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Stabilization of Microtubules by Encapsulation of GFP Using Tau-Derived Peptide

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We constructed GFP-encapsulated microtubules (MTs) using Tau-derived peptide which binds to their interior. The encapsulation of GFP dramatically increased the rigidity of MTs, resulting in their enhanced velocity on a kinesin-coated substrate. Moreover, the GFP-encapsulated MTs were significantly more stable compared to normal MTs.

Cytoskeletal filaments, including microtubules (MTs), F-actin, and intermediate filaments change their length, stability, and rigidity as needed in order to perform various functions such as cellular structural support, cell division, and cell motility.[1–3] MTs are hollow tube assemblies formed by the polymerization of tubulin dimers and their dynamic flux between polymerization and depolymerization, their stability and rigidity, and their binding to motor proteins (kinesin and dynein), play critical roles in their function.[3–5] Based on the unique properties, MTs have been utilized for construction of various nanomaterials and nanodevices including cargo delivery systems, active network gels, and molecular robots.[6] However, modulation of the properties of MTs such as stability, mechanical rigidity, motility on the kinesin-coated plate remains challenging. In nature, the structural properties of MTs are precisely modulated. For instance, cytoplasmic MTs reversibly switch between phases of polymerization and depolymerization, whereas doublet MTs, which constitute the cytoskeleton core of both cilia and flagella, require high stability and rigidity to exert cell motility. How do they modulate their stability and rigidity? One approach in nature is the binding of microtubule-associated proteins (MAPs) to MTs.[4,5] Most well-studied MAPs, such as MAP2 and MAP4, bind to the outer surface of MTs. Similarly, the binding of synthetic dendrons with multiple guanidinium ions to the outer surface was found to stabilize MTs against depolymerization.[7]

Recently, additional proteins have been discovered that bind to the inside of MTs. In addition to cytoplasmic MTs,[8,9] these proteins, called microtubule inner proteins (MIPs), have been observed in the doublet MTs of cilia and flagella.[10–15] It is presumed that the binding of MIPs to the inside of doublet MTs dramatically increases their mechanical rigidity and stability against depolymerization.[10–15] Doublet MTs can withstand intense mechanical forces during ciliary/flagellar beating, and are stable under conditions that induce depolymerization of singlet MTs, such as low temperatures and high salt concentrations.[15] Despite the assumed importance of MIPs, their effects on the mechanical properties of MTs are not well-studied. The introduction of exogenous proteins inside MTs could not only provide insight into the function of natural MTs, but also generate artificial MTs with exceptionally high stability and rigidity. The stabilized MT analogs could be used as new scaffolds for nanomaterial applications. We tackled this challenging issue by using our MT-binding peptide.

We previously developed a Tau-derived peptide, **TP** (CGGGKKHVPGGGSVQIVYKPVDL), as a binding motif to the internal domains of MTs.[16] Tau protein is hypothesized to bind tubulin by cross-linking the external and internal tubulin lattice and stabilize MTs.[17] We designed **TP** based on a repeat domain of Tau that is involved in binding to the inner surface of MTs through interaction with a taxol-binding pocket on tubulin. Binding of **TP** to the interior of MTs can be achieved by preincubating the peptide with tubulin, followed by subsequent polymerization of the peptide-tubulin complex.[16] Thus, conjugation of exogenous proteins to **TP** is a promising strategy for their encapsulation inside MTs. Here we report the synthesis of MT-encapsulated green fluorescent protein (GFP) via construction of a **TP**-conjugated GFP (**TP-GFP**) (Figs. 1a and 1b). We chose GFP as a model protein due to its detectability by

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†Electronic Supplementary Information (ESI) available: Experimental details and supporting figures. A movie that illustrates motility of **TP-GFP**-MTs and unbound MTs (Movie S1). See DOI: 10.1039/x0xx00000x

fluorescence, small size, and capacity for the modification of surface amino acids.[18] Interestingly, we found that encapsulation of GFP increased the rigidity and stability of MTs, and enhanced their motility on a kinesin-coated substrate (Fig. 1c).

To construct **TP-GFP**, we utilized the split GFP system developed by Waldo *et al*.[18,19] In this system, the C-terminal fragment of β-strand 11 of superfolder GFP (GFP11) and truncated N-terminal GFP1–10 spontaneously assemble to form a fluorescent mature GFP. We then synthesized the TP-GFP11 peptide, which consisted of GFP11 and **TP** connected by a 4-amino-acid linker (GGGS, Fig. 1a) and confirmed its structure using MALDI-TOF-MS (Fig. S1a, ESI†). GFP1–10 was expressed as a soluble fraction in *E. coli* by fusing with a soluble maltose-binding protein (MBP) and purified after removal of MBP by the treatment of a TEV protease (Fig. S2, ESI†). Successful assembly of the mature **TP-GFP** was monitored using fluorescence, which was observed following incubation of the TP-GFP11 peptide with GFP1–10 for 5 h (Fig. S3a, ESI†). A dissociation constant (K_d) of 1.1 ± 0.70 μM for the mature assembly was determined using fluorescence titrations (Fig. S3b, ESI†). This K_d value was similar to that of the unmodified GFP11 (Figs. S3c and S3d, ESI†, K_d = 0.83 ± 0.44 μM); thus, the introduction of the **TP** moiety has minimal effect on the reconstruction of mature GFP. In the following experiments, we used **TP-GFP** reconstructed from GFP1–10 and 5 equivalents of TP-GFP11 peptide.

Binding of **TP-GFP** to MTs was characterized by confocal laser scanning microscopy (CLSM). To monitor MTs, red fluorescent tetramethylrhodamine (TMR) was conjugated to tubulin (tubulin-TMR). **TP** is known to bind to the inside of MTs following preincubation with tubulin and subsequent MT formation (the "Before" method);[16] therefore, we employed this approach for the encapsulation of **TP-GFP** (Fig. 1b). In this method, tubulin and tubulin-TMR were preincubated with **TP-GFP**, followed by incubation with GMPCPP, a slowly hydrolysable GTP analog used to form stable MTs to induce polymerization. Colocalization of green **TP-GFP** with red MTs suggested successful binding of **TP-GFP** to MTs (Fig. 2a). Additionally, when **TP-GFP** was added to preformed MTs (the "After" method), binding of **TP-GFP** to MTs was still observed (Fig. S4, ESI†). Furthermore, the GFP constructed from GFP1–10 and GFP11 without **TP** did not bind MTs following either the "Before" or "After" methods (Fig. S5, ESI†), which indicated that the **TP** moiety is necessary for GFP binding to MTs.

Unfortunately, it is difficult to directly observe **TP-GFP** encapsulated in MTs by transmission electron microscopy (TEM; Fig. S6, ESI†). Thus, the binding of a fluorescent ATTO 647N-labeled anti-GFP single domain antibody was used to elucidate the nature of the binding of **TP-GFP** to MTs using the "Before" method (Fig. 2b). Presumably, the anti-GFP antibody would not bind encapsulated **TP-GFP** due to the low accessibility of the antibody to the MT interior. The low fluorescence was observed from the anti-GFP antibody when **TP-GFP**-MTs were prepared by the "Before" method (Fig. S7a, ESI†). In contrast, intense fluorescence from the anti-GFP antibody was observed when using the "After" method (Fig. S7b, ESI†). The lower ratio of ATTO 647N to **TP-GFP** fluorescence

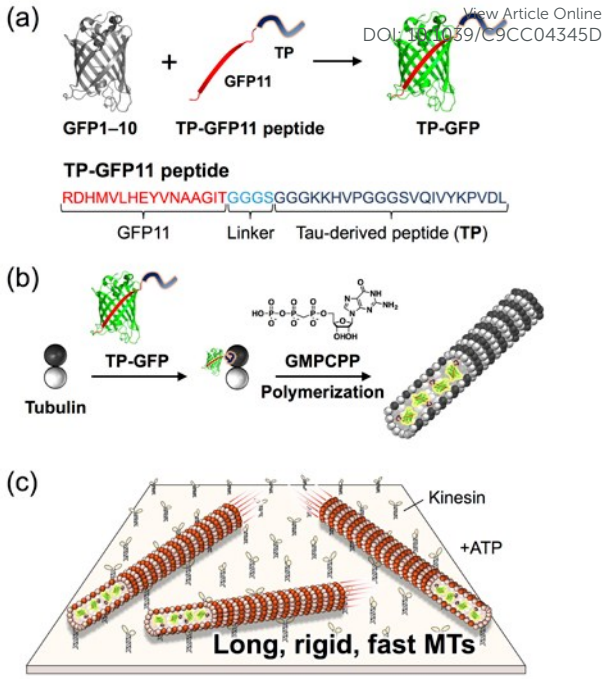

Fig. 1 (a) Construction of Tau-derived peptide-conjugated green fluorescent protein (**TP-GFP**) by reassembly of GFP1–10 and TP-GFP11 peptide. (b) Binding of **TP-GFP** inside microtubules (MTs) by preincubation with tubulin and subsequent polymerization. (c) **TP-GFP**-MTs gliding on kinesins.

intensity for MTs prepared using the "Before" method suggested that the majority of **TP-GFP** proteins were bound to the MT interior; however, the "After" method resulted in **TP-GFP** binding to the outside of MTs, which could therefore be detected using the anti-GFP antibody (Fig. 2c). The different binding sites of **TP-GFP** in the "Before" and "After" methods are in good agreement with the binding of **TP** to MTs.[16]

To further confirm the binding specificity of **TP-GFP**, a substitution experiment using an anti-tubulin antibody was performed according to our previously reported method (Fig. S8, ESI†).[16] In brief, **TP-GFP** bound to the MT exterior can be displaced using an anti-tubulin antibody, which selectively binds to the C-terminal region of tubulin, located on the outer surface of MTs. The fluorescence of **TP-GFP** bound to TMR-labeled MTs prepared using the "Before" method was not significantly influenced by the addition of this antibody, indicating that the main binding site of **TP-GFP** was not the outer surface. In contrast, a large decrease in fluorescence from **TP-GFP** resulted from treatment of MTs prepared using the "After" method with the anti-tubulin antibody, indicating that binding of **TP-GFP** predominantly occurred on the outer surface.

The amount of bound **TP-GFP** on MTs prepared using the "Before" method was estimated by measuring the fluorescence of free **TP-GFP** in the supernatant after precipitation of **TP-GFP**-MTs via ultracentrifugation (Fig. S9, ESI†). Under typical encapsulation conditions using 2.5 μM tubulin and 5 μM **TP-GFP**, the ratio of bound **TP-GFP** to tubulin dimer was 0.31. Since most **TP-GFP** was probably bound to the inside of MTs under these conditions (Figs. 2c and S8, ESI†), we concluded that approximately 30% of tubulin dimers possessed bound **TP-**

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GFP. When the concentration of **TP-GFP** was increased to 10 μM, the ratio of bound **TP-GFP** to tubulin dimer incr eased to 1.26 (Fig. S9, ESI†), indicating that at higher concentrations **TP-GFP** may also bind to the outer surface of MTs.

Next, we investigated how the length, rigidity, and velocity of MTs on a kinesin-coated substrate was influenced by **TP-GFP** (Fig. 1c). Motility assay were performed using a flow cell made from cover glass. MT motility was initiated by the addition of ATP, and monitored under a fluorescence microscope (Fig. 3a and Movie S1). We found that internal binding of 5 μM **TP-GFP** dramatically increased contour (1.7-fold) and persistence (L_p, 4.0-fold) lengths, which have previously been used as a measure of MT rigidity,[20] compared to unbound MTs (Figs. 3b and 3c). Notably, the velocity of MTs with **TP-GFP** was increased by 22% compared to unbound MTs (0.84 ± 0.02 to 0.69 ± 0.03 μm/sec, respectively, Fig. 3d). This enhanced velocity was interesting, given that conjugation of metal nanoparticles on the outer surface is known to slow MT motion.[21] It is well-known that MTs polymerized in the presence of GMPCPP have a higher flexural rigidity compared to GTP-MTs, and that these rigid MTs move faster compared to their more flexible derivatives.[22] Furthermore, kinesins move faster along rigid GMPCPP-MTs, compared to flexible GTP-MTs.[23] Thus, it is reasonable to assume that the increased rigidity of our MTs resulting from the encapsulation of **TP-GFP** is responsible for their enhanced velocity. Conversely, the velocity of MTs with TMR-labeled **TP** (**TP-TMR**) was similar to that of unmodified MTs (Fig. S10, ESI†), suggesting that the GFP scaffold has an important role in the increased velocity of **TP-GFP**-bound MTs. The properties of MTs were also dependent on the concentration of **TP-GFP** (Fig. S11, ESI†). MT contour length and L_p increased with increasing concentration of **TP-GFP** (Figs. S11a and S11b, ESI†); however, velocity decreased when the concentration of **TP-GFP** was increased from 5 μM to 10 μM (Fig. S11c, ESI†). Given that **TP-GFP** possibly binds to both the inner and outer surfaces of MTs at higher concentrations (Fig. S9, ESI†), it is possible that its binding to the MT exterior slows their motion under these conditions.

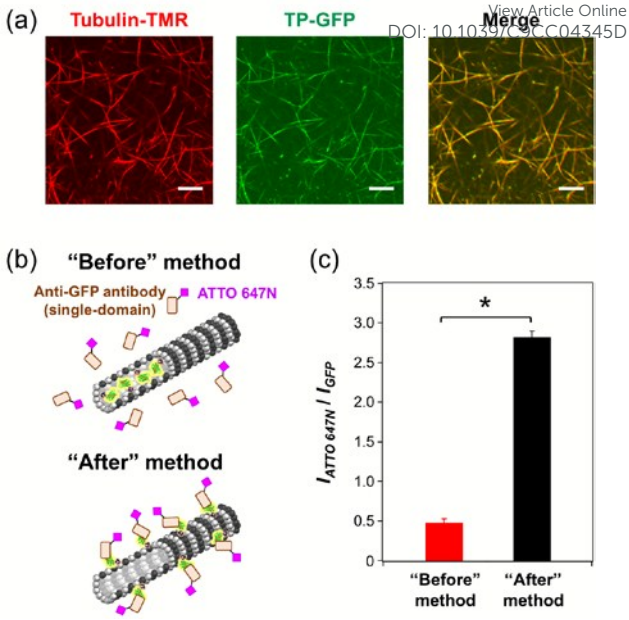

Fig. 2 (a) Confocal laser scanning microscopy (CLSM) images of tetramethylrhodamine (TMR)-labeled MTs (2.5 μM) incubated with **TP-GFP** (5 μM) using the "Before" method (scale bar: 10 μm). (b) Binding analysis of ATTO 647N-labeled anti-GFP antibody to **TP-GFP**-bound MTs prepared using the "Before" and "After" methods. (c) $I_{ATTO\ 647N}/I_{GFP}$ of each **TP-GFP**-bound MTs (2.5 μM tubulin, 5 μM **TP-GFP**) treated with anti-GFP antibody (1 μM), determined from CLSM images. Error bars represent the standard error of the mean (N = 20). *P < 0.01, t-test.

To evaluate the stability of **TP-GFP**-MTs, we performed turbidity measurements, as increases in turbidity are indicative of the rate of MT formation. The addition of **TP-GFP** to tubulin in the presence of GTP at 37°C dramatically increased turbidity to a similar level to that of the addition of taxol, which is an anticancer drug that binds the same site as **TP**, indicating an enhancement in tubulin polymerization (Fig. 4). Notably, the addition of TP-GFP11 peptide without GFP1–10 produced only a moderate enhancement of tubulin polymerization; hence, both the **TP** and complete GFP scaffolds of **TP-GFP** are required to

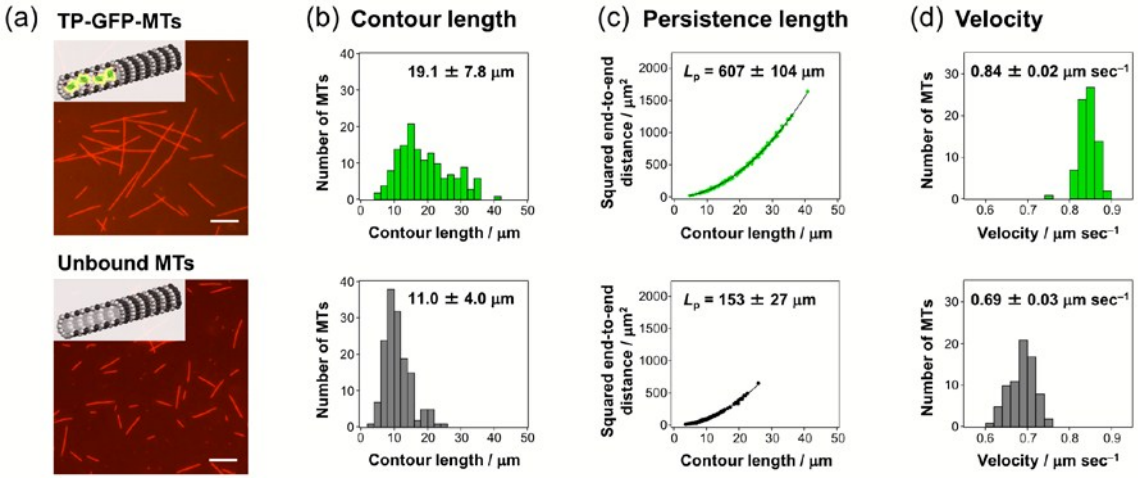

Fig. 3 Properties of **TP-GFP** encapsulated TMR-labeled MTs (2.5 μM) prepared using the "Before" method with 5 μM **TP-GFP** and unbound control MTs on kinesin-coated substrates. (a) Fluorescence microscopy images showing individual MT (scale bar: 10 μm). (b) Distribution of contour length (N = 150), (c) persistence length (L_p, N = 150), and (d) velocity (N = 75) for MTs with **TP-GFP** (**TP-GFP**-MTs: green, unbound MTs: black). In Fig. 3c, black curves indicate the statistically estimated result (see ESI† for detail).

stabilize higher rates of MT polymerization. The thermal stability of formed **TP-GFP**-MTs is estimated to be similar to the unbound MTs (Fig. S12, ESI†). When the temperature was decreased to 4°C to initiate depolymerization, **TP-GFP**-MTs remained partially stable, as similar to taxol-treated MTs, compared to unmodified MTs (Fig. 4). These results show that binding of **TP-GFP** increased the stability of MTs against depolymerization, possibly due to the binding of the GFP scaffold to the inside wall of MTs. The secondary structure of tubulin was minimally affected by the binding of **TP-GFP**, as evaluated by circular dichroism (CD) spectroscopy (Fig. S13, ESI†). Using molecular mechanics calculations, we predicted that, when the **TP** moiety of **TP-GFP** binds to MT taxol-binding pockets, the GFP scaffold of **TP-GFP** also interacts with the MT wall via electrostatic interactions and hydrogen bonding (Fig. S14, ESI†). Thus, it is possible that this cooperative binding increases the stability and rigidity of MTs in a similar manner to that of natural MIPs.[13–15]

In conclusion, we developed GFP-encapsulated MTs by conjugation of the MT-binding peptide **TP** to GFP using a split GFP system and subsequently elucidated the effects of this protein's encapsulation on the properties of MTs. We demonstrated that the binding of this GFP conjugate significantly increased the stability and rigidity of MTs, resulting in enhanced velocity. Based on this study, we now aim to investigate the effects of encapsulating various exogenous proteins using **TP** to elucidate the structure and functions of natural MTs. In addition, chemical design of TP-GFP11 peptide, such as introduction of non-natural amino acids, enables further functionalization such as light-induced release of GFP from MTs by using a photocleavable amino acid.

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Conflicts of interest

There are no conflicts to declare.

References

1 D. A. Fletcher and R. D. Mullins, *Nature*, 2010, **463**, 485.
2 L. Blanchoin, R. Boujemaa-Paterski, C. Sykes and J. Plastino, *Physiol. Rev.*, 2014, **94**, 235.
3 H. Hess and J. L. Ross, *Chem. Soc. Rev.*, 2017, **46**, 5570.
4 E. Mandelkow and E. M. Mandelkow, *Curr. Opin. Cell Biol.*, 1995, **7**, 72.
5 T. Hawkins, M. Mirigian, M. Selcuk Yasar and J. L. Ross, *J. Biomech.*, 2010, **43**, 23.
6 H. Hess and V. Vogel, *Rev. Mol. Biotechnol.*, 2001, **82**, 67; A. M. R. Kabir and A. Kakugo, *Polym. J.*, 2018, **50**, 1139; G. D. Bachand, E. D. Spoerke and M. J. Stevens, *Biotechnol. Bioeng.*, 2015, **112**, 1065; T. Sanchez, D. T. N. Chen, S. J. DeCamp, M. Heymann and Z. Dogic, *Nature*, 2012, **491**, 431; Y. Sato, Y. Hiratsuka, I. Kawamata, S. Murata and S.-i. M. Nomura, *Sci. Rob.*, 2017, **2**, eaal3735; J. J. Keya, R. Suzuki, A. M. R. Kabir, D. Inoue, H. Asanuma, K. Sada, H. Hess, A. Kuzuya and A. Kakugo, *Nat. Commun.*, 2018, **9**, 453; J. J. Keya, A. M. R. Kabir, D. Inoue, K. Sada, H. Hess, A. Kuzuya and A. Kakugo, *Sci. Rep.*, 2018, **8**, 11756; K. Matsuda, A. M. R. Kabir, N. Akamatsu, A. Saito, S. Ishikawa, T. Matsuyama, O. Ditzer, M. S. Islam, Y. Ohya, K. Sada, A. Konagaya, A. Kuzuya and A. Kakugo, *Nano Lett.*, 2019, **19**, 3933.
7 K. Okuro, K. Kinbara, K. Tsumoto, N. Ishii and T. Aida, *J. Am. Chem. Soc.*, 2009, **131**, 1626; M. Garzoni, K. Okuro, N. Ishii, T. Aida and G. M. Pavan, *ACS Nano*, 2014, **8**, 904.
8 B. K. Garvalov, B. Zuber, C. Bouchet-Marquis, M. Kudryashev, M. Gruska, M. Beck, A. Leis, F. Frischknecht, F. Bradke, W. Baumeister, J. Dubochet and M. Cyrklaff, *J. Cell Biol.*, 2006, **174**, 759.
9 C. Bouchet-Marquis, B. Zuber, A.-M. Glynn, M. Eltsov, M. Grabenbauer, K. N. Goldie, D. Thomas, A. S. Frangakis, J. Dubochet and D. Chrétien, *Biol. Cell*, 2007, **99**, 45.
10 H. Sui and K. H. Downing, *Nature*, 2006, **442**, 475.
11 D. Nicastro, C. Schwartz, J. Pierson, R. Gaudette, M. E. Porter and J. R. McIntosh, *Science*, 2006, **313**, 944.
12 D. Nicastro, X. Fu, T. Heuser, A. Tso, M. E. Porter and R. W. Linck, *Proc. Natl. Acad. Sci. U.S.A.*, 2011, **108**, E845.
13 M. Ichikawa, D. Liu, P. L. Kastritis, K. Basu, T. C. Hsu, S. Yang and K. H. Bui, *Nat. Commun.*, 2017, **8**, 15035.
14 M. Owa, T. Uchihashi, H.-A. Yanagisawa, T. Yamano, H. Iguchi, H. Fukuzawa, K.-I. Wakabayashi, T. Ando and M. Kikkawa, *Nat. Commun.*, 2019, **10**, 1143.
15 M. Ichikawa and K. H. Bui, *Bioessays*, 2018, **40**, 1700209.
16 H. Inaba, T. Yamamoto, A. M. R. Kabir, A. Kakugo, K. Sada and K. Matsuura, *Chem. Eur. J.*, 2018, **24**, 14958.
17 S. Kar, J. Fan, M. J. Smith, M. Goedert and L. A. Amos, *EMBO J.*, 2003, **22**, 70.
18 J. D. Pédelacq, S. Cabantous, T. Tran, T. C. Terwilliger and G. S. Waldo, *Nat. Biotechnol.*, 2006, **24**, 79.
19 S. Cabantous, T. C. Terwilliger and G. S. Waldo, *Nat. Biotechnol.*, 2005, **23**, 102.
20 F. Gittes, B. Mickey, J. Nettleton and J. Howard, *J. Cell Biol.*, 1993, **120**, 923.
21 M. Platt, G. Muthukrishnan, W. O. Hancock and M. E. Williams, *J. Am. Chem. Soc.*, 2005, **127**, 15686.; B. M. Hutchins, M. Platt, W. O. Hancock and M. E. Williams, *Micro Nano Lett.*, 2006, **1**, 47; M. Bachand, A. M. Trent, B. C. Bunker and G. D. Bachand, *J. Nanosci. Nanotechnol.*, 2005, **5**, 718.
22 R. D. Vale, C. M. Coppin, F. Malik, F. J. Kull and R. A. Milligan, *J. Biol. Chem.*, 1994, **269**, 23769.
23 M. Morikawa, H. Yajima, R. Nitta, S. Inoue, T. Ogura, C. Sato and N. Hirokawa, *EMBO J.*, 2015, **34**, 1270; T. Torisawa, D. Taniguchi, S. Ishihara and K. Oiwa, *Biophys. J.*, 2016, **111**, 373.

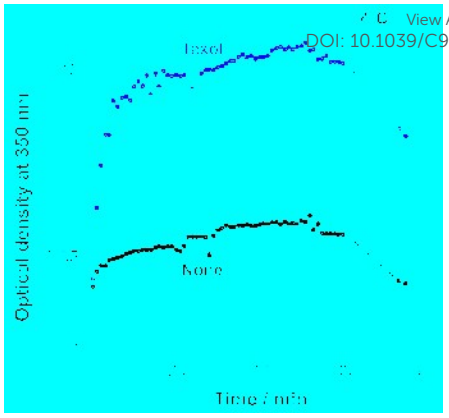

Fig. 4 Changes in turbidity over time as a result of tubulin polymerization. Optical density at 350 nm was measured with 2.5 μM tubulin and 1 mM GTP in the absence (black) or presence of 25 μM taxol (blue), **TP-GFP** consisting of 5 μM GFP1–10 and 25 μM TP-GFP11 peptide (red), or 25 μM TP-GFP11 peptide (green) at 37°C. After 60 min of measurements, samples were cooled to 4°C for 15 min and remeasured.

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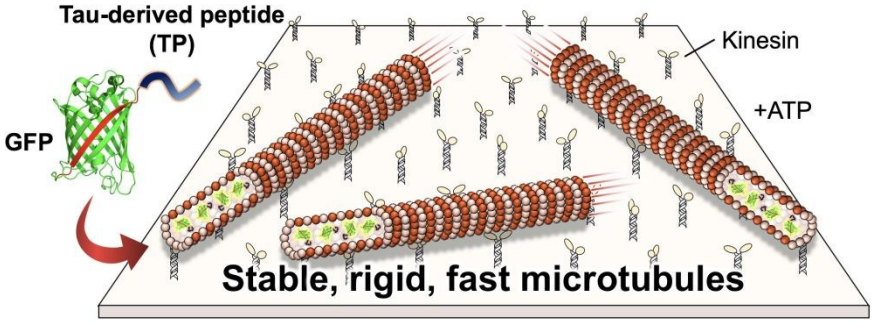

Encapsulation of GFP inside microtubules by using Tau-derived peptide increased the stability, rigidity, and velocity of microtubules.