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Microtubule bundle formation driven by ATP: Effect of the concentrations of kinesin, streptavidin, and microtubules

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Abstract

Recently, a method was established for the formation of microtubule (MT) assemblies by an active self-organization (AcSO) process, in which MTs were crosslinked during sliding motion on a kinesin-coated surface, and this was coupled with adenosine triphosphate (ATP) hydrolysis. Streptavidin (ST) was the glue used to crosslink biotin-labeled MTs. Although most of the MT assemblies were in the bundle form, they varied in size, shape, and motility, depending on the initial conditions used. In this paper, we systematically examined the effects of the concentrations of kinesin, ST, and MT on the formation of MT bundles under the initial conditions of the process.

1. Introduction

Linear motor protein systems such as actin-myosin and microtubule-kinesin are proposed to be the building blocks of ATP-fueled biomachines [1–7]. The greatest difficulty with such systems is the integration of their motilities with large structures such as those in cells and tissues. In previous papers, we reported that large linear-shaped actin bundles that consist of several tens of filamentous actin could be obtained through electrostatic interactions with synthetic polymers carrying positive charges [8–12]. In the presence of ATP, these bundles showed motility on a surface coated with myosin. Recently, a method was developed to integrate microtubules (MTs) into a bundle structure on an MT-based motor protein “kinesin-coated surface” by employing a streptavidin (ST)–biotin interaction during the sliding motion of MTs in the presence of ATP (Figure 1) [13]. Using this method, we reported that large ring-shaped MT bundles could be produced by integrating and simultaneously crosslinking MTs prepared in the presence of guanosine-5'-triphosphate (GTP) during the sliding motion of MTs on a surface coated with kinesin [14]. Interestingly, these ring-shaped MT bundles with diameters of 1–12 μm showed preferential rotation depending on the number of protofilaments (PFs) in the MT. The ratio of the rings rotating in the counterclockwise (CCW) direction to those rotating in the clockwise (CW) direction was 14/1 ($n_{\text{CCW}}/n_{\text{CW}} = 222/16$) when the majority of the MTs used were composed of 14 PFs. Meanwhile, when MTs with more than 14 PFs

were used, both the CCW/CW ratio and the number of ring-shaped MT bundles dramatically decreased. The preferential rotation of the MT bundle was therefore attributed to the lattice structure of the MTs. In a previous paper, we also reported that straight MT bundles could be selectively obtained by the active self-organization (AcSO) process using rigid MTs prepared by polymerization with guanylyl-(α , β)-methylene-diphosphonate (GMPCPP) [15]. MTs prepared in the presence of GMPCPP (GMPCPP-MT) are structurally stable, and the flexural rigidity of GMPCPP-MT is reported to be ~2-fold higher than that of GTP-MTs due to the strong lateral interactions between PFs [16]. Of these bundles, 94% were motile 4 h after ATP addition, and the velocity was similar to that of single GMPCPP-MTs just after ATP addition. This was in contrast to the bundles formed with GTP-MTs, of which 44% were motile. The high motility of these bundles suggested that there could be a highly ordered structure with unipolarity. These results indicated that the functions and morphologies of MT bundles are influenced by the nature (structure, mechanical property, and so on) of the single MTs used in the AcSO process. In this paper, we systematically studied the effects of unknown parameters such as the concentrations of the kinesin “motor,” ST “glue,” and MT “assembly component,” represented as C_k , C_{ST} , and C_{MT} , respectively, on the formation and motility of MT bundles. This study may help in the application of motor proteins, including the MT-kinesin and actin-myosin systems, as the building blocks of ATP-fueled biomachines.

2. Material and Methods

2.1. Preparation of tubulins and kinesin

Tubulin was purified from porcine brain using a high-concentration PIPES buffer. High-molarity PIPES buffer (HMPB) and Brinkley BR buffer 1980 (BRB80) were prepared using the dipotassium salt (PIPES-2K) (Sigma), and the pH was adjusted with HCl [17]. GFP-fused kinesin-1 consisting of the first 560 amino acids (K560-GFP) was prepared as described earlier by partially modifying the expression and purification methods [18].

2.2. Preparation of labeled tubulins

Biotin-tubulin was prepared using biotin-XX-SE (Invitrogen) according to standard techniques [19]. The labeling stoichiometry was approximately 2 per tubulin, which was estimated by spectrometric titration using the 2-(4'-hydroxyphenylazo)benzoic acid (HABA) dye (Dojindo) [20]. Rhodamine-labeled tubulin was prepared using tetramethylrhodamine succinimidyl ester (TAMRA-SE) (Invitrogen) according to standard techniques [21]. Rhodamine-tubulin was obtained by chemical crosslinking, and the labeling ratio was 1.2. This ratio was determined by measuring the absorbance of the protein at 280 nm and that of tetramethylrhodamine at 555 nm.

2.3. Motility assay 1: Effect of C_k

Biotin- and rhodamine-labeled MTs were obtained by polymerizing biotin-tubulin, rhodamine-tubulin, and native tubulin (molar ratio of 45:10:45; final concentration was 24 μ M) in polymerization buffer (80 mM PIPES-2K, 1 mM EGTA, 5 mM $MgCl_2$, 1 mM guanosine-5'-triphosphate (GTP), and 5% DMSO; pH adjusted to 6.8 with HCl) at 37°C for 30 min. The solution containing the MTs was then diluted 100-fold with the stabilizing buffer (80 mM PIPES-2K, 1 mM EGTA, 1 mM $MgCl_2$, 1% DMSO, and 10 μ M paclitaxel; pH adjusted to 6.8 with HCl) and gently pipetted 10 times. Hereafter, C_{MT} represents the concentration of tubulins. The concentration of diluted MTs was 0.24 μ M. These were incubated at ~25°C until the preparations for the motility assays were complete, which generally took 12 h.

Flow cells were prepared by placing a cover glass ($18 \times 18 \text{ mm}^2$; Matsunami) on a slide glass ($26 \times 76 \text{ mm}^2$) equipped with a pair of spacers to form a chamber of approximate dimensions $4 \times 18 \times 0.1 \text{ mm}^3$ ($W \times L \times H$). A single layer of Parafilm was used to fix the spacer-separated glasses by heating. The flow cell was filled with 0.2 mg ml^{-1} anti-GFP antibody (Invitrogen) for 15 min, and this was followed by a wash with $48 \text{ }\mu\text{l}$ of casein solution (80 mM PIPES-2K, 1 mM EGTA, 1 mM MgCl_2 , and $\sim 0.5 \text{ mg ml}^{-1}$ casein; pH adjusted to 6.8 with HCl). After incubating with the casein solution for 5 min to block the remaining glass surface, $24 \text{ }\mu\text{l}$ of K560-GFP solution (0.63, 6.3, or 63 nM in kinesin solution; $\sim 80 \text{ mM}$ PIPES-2K, $\sim 40 \text{ mM}$ NaCl, 1 mM EGTA, 1 mM MgCl_2 , 0.5 mg ml^{-1} casein, 1 mM DTT, 4.5 mg ml^{-1} D-glucose, 50 U ml^{-1} glucose oxidase, 50 U ml^{-1} catalase, $10 \text{ }\mu\text{M}$ paclitaxel, and $\sim 1\%$ DMSO; pH 6.8) was introduced, and this was incubated for 5 min to allow kinesins to bind to the antibody. Hereafter, C_k represents the kinesin concentration. The flow cell was washed with $32 \text{ }\mu\text{l}$ of motility buffer (80 mM PIPES-2K, 1 mM EGTA, 2 mM MgCl_2 , 0.5 mg ml^{-1} casein, 1 mM DTT, 4.5 mg ml^{-1} D-glucose, 50 U ml^{-1} glucose oxidase, 50 U ml^{-1} catalase, $10 \text{ }\mu\text{M}$ paclitaxel, and $\sim 1\%$ DMSO; pH 6.8). A diluted solution ($24 \text{ }\mu\text{l}$) of 9.6 nM MTs in motility buffer was introduced, and this was incubated for 5 min, followed by washing with $32 \text{ }\mu\text{l}$ of motility buffer. Biotins on the MT surface were partially covered with streptavidin-FITC (ST) (Wako) by incubating biotin with the ST solution (9.6 nM, which is equimolar to the MT solution, in motility buffer) for 10 min. This was followed by washing with $50 \text{ }\mu\text{l}$ of motility buffer. Hereafter, C_{ST} represents the concentration of the ST solution. Finally, the motility assay was initiated by applying $24 \text{ }\mu\text{l}$ of ATP solution (motility buffer supplemented with 5 mM ATP). Solutions that had three different C_k values were prepared simultaneously, and ATP additions were delayed for 15 min. To avoid drying, the flow cell was sealed with grease. Since biotin- and rhodamine-labeled MT contain 45% biotin-labeled tubulin, which has 2 biotins per tubulin heterodimer, there was 21.6 nM biotin on the MT surface at a C_{MT} of 24 nM. On the other hand, a streptavidin molecule has 4 biotin-binding sites. Thus, 4.8 nM of streptavidin had 19.2 nM of biotin-binding sites. This is almost equivalent to the biotins in 21.6

nM biotin- and rhodamine-labeled MTs, although the amounts of MT and ST, which were substantially bound to the surfaces in the flow cell, were not considered here.

2.4. Motility assay 2: Effect of C_{ST}

Motility assay 2 was basically carried out using the same method as that described for motility assay 1. The C_{ST} was varied as follows: 0, 0.48, 4.8, 48, 480, and 4800 nM. The C_k and C_{MT} were fixed at 63 nM and 24 nM, respectively. The diluted MTs after polymerization were incubated at 25°C for 24 h prior to the preparation of MTs in the motility buffer. The flow cells with the solutions at two different C_{ST} values were prepared simultaneously, except that ATP addition was delayed for 15 min to allow the observations to be recorded. Three sets of these paired conditions were used to examine the solutions at six different C_{ST} values.

2.5. Motility assay 3: Effect of C_{MT}

The procedure used in motility assay 1 was modified as follows. The C_k was fixed at 63 nM. The C_{MT} in the motility buffer was varied as follows: 1.9, 9.6, 48 and 240 nM. MTs were diluted 50 fold after polymerization and incubated for 16 h until the preparation of these solutions. Concomitantly, the C_{ST} was also varied to ensure that the molar ratio was equal to that of the MTs. ATP addition was delayed in the same manner as that described above.

2.6. Microscopic image capture

For the MT motility assays, rhodamine-labeled MTs were illuminated with a 100-W mercury lamp and visualized by epifluorescence microscopy using a PlanApo 60×/1.40 objective (Olympus). The images were captured using a cooled-CCD camera (Cascade II, Nippon Roper) connected to a PC. To capture a field of view for more than several minutes, ND filters were inserted into the illuminating light path of the fluorescent microscope to avoid photobleaching of rhodamine-labeled MTs. The motions of the MTs were analyzed using image analysis software (Image Pro Plus 5.1J, Media Cybernetics). Although the inner surface of the cover glass was observed through the glass, the captured images corresponded to those observed from the reverse of the flow cell.

2.7. Image analysis for density measurements

The fluorescence microscopy images of MTs and MT bundles (or MT complexes) captured 15 min and 4 h after (or before) ATP addition were analyzed using Image Pro 5.1J. “MT bundles” and “MT complexes” were the terms used for MTs observed after and before ATP addition, respectively. MT bundles (or MT complexes) were distinguished as segments that were obviously brighter than the other MTs. Small objects, whose lengths and widths were indistinguishable, were not counted. In cases where the MTs crossed each other, the lengths were measured as follows. When MTs overlapped at a point, they were measured independently. When they overlapped lengthwise in a segment, the longer MTs were measured as a single piece, and the others were measured only in the nonoverlapping sections. In the case of the ring-shaped MT bundles, the circumferential lengths of the bundles were measured. For MTs that were partially invisible at the end of the viewing field, only the visible parts were measured. The total contour lengths of MTs per field of view ($135 \times 135 \mu\text{m}^2$) were measured manually and converted to the length per unit field. The total length of the MTs including the MT bundles (or MT complex) per unit area was termed as the “total sum length of the MTs” (L_{total}), and the total lengths of the only MT bundles (or MT complex) per unit area were termed as the “total bundle length” (L_{bundle}). To calculate the individual lengths for the histogram and the average lengths, we excluded the MTs and MT bundles (or MT complexes) that were not entirely visible.

2.8. Motility analysis of microtubule bundles

The number ratios of motile single- or bundled-MTs to the whole were calculated. The MTs that showed linear sliding motion over a distance of more than $\sim 0.5 \mu\text{m}$, which corresponded to 2 pixels in digital data, were judged to be motile MTs. The frame rates of the movies were 5.1 s (for studying C_k), 3.6 s (for C_{ST}), and 3.6 s (for C_{MT}). The observation time per movie was 122 s (for studying C_k), 178 s (for C_{ST}), and 87 s (for C_{MT}).

To measure the velocity of the MTs, the track of the MTs in the 10 sequential images with 15.5-s intervals was manually traced using the software.

3. Results and Discussion

3.1. MT bundle formation: Effect of C_k

Figure 1 shows a schematic illustration of the AcSO system of MT bundles in which MTs are crosslinked by the ST-biotin interaction during sliding motion on a kinesin-coated surface, and the process is driven by ATP hydrolysis [13]. The AcSO system was initiated by ATP addition, as shown in Figure 2. First, we studied the effect of C_k on the AcSO process when the C_{ST} (9.6 nM) and C_{MT} (9.6 nM) were kept constant. A C_{MT} of 9.6 nM was used to avoid annealing of the MTs before starting the AcSO process. The amount of GFP-fused kinesins coated on the glass surface was changed by controlling the C_k of the applying solution from 0.63 to 63 nM, while the concentration of the anti-GFP antibody solution was kept constant at 0.2 mg ml⁻¹ (~1.3 μ M).

Fluorescence microscopy images of the MTs prior to ATP addition are shown in Figure 3a “before.” In this case, some of the biotins on the surface of the MTs were modified by the STs. Unexpectedly, the MT complex that was formed during the flow to prepare kinesin-bound MTs with ST modification was observed even prior to ATP addition. The lengths of the MTs attached to the surface of kinesins before starting the AcSO process were measured as the initial condition. Figure 3b “before” shows the histograms of the lengths of the MTs (*black*) and MT complexes (*shaded*). The density of the MTs was also evaluated as the total sum length of the MTs per unit field (L_{total} : mm⁻¹). At C_k values of 0.63, 6.3, and 63 nM, the L_{total} values were 6.3, 9.1, and 13.2 mm⁻¹, respectively, while the average lengths of the MTs were 6.0 ± 4.8 ($n = 177$), 6.6 ± 5.3 ($n = 236$), and 7.2 ± 5.9 μ m ($n = 286$), respectively. Thus, the densities and lengths of MTs and MT complexes increased with an increase in the C_k . The higher kinesin density enabled it to bind longer MTs against the flow during preparation.

AcSO was initiated by adding 5 mM ATP, and moving MT bundles were observed after 15 min and 4 h of running (Figure 3a, “15 min” and “4 h”). Bundle formation was confirmed by the increase in the fluorescence intensity of the MTs in the images. The lengths of the MTs (*black*) and MT bundles (*shaded*) run for 15 min and 4 h are shown in Figure 3b “15 min” and “4 h,” respectively.

In the histogram shown in Figure 3b “15 min,” some MT bundles were observed under each set of conditions, although no clear tendency was observed. An increase in the number of MT bundles (*shaded*) with an increase in the C_k was observed in the histogram of Figure 3b “4 h.” The longest MT bundle (~50 μm) was observed at a C_k of 63 nM. The total lengths (L_{bundle}) of the MT bundles (or the MT complex) before (“before”) and after (“15 min” and “4 h”) ATP addition per unit area are shown in Figure 3c. After 4 h of the AcSO process, the L_{bundle} at a C_k of 63 nM was higher than that at a C_k of 6.3 nM, while no distinct tendency was observed 15 min after ATP addition. At a C_k of 0.63 nM, the MTs did not move, as shown in Figure 3f, and AcSO did not progress.

To determine the fraction of MT bundles in this system, the total length of the MT bundles was divided by the total sum lengths, i.e., $(L_{\text{bundle}}/L_{\text{total}}) \times 100$ (shown in Figure 3d). Prior to ATP addition, less than 7% of the MTs were in the complex form, even at the highest C_k of 63 nM. After 4 h of ATP addition, the fraction of MT bundles increased to 46% and 39% at C_k values of 6.3 and 63 nM, respectively, although the fraction of MT bundles was only 3% at a C_k of 0.63 nM. This indicates that the movement of MTs is important in the formation of MT bundles. However, as shown in Figure 3e, 4 h after ATP addition, only 50% of the MTs and MT bundles were moving at a C_k of 6.3 nM, whereas 90% of these were still actively moving at a higher C_k (63 nM). In comparison to the number of short MTs at a higher kinesin density (C_k of 63 nM), a lower kinesin density (C_k of 6.3 nM) could also lead to fewer short MTs at 4 h, as shown in Figure 3b. However, the MT bundle fraction at a C_k of 6.3 nM was slightly higher than that at a C_k of 63 nM after 4 h of AcSO (Figure 3d) despite the lower value of L_{bundle} at a C_k of 6.3 nM. This could be attributed to the decrease in the L_{total} at a C_k of 6.3 nM due to the detachment of short MTs from the kinesin-coated surface. In Figure 3f, the velocities of the MTs and MT bundles were plotted against their lengths. Most MTs did not show sliding motion at a C_k of 0.63 nM. The sliding motions of MTs and MT bundles were observed at a C_k higher than 6.3 nM. However, sliding motion of MTs and MT bundles shorter than 2 μm was not observed at a C_k of 6.3 nM. This result also implies that the kinesins are separated from each

other by a distance that is longer than the length of the short MTs and MT bundles (by $\sim 2 \mu\text{m}$). If it is assumed that these short MTs and MT bundles are detached from the kinesin surface by the AcSO process, the results would be consistent with the decrease in the fraction of short MTs at a C_k of 6.3 nM at 4 h after the process was initiated (Figure 3b).

Thus, to obtain more MT bundles with motility, a high C_k is required, although MT bundles can be formed even at a low C_k , as shown in Figure 3b.

3.2. MT bundle formation: Effect of C_{ST}

Next, we studied the effect of the C_{ST} on the AcSO process when C_{MT} (24 nM) and C_k (63 nM) were kept constant. The biotin coverage ratio of the ST was changed by controlling the C_{ST} of the applied solutions in the range from 0 to 4800 nM.

Fluorescence microscopy images of ST-modified MTs are shown in Figure 4a “before.” Figure 4b “before” shows the histograms of the lengths of the MTs (*black*) and MT complexes (*shaded*). The L_{total} values were almost constant and ranged from 15 to 18 mm^{-1} . The average lengths of the MTs were also insensitive to the C_{ST} and ranged between 10 and 12 μm . In other words, the average number density of the MTs on the kinesins was approximately 2500 mm^{-2} .

After 15 min and 4 h of the AcSO process, only a few MT bundles were observed, even when the C_{ST} was 0 nM (Figure 4a, “15 min” and “4 h”). This might be attributed to the hydrophobicity arising from rhodamine modification on the MT surface. At a C_{ST} higher than 0.48 nM, the assembly of MTs of various shapes, such as linear, ring, and buckling, was observed (shown in “4 h”). The lengths of the MTs (*black*) and MT bundles (*shaded*) run for 4 h are shown in “4 h” of Figure 4b. The lengths of the MTs were obviously shorter during the sliding motions than the lengths in “before” or “15 min.” A similar phenomenon was reported earlier [22] and was explained on the basis of the “breakage” or “shrinkage” of the MTs moving on the kinesin-coated surface. The number of shortened MTs also increased with an increase in the C_{ST} ; most of the MTs and MT bundles

were less than 10 μm in length at an ST of 4800 nM. It can be assumed that MTs decorated with more STs could be easily broken by the increased nonspecific interactions and by steric hindrance with other MTs or kinesins. The histogram shown in the right side of “4 h” in Figure 4b, which is in magnified expression in the vertical axis, shows the number of MT bundles that increased with an increase in the C_{ST} . At a C_{ST} higher than 48 nM, it was difficult for MT bundles longer than 25 μm to form. Meanwhile, the number of MT bundles in the “buckling” shape increased with an increase in the C_{ST} . At a higher C_{ST} , the difficulty in forming longer MT bundles could be attributed to the shortening of the MTs. The frequent “buckling” shape could also be explained on the basis of the nonspecific interaction between STs and kinesins on the glass surface. Figure 4c shows the effect of the C_{ST} on L_{bundle} (“before,” “15 min,” and “4 h”). Without ST, L_{bundle} decreased with time, indicating that the MT complexes were broken by the driving force of the kinesins. At 4 h after ATP addition, the L_{bundle} increased with an increase in the C_{ST} and almost plateaued above 480 nM; the L_{bundle} at a C_{ST} of 0 nM was almost negligible in comparison with the plateau at 4 h. In contrast, no significant change in L_{bundle} was observed prior to ATP addition. Therefore, it can be considered that the excess amount of ST modified on the MTs via biotin locally inhibited the sliding motion of the MT bundles and led to the formation of a “buckling” shape or breakage of the MT bundles, although the L_{bundle} was constant at a C_{ST} higher than 4.8 nM.

The fractions of MT complexes and MT bundles in this system are shown in Figure 4d. Prior to ATP addition, approximately 20% of the MTs were in the complex form. After initiating the AcSO process, the fraction of MT bundles increased with an increase in C_{ST} . This tendency was more pronounced at 4 h. The fraction of MT bundles was approximately 40% at ST concentrations higher than 480 nM, whereas it was only approximately 4% at a C_{ST} of 0 nM. Thus, the higher C_{ST} resulted in both a larger L_{bundle} and a larger fraction of MT bundles. However, the longest MT bundles were observed at a C_{ST} of ~ 48 nM. This might also be coupled to the motility of the MT bundles at various C_{ST} values. Figure 4e shows the percentage of motile MTs and MT bundles.

Although the presence of ST (0.48–4.8 nM) had no influence on the motility of the MT bundles, 50% of these showed no sliding motion at a C_{ST} of 48 nM, and almost no MT bundles moved at an ST concentration higher than 480 nM. Thus, no MT bundle formation was observed at a C_{ST} of 0 nM in the movie recorded in this assay.

3.3. MT bundle formation: Effect of C_{MT}

We next investigated the effect of C_{MT} on the AcSO process at a C_k of 63 nM. Based on the effect of C_{ST} on MT bundle formation, the ratio of C_{ST} and C_{MT} (ST/MT) was maintained at 1/1 so that the MT bundles were motile. The C_{MT} was varied from 1.92 to 240 nM. An increase in C_{MT} is expected to raise the frequency of collisions between MTs during sliding motion, which may facilitate the formation of a large MT assembly.

Figure 5a “before” shows the fluorescence microscopy images of MTs bound to kinesins without ATP. Figure 5b “before” shows histograms of the lengths of the MTs (*black*) and MT complexes (*shaded*). The L_{total} values were varied from 2 to 175 mm^{-1} to change the C_{MT} from 1.92 to 240 nM. The average lengths of the MTs were also constant and ranged between 7.4 and 8.3 μm .

Figure 5a (“15 min” and “4 h”) shows fluorescence images of MTs and MT bundles after ~15 min and 4 h of the AcSO process. Various shapes such as linear, ring, and buckling were observed under all conditions at 4 h after ATP addition. The length distribution of these MTs and MT bundles is shown in the histograms in Figure 5b (“15 min” and “4 h”). Shortening of the MTs was also observed after 4 h of the AcSO process. The shortening was enhanced at a higher C_{MT} . This result supported the notion that entanglement of MTs leads to the shortening of the MTs during AcSO. In the case of the MT complexes, the numbers of them which were observed prior to ATP addition decreased during the first 4 h of the AcSO process. The average length of the MT bundles was insensitive to the C_{MT} (1.9–48 nM) and ranged between 9.2 and 11.1 μm . However, at an MT concentration of 240 nM, elongation of the MT bundles was observed, and the longest MT bundle was

~50 μm .

The effect of C_{MT} on L_{bundle} is shown in Figure 5c (“before,” “15 min,” and “4 h”). Prior to ATP addition, the L_{bundle} increased with an increase in the C_{MT} (Figure 5c, “before”). A decrease in the L_{bundle} with time was observed after the AcSO process (“15 min” and “4 h”) at C_{MT} values of 1.9 and 2.4 nM. This could be attributed to the breakage of the MT complex during the AcSO process. At C_{MT} values of 48 nM (“15 min” and “4 h”) and 240 nM (“15 min”), the L_{bundle} values were higher than those at “before,” indicating that the MT bundle was produced by AcSO. However, at a C_{MT} of 240 nM (“4 h”), the L_{bundle} was shorter than that at “before” or “15 min.” This result implies that the MT bundles were thickened due to their integration with an increased density of MT bundles.

The fractions of MT complexes and MT bundles were also investigated (Figure 5d). Prior to ATP addition, approximately 20% of the MTs were in the complex form. After the AcSO process had proceeded for 4 h, the fraction of MT bundles increased with an increase in C_{MT} ; this was also observed 15 min after ATP addition (Figure 5b, “15 min”). The MT complex formed prior to ATP addition seems to be broken because the MT bundle fraction decreased at C_{MT} values of 1.9 and 9.6 nM during the AcSO process. It should be noted that the “MT bundle fraction” includes the MT complex (“before”) and MT bundles (“15 min” and “4 h”). On the other hand, the MT bundles were surely formed through the process when the C_{MT} was as high as 240 nM. The formation of MT bundles was also confirmed by the presence of long MT bundles (as long as ~30 μm), which were not observed prior to ATP addition. Assuming that the decrease in the fraction of initial MTs was due to the consumption of MTs to thicken the MT bundles, the widths of the bundles were roughly estimated to be 1.5 and 3.1 of MTs at C_{MT} values of 48 and 240 nM, respectively; these values were obtained by dividing the L_{total} of “before” with that of “4 h.” To accurately determine the thickness of the MT bundles, transmission electron microscopy data is required.

Figure 5e shows the percentages of MTs and MT bundles exhibiting sliding motion after 4 h of the AcSO process. At a C_{MT} of 1.9 nM, no MT bundle was moving in the movie. At C_{MT} values

of 9.6 and 48 nM, 83% of the MT bundles were moving. At a C_{MT} of 240 nM, only 17% of the MT bundles showed sliding motion. Moreover, 24% showed fishtailing motion with a “buckling” shape, whereas approximately 59% had not moved.

3.4. What are the best conditions?

To discuss the effects of C_{ST} and C_{MT} on the AcSO of MTs, the motile MT-bundle length per unit area ($L_{m-bundle}$) at a C_k of 64 nM was plotted against various C_{ST}/C_{MT} ratios at a constant C_{MT} (24 nM) and against various C_{MT} values at a constant C_{ST}/C_{MT} ratio (mol/mol) in a 3D diagram (Figure 6). In this case, C_{ST} was normalized by C_{MT} as C_{ST}/C_{MT} (mol/mol), and the motile MT-bundle length per unit area ($L_{m-bundle}$) was obtained by multiplying the percentage of moving MT bundles (Figure 4e and 5e) by L_{bundle} (Figure 4c and 5c) at 4 h after ATP addition. As shown in the diagram, it was confirmed that an optimum C_{ST}/C_{MT} and C_{MT} condition existed at around 1 mol/mol (the labeling stoichiometry of biotins to MTs was 0.9) and 48 nM, and this facilitated the formation of large motile MT bundles. In this study, C_{ST}/C_{MT} was evaluated using the concentrations of the solutions applied in the flow cell and disregarding the adsorption efficiency of biotin to the MT. If C_{ST}/C_{MT} is equal to the actual biotin coverage ratio of ST, the optimum C_{ST}/C_{MT} is expected to be ~0.5 prior to the AcSO process as reported earlier [13]. This is because STs will hinder the crosslinking reaction at a C_{ST}/C_{MT} higher than 0.5, and the crosslinking points will monotonously decrease at a C_{ST}/C_{MT} value less than 0.5. Thus, the biotin coverage ratio of the ST under this experimental condition was estimated to be >50%. At C_{MT} values less than 48 nM, the lower collision probability between MTs might decrease the production of MT bundles. Meanwhile, as the MT concentration increases, the probability of simultaneously encountering multiple filaments would also increase, which might facilitate the formation of the MT assembly in an antiparallel alignment that is not motile.

4. Conclusions

In this study, we reported that during the AcSO of MTs, a higher C_k was favorable for

producing motile MT bundles in the C_k range of 0–63 nM. Moreover, at the highest C_k (63 nM), there was an optimum C_{ST}/C_{MT} of approximately 1 mol/mol at which the AcSO process occurred effectively. We also found that a higher C_{MT} effectively produced a longer MT bundle, which was confirmed by the MT bundle fraction. However, there was an optimum C_{MT} of approximately 48 nM that produced MT bundles with the ability to move.

This study on MT bundle formation may be useful for designing novel micromachine systems with the “kinesin and microtubule” motor proteins. The concept of molecular machines has to be extended for such a development to occur [23]. Furthermore, combination with a technique to orient MTs in a 3D structure [24] may increase the feasibility of developing ATP-fueled biomachines of much larger dimensions.

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References

- [1] Limberis L and Stewart R J 2000 *Nanotechnology* **11** 47–51.
- [2] Hess H and Vogel V 2001 *Rev. Mol. Biotech.* **82** 67–85.
- [3] Böhm K J, Stracke R, Mühlig P and Unger E 2001 *Nanotechnology* **12** 238–244.
- [4] Yokokawa R, Yoshida Y, Takeuchi S, Kon T and Fujita H 2006 *Nanotechnology* **17** 289–294.
- [5] Diehl M R, Zhang K, Lee H J and Tirrell D A 2006 *Science* **311** 1468–1471.
- [6] Kim T, Kao M-T, Meyhöfer E and Hasselbrink E F 2007 *Nanotechnology* **18** 025101(9pp).
- [7] Suzuki H, Oiwa K, Yamada A, Sakakibara H, Nakayama H and Mashiko S 1995 *Jpn. J. Appl. Phys.* **34** 3937–3941.
- [8] Kakugo A, Sugimoto S, Gong J P and Osada Y 2002 *Adv. Mater.* **14** 1124–1126.
- [9] Kakugo A, Shikinaka K, Matsumoto K, Gong J P and Osada Y 2003 *Bioconjugate Chem.* **14** 1185–1190.
- [10] Kwon H J, Kakugo A, Shikinaka K, Osada Y and Gong J P 2005 *Biomacromol.* **6** 3005–3009.
- [11] Kwon H J, Tanaka Y, Kakugo A, Shikinaka K, Furukawa H, Osada Y and Gong J P 2006 *Biochemistry* **45** 10313–10318.
- [12] Shikinaka K, Kwon H J, Kakugo A, Furukawa H, Osada Y, Gong J P, Aoyama Y, Nishioka H, Jinnai H and Okajima T 2008 *Biomacromol.* **9** 537–542.
- [13] Hess H, Clemmens J, Brunner C, Doot R, Luna S, Ernst K-H and Vogel V 2005 *Nano Lett.* **5** 629–633.
- [14] Kawamura R, Kakugo A, Shikinaka K, Osada Y and Gong J P 2008 *Biomacromol.* **9** 2277–2282.
- [15] Kawamura R, Kakugo A, Osada Y and Gong J P 2010 *Langmuir* **26** 533–537.
- [16] Vale R D, Coppin C M, Malik F, Kull F J and Milligan R A 1994 *J. Biol. Chem.* **269** 23769–23775.
- [17] Castoldi M and Popov A V 2003 *Protein Expr. Purif.* **32** 83–88.
- [18] Case R B, Pierce D W, Hom-Booher N, Hart C L and Vale R D 1997 *Cell* **90** 959–966.
- [19] Hyman A, Drechsel D, Kellogg D, Salser S, Sawin K, Steffen P, Wordeman L and Mitchison T 1991 *Methods. Enzymol.* **196** 478–485.
- [20] Green N M 1970 *Methods. Enzymol.* **18** 418–424.
- [21] Peloquin J, Komarova Y and Borisy G 2005 *Nat. Methods* **2** 299–303.

- [22] Brunner C, Ernst K-H, Hess H and Vogel V 2004 *Nanotechnology* **15** S540–S548.
- [23] Hess H, Bachand G D and Vogel V 2004 *Chem. Eur. J.* **10** 2110–2116.
- [24] Kakugo K, Tamura Y, Shikinaka K, Yoshida M, Kawamura R, Furukawa H, Osada Y and Gong J P 2009 *J. Am. Chem. Soc.* **131** 18089–18095.

Figures and Captions

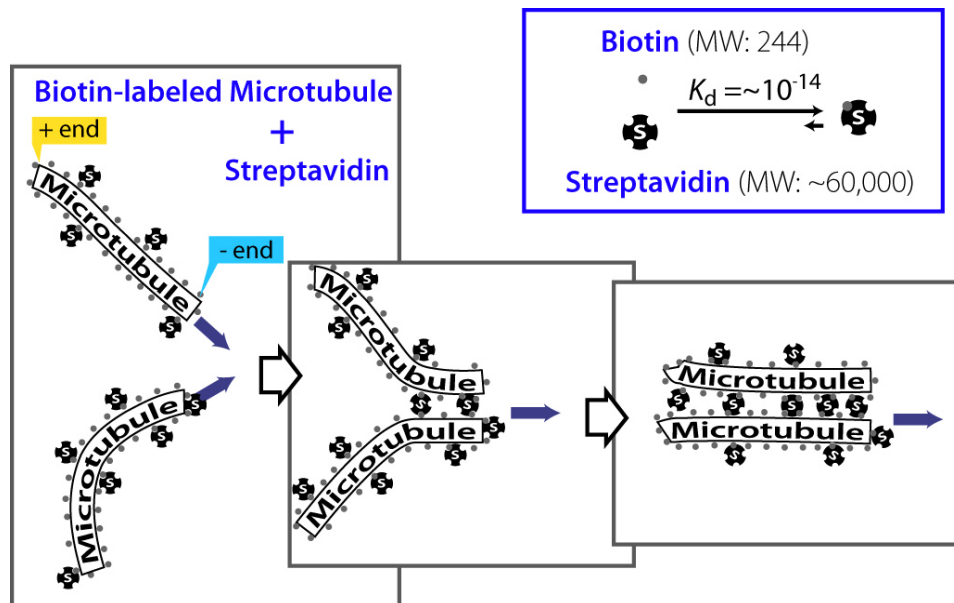


Figure 1: Schematic illustration of the active self-organization of MTs.

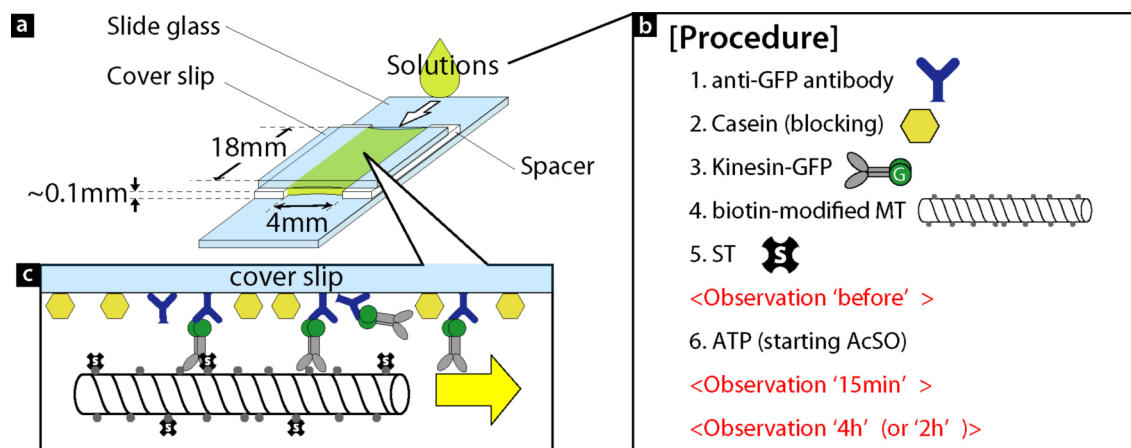


Figure 2: Schematic illustration of the procedures involved in the active self-organization of MTs. (a) Dimensions of the flow cell. (b) Solutions introduced in the flow cell. The numbers represent the order in which each component solution was introduced into the flow cell; the unbound components at the surface of the flow cell were washed out before introducing the next solution or recording the observations. (c) Illustration of the MTs driven on kinesins coated on the glass surface.

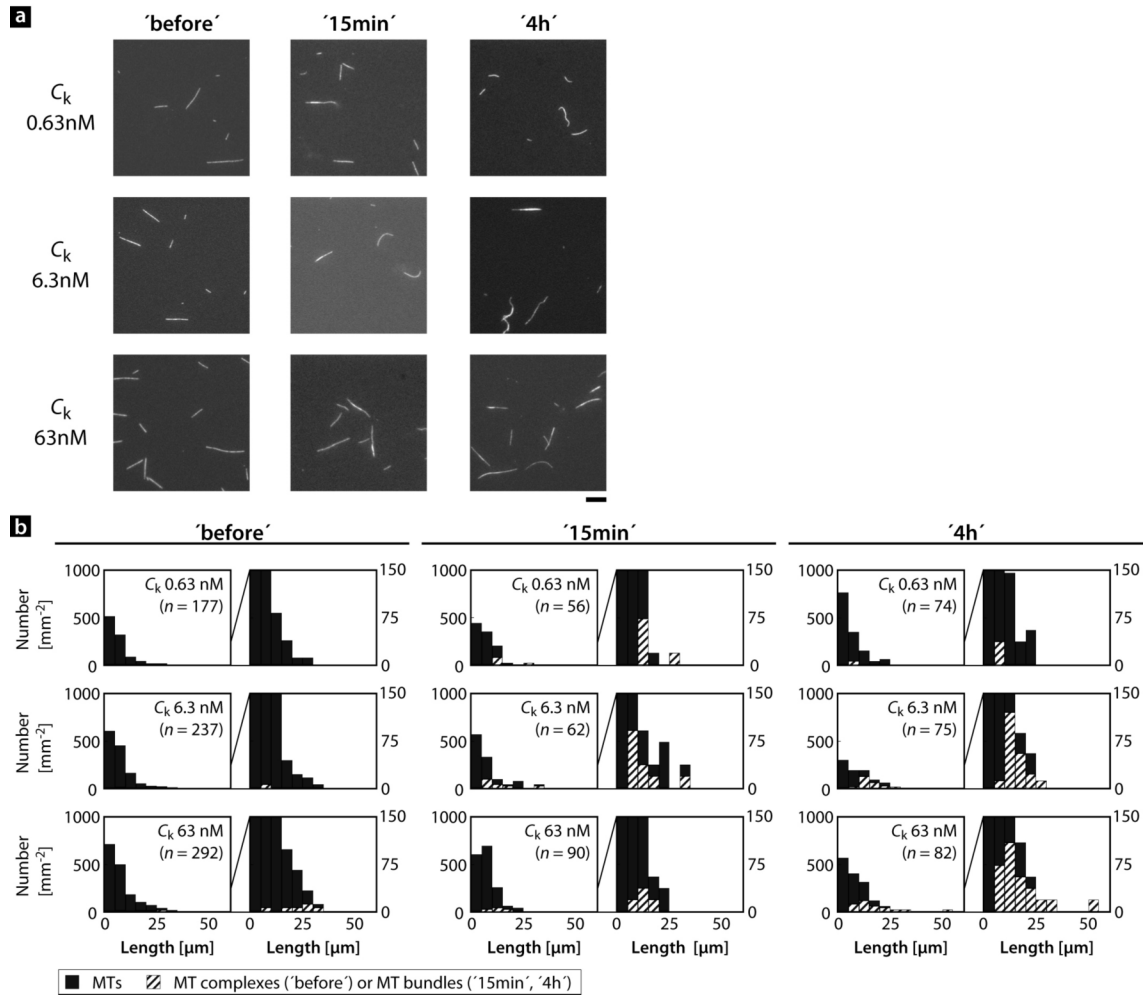


Figure 3a-e: Effect of the kinesin concentration (C_k) on bundle formation. (a) Fluorescence images of single MTs and MT complexes ("before") or MT bundles ("15 min" and "4 h"). Scale bar, 10 μm . (b) MT length histograms representing numbers per unit area. The sample number is represented by n .

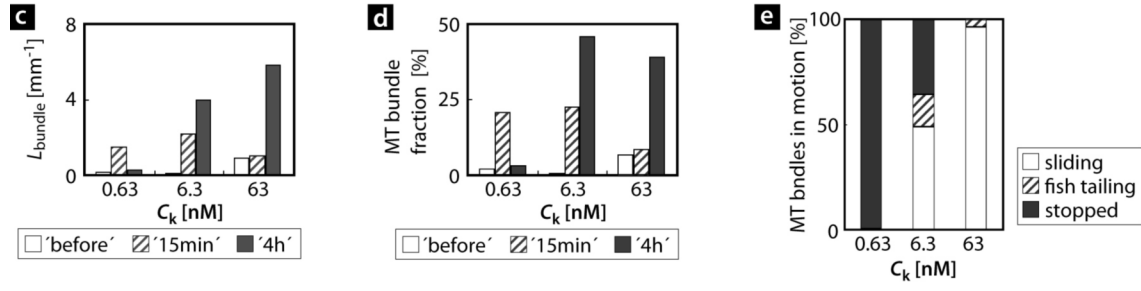


Figure 3c-e: (c) Total bundle lengths per unit area (L_{bundle}). (d) MT bundle fractions, defined as the percentage of the total bundle length (L_{bundle}) to the total sum length of single MTs including MT complexes, and MT bundles (L_{total}). (e) Percentage of MT bundles in motion calculated by the number ratio of MT bundles in motion to all MT bundles.

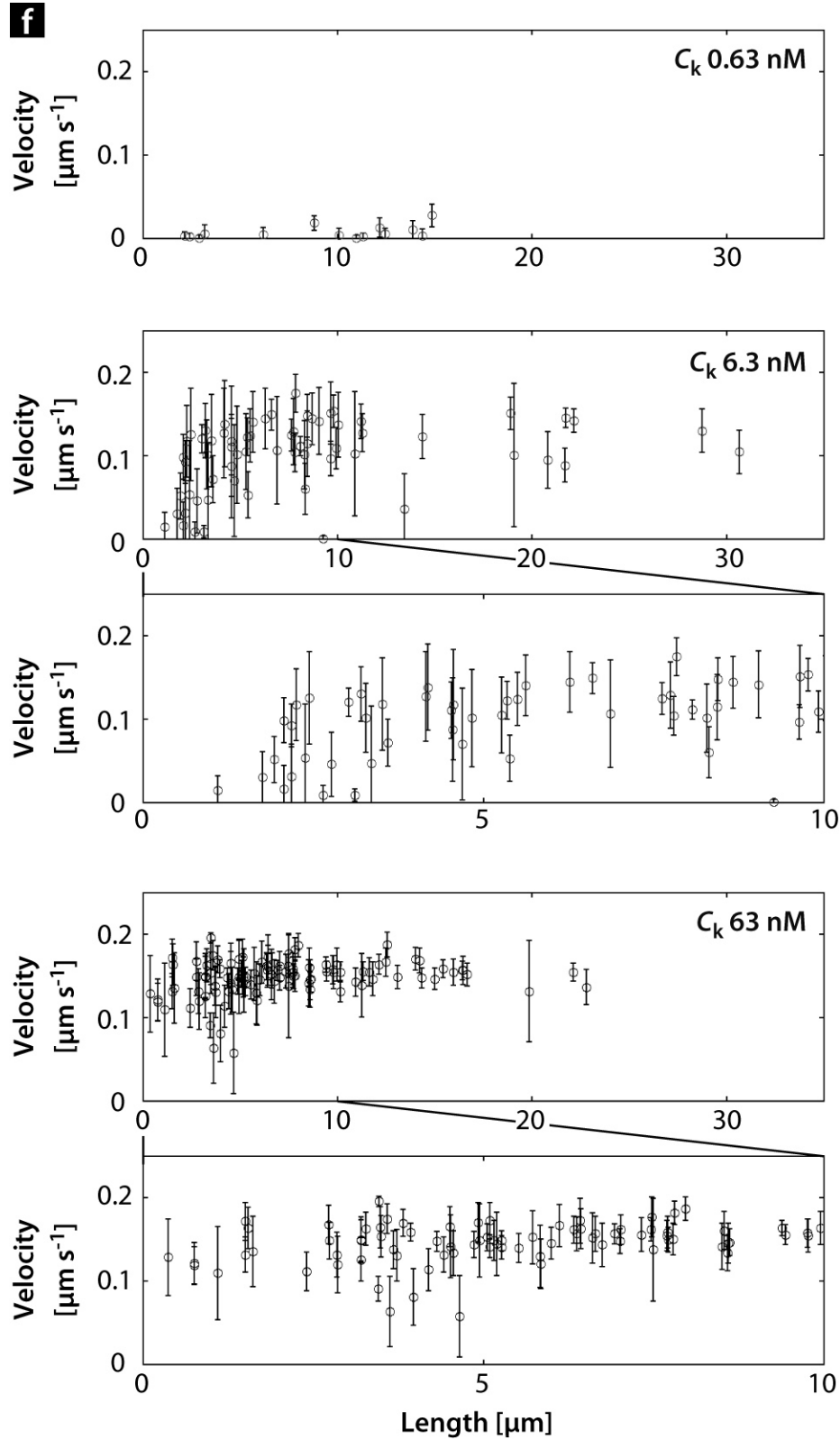


Figure 3f: Dependence of the sliding velocity of MT on the filament length at various C_k values.

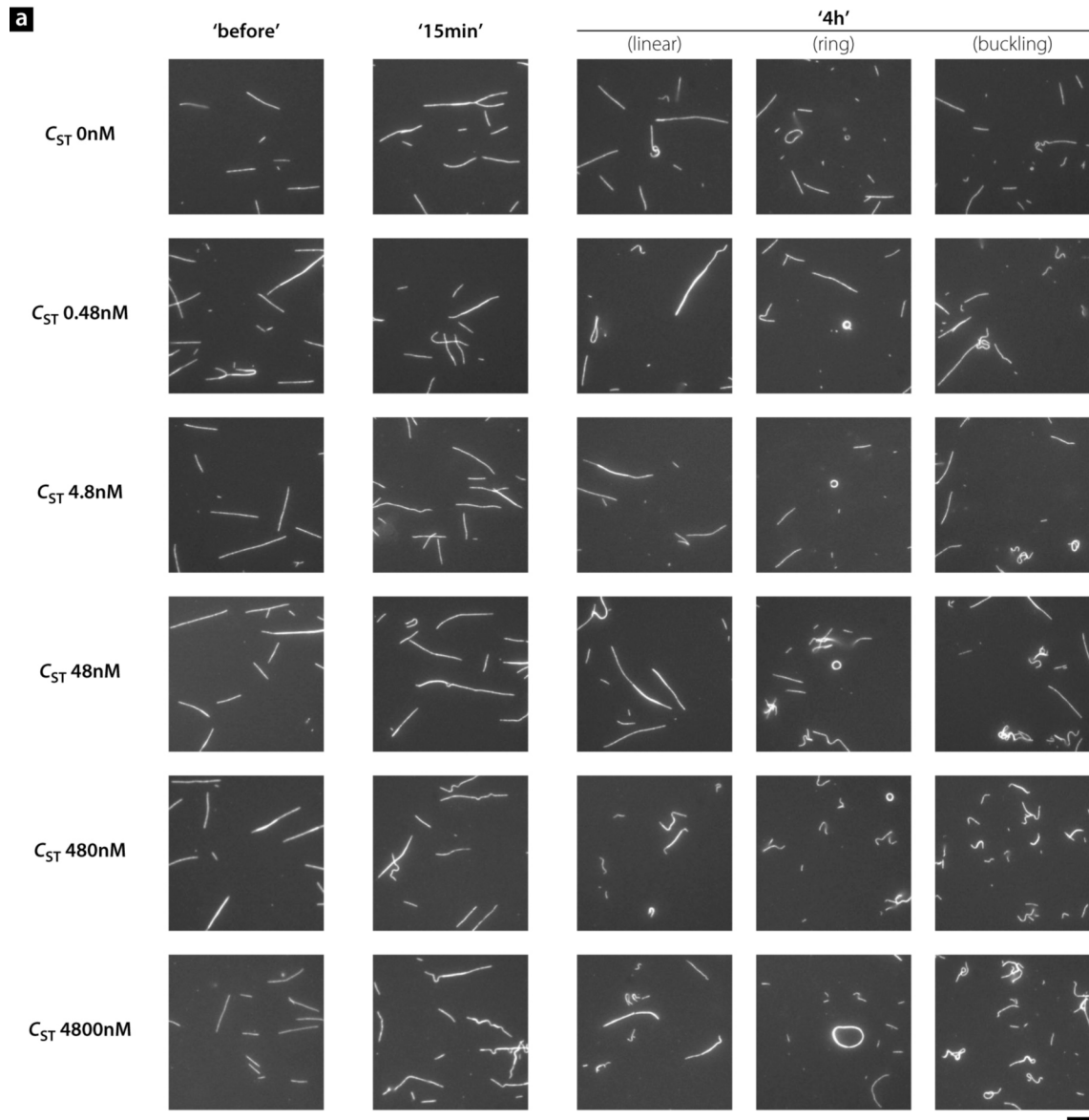


Figure 4a: Effect of the C_{ST} on bundle formation. (a) Fluorescence images of MTs and MT complexes ("before") or MT bundles ("15 min" and "4 h"). Scale bar, 10 μm .

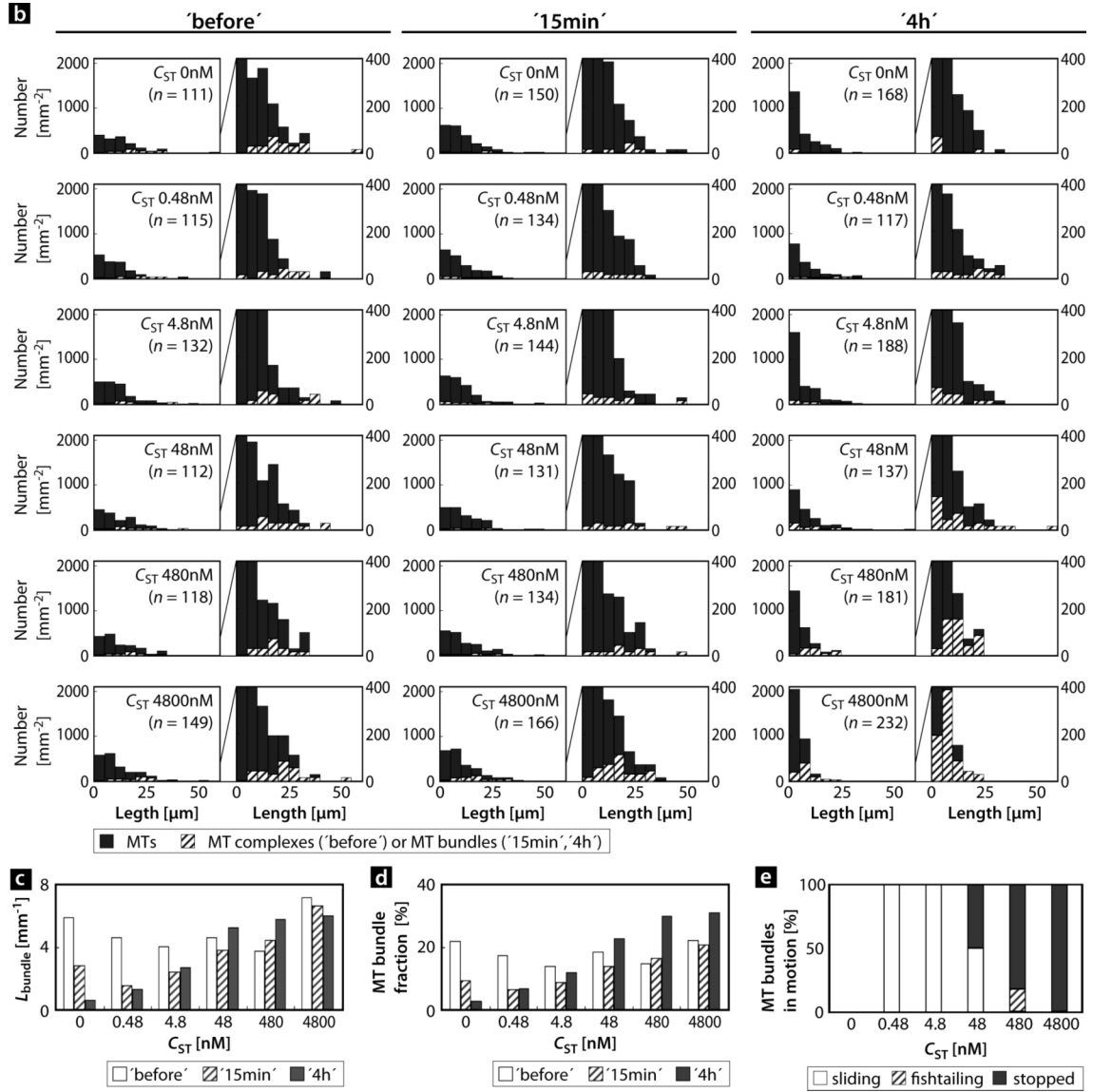


Figure 4b-e: Effect of the C_{ST} on bundle formation. (b) MT length histograms representing numbers per unit area. The C_{ST} and number of counted single MTs, MT complexes, and MT bundles are shown in the graphs. (c) Total bundle lengths per unit area (L_{bundle}). (d) MT bundle fractions that are defined as the percentage of L_{bundle} to L_{total} . (e) Percentage of MT bundles in motion, calculated as the number ratio of MT bundles in motion to all MT bundles.

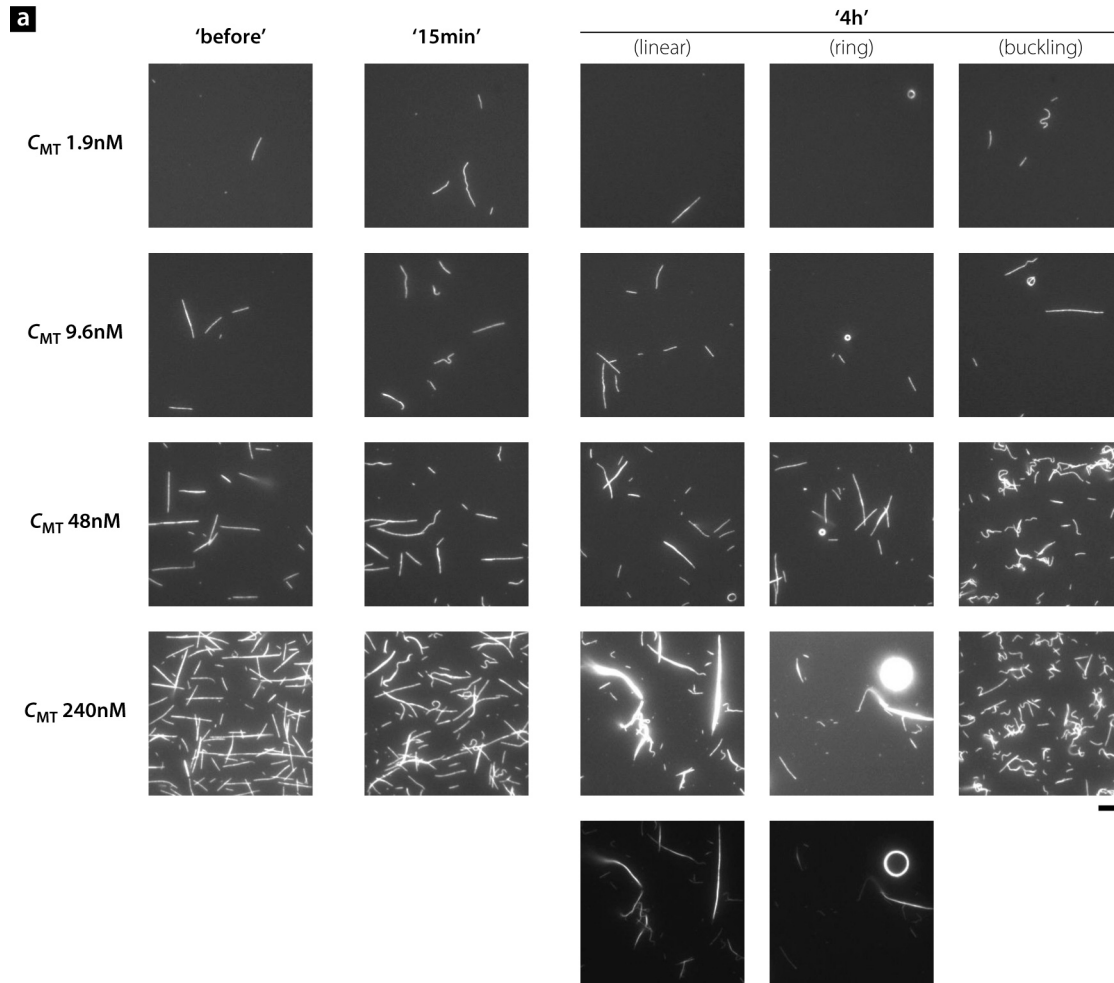


Figure 5a: Effect of the C_{MT} on bundle formation. (a) Fluorescence images of MTs and MT complexes ("before") or MT bundles ("15 min" and "4 h"). Scale bar, 10 μm .

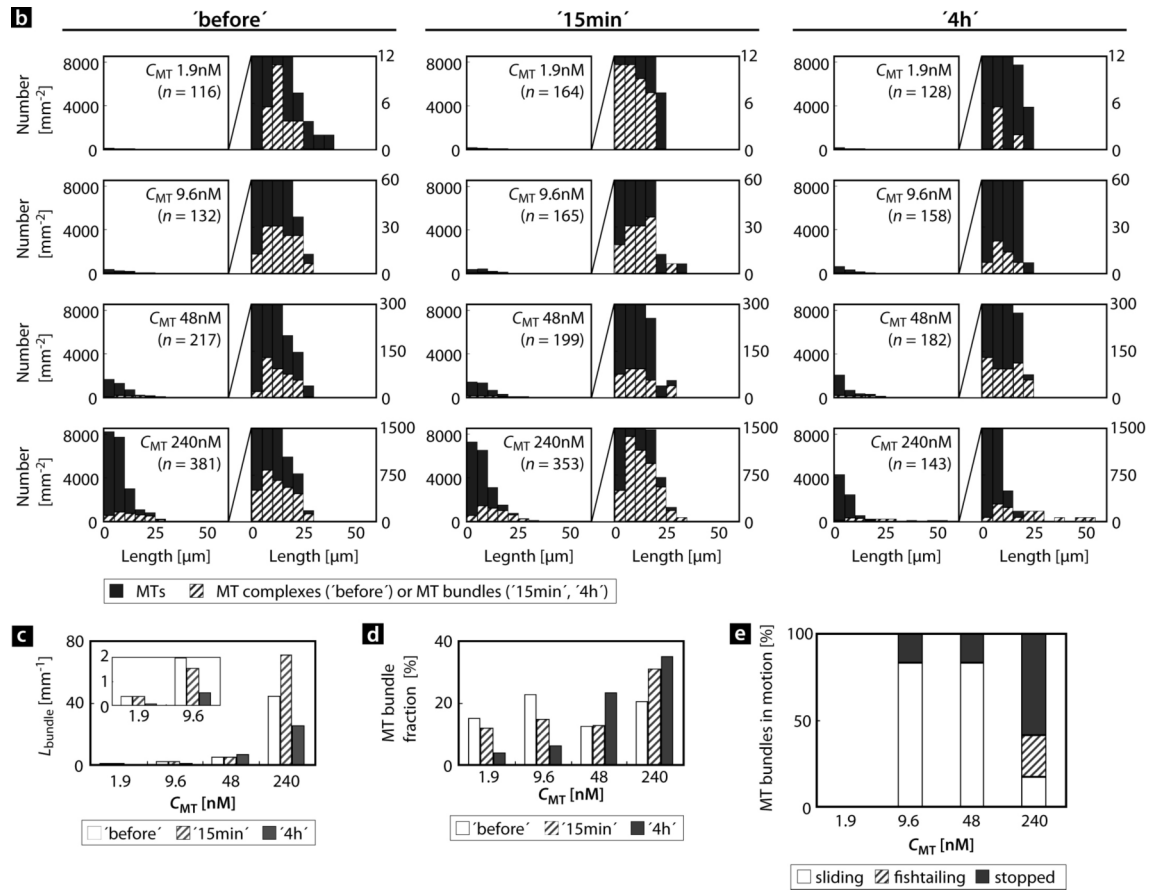


Figure 5b-e: Effect of the C_{MT} on bundle formation. (b) MT length histograms represented as numbers per unit area. (c) Total bundle lengths per unit area (L_{bundle}). (d) MT bundle fractions that are defined as the percentages of L_{bundle} to L_{total} . (e) Percentage of MT bundles in motion calculated as the number ratio of MT bundles in motion to all MT bundles.

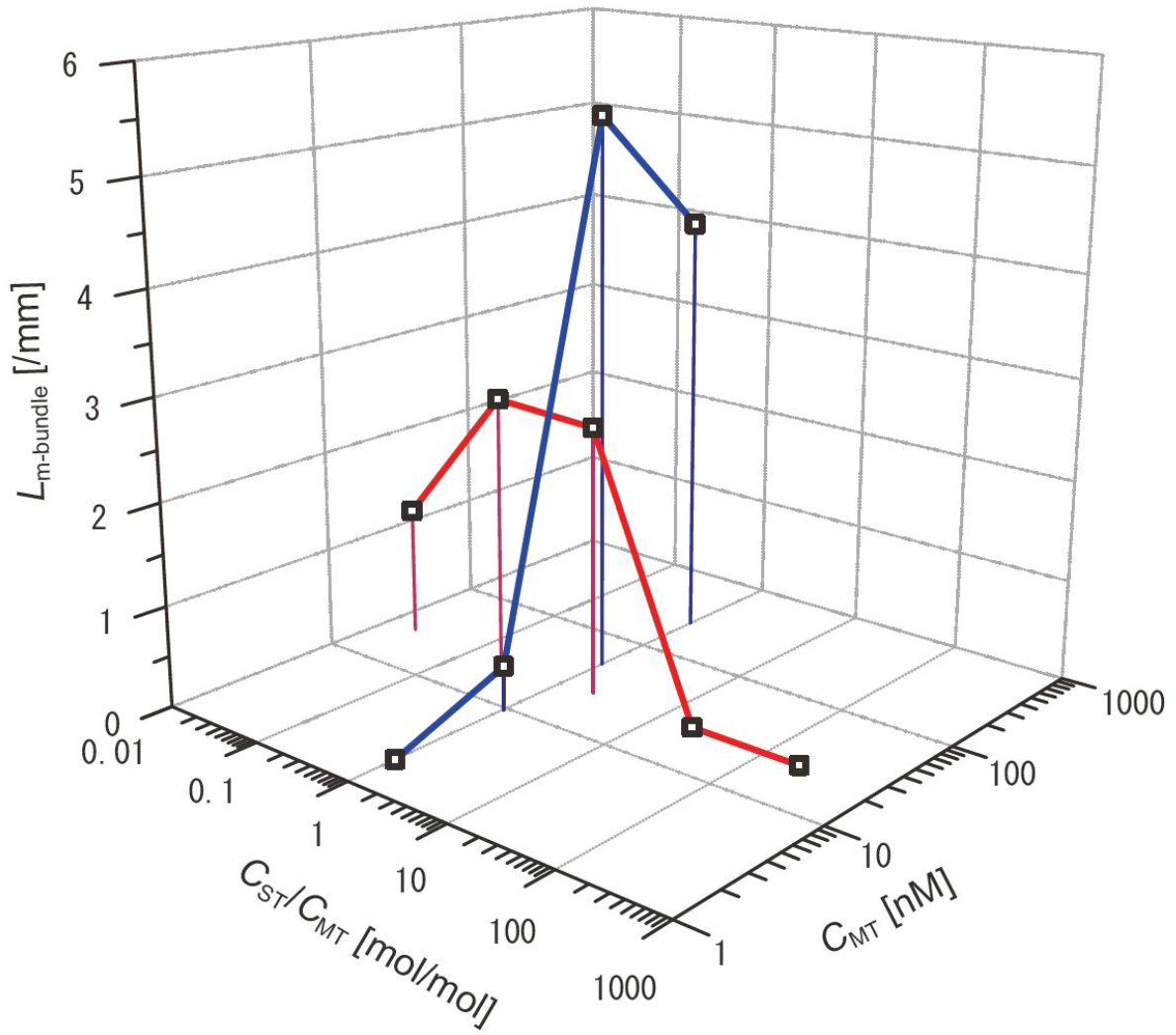


Figure 6: Estimation of the motile MT-bundle length per unit area at various C_{MT} and C_{ST}/C_{MT} ratios. Motile MT-bundle length per unit area ($L_{m-bundle}$) versus C_{MT} was calculated by multiplying L_{bundle} (Figure 5c) and the percentage of moving MT bundles (Figure 5e) (blue line). The red line represents $L_{m-bundle}$ versus the C_{ST}/C_{MT} ratio; in this case, C_{ST} was normalized by C_{MT} for comparison.