Effect of Microtubule Immobilization by Glutaraldehyde on Kinesin-Driven Cargo Transport

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ABSTRACT

The glutaraldehyde fixation method for fixing tissues is attractive for its ease of use and straightforward surface chemistry. We investigated the effect of glutaraldehyde-induced microtubule immobilization on kinesin-driven cargo transport along microtubules and found that at low glutaraldehyde concentrations, the microtubule-kinesin interaction remains unperturbed. Such findings may facilitate the application of the glutaraldehyde fixation method for many *in vitro* studies aiming to build nanodevices powered by the microtubule-motor protein interaction.

KEYWORDS: Microtubule, Glutaraldehyde, Kinesin, Cargo transport, *In vitro*

INTRODUCTION

Glutaraldehyde (GA) is a bifunctional aldehyde with aldehyde groups at either end of the molecule. It has the potential to react with the side chains of proteins to form reactive hydroxymethyl groups.[1] Therefore, GA has long been used as a fixative for tissue.[2] This fixation process is straightforward and easy to use and has high reliability in protein immobilization.[3] The treatment of fragile protein crystals by GA can render them mechanically stable while retaining approximately 30-70% of their original enzyme activity.[4] Therefore, GA-induced protein fixation is widely used in optical trapping assays.[5] Construction of adenosine triphosphate (ATP)-fueled soft gel machines is promoted by the GA-induced crosslinking of cytoskeletal filaments, actin, and its associated motor protein, myosin.[6,7] Biological motors can directly convert the chemical energy of ATP hydrolysis into mechanical energy with high efficiency.[8] The most rigid cytoskeletal component, microtubule, together with its associated motor proteins, kinesin and dynein, is also being exploited in creating devices powered by motor proteins.[9–11] Recently, the microtubule/kinesin system has been developed for applications in molecular robotics.[12,13] For a variety of experiments based on microscopic observations, microtubule fixation to the substrate is a prerequisite.

Moreover, the recent aim of microtubule stabilization for their application in nanodevices[14,15] has also been accomplished by light crosslinking with GA.[16] While GA-induced crosslinking to stabilize microtubules and its effect on kinesin transport have been reported,[16] how microtubule immobilization on a substrate using GA may affect kinesin transport remains elusive. Therefore, in the present work, we explore the application of GA to immobilize microtubules over a substrate and investigate its effect on the interaction between microtubules and kinesins *in vitro*. The effect of GA-induced immobilization on the microtubule structure and thereby on the microtubule-kinesin interaction was explored by observing cargo (quantum dot) transport by multiple kinesins along immobilized microtubules in the presence of different concentrations of GA. We find that the microtubule-kinesin interaction is preserved up to a critical concentration of GA applied, while at concentrations higher than that level, the cargo transport phenomenon is hindered. Such findings are essential in applications where surface

immobilization of microtubules without perturbation of their inherent properties is required.

EXPERIMENTAL

Materials and methods

Chemicals

All chemicals were purchased from Sigma unless mentioned otherwise; 25% glutaraldehyde was purchased from Fujifilm Wako Pure Chemical Corporation (Product code 079-00533).

Purification, labeling, and polymerization of tubulin

Tubulin was purified from fresh porcine brain using a high-concentration PIPES buffer (1 M PIPES, 20 mM EGTA, and 10 mM $MgCl_2$; pH adjusted to 6.8 using KOH).[17] We obtained ATTO 550 fluorescent dye-labeled tubulin following a standard protocol.[18] Microtubules were polymerized from 56 μM tubulin (20% ATTO 550-labeled tubulin and 80% nonlabeled tubulin) in BRB80 (80 mM K-PIPES, 1 mM $MgCl_2$, and 1 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA); pH 6.8) in the presence of 5 mM guanosine 5’-triphosphate (GTP), 20 mM $MgCl_2$, and 25% dimethyl sulfoxide at 37 °C for 30 minutes.

Expression and purification of kinesin

The recombinant conventional kinesin-1 construct consisting of human kinesin (residues 1-465), an *N*-terminal histidine tag, and a *C*-terminal Avi-tag was purified as described in previously published reports, with partial modification.[19]

Preparation of kinesin-quantum dot conjugates

To investigate kinesin-driven cargo transport, we used streptavidin-conjugated Qdot™ 525 (Q10143MP, Invitrogen, USA) as cargo. The Qdots were mixed with kinesins at a molar concentration ratio of 1:10 and incubated in ice for 15 minutes. The solution of the conjugates was diluted just before applying it to the flow cell by using 1 mM ATP solution

with oxygen scavengers (1 mM ATP, 3 mg/mL casein, 20 mM DTT, 40 µM paclitaxel and scavengers: glucose, glucose oxidase, and catalase in BRB80 comprising ~80 mM PIPES, 1 mM EGTA, and 1 mM $MgCl_2$; pH 6.8) to obtain final Qdot and kinesin concentrations equal to 5 and 50 nM, respectively.

Qdot transport assay by kinesin along microtubules immobilized by glutaraldehyde

A Qdot transport assay by kinesins along microtubules was performed *in vitro*. A flow cell was prepared by making a narrow channel using double-sided tape on a 28 mm×50 mm glass slide covered with an 18 mm×18 mm coverslip. The double-sided tape served as a spacer. The narrow channel was exposed to air plasma for 4 minutes (10 Pa, 8 mA) in a plasma etcher (SEDE-GE; Meiwafosis Co. Ltd.). At room temperature, 0.5 mg/mL protein-A (P6031, Sigma-Aldrich, USA), followed by a 73 mg/mL anti-tubulin antibody solution (T3526, Sigma-Aldrich, USA), was applied to the flow cell. The flow cell was washed with solution A (~80 mM PIPES, 1 mM EGTA, 1 mM $MgCl_2$, 0.5 mg/mL casein, 1 mM DTT, and 10 mM paclitaxel; pH 6.8). Next, paclitaxel-stabilized, rhodamine-labeled microtubule solution was introduced and incubated for 5 minutes, followed by washing with solution A. Microtubules were chemically immobilized by exposure to 0.00% to 0.50% (v/v) GA solutions in BRB80 for 4 minutes. The glutaraldehyde fixing reaction was quenched by 0.1 M glycine with 5 minutes of incubation. The preincubated solution of kinesin-Qdot conjugates in the presence of 1 mM ATP was introduced. The flow cell was observed immediately after the addition of ATP. All experiments were performed at 25 °C.

Fluorescence microscope observation

Samples were illuminated with a 100 W mercury lamp and visualized by a two-color epifluorescence microscope (Eclipse Ti; Nikon) equipped with an oil-coupled Plan Apo 60×1.40 objective (Nikon). Filter blocks with UV-cut specifications (TRITC: EX540/25, DM565, BA606/55; Q525: EX435/40, DM510, 525/15; Nikon) were used in the optical path of the microscope to eliminate the UV part of radiation and minimize its harmful effect on the samples. Images were captured using a cooled complementary metal-oxide semiconductor (CMOS) camera (Neo CMOS; Andor) connected to a PC. Observation of

the Qdot assay was performed by capturing images at a rate of 1 frame/3 s with an exposure time of 300 ms.

Data analysis

The fluorescence images were analyzed by NIS-Elements BR software (Nikon) and Fiji 1.52J software (National Institutes of Health, USA). Velocities of the kinesin-Qdot conjugates were determined using the ImageJ plugin MTrackJ. Statistical analyses were performed with Prism 8 (GraphPad).

RESULTS AND DISCUSSION

To investigate the effect of GA on immobilized microtubules and their interaction with kinesins *in vitro*, we observed the transport of Qdots conjugated to multiple kinesins along immobilized microtubules on glass. For this experiment, we fixed the microtubules to a glass substrate through their interaction with the anti-tubulin antibody. First, the substrate was coated with protein A, followed by the anti-tubulin antibody solution and subsequent washing. The paclitaxel-stabilized microtubules were fixed to the substrate through interaction with the anti-tubulin antibody. To investigate the effect of GA, we applied different concentrations of GA solutions (0.05%, 0.1%, 0.2% and 0.5% v/v) to the flow cell to immobilize the microtubules on the substrate. Next, as a blocking agent, 0.1 M glycine solution was flowed into the flow cell to remove free GA molecules. Then, the solution of the preincubated Qdot-kinesin conjugates in the presence of 1 mM ATP was introduced, and kinesin-driven Qdot transport was monitored along the immobilized microtubules (*see materials and methods*).

Fig. 1 shows a schematic illustration of how the immobilization of microtubules was carried out on a glass substrate induced by GA. For comparison, we carried out a control experiment in which no GA was applied and monitored kinesin-driven Qdot transport along microtubules immobilized only using protein A and anti-tubulin interactions. We compared the multiple kinesin-driven Qdot transport along the microtubules fixed by different concentrations of GA with that of the control experiment. The time-lapse images of the transport of Qdots conjugated to multiple kinesins along microtubules immobilized in the absence and in the presence of GA (0.10% v/v), respectively, are shown in Fig. 2A

and 2B. The presence of curved microtubules in Fig. 2A may indicate that the use of only protein A-anti-tubulin could not efficiently immobilize the microtubules. The moving Qdots were tracked to determine any alteration in the kinesin-driven transport behavior caused by the GA-induced immobilization of microtubules. Fig. 2C shows the time-displacement profiles of the representative events of kinesin-driven Qdot transport along microtubules that are immobilized on the glass substrate in the presence of different concentrations of GA. The time-displacement profiles of kinesin-driven Qdots in the absence and presence of 0.10% GA nearly overlap, indicating the minimal effect of GA on the microtubule-kinesin interaction. It is worth mentioning that the microtubules immobilized using no or low GA concentrations (< 0.10% GA) showed the presence of fully immobilized and partially immobilized microtubules. An example is shown in Fig. 2D. The alteration in the microtubule shape with time during kinesin-driven transport along the microtubule suggests that the interaction between microtubules and anti-tubulin was weaker than the force generated by multiple kinesin motility. Evidence of microtubule crosslinking by GA at low concentrations can be obtained from the count of the number of fully immobilized and partly immobilized microtubules in the area of 1500×1500 pixels in the fluorescence images. The results are given in Table 1.

Table 1. Calculation of % microtubules fully immobilized in an area of 1500×1500 pixels induced by different GA concentrations.

% (v/v) GA	No. of microtubules		% fixed microtubules
	Fully immobilized	Partly immobilized	
0.00	1	34	3
0.05	29	7	81
0.10	28	0	100

We tracked the movement of the Qdots transported by multiple kinesins to determine the instantaneous velocities of each of the moving Qdots. The mean of the instantaneous velocities gave the velocity of each Qdot. Fig. 3 presents the distributions of the velocities of Qdots transported by multiple kinesins along the microtubules in the presence of varying GA concentrations. The mean velocity of kinesin-Qdot conjugates along microtubules in the control experiment was determined to be 210±90 nms^{-1} (mean ± standard deviation, n=15). The intrinsic velocity of the reconstructed kinesin used in our

work was reported to be ~250 nms^{-1},[19–21] which is similar to what we observed in our control experiment. As shown in Fig. 3, the velocities of Qdot transport along the microtubules immobilized in the presence of 0.05% and 0.10% (v/v) GA were 200±50 nms^{-1} (n =15) and 220±50 nms^{-1} (n = 15), respectively. However, a further increase in the GA concentration caused a decrease in the velocities of the moving Qdots (130±30 nms^{-1} (n = 15) at 0.20% and 80±20 nms^{-1} (n = 15) at 0.50% (v/v) GA).

For a Qdot driven by multiple kinesins along the microtubules, the average distance traveled before it is detached from the microtubule surface is defined as the run length. Fig. 4 shows the distributions of the run lengths of Qdots transported by multiple kinesins along the microtubules immobilized in the presence of varying GA concentrations. The mean velocities and the mean run lengths as functions of the applied GA concentrations are summarized in Fig. 5A and B. Statistical tests were carried out to determine the significant differences in the velocities in comparison to the control experiment (Fig. 5A). We find that the Qdot velocities along microtubules in the presence of 0.05% and 0.10% (v/v) GA were not significantly different from the control experiment (ordinary one-way ANOVA followed by Dunnett's multiple comparison test, $p < 0.001$). However, the higher concentration of the applied GA significantly lowered the transport velocities.

Similarly, ordinary one-way ANOVA followed by Dunnett's multiple comparison test was carried out to determine the significance in the difference in run lengths in comparison to the control experiment (in the absence of the GA). While the run lengths of the moving Qdots in the presence of up to 0.10% GA did not change statistically, a higher GA concentration caused longer run lengths. This observation suggests that kinesin-driven Qdot transport along microtubules immobilized in the presence of GA was uninterrupted within the GA concentration limit of 0.10%. However, at higher GA concentrations, the motility of the moving Qdots was retarded. We speculate that in the presence of a high GA content, the subsequent addition of 0.1 M glycine used in our experiment may have not effectively quenched the free GA molecules. The existing free GA may give rise to high intramolecular crosslinking of the microtubule surface.[22] Thus, Qdot transport by kinesins along the microtubules was hindered. Nevertheless, it is still possible for GA molecules to cause intramolecular crosslinking in microtubules at low

concentrations. However, the unaltered velocities or run lengths of the kinesin-driven Qdots at such low GA concentrations suggest that such a microtubule-GA interaction is not able to affect the microtubule-kinesin interaction for the transport of Qdots. The free GA content at high GA concentrations may be expected to enhance the nonspecific binding of kinesins on microtubules, thus slowing the rate-limiting step of the ATP hydrolysis process. Therefore, the velocity of the Qdots driven by kinesins is slowed. As an effect of the assumed nonspecific binding, kinesins may bind with longer association time and traverse a longer run length along the microtubules.

CONCLUSION

In conclusion, we have provided a systematic investigation of the effect of a widely used crosslinking agent, GA, on microtubules. We used GA not to stabilize microtubules against an adverse environment[16] but to immobilize the protein on a solid substrate. In the absence of GA, the interaction between microtubules and anti-tubulin was found to be too weak to withstand the force generated by kinesin-driven Qdot transport. GA-induced crosslinking could facilitate microtubule immobilization. We found a critical concentration of GA up to which the native microtubule condition was preserved. However, at a higher GA content, the detrimental effect on the microtubules caused an increase in run lengths and a decrease in the velocities of the moving kinesin-Qdot conjugates. These findings will find application in establishing a damage-free method of fixing microtubules on a substrate for extensive use in various microscopic observation systems.

ACKNOWLEDGMENTS

This work was financially supported by the Future AI and Robot Technology Research and Development Project from New Energy and Industrial Technology Development Organization (NEDO), Japan, Grant-in-Aid for Scientific Research on Innovative Areas “Molecular Engine” (JP18H05423) and Grant-in-Aid for Scientific Research (A) (JP18H03673) to A.K. from the Japan Society for the Promotion of Science (JSPS).

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Figure legends

Fig. 1. A schematic illustration showing the microtubule immobilization method using the protein A-anti-tubulin interaction in the presence of GA. The microtubule is fixed on the substrate due to its interaction with anti-tubulin. GA molecules may introduce crosslinking between the substrate to anti-tubulin and/or anti-tubulin to microtubules or between the protofilaments of a microtubule. Qdots conjugated to multiple kinesins are transported toward the plus end of the immobilized microtubule in the presence of ATP.

Fig. 2. (A) Time-lapse images of a kinesin-driven Qdot along microtubules immobilized by only protein A-anti-tubulin. The curved microtubules may indicate that the use of only protein A-anti-tubulin may not efficiently immobilize the microtubules. Scale bar: 10 μm. **(B)** Time-lapse images of a kinesin-driven Qdot along microtubules immobilized by protein A-anti-tubulin in the presence of 0.1% (v/v) glutaraldehyde. The (+) and (-) signs in the first fluorescence images indicate the plus end and the minus end of the microtubules, respectively. The positions of the Qdot in consideration are indicated using the vertical arrows. Scale bar: 10 μm. **(C)** Time-displacement profiles of the moving Qdots transported by multiple kinesins along immobilized microtubules in the absence and presence of different concentrations of GA. The representative events from each case are shown in the figure. **(D)** Time-lapse images of a kinesin-driven Qdot along microtubules immobilized by protein A-anti-tubulin (no GA was used for immobilization). The images show an event where the microtubule was not efficiently immobilized on the substrate. Kinesin-driven Qdot transport along the microtubule in the presence of ATP has caused a change in the shape of the free end of the microtubule. Scale bar: 10 μm.

Fig. 3. The number frequency distribution of the velocities of the Qdots transported by multiple kinesins along the microtubules in the presence of different concentrations of GA. The distributions were fitted to Gaussian distribution curves using GraphPad Prism 8.2.1 software. The mean velocities obtained from the fit ($mean_{fit}$), arithmetic mean ($mean_{math}$), and goodness of fit are all provided in the legends of the plots. Fifteen samples were analyzed for each case.

Fig. 4. The number frequency distribution of the run lengths of the Qdots transported by multiple kinesins along the microtubules in the presence of different concentrations of GA. The distributions were fitted to Gaussian distribution curves using GraphPad Prism 8.2.1 software. The mean velocities obtained from the fit ($mean_{fit}$), arithmetic mean ($mean_{math}$), and goodness of fit are all provided in the legends of the plots. Fifteen samples were analyzed for each case.

Fig. 5. (A) Mean velocities of the Qdots transported by multiple kinesins in the presence of different concentrations of GA. Values are obtained from the Gaussian fitting of the velocity distributions given in Fig. 3. Ordinary one-way ANOVA followed by Dunnett's multiple comparison test was carried out to determine the significance in the difference of velocities with the control experiment (in the absence of the GA). ns = nonsignificant, **p<0.01, ****p<0.0001. **(B)** Mean run lengths of the Qdots transported by multiple kinesins in the presence of different concentrations of GA. Values are obtained from the Gaussian fitting of the run length distributions given in Fig. 4. Ordinary one-way ANOVA followed by Dunnett's multiple comparison test was carried out to determine the significance in the difference of velocities with the control experiment (in the absence of

the GA). ns = nonsignificant, **p<0.01, and ****p< 0.0001. For both Fig. 5A and 5B, the number of events analyzed was 15 in all cases.