bead bound to HMM, successively moved (Fig. 4, filled circles) and their displacement was considerably larger than that of G-actin (Fig. 4, open circles). Figure 5A shows the time development of displacement of the microspheres in the three kinds of preparation. The displacement of G-actin-bead almost obeyed to Brownian motion ($<x^2> = 4Dt$, where x and t denote the displacement and elapsed time, respectively, and D is the diffusion coefficient, 4.9×10^{-13} m^2/s for a sphere of diameter 1.0 μm). This feature demonstrates that G-actin-bead could not interact with HMM heads. This finding can be explained by the fact that a little amount of actin monomer was bound to the microspheres (Fig. 2, lane c). Moreover, it might be difficult for myosin heads to bind to actin monomers because myosin heads generally interact with actin filaments, whose conformation is distinct from that of G-actin[10].

On the other hand, the displacements of F-actin-bead and G-F-actin-bead increased linearly with time. It is likely that the translocation of F-actin-bead and G-F-actin-bead resulted from actin-myosin interactions via ATP hydrolysis. In fact, in the absence of ATP, these actin-bound microspheres were fixed on HMM (data not shown), owing to the strong binding between actin filaments and myosin heads, namely the "rigor state"[15]. Furthermore, in the presence of 10 mM KCl, G-F-actin-bead moved over a longer distance than F-actin-bead did. Figure 5B shows the KCl-dependence of the displacement of G-F-actin-bead. The displacement increased with increasing KCl concentrations over the range of 0–50 mM, whereas above 75 mM KCl, the displacement declined to the level of thermal fluctuations. One explanation for this

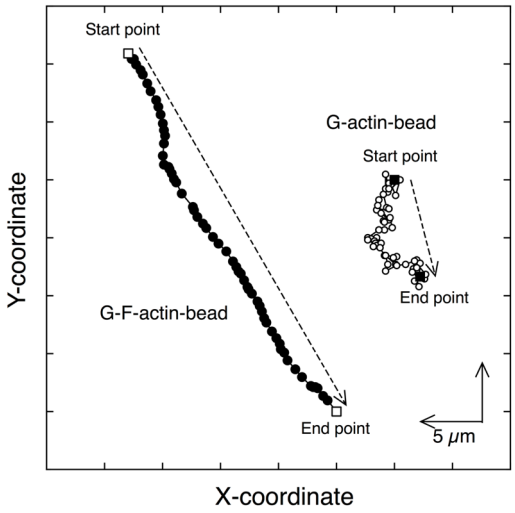

Figure 4: Typical traces of translocation of the microspheres in case of G-actin-bead (open symbols) and G-F-actin-bead (filled symbols). Each point was plotted at intervals of 0.5 s for 30 s. Square symbols denote the start point and end point. The dotted arrows indicate the direction of translocation. Scale bar at the right bottom indicates a distance of 5 μm.

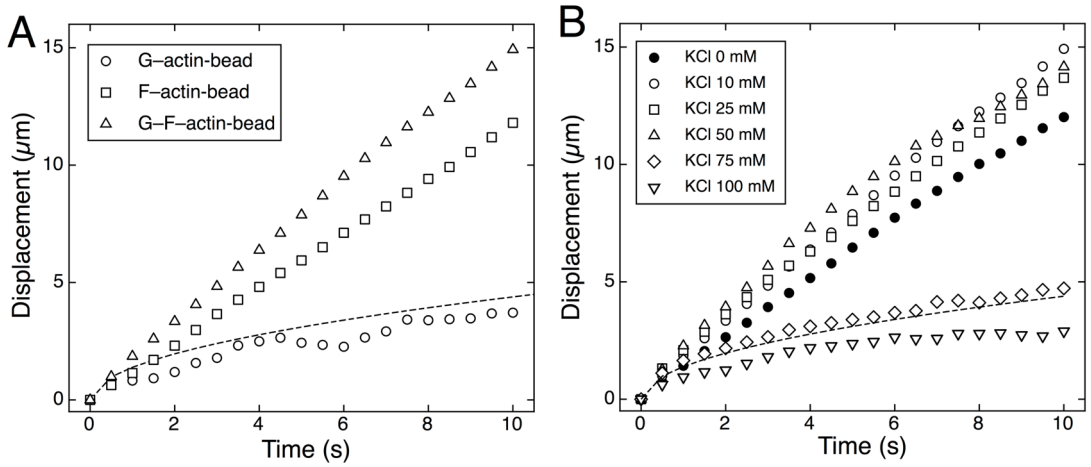

Figure 5: (A) Time development of displacement of microspheres for G-actin-bead, F-actin-bead, and G-F-actin-bead. F-actin-bead and G-F-actin-bead were evaluated at 10 mM KCl. (B) KCl-dependence of the displacement of G-F-actin-bead. At each condition, the data taken from 20 to 70 microspheres were averaged. The dotted curve line indicates theoretical values for the Brownian motion of a microsphere of diameter 1.0 μm.

phenomenon is that actin filaments dissociated from microspheres because the electrostatic binding tended to become weaker as the KCl concentration increased. Another explanation is the decrease of actin-myosin binding, which is governed by electrostatic interactions. The former explanation is unlikely because SDS-polyacrylamide gel electrophoresis showed KCl-independent binding of actin to microspheres under these experimental conditions (Fig. 2, lane g and i). Furthermore, sliding movement of single actin filaments on myosin molecules is observed at 0–50 mM KCl, but not at KCl concentrations above 75 mM[12, 16]. KCl-dependence of the sliding movement is quite similar to that of translocations in this study. Therefore, it is likely that, at higher KCl concentrations, the weakening of actin-myosin interactions caused the suppression of translocation.

3.3 KCl-dependence of the rate and the linearity of translocation

Figure 6 summarizes certain properties of translocation over the concentration range of 0–100 mM KCl. The effect of phalloidin, which is a stabilizer of F-actin, on the translocation is also shown in the figure. The above-mentioned F-actin-bead and G-F-actin-bead were prepared with phalloidin (standard). With respect to the translocation rate (distance between the start point and end point divided by the elapsed time), G-F-actin-bead with phalloidin was observed to be the fastest in the three cases that we tested (Fig. 6A). The maximum rate (approximately 1.5 μm/s) was observed in the range of 10–50 mM KCl.

However, this translocation rate of the microspheres was about three times slower than the velocity of single actin filaments (approximately 5 μm/s) in our *in vitro* motility assay. As the orientation of actin filaments is considered not be the same in all filaments bound on microspheres, the filaments that are positioned in a direction opposite to that of movement might act as friction components, which decrease the rate of motility. Alternatively, a lowering of the flexibility of actin filaments due to the restriction on microspheres might decrease the driving efficiency of actin-myosin interactions[17]. Additionally, the absence of phalloidin further decreased the translocation rate. In particular, in the absence of KCl, G-F-actin-bead without phalloidin decreased the translocation rate to the level of thermal fluctuations (0.45 μm/s). The presence of metal ions facilitates actin polymerization, therefore, the absence of both KCl and phalloidin might lead to the destabilization or depolymerization of actin filaments on the surface of microspheres as well as the G-actin-bead. Compared with G-F-actin-bead, F-actin-bead had a tendency for slower translocation rates. This property might explain the relatively lesser quantity of bound actin (3.8%) in F-actin-bead compared with that (4.4%) in G-F-actin-bead. In fact, the translocation rate increased from 1.0 to 2.2 μm/s with an increase in actin concentrations mixed with microspheres in the range of 0.02–0.5 mg/mL (data not shown).

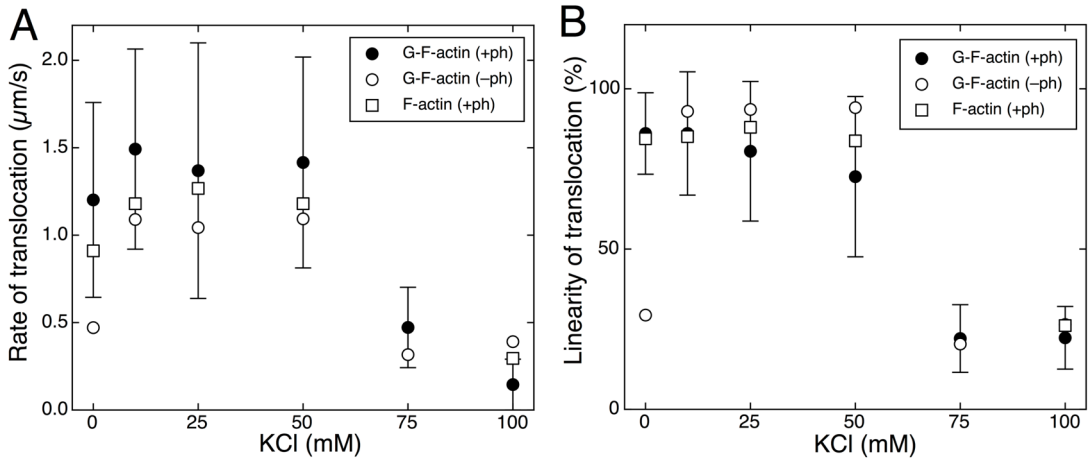

Figure 6: The rate (A) and linearity (B) of translation in the concentration range of 0–100 mM KCl. Filled circles, open circles, and open squares represent the case of G-F-actin-bead with phalloidin, G-F-actin-bead without phalloidin, and F-actin-bead, respectively. The rate was defined as the distance between the start point and end point divided by the elapsed time (10 s). The linearity was defined as the ratio of the displacement to the overall distance. Error bars indicate the standard deviation of 20–70 microspheres at each concentration in the case of G-F-actin-bead with phalloidin. Standard deviation in other cases (not shown) was at the same level as these values.

The standard deviations of the rates were considerably large. For instance, the actin-bound microspheres may occasionally move actively, as fast as single actin filaments, whereas some fractions may suffer from a resistance owing to improper binding to the HMM surface, resulting in a slower translocation rate.

Figure 6B shows the linearity of translocation, which we defined as the ratio of displacement to the overall distance. In the range of 0–50 mM KCl, the linearity of F-actin-bead and G-F-actin-bead with phalloidin was above 70–90%, which is higher than the 50% linearity of movement of single actin filaments[11]. The linearity may be caused by suppressing the bending motion of actin filaments restricted on the surface of microspheres. On the other hand, further increases in KCl concentration (≥ 75 mM) declined linearity to 30%, indicating low orienting motions such as thermal fluctuations. Then, it was found that lower linearity corresponds to slower rate of translocation, and the overall distance for thermal fluctuations (15 μm during 10 s) is almost compatible with that for actin-myosin-driven microspheres (17 μm during 10 s). In other words, long-distance translocation of microspheres results from a rectification of displacement at the level of thermal fluctuations through actin-myosin interactions.

4. Conclusion

This study shows that actin filaments (F-formed actin) have the potential to bind to cationic polystyrene microspheres, which is similar to the case of the cationic liposomes reported previously[11]. In contrast, G-formed actin monomers did not bind efficiently to the microspheres. In the presence of ATP, these actin-bound microspheres were translocated by the actin-myosin-driven force at the rate of approximately 1.5 μm/s in the concentration range of 0–50 mM KCl. Additionally, the translocation had a tendency to be linear, which is similar to that observed in actin-myosin-driven liposomes. These results demonstrate that, in addition to biological matter, actin filaments can bind to artificial matter, and these complexes can interact with myosin heads. Further studies are needed to align the direction of actin filaments and to control the direction of movement. Subsequently, this simple method, which does not use other binding components, may be used for the active transport of various micro-objects.

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Disclosure

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