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COMMUNICATION

Controlling the kinetics of interaction between microtubules and kinesins over a wide temperature range using the deep-sea osmolyte trimethylamine N-oxide

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Trimethylamine N-oxide is found effective in regulating the interaction between microtubules and kinesins over a wide temperature range. Lifetime of motility of microtubules on kinesins at high temperatures is prolonged using trimethylamine N-oxide. Activation energy of microtubule motility is modulated by trimethylamine N-oxide and upon prolonging the lifetime of motility.

Kinesin is a biomolecular motor protein that plays the key roles in intracellular transportation in cooperation with its associated protein microtubule (MT) by consuming adenosine triphosphate (ATP).¹ Because of nanometer scale, high energy efficiency, engineering properties, etc. MTs and kinesin have appeared promising components in biomimetic engineering and have been considered as potential candidates for constructing hybrid bio-nanodevices.² Nowadays, kinesin and MTs are successfully reconstructed and employed in 'in vitro motility assay' (Figure 1A), which forms the basis for nano-transportation,³ biosensing,⁴ surface imaging,⁵ characterizing surface mechanical deformation,⁶ force measurement,⁷ single molecule manipulation,⁸ swarm robotics,⁹ creation of artificial muscle¹⁰ etc. Like other enzymes the activity of kinesins is extremely sensitive to temperature. Although in living organism kinesins function well at temperatures as high as ≥ 40 °C,¹¹ the reconstructed kinesins undergo rapid denaturation at or above ~ 30 °C,¹² or when they are employed for prolonged operation even at room temperature (~ 22 – 25 °C).¹³ Thermal denaturation of reconstructed kinesins poses a threat to the hybrid devices designed based on in vitro motility assay. Stable and prolonged operation of an in vitro motility assay of MTs with respect to temperature changes or operating temperature extreme has been a challenging task.

Living organisms are shaped through natural selection process over time to acquire amazing adaptation ability and defense systems that would help them survive against chemical, physical or environmental threats.¹⁴ Enhanced synthesis and accumulation of osmolytes is one of the noteworthy approaches adopted by the living organisms to combat inactivation or damage of proteins and enzymes by hydrostatic pressure, temperature or other perturbants in harsh environments.^{15–17} In this work, being motivated by the promises of a deep-sea osmolyte trimethylamine N-oxide (TMAO),^{15–18} we have explored its utility in protecting MT-kinesin from thermal denaturation. From the results of motility

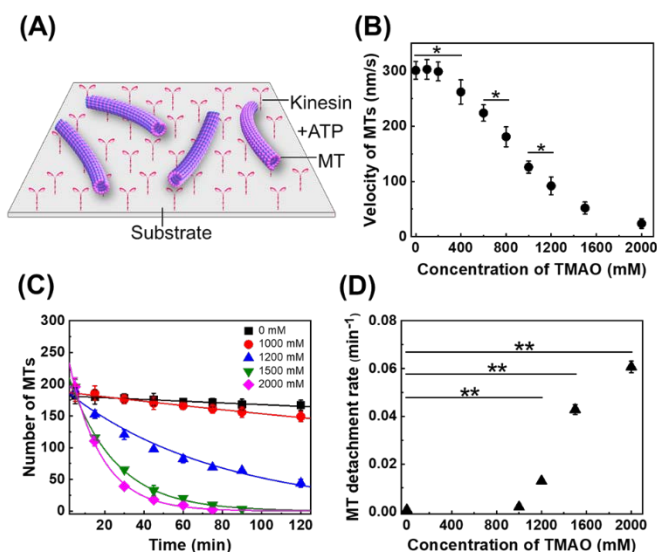


Figure 1. (A) Schematic representation of an in vitro motility assay of MTs on a kinesin coated substrate. Streptavidin is not shown for simplicity. (B) Effect of TMAO on the motility of MTs in the in vitro motility assay performed at 22 °C. (C) Change in number of MTs per field of view ($237.6 \times 281.6 \mu\text{m}^2$) with time in the motility assay performed at 22 °C in the presence of various TMAO concentrations as mentioned in the legends. Each line is a fit to the equation $N_t = N_0 \times \exp(-k_{off} \times t)$, where ' N_t ', ' N_0 ', ' k_{off} ', and ' t ' represents number of MTs at any time, initial number of MTs, detachment rate constant of MTs from the motility assay substrate, and time respectively. R^2 values of fitting were 0.9443, 0.9859, 0.9696, 0.9956, and 0.9919 for 0 mM, 1000 mM, 1200 mM, 1500 mM and 2000 mM TMAO respectively. (D) Change in detachment rate constant of MTs from motility assay substrate, as determined from the fitting of data shown in (C), with the change of TMAO concentration in motility assay. From Student's t-test significant difference between the data sets was observed at $P < 0.0001$ indicated by * for (B) and $P < 0.001$ indicated by ** for (D). Error bars: standard deviation.

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assay of MTs on kinesins demonstrated at 22–46 °C we show that, TMAO can control the kinetics of interaction between MTs and kinesins over such wide temperature range and prolongs the lifetime of motility of the kinesin-driven MTs.

First, we examined the effect of TMAO on motility of MTs driven by kinesins at room temperature (~22 °C, Figure 1A). TMAO (0–2000 mM) was employed in the motility assay by mixing with motility buffer in which the concentration of the fuel (ATP) was constant at a saturating concentration (5 mM). Decrease in MT velocity (Figure 1B) with increasing the concentration of TMAO and detachment of MTs from the motility assay substrate at relatively high TMAO concentrations (>1000 mM) (Figure 1C and 1D) are evident. Next, we raised the temperature of MT motility assay from 22 °C to 37 °C and 46 °C sequentially both in the absence and presence of 200 mM TMAO. Here, the concentration of TMAO was set to 200 mM as the velocity of MTs was not noticeably affected at this TMAO concentration. MTs started to detach from the substrate after the temperature was raised to 37 °C in the absence of TMAO (Figure S1), that agrees to a previous report.¹² The detachment of MTs was enhanced at 46 °C which indicates that detachment was the result of thermal damage of kinesins and/or MTs at 37 °C and 46 °C. By performing motility assay at 22 °C using MTs pre-incubated at 37 °C and 46 °C for one hour we confirmed no detachment of MTs from the substrate. Therefore, it was the thermal damage kinesins that caused detachment of MTs when temperature of the motility assay was raised to 37 °C and 46 °C. Detachment of MTs was substantially suppressed at 37 °C and 46 °C in the presence of 200 mM TMAO (Figure S1). This result indicates that the deep-sea osmolyte TMAO can protect kinesins from thermal damage at the above high temperatures. Next, motility assay of MTs were separately performed by varying the temperature in a range of 22 °C to 46 °C and the concentration of TMAO from 0 to 1000 mM. Detachment of MTs from the motility assay substrate at 37 °C and 46 °C can be considered as the signature of thermal damage of kinesins. Therefore, lifetime of motility assay at different temperatures and TMAO concentrations was investigated by monitoring change in MT density with time until all the MTs detached from the substrate. At 22 °C, in the absence of TMAO, number of MTs per field of view, and velocity of MTs did not change noticeably after ~120 min of initiation of motility (Figure 2A and 3A), which agrees well to our previous work.¹³ MT density and velocity did not change appreciably with time at 22 °C in the presence of 200 mM, 600 mM, and 1000 mM TMAO (Figure 2A, and Figure 3A). At 32 °C the number of MTs per field of view on the kinesin coated substrate decreased with time (Figure 2B and Figure S2). In the absence of TMAO, all the MTs detached from the substrate by ~360 min after the temperature was raised. In the presence of 200 mM and 600 mM TMAO, motile MTs were observed for ~480 min and ~600 min respectively at 32 °C. Upon increasing the TMAO concentration further to 1000 mM the motility assay system survived until ~390 min. MTs exhibited faster motility at 32 °C compared to at 22 °C (Figure 3B). No remarkable change in velocity of MTs with time could be observed within the lifetime of the assay system at 32 °C for any of the TMAO concentrations employed. Once all the MTs were

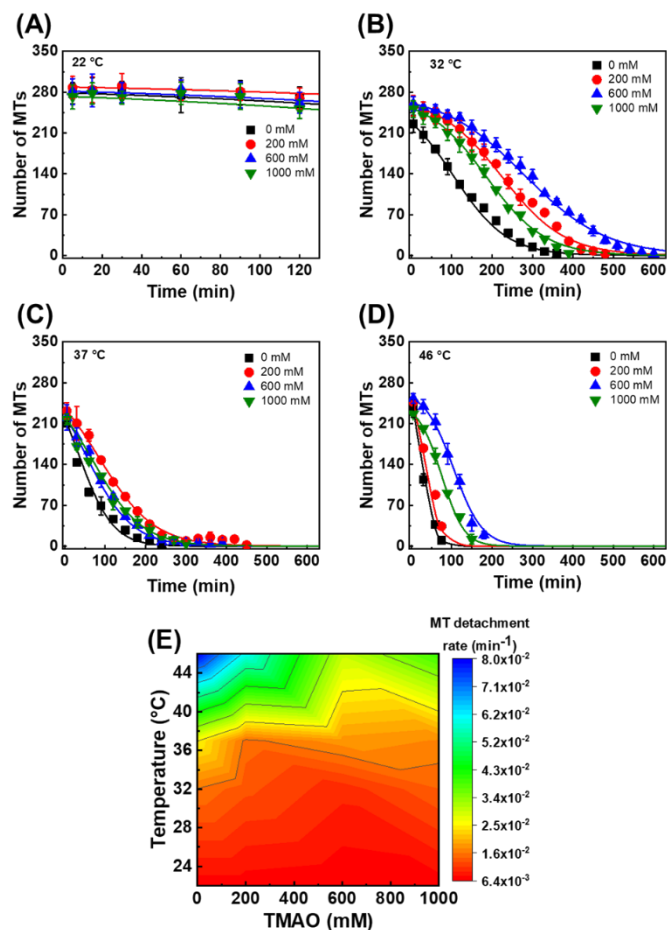


Figure 2. Change in number of MTs per field of view (237.6×281.6 μm²) with time in the motility assay performed at (A) 22 °C, (B) 32 °C, (C) 37 °C, (D) 46 °C in the presence of various TMAO concentrations as mentioned in the legends. Error bars: standard deviation. Each line is a fit to the equation -1. Data on rate constants for MT detachment are provided in Table S1 of supporting information. (E) Contour plot shows change in rate of MT detachment (per field of view) at different temperatures and TMAO concentrations. The detachment rate constants were obtained from the fitting of data shown in Figure 2 (A)–(D).

detached from the substrate, we applied newly polymerized MTs to the flow cell. Nonetheless, no new MTs were attached to the substrate which confirms that motor domain of kinesins lost their ability to interact with MTs due to prolonged operation at 32 °C. When MTs preincubated at 32 °C (also at 37 °C and 46 °C) for 6 hours were used, excellent binding of the MTs to the kinesin coated substrate and motility was confirmed at room temperature. Therefore, changes in kinesin, not in MTs, were responsible for detachment of MTs with time from motility assay substrate at 32 °C. At 37 °C number of MTs per field of view decreased more rapidly than at 32 °C (Figure 2C and Figure S3) and velocity of MTs fluctuated with time (Figure 3C). The lifetime of motility assay was ~240 min in the absence of TMAO, which was prolonged to ~450 min, ~390 min, and ~300 min using 200 mM, 600 mM and 1000 mM TMAO respectively. At 46 °C the number of MTs and MT velocity decreased drastically in all the TMAO concentrations (Figure 2D, Figure S4 and Figure 3D). Presence of TMAO prolonged the lifetime of motility assay significantly, which was ~75 min, ~180 min and ~150 min for 0 mM, 200 mM, 600 mM and 1000 mM TMAO respectively.

We use a logistic function, as mentioned by equation (1), to understand the kinetics of MT detachment:

$$N(t) = N_0 \left(1 - \frac{N_f}{N_f + (N_0 - N_f) \exp(-kt)} \right) \quad (1)$$

Here, $N(t)$ is the number of MTs on the substrate at any time ' t ', N_0 is the number of MTs on the substrate at $t=0$, N_f is the number of MTs in the solution at $t=0$, and k is the initial rate of detachment of MTs. In the function, we have assumed that the dissociation rate is proportional to $k(1-N(t)/N_0)N(t)$, indicating that the rate linearly decreases with increasing $N(t)$ and becomes zero when all of MTs disappear from the surface. The experimental data shown in Figure 2 are fitted well by the equation (1). At 22 °C, the detachment of MTs was negligible, while at or above 32 °C $N(t)$ decreased with time. The sigmoidal feature of the logistic function became evident when the concentration of TMAO was increased. On the other hand, the profile turned to be single exponential type when the temperature is increased further (37 °C, 46 °C). The detachment rate of MTs decreased at higher concentrations of TMAO or at the lower temperatures (Figure 2E and Table S1). Thus, TMAO successfully prolongs the lifetime of in vitro motility assay at high temperatures (Figure 4A).

Density of kinesins on the motility assay substrate¹⁹ decreased with time and the rate increased with increasing the temperature of the motility assay (Figure S5 and Figure 4B). Possibly, kinesins were detached from the substrate due to thermal damage of their tail part, which is responsible for binding to the substrate or cargo in cells.²⁰ Since the lifetime of motility assay became shorter with increasing temperature (Figure 4A), decrease in kinesin density due to denaturation is directly connected to the detachment of MTs. The detachment rate of kinesin was suppressed by TMAO (Figure S5 and Figure 4B). Hence, prolonged lifetime of MT motility at high temperatures could be accounted for by the suppressed denaturation of kinesins in the presence of TMAO. However, by the time at which all the MTs detached from motility assay

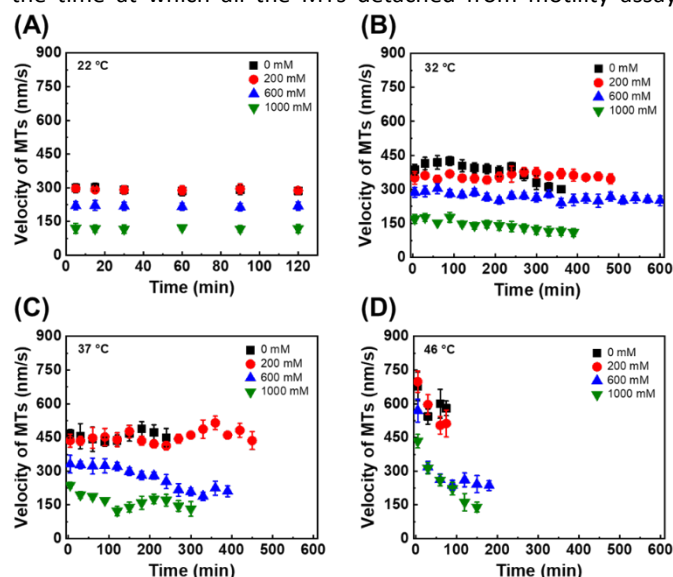


Figure 3. Velocity of MTs versus time obtained from the in vitro motility assay performed at (A) 22 °C, (B) 32 °C, (C) 37 °C, (D) 46 °C in the presence of different TMAO concentrations indicated by the legends. The number of MTs considered for analysis was 50 except the cases where enough MTs were not available due to detachment. Error bars: standard deviation.

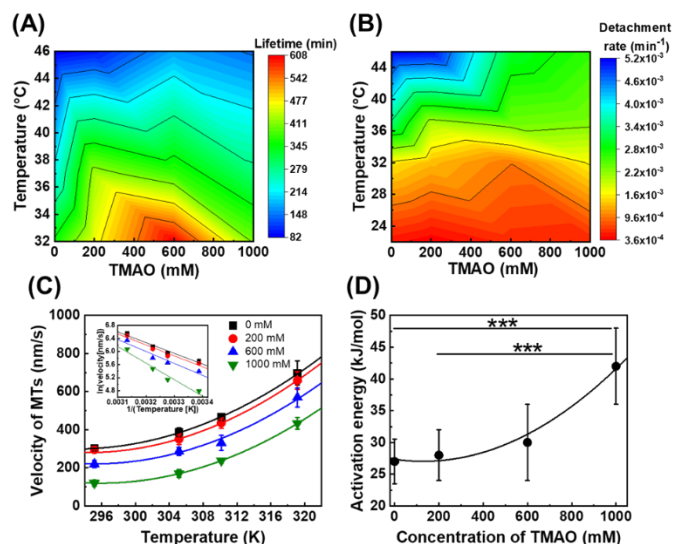


Figure 4. (A) Summarized data show effectiveness of TMAO in prolonging the lifetime of in vitro motility assay of MTs over the range of temperatures from 32 °C to 46 °C. (B) Rate of detachment of kinesins per μm^2 area of the motility assay substrate over the temperature range from 22 °C to 46 °C in the presence of different TMAO concentrations. (C) Dependence of gliding velocity of MTs on temperature at different TMAO concentrations indicated by the legends. The velocity was measured after 5 min of raising the temperature. The data were fitted to a polynomial function, $v = a + b_1 \cdot T + b_2 \cdot T^2$, where ' v ' is velocity of MTs, ' T ' is temperature in Kelvin, ' a ' is intercept, ' b_1 ' and ' b_2 ' are fit parameters. The values of ' a ', ' b_1 ', ' b_2 ' and ' R^2 ' for fitting of data are provided in Table S3 of supporting information. Inset shows corresponding Arrhenius plot. Each line is a fit to equation $\ln v = C - \frac{m}{T}$, where ' v ' is velocity of MTs in nm/s, ' C ' is the intercept, ' m ' is the slope from which activation energy was calculated, ' T ' is temperature in Kelvin. R^2 values for the fitting were 0.9676, 0.9562, 0.9335, 0.9626 for 0 mM, 200 mM, 600 mM and 1000 mM TMAO respectively. (D) Change in activation energy for the motility of MTs on kinesins upon changing the concentration of TMAO in the motility assay. The data were fitted to a polynomial function $E_a = 27 - 0.005C + 0.00002C^2$, where E_a is the activation energy, ' C ' is the concentration of TMAO. Significant difference between the data sets was observed at $P < 0.01$ indicated by ***. Error bars: standard deviation.

substrate, there was enough kinesin density on the substrate to bind and propel MTs (Figure S5).²¹ Despite that, no new MTs were attached to the kinesin coated substrate. Therefore, thermally denatured motor domain of kinesins appears to be the main reason behind the impairment of motility assay at high temperatures. To confirm, we performed an in vitro motility assay at 55 °C where gliding MTs were detached from the motility assay substrate, even in the presence of TMAO, within ~5 min after the temperature was increased. However, fluorescence intensity and density of kinesin on the substrate did not decrease noticeably within this time. Hence, thermal damage of MT-binding domain of kinesins is mainly responsible for termination of MT motility at high temperatures.

Steady velocity of MTs at 22 °C, 32 °C indicates sustained enzymatic activity of kinesins at these temperatures (Figure 3A and 3B). Fluctuation and reduction in velocity of MTs with time could be observed at 37 °C and 46 °C respectively (Figure 3C and 3D). Velocity of MTs, measured just after 5 min of increasing the temperature of motility assay, fits well to Arrhenius equation (Figure 4C and Table S2). The activation energy of MT motility in the absence of TMAO was estimated to be 27 ± 3 kJ/mol (fit value $\pm$ standard error of the fit) which is within the range reported in literature (10–100 kJ/mol).¹² However, in the presence of 200 mM, 600 mM, and 1000 mM TMAO the activation energy was 28 ± 4 kJ/mol, 30 ± 6 kJ/mol, and 42 ± 6 kJ/mol respectively (Figure 4D). The activation energy at 1000

mM TMAO was significantly higher ($p < 0.01$) compared to that at 0 mM and 200 mM TMAO. TMAO might have induced changes in kinesin structure which affected the activation energy of MT motility.²² From the velocity of MTs just before the termination of motility assay (Figure S6 and Table S3), the activation energy was found to decrease significantly at 1000 mM TMAO compared to that at 0 mM and 200 mM TMAO ($p < 0.001$). Prolonged operation at high temperatures might have incurred structural changes in kinesin which is different from that induced by TMAO. Finally, it is to note that, decrease in the number of MTs on the motility assay substrate at 22–46 °C was not contributed by depolymerization of the MTs.²³

In conclusion, kinetics of interaction between MTs and kinesins over a wide temperature range is regulated using a naturally occurring osmolyte TMAO. Lifetime of motility of MTs on kinesins at high temperatures is significantly prolonged using TMAO, which is ascribed to the TMAO induced suppression of denaturation of kinesins at high temperatures. Future explorations are now encouraged to confirm role of osmolytes in regulating stability and activity of biomolecular motors and other proteins and enzymes in cells. This work will also contribute to biomimetic engineering²⁴ and sustainable development of hybrid devices based on reconstructed proteins or enzymes and foster their applications in nanotechnology and materials science.^{25–27}

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Conflicts of interest

There are no conflicts to declare.

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