Biochemical and Biophysical Research Communications 402 (2010) 66–69

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Temperature dependence rigidity of non-taxol stabilized single microtubules

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ARTICLE INFO

Article history:
Received 21 September 2010
Available online 27 October 2010

Keywords:
Microtubule
Flexural rigidity
Temperature
Anisotropic property
Conformation change

ABSTRACT

Because microtubules are the structural elements of cells, it is essential to study the mechanical properties of single microtubules under physiological conditions. Previously, we measured the effect of temperature on the flexural rigidity of a single taxol-stabilized microtubule and found that the flexural rigidity is 2.5×10^{-24} Nm^2, independent of temperature in the 20–35 °C range. Employing the same technique here, we have measured the flexural rigidity of microtubules polymerized in the presence of guanylyl-(a,b)-methylene-diphosphonate (GMPCPP, the slowly hydrolyzable GTP analogue) and in the presence of GTP only; both of the states were taxol-free. The obtained values were approximately 5-fold (for GMPCPP) and three- to 4-fold (for GTP) greater than those of taxol-stabilized microtubules. Interestingly, rigidity decreased as temperature increased, that is, temperature dependence was only observed in taxol-free microtubules. Length dependence was also observed. These results indicate that the transition of microtubule's rigidity is associated with the tubulin conformation change from a GTP-bound state to a GDP-bound state in the α/β subunit. We discuss the relationship of the regulation mechanism of the microtubules in the cell body to the changes in rigidity through hydrolysis.

1. Introduction

Microtubules are rigid tubular polymers composed of numerous tubulin heterodimers, which are one of the elements that makes up the eukaryotic cytoskeleton in conjunction with actin and intermediate filaments. Microtubules resist various forces to maintain the cell's shape, and they also function as a track for motor proteins, such as kinesin and dynein, which generate the force required for cell movement and changes in shape [1,2]. Microtubules within the cell are highly dynamic under physiological conditions. They can rapidly polymerize and depolymerize at their free ends. This dynamic instability depends on GTP hydrolysis and is regulated by proteins called microtubule-associated proteins (MAPs) [3]. The detailed mechanism of this regulation, however, remains unclear. Furthermore, part of the microtubule network has a miotic spindle form that pulls apart chromosomes during cell division; this has led to microtubules becoming a major target of several anticancer therapies [4]. In this context, an investigation of the mechanical properties and regulation mechanisms of purified microtubules is essential.

Flexural rigidity is an important mechanical property of microtubules that has been estimated by various methods, including thermal fluctuations, hydrodynamic flows, optical tweezers, atomic force microscope (AFM) probes, and motor proteins; these methods are reviewed in Kasas and Dietler [5] and Hawkins et al. [6]. However, the reported flexural rigidity for taxol-or paclitaxel-stabilized microtubules varies in a wide range from 1.9×10^{-24} Nm^2 [7] to 21.5×10^{-24} Nm^2 [8]. It is therefore important to clarify why the estimated flexural rigidity of different microtubules is scattered over such a wide range. Until recently, an isotropic elastic beam model was commonly used to investigate the mechanical properties of microtubules. However, recent papers [9–11] have suggested that microtubules have an anisotropic property, which means that they are not homogeneous plastic beams and that the longitudinal bonds between tubulin dimers within the protofilament are stronger than the lateral inter-protofilament bonds. Studies of surface reconstructions of microtubules also support this idea [12,13] because they are far from smooth, isotropic cylinders. This anisotropic property has been used to explain the aforementioned wide range of measured rigidities because the microtubule length dependence on flexural rigidity is generated by its anisotropy [9–11]. Previously, we measured the effect of temperature on the flexural rigidity of a single taxol-stabilized microtubule and found that the flexural rigidity is 2.54×10^{-24} Nm^2, independent of temperature in the 20–35 °C range [14]. A slight length dependency was observed, but it was not statistically significant. The temperature-dependent elasticity of microtubules was recently investigated using an AFM, and remarkable results were obtained [15]. However, the microtubules used in the study were fixed by glutaraldehyde, and thus the possibility remains that the experimental results do not relate to those under physiological conditions. Using the same technique in this study, we measured the flexural rigidity of taxol-free (GTP only) microtubules and microtubules polymerized in the presence of GMPCPP in the temperature range from 20–35 °C. In this way,

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doi:10.1016/j.bbrc.2010.09.112

we were able to measure the flexural rigidity of single microtubules under more physiological conditions.

2. Materials and methods

2.1. Preparation of microtubules

Tubulin was extracted from porcine brain as previously described [14,16]. Protein concentration was determined by a Bio-Rad protein assay (Nippon Bio-Rad Laboratories, Higashi-Nippori, Japan), which was calibrated using bovine serum albumin as the standard. Microtubules were prepared by polymerizing 20 μM tubulin in 80 mM piperazine-1,4-bis-2-ethanesulfonic acid (PIPES) (pH 6.8), 1 mM EGTA, 5 mM $MgCl_2$, 1 mM GTP, or 1 mM GMPCPP, and 36% glycerol at 37 °C for 30 min.

2.2. Measurement of the flexural rigidity of single microtubules by thermal fluctuations

The method to measure the flexural rigidity of single microtubules by thermal fluctuations was performed as described previously with slight modifications [14]. The polystyrene beads (diameter 1.0 μm; carboxylate-modified, Molecular Probes) in an assay buffer containing 2 mM $MgCl_2$, 80 mM PIPES (pH 6.8), and 1 mM ethylene glycol tetraacetic acid (EGTA) were introduced into a flow chamber and incubated for 1 min to allow the attachment of beads to the glass surface. Subsequently, the chamber was washed twice with an assay buffer to remove unadhered beads. The assay buffer containing the microtubules (for GTP-state experiments, 1 mM GTP was added to the assay buffer) was then introduced into a flow chamber and incubated for 2 min to allow the binding of microtubules to the beads. The assay buffer with 0.7 mg/ml filtered casein was then introduced into the flow chamber to prevent microtubules from sticking to surfaces (Fig. 1). Because we employed the non-specific interactions between microtubules and beads in this experiment, the introduced microtubules attached all over the flow chamber including to the beads. For the analysis of flexural rigidity, the attached and fluctuating microtubules were appropriately selected. In the experiment with the GTP-state, we need to introduce approximately 100 times the number of microtubules into the flow chamber to find a suitable number of the appropriate microtubules because of their instability, especially in the absence of free tubulin; the microtubules that kept to a constant length were selected for measurements. The microtubules were observed under a dark-field microscope (BX-51: Olympus Co., Tokyo, Japan) using a 1.2–1.4 numerical aperture condenser (U-DCW: Olympus Co., Tokyo, Japan). In this study, the flexural rigidity of microtubules was estimated by measuring the thermal fluctuations of the free end of a microtubule, the other end of which was attached near its tip to a polystyrene bead. Only the microtubules on which the fluctuations on both ends showed no correlation with each other were used in the analysis. In these microtubules, the positions of the free end followed a nearly Gaussian distribution, which confirmed that the other end of the microtubule was firmly fixed to the bead. According to Cassimeris et al. [17], this type of analysis of flexural rigidity relies upon the following equation:

$$EI = \frac{k_B T L^3}{3\langle d^2 \rangle} \tag{1}$$

where E is the Young's modulus, I is the geometrical moment of inertia of the cross section, L is the contour length of a microtubule between the tip of the free end and the attachment point, and $\langle d^2 \rangle$ is the mean-square deflection of the free end of the microtubule. The other parameters are the Boltzmann constant (k_B) and absolute temperature (T). Here, we term EI the flexural rigidity. Single microtubules (5–20 μm in length) that were attached to a bead at one end and freely bending due to thermal fluctuations at the other were selected for analysis. Point (x_0, y_0) corresponds to the attachment point. The 60 collected points $((x_1, y_1) \ldots (x_{60}, y_{60}))$ represent the positions of the fluctuating tip of the microtubule in each of the 60 frames (see Fig. 1B in [14]). The 60 points represent a spread of the different positions of the microtubule's tip. The mean position (x_{mean}, y_{mean}) is thus calculated as

$$x_{mean} = \frac{1}{60}\sum_{n}^{60} x_n \tag{2}$$

$$y_{mean} = \frac{1}{60}\sum_{n}^{60} y_n \tag{3}$$

The length L of the microtubule is calculated as

$$L = \sqrt{\{(x_0 - x_{mean})^2 + (y_0 - y_{mean})^2\}} \tag{4}$$

The mean-square deflection $\langle d^2 \rangle$ is calculated as

$$\langle d^2 \rangle = \frac{1}{60}\sum_{n}^{60}\{(x_n - x_{mean})^2 + (y_n - y_{mean})^2\} \tag{5}$$

For the experiments at 20 and 25 °C, the room temperature was controlled using an air conditioner. For the experiments at 30 and 35 °C, the temperature of the microscope stage was maintained using a homemade air incubator (accuracy ±1 °C). The temperature was measured using a thermometer attached to the surface of the experimental chamber.

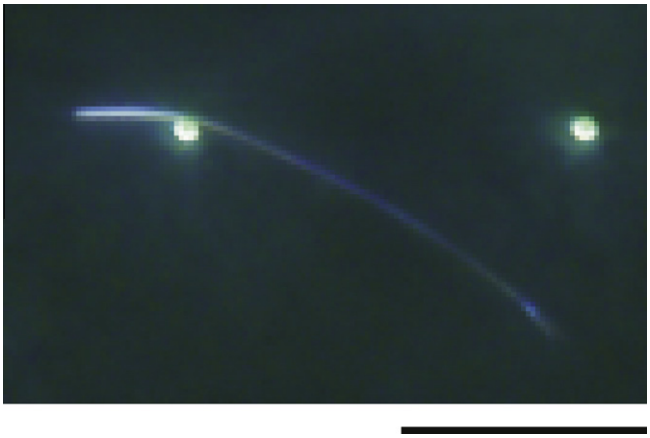

Fig. 1. Measurements of the flexural rigidity of single microtubules by observing their thermal bending motion. A dark-field micrograph showing a microtubule, both ends of which are free to fluctuate in solution. The microtubule was attached near one end to a casein-coated bead adsorbed onto a glass surface. Scale bar: 5 μm.

3. Results and discussion

3.1. Length dependency of the flexural rigidity of single microtubules

First, we measured the length dependency of the flexural rigidity of non-taxol stabilized single microtubules. The flexural rigidities of different lengths of microtubules with GTP, polymerized with GMPCPP, and taxol at each temperature are shown in Fig. 2. Note that the buffer for experiments with taxol-stabilized microtubules was GTP-free. Even though the length dependency of the rigidity of taxol-stabilized microtubules was not statistically significant [14], the data for GTP and GMPCPP from around 5–20 μm revealed that the rigidity increased approximately 2-fold at each temperature (Fig. 2). Interestingly, length dependence was

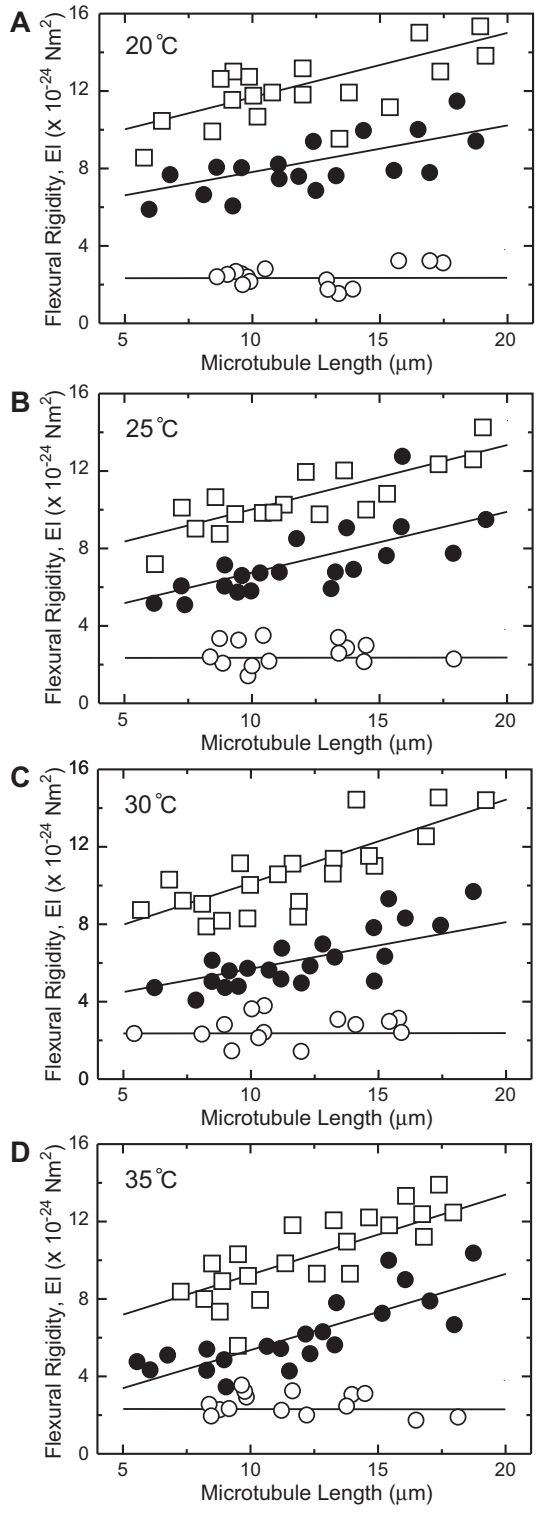

Fig. 2. Length dependency of the flexural rigidity of single microtubules. The flexural rigidity of single microtubules is determined from the thermal bending motion. The dependence of the flexural rigidity of single microtubules on the length of microtubules is shown for various temperatures: 20 °C (A), 25 °C (B), 30 °C (C), and 35 °C (D). The average values of the flexural rigidity (average ± s.d.) were 12.0 ± 1.70 (n = 19), 10.6 ± 1.69 (n = 17), 10.3 ± 2.18 (n = 21), and 10.1 ± 1.99 (n = 25) × 10^{-24} Nm^2 for GMPCPP-stabilized microtubules (squares) at 20 °C, 25 °C, 30 °C, and 35 °C, respectively. In the presence of GTP (closed circles), the values were 8.12 ± 1.52 (n = 18), 7.30 ± 1.95 (n = 20), 6.26 ± 1.55 (n = 22), and 6.10 ± 1.92 (n = 21) × 10^{-24} Nm^2 at 20 °C, 25 °C, 30 °C, and 35 °C, respectively. The values of the flexural rigidity of taxol-stabilized microtubules (open circles) were previously reported as 2.46 ± 0.36 (n = 15), 2.54 ± 0.46 (n = 14), 2.57 ± 0.58 (n = 14), and 2.36 ± 0.30 (n = 15) × 10^{-24} Nm^2 at 20 °C, 25 °C, 30 °C, and 35 °C, respectively [14].

observed only in non-taxol microtubules. The linear fit (shown by the thin line) yielded $EI = (0.286L + 8.64) \times 10^{-24}$ Nm^2 (where E is the Young's modulus, I is the geometrical moment of inertia of the cross section, and L is the length of the microtubules in micrometers) for 20 °C of GMPCPP-stabilized microtubules (other data not shown). This relatively small length dependency compared to other reports [9,10,18] may be attributed to different methods of estimation based on simple theoretical analyses. The average values of the flexural rigidity (average ± s.d.) that were obtained were 12.0 ± 1.70 (n = 19), 10.6 ± 1.69 (n = 17), 10.3 ± 2.18 (n = 21), and 10.1 ± 1.99 (n = 25) × 10^{-24} Nm^2 for GMPCPP-stabilized microtubules at 20 °C, 25 °C, 30 °C, and 35 °C, respectively. In the presence of GTP, the values were 8.12 ± 1.52 (n = 18), 7.30 ± 1.95 (n = 20), 6.26 ± 1.55 (n = 22), and 6.10 ± 1.92 (n = 21) × 10^{-24} Nm^2 at 20 °C, 25 °C, 30 °C, and 35 °C, respectively. These values were approximately 5-fold (for GMPCPP) and 3- to 4-fold (for GTP) higher than those of taxol-stabilized microtubules; these values were also in good agreement with those obtained elsewhere [6]. Furthermore, these results reveal the aforementioned anisotropic property of microtubules, which becomes more prominent in under taxol-free (physiological) conditions. This suggests the possibility that taxol changes the mechanical properties of microtubules. We will discuss an interpretation of this effect later.

3.2. Temperature dependence of the flexural rigidity of microtubules

Next, to avoid the influence of length dependence, we used microtubules of nearly the same length (from 8 to 12 μm) and measured the temperature dependence of their flexural rigidity from 20 to 35 °C. Interestingly, flexural rigidity decreased as temperature increased, as shown in Fig. 3. The obtained average values of the flexural rigidity (average ± s.d.) were 11.9 ± 1.10 (n = 10), 10.5 ± 0.98 (n = 8), 9.56 ± 2.29 (n = 10), and 8.90 ± 1.78 (n = 10) × 10^{-24} Nm^2 for GMPCPP-stabilized microtubules at 20 °C, 25 °C, 30 °C, and 35 °C, respectively. In the presence of GTP, the values were 8.12 ± 0.76 (n = 8), 6.60 ± 1.12 (n = 8), 5.41 ± 0.77 (n = 11), and 4.81 ± 0.80 (n = 8) × 10^{-24} Nm^2 at 20 °C,

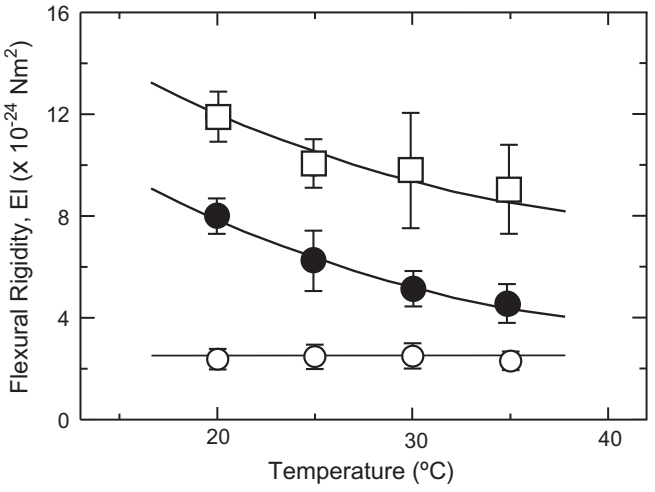

Fig. 3. Temperature dependence of the flexural rigidity of microtubules obtained from the data in Fig. 2. Microtubules of nearly the same length (from 8 to 12 μm) were used for the analysis. The error bars represent s.d. The obtained average values of the flexural rigidity (average ± s.d.) were 11.9 ± 1.10 (n = 10), 10.5 ± 0.98 (n = 8), 9.56 ± 2.29 (n = 10), and 8.90 ± 1.78 (n = 10) × 10^{-24} Nm^2 for GMPCPP-stabilized microtubules at 20 °C, 25 °C, 30 °C, and 35 °C, respectively. In the presence of GTP, the values were 8.12 ± 0.76 (n = 8), 6.60 ± 1.12 (n = 8), 5.41 ± 0.77 (n = 11), and 4.81 ± 0.80 (n = 8) × 10^{-24} Nm^2 at 20 °C, 25 °C, 30 °C, and 35 °C, respectively. The previously reported values of the flexural rigidity of taxol-stabilized microtubules (from 5 to 18 μm) showed no change in rigidity [14].

25 °C, 30 °C, and 35 °C, respectively. This result corresponds with that reported previously by Kis et al. [15,19]. Based on the results of their AFM experiments, they state that this temperature dependence is due to the changing shear modulus, which implies that the lateral interaction between the protofilaments is strongly determined by temperature [15]. While there is no reason to doubt their conclusion, some other mechanism may be at work because this trend is predominant only in the non-taxol condition in our experiments. The hydrolysis from GTP to GDP in tubulin dimers is triggered by polymerization and GTP-tubulin forms stable filaments. However, GDP-tubulin in microtubules is highly unstable and rapidly depolymerizes [3]. It is thus reasonable to postulate that hydrolysis is strongly linked to flexural rigidity. Because hydrolysis is an enzyme reaction, the rate will be proportional to temperature. As temperature increases, the decrease of GTP-tubulin in microtubules may be reflected in the rigidity, although the exact hydrolysis rates of GTP-tubulin and GDP-tubulin in the microtubule are not known. This idea is supported by the fact that GTP hydrolysis is sometimes incomplete during polymerization, that is, GTP-tubulin remnants remain even in older parts of microtubules when using a new antibody against GMPCPP [20]. Furthermore, this idea explains why the microtubules stabilized by taxol have no temperature dependence; it is because almost all of the tubulin in taxol-stabilized microtubules would be GDP-tubulin (note that there was no GTP in the experimental buffer in the measurements for taxol-stabilized microtubules, as mentioned above).

Taxol binds stoichiometrically to the β-subunit of the tubulin dimer and is commonly used in vitro to stabilize microtubules against the depolymerization induced by Ca^{2+}, cold temperature, and dilution after GTP hydrolysis has occurred [21]. Most research groups (including our group) agree that taxol decreases the rigidity of microtubules [6], although one group has reported the opposite [22]. Mechanochemical models, and molecular simulation studies have proposed that taxol may function by reducing the flexural rigidity of microtubules [23,24]. These authors also postulated that this increase in flexibility allows the microtubule to counteract the conformational changes induced by nucleotide hydrolysis and keeps the protofilaments in a straight conformation, which results in a stable microtubule. This idea is also supported by the recent observation that individual protofilaments bound with taxol are significantly less curved or kinked than protofilaments bound with either GDP or GMPCPP [25]. In this study, we showed that a length and temperature dependence on rigidity were not observed in taxol-stabilized microtubules. Our results suggest that the mechanical properties of microtubules must be changed through taxol binding despite the fact that the conformational change is counteracted by GTP hydrolysis, which implies that the tubulin itself was in a GDP state. Thus, microtubules would be more likely to have an anisotropic property in GTP-bound tubulin. To clarify this, future studies should focus on visualizing the GTP (not GMPCPP) state of tubulin, and the moment of the conformational change induced by hydrolysis must also be captured. This may shine new light on microtubules regulated not only by MAPs but also by hydrolysis. Such research would clarify how microtubule rigidity is regulated in the cell.

References

[1] J. Howard, Mechanics of Motor Proteins and the Cytoskeleton, Sinauer Associates, Sunderland, MA, 2001.
[2] K. Kawaguchi, Energetics of kinesin-1 stepping mechanism, FEBS Lett. 582 (2008) 3719–3722.
[3] J. Howard, A.A. Hyman, Growth, fluctuation and switching at microtubule plus ends, Nat. Rev. Mol. Cell Biol. 10 (2009) 569–574.
[4] M. Kavallaris, Microtubules and resistance to tubulin-binding agents, Nat. Rev. Cancer 10 (2010) 194–204.
[5] S. Kasas, G. Dietler, Techniques for measuring microtubule stiffness, Curr. Nanosci. 3 (2007) 79–96.
[6] T. Hawkins, M. Mirigian, Y.M. Selcuk, J.L. Ross, Mechanics of microtubules, J. Biomech. 43 (2010) 23–30.
[7] H. Felgner, R. Frank, M. Schliwa, Flexural rigidity of microtubules measured with the use of optical tweezers, J. Cell Sci. 109 (1996) 509–516.
[8] F. Gittes, B. Mickey, J. Nettleton, J. Howard, Flexural rigidity of microtubules and actin filaments measured from thermal fluctuations in shape, J. Cell Biol. 120 (1993) 923–934.
[9] C. Li, C.Q. Ru, A. Mioduchowski, Length-dependence of flexural rigidity as a result of anisotropic elastic properties of microtubules, Biochem. Biophys. Res. Commun. 349 (2006) 1145–1150.
[10] F. Pampaloni, G. Lattanzi, A. Jonás, T. Surrey, E. Frey, E.L. Florin, Thermal fluctuations of grafted microtubules provide evidence of a length-dependent persistence length, Proc. Natl. Acad. Sci. USA 103 (2006) 10248–10253.
[11] I.A. Schaap, B. Hoffmann, C. Carrasco, R. Merkel, C.F. Schmidt, Tau protein binding forms a 1 nm thick layer along protofilaments without affecting the radial elasticity of microtubules, J. Struct. Biol. 158 (2007) 282–292.
[12] E. Nogales, S.G. Wolf, K.H. Downing, Structure of the alpha beta tubulin dimer by electron crystallography, Nature 391 (1998) 199–203.
[13] E. Nogales, M. Whittaker, R.A. Milligan, K.H. Downing, High-resolution model of the microtubule, Cell 96 (1999) 79–88.
[14] K. Kawaguchi, S. Ishiwata, T. Yamashita, Temperature dependence of the flexural rigidity of single microtubules, Biochem. Biophys. Res. Commun. 272 (2008) 895–899.
[15] A. Kis, S. Kasas, A.J. Kulik, S. Catsicas, L. Forró, Temperature-dependent elasticity of microtubules, Langmuir 24 (2008) 6176–6181.
[16] K. Kawaguchi, S. Ishiwata, Nucleotide-dependent single- to double-headed binding of kinesin, Science 291 (2001) 667–669.
[17] L. Cassimeris, D. Gard, P.T. Tran, H.P. Erickson, XMAP215 is a long thin molecule that does not increase microtubule stiffness, J. Cell Sci. 114 (2001) 3025–3033.
[18] M. Kurachi, M. Hoshi, H. Tashiro, Buckling of a single microtubule by optical trapping forces: direct measurement of microtubule rigidity, Cell Motil. Cytoskeleton 30 (1995) 221–228.
[19] A. Kis, S. Kasas, B. Babic, A.J. Kulik, W. Benoit, G.A. Briggs, C. Schonenberger, S. Catsicas, L. Forro, Nanomechanics of microtubules, Phys. Rev. Lett. 89 (2002) 248101.
[20] A. Dimitrov, M. Quesnoit, S. Moutel, I. Cantaloube, C. Poüs, F. Perez, Detection of GTP-tubulin conformation in vivo reveals a role for GTP remnants in microtubule rescues, Science 322 (2008) 1353–1356.
[21] P.B. Schiff, J. Fant, S.B. Horwitz, Promotion of microtubule assembly in vitro by taxol, Nature 277 (1979) 665–667.
[22] B. Mickey, J. Howard, Rigidity of microtubules is increased by stabilizing agents, J. Cell Biol. 130 (1995) 909–917.
[23] V. Van Buren, L. Cassimeris, D.J. Odde, Mechanochemical model of microtubule structure and self-assembly kinetics, Biophys. J. 89 (2005) 2911–2926.
[24] A. Mitra, D. Sept, Taxol allosterically alters the dynamics of the tubulin dimer and increases the flexibility of microtubules, Biophys. J. 95 (2008) 3252–3258.
[25] C. Elie-Caille, F. Severin, J. Helenius, J. Howard, D.J. Muller, A.A. Hyman, Straight GDP-tubulin protofilaments form in the presence of taxol, Curr. Biol. 17 (2007) 1765–1770.