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Analysis of the migration behaviour of single microtubules in electric fields

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Abstract

By video contrast microscopy, individual microtubules formed from pure tubulin in the presence of taxol were studied in constant electric fields. At nearly physiological conditions, i.e., in a buffer at pH 6.8 and 120 mM ionic strength, suspended microtubules moved towards the anode with an electrophoretic mobility of approximately $2.6 \times 10^{-4}\,cm^2/V\,s$, corresponding to an unbalanced negative charge of 0.19 electron charges per tubulin dimer. Strikingly, this value is lower by a factor of at least 50 than that calculated from crystallographic data for the non-assembled tubulin dimer. Moreover, the taxol-stabilized microtubules had an isoelectric point of about pH 4.2 which is significantly lower than that known for the tubulin monomers. This indicates that microtubule formation is accompanied by substantial changes of charge distribution within the tubulin subunits. Constant electric fields were shown to affect also the orientation of microtubules gliding across a kinesin-coated surface at pH 6.8.

Keywords: Tubulin; Kinesin; Electrophoretic mobility; Isoelectric point; Orientation; Effective charge; Dipole moment

Microtubules are proteinaceous eukaryotic organelles essentially involved in various obligatory cellular mechanisms, such as chromosome segregation, maintenance of cell shape, and intracellular vesicle transport. They represent hollow cylinders with outer diameters of about 25 nm and lengths in the micrometer range and consist of tubulin which is a globular protein, existing in an alpha and a beta form. Under native conditions, both forms constitute heterodimers. The dimers associate in a chain-like manner resulting in so-called protofilaments [1]. Due to the dimeric character of tubulin and the strong alpha–beta alternation one end of the protofilament is terminated by an alpha subunit and at the opposite one by a beta subunit. This provides the protofilament with a certain kind of polarity. The microtubule wall is organized in such a way that the protofilaments are associated laterally with same polarity. Therefore, the microtubule also appears as a polar structure with a plus and a minus end. Under conditions of steady state, at the plus end more dimers are added than lost and at the minus end more dimers are released than new ones re-associate. This (chemical) polarity is a very important feature for microtubule functioning. It is the basic property for direction-dependent cellular events, e.g., vesicle transport.

At physiological temperature and in the presence of magnesium ions and GTP, purified tubulin is able to reconstitute microtubules. The potency of tubulin to self-assemble in vitro into microtubules and polymorphic assemblies [1] might provide a basis to produce novel nanometer-structured materials. An increasing number of publications [2–4] strengthens expectations that microtubules can be involved in such bio nanotechnological developments. One of the key problems in this regard is the alignment of a large number of microtubules with parallel and especially isopolar orientation. Based on the postulated dipole character of tubulin [5–7], we tried to use electric fields to obtain arrays of parallel and/or isopolar microtubules and to steer microtubules gliding across a kinesin-coated surface into a desired direction.

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In this context, we used for the first time video-enhanced microscopy to analyse the behaviour of both individual microtubules in suspension and individual microtubules gliding across a kinesin-coated surface in electric fields in cell-free environment. The results are also believed to contribute to a better understanding of electric properties of microtubules and consequently to improve the understanding of molecular mechanisms of microtubule-dependent processes within cells.

Materials and methods

Microtubule preparation. The tubulin was purified from porcine brain homogenates by two cycles of temperature-dependent disassembly/reassembly [8], followed by phosphocellulose column chromatography [9]. To study suspended microtubules in the electric fields, the tubulin (6.3 mg/ml) was supplemented with 1 mM GTP and 10 μM paclitaxel (Sigma–Aldrich Chemie, Taufkirchen, Germany) as assembly promoter and incubated for 20 min at 37 °C.

Kinesin preparation. The kinesin was purified from porcine brain high-speed homogenate supernatants by a combined procedure of ion exchange chromatography, microtubule affinity-binding, and gel filtration [10], using a buffer consisting of 50 mM imidazole, 50 mM KCl, 0.5 mM $MgCl_2$, 0.5 mM EGTA, 0.1 mM EDTA, and 0.5 mM dithiothreitol, pH 6.8 (IEEM buffer).

Microtubule suspensions in the electric field. To study microtubule suspensions in electric fields a special observation chamber was constructed. Therein, a microscopic slide was covered by double-adhesive tape (100 μm thick) in which a stripe 2.2 mm in width and about 50 mm in length was cut resulting in a channel with corresponding dimensions (Fig. 1). For reasons of symmetry concerning the electroosmotic flow, this channel was covered by a microscopic slide of the same glass material as the support. Two holes of about 1 mm diameter were drilled near the ends by a diamond tool into the cover. Two plastic blocks with central holes of about 1-ml volume, functioning as buffer reservoirs, were connected with the channel via the cover holes. The electric contact from the reservoirs to the power supply was provided by platinum wires. The magnitude of dc voltage applied could be varied in the range between 0 and 100 V, resulting in a maximum field strength of about 20 V/cm.

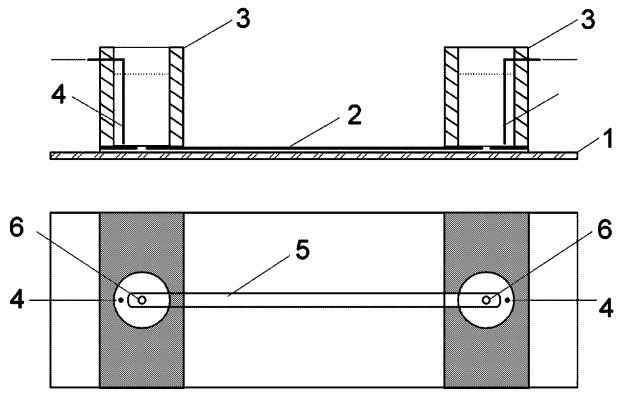

Fig. 1. Device for investigation of microtubules in electric fields. Side view (upper part), top view (lower part). (1) Microscopic slide, (2) cover with holes, (3) buffer reservoirs, (4) platinum wires, (5) channel ($50 \times 2.2 \times 0.1\,mm^3$ or 0.04 mm, for microtubule suspensions or gliding microtubules), (6) holes in the cover.

To observe individual microtubules during measurement, the microtubule stock suspension (6.3 mg/ml tubulin) was diluted 40–100-fold with PEM buffer (20 mM PIPES, 80 mM NaCl, 1 mM EGTA, 0.5 mM $MgCl_2$, pH 6.8), containing 10 μM paclitaxel. The ionic strength of this buffer corresponds to about 120 mM. In both cases, suspended and gliding microtubules (see below), the holes in the glass cover were sealed with agarose gel (1% in PEM buffer) to prevent fluid flow due to unequal buffer levels in the reservoirs, but maintaining the passage of the electric current through the channel.

The electrophoretic mobility (B) depends on viscosity of the buffer and consequently on temperature. Therefore, to obtain quantitative data on electric properties of microtubules the viscosity and the specific resistance of the buffer were measured as a function of temperature. The different experiments were performed at 25 ± 2 °C. The expected variation of viscosity within this range is about 10%. The influence of temperature within the range between 23 and 27 °C on the electrophoretic mobility was neglected as insignificant in this case.

The microtubules were visualized by an Axiophot microscope (ZEISS, Jena, Germany) equipped with a Chalnicon video camera, type C2400-01, and the image processing system Argus 20 (both obtained from Hamamatsu Photonics Deutschland, Herrsching, Germany). Image processing was performed following instructions of Weiss and Maile [11]. For suspended microtubules a special phase contrast objective (40×/0.60) enabling to adjust the thickness of the cover glass was used.

For determination of the electrophoretic mobility of suspended microtubules, the velocity of single microtubules in the electric field was measured. To exclude the influence of the electroosmotic flow, the measurements had to be carried out in so-called stationary layers in which the electroosmotic flow is zero. The exact level of the two layers was calculated according to Komagata [12]. The measurements were done in the lower stationary layer situated at one fifth of the channel height.

The velocity of migrating microtubules was determined using the speed function of the Argus image processor. To obtain precise results, only a low field strength of 1–5 V/cm could be applied, otherwise the velocities were too high.

Parallel to the measurement of microtubule migration velocity, the electric current was determined. The effective electric field strength was calculated using the formula

$$E = \frac{I\rho}{bh}, \tag{1}$$

where I is the electric current, ρ is the specific resistance of the buffer, b and h are the channel width and height, respectively.

Because of fluctuations of some non-specific particles within the samples caused by Brownian motion the quality of the phase contrast image became rapidly poor. Additionally many microtubules disappeared from the focal plane during the measurement. Due to these effects, the velocities of only 10 microtubules were measured in any given experiment, but in total $n = 14$ different experiments each with freshly prepared specimens were performed.

Gliding microtubules in the electric field. Using IEEM buffer, paclitaxel-stabilized microtubules and kinesin were mixed at room temperature resulting in final concentrations of 40 μg/ml tubulin and 70 μg/ml kinesin. Following the addition of bovine serum albumin (final concentration: 5 mg/ml), paclitaxel (10 μM), and magnesium ATP (0.5 mM) and a 10-min preincubation at room temperature, this mixture was placed into the channel (pretreated with 5 mg/ml albumin for 5 min) similar to that described for the study of microtubule suspensions in the electric field. However, the channel was only 40 μm high and a glass slip (170 μm thick) of 60 mm length with two drilled holes was used as cover. To observe gliding microtubules an objective 100×/1.3 for differential interference contrast was used.

For the statistical analysis of the gliding direction, a large number of microtubules were observed moving across a virtual line oriented perpendicular to the field direction. All microtubules passing the line

from left to right and vice versa were counted (independently of the angle under which the line was crossed).

Results

Calculation of tubulin dimer charge and tubulin dipole moments

The overall charge and the dipole moment of the tubulin dimer were determined on the basis of the crystallographic data published for the tubulin by Nogales et al. [13], which are available from the protein data bank (PDB entry, tub) in connection with the CHARMM and TINKER software packages [14]. Excluding the C-terminal region, the tubulin dimer was found to carry 10 negative elementary charges. In addition, the dielectric constant of tubulin, which is $\varepsilon = 4.0$, as well as the charge distribution on the protein (Fig. 2) were calculated using TINKER. The corresponding results are summarized in Table 1.

Experimental determination of tubulin net charge within microtubules

To quantitatively characterize the charge of tubulin dimers within microtubules under defined buffer conditions, the migration of suspended microtubules in electric fields was investigated. After application of an electric field microtubules in a buffer with pH 6.8 were seen to move immediately to the positive electrode indicating a negative effective charge. The velocity of this movement varied between 1 and 8 μm/s in dependence on the electric field strength and the ionic strength (Fig. 3). The electrophoretic mobility of the microtubules was numerically determined using the formula

$$B = \frac{v}{E}, \tag{2}$$

where v is the migration velocity and E the electric field strength.

At nearly physiological conditions, i.e., at pH 6.8 (see e.g. [15]) and a ionic strength of 120 mM (see e.g. [16]), our results yield an electrophoretic mobility of $(2.6 \pm 0.4)\ 10^{-4}\,\mathrm{cm^2/V\,s}$. This value was found on an approximately constant level within the pH range from 5.1 to 8.1 (Fig. 4A). Lowering the pH to 4.5 results in about a 70% decrease of the mobility. At pH 4.1, the microtubules moved with an electrophoretic mobility of $(0.3 \pm 0.1)\ 10^{-4}\,\mathrm{cm^2/V\,s}$ to the cathode, indicating an excess of unbalanced positive charges. Interpolation of the pH dependency between pH 4.1 and 4.5 provided an isoelectric point of 4.2 for taxol-stabilized microtubules. Moreover, lowering the ionic strength range from 120 to

Table 1
Electrostatic properties of tubulin calculated on the basis of the tubulin sequence

Property	Calculated value for the α-monomer	Value calculated by Brown [34] for the dimer
Charge (elementary charges)	−5	−10
Total dipole moment (Debye)	566	1714
Dipole components		
p_x	115	337
p_y	−554	−1669
p_z	−6	198

Notes. The tubulin sequence was taken from the paper of Nogales et al. [13]. The tail region was not included into the calculation.

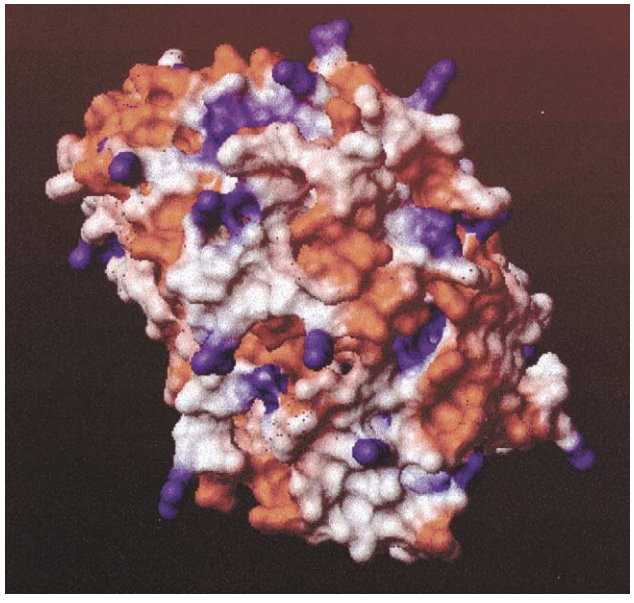

Fig. 2. Electric charge distribution on the surface of the α-tubulin monomer. Red colour indicates positive charge, blue negative, and white neutral areas.

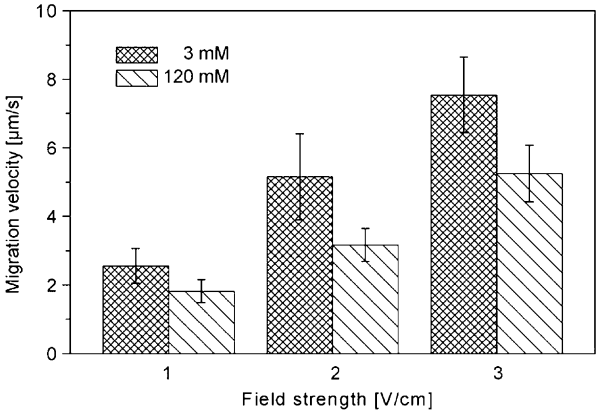

Fig. 3. Dependence of the velocity of microtubule migration in electric fields on field strength at different ionic strength. PEM buffer, pH 6.8 (undiluted and 40-fold diluted, respectively); microtubules (0.15 mg/ml tubulin) stabilized by 10 μM paclitaxel. The bars indicate the standard deviation (SD).

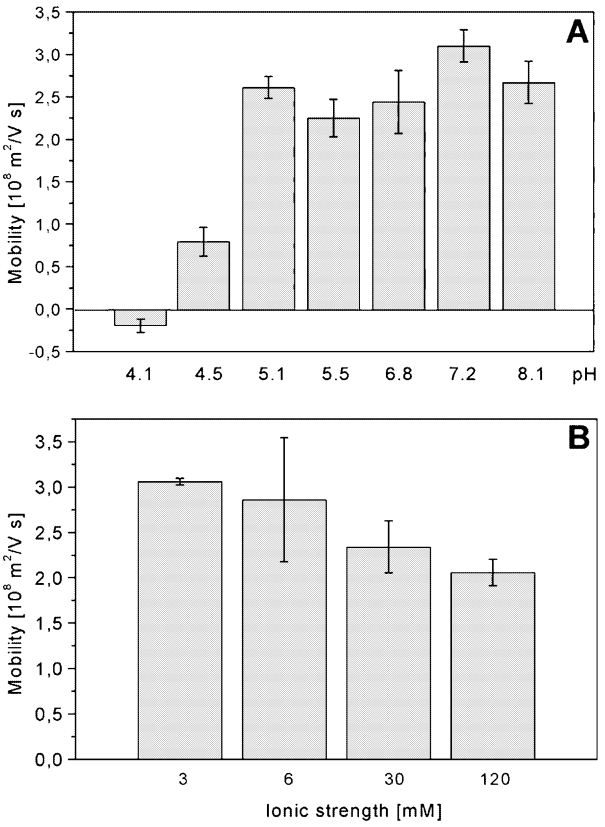

Fig. 4. Electrophoretic mobility of the microtubules at different pH (A) and different ionic strength (B). A—20 mM phosphate buffer, 41 mM ionic strength, microtubules (0.06 mg/ml tubulin), 10 μM paclitaxel; B—PEM buffer, pH 6.8 (40-, 20-, 4-fold diluted, and undiluted, respectively); microtubules (0.15 mg/ml tubulin), 10 μM paclitaxel. The bars indicate the standard deviation (SD).

3 mM resulted in an increase of motility by about 35% (Fig. 4B). Unfortunately, measurements at ionic strength below 3 mM could not be performed as the microtubules disassembled under these conditions. In addition, no differences in their migration characteristics could be found between the shortest (2–3 μm) and longest (about 15 μm) microtubules.

At the existence of an unbalanced charge, two forces affect microtubule movement in electric fields: firstly, the electric force

$$F_e = qE, \tag{3}$$

where q is the charge and secondly, the force of the viscous medium acting on the migrating microtubules

$$F_v = fL\eta v, \tag{4}$$

where f is a dimensionless factor, regarding the shape and average orientation of microtubules, L, v are microtubule length and velocity, respectively, and η is the dynamic viscosity of the buffer. For a cylinder freely moving in a fluid the dimensionless coefficient f depends on the length-to-diameter ratio $L/2r$. Using the formulas given by Hunt et al. [17]

$$f_{par} = \frac{2\pi}{\ln(L/2r) - 0.2} \tag{4a}$$

and

$$f_{per} = \frac{4\pi}{\ln(L/2r) + 0.84} \tag{4b}$$

one obtains the coefficients $f_{par} = 1.23$ and $f_{per} = 2.05$ for a microtubule with $2r = 25$ nm and $L = 5$ μm for the cases of parallel and perpendicular motion to the cylinder axis, respectively. However, it is known that microtubules reveal on their surface flexible protrusions composed of about 12–16 amino acids at the C-termini of the tubulin monomers. These protrusions can be as long as 4 nm which increases the efficient diameter of the microtubule and as a consequence its drag coefficient. The corresponding correction for the C-termini with $2r = 33$ nm yields $f_{par} = 1.30$ and $f_{per} = 2.14$. Calculating the effective value of f as

$$f = (f_{par} + 2f_{per})/3 \tag{4c}$$

yields $f = 1.86$.

In the stationary state when the electric force and the counteracting frictional force are balanced ($F_e = F_v$) the equation

$$\frac{q}{L} = f\eta B \tag{5}$$

can be used to calculate the charge per length q/L from B. With $\eta = 0.925 \times 10^{-3}$ kg/m s for the dynamic viscosity of the buffer at a temperature of 25 °C, $f = 1.86$, and $B = 2.6 \times 10^{-8}$ m^2/V s the calculation gives $q/L = 4.5 \times 10^{-17}$ A s/μm, or expressed in multiples of the elementary charge e^- ($= 1.6 \times 10^{-19}$ A s), approximately $q/L = 280$ e^- per micrometer. Under our conditions, i.e., in absence of microtubule-associated proteins, the mean number of protofilaments for a population of microtubules is about 12 [18,19]. With this number and the dimer length of 8 nm, the calculation yields about 0.19 e^- as the average charge per tubulin dimer. The electric force acting on a microtubule of $L = 5$ μm at a field strength of $E = 10$ V/cm was calculated to be $F_e = 0.2$ pN.

Contrary to our expectation based on reports about the dipole character of tubulin [5–7], there was no pronounced tendency for an orientation of the microtubule axis in field or any other direction. This was also not the case at the highest field strength used which was about 20 V/cm. Under extended application of the maximum field strength the microtubules disassembled under our conditions.

Gliding microtubules in the electric field

Depending on microtubule length and the density of kinesin bound to the surface, an immediate or a delayed influence of the electric field became visible. Usually, it

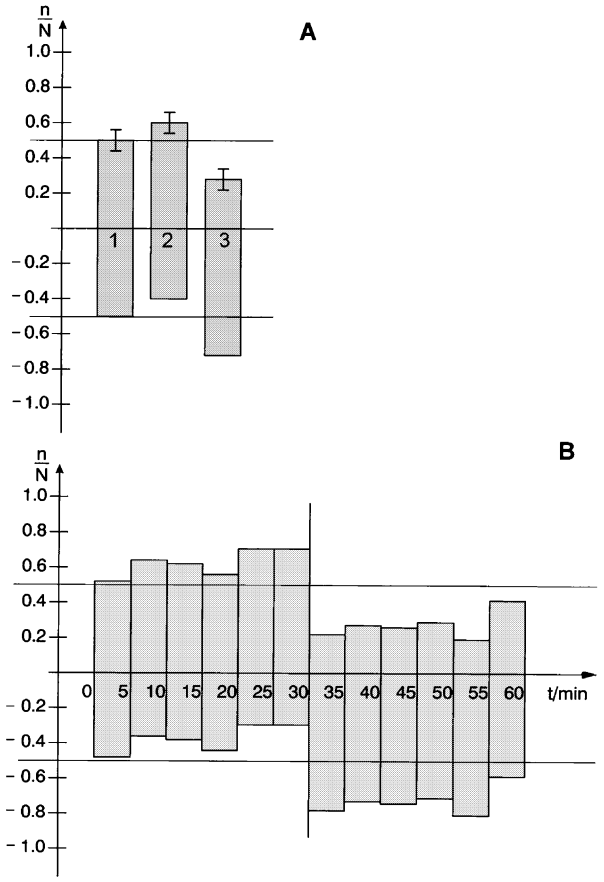

Fig. 5. Influence of electric fields on gliding microtubules. n/N is the fraction of microtubules gliding to the right (positive values) or left (negative values). Field strength 12.5 V/cm. (A) 1 control without field, 2 after field application, 3 after reversal of field polarity. (B) Time dependence of the gliding direction after switching on the field. At $t = 30$ min the field was reversed.

took 1–2 min until a directional change was observed after switching on the field or after changing field direction. However, at low kinesin densities (about 400 molecules/μm^2), assuming that all kinesin was bound to the glass, immediately more microtubules started to move from the negative electrode towards the positive one than vice versa. The results of a representative experiment are illustrated in Fig. 5.

Discussion

By experiments with suspended microtubules in electric fields, the present study demonstrates that at physiological pH (about 6.8) microtubules have a negative net charge. For the first time, the net charge of the tubulin dimer within microtubules has been determined: it amounts on average 0.19 elementary charges per dimer. It is striking that this value is at least about 50 times lower than that calculated for the tubulin dimer on the basis of the crystallographic data [13].

Microtubules are known to be maintained within a cell at nearly neutral pH and to be surrounded by numerous cations, interacting with the negative charge surface of the tubulin. Also in cell-free environment it is necessary to add physiologically relevant cations (Mg^{2+}, monovalent alkaline ions) to stabilize tubulin and microtubules. The discrepancy between the theoretically determined tubulin dimer charge and that determined by measurements of microtubules migrating in electric fields is assumed to be mainly due to masking of free charges by the sodium ions from the buffer. At least, for the C-terminal region of the tubulin dimer it has been reported that monovalent cationic counter ions tend to neutralize the charges [20]. Measurements at different ionic strengths (3–120 mM) seem to corroborate this hypothesis (Fig. 4B). It has to be taken into account that at 3 mM the concentration of cations still exceeds the concentration of tubulin dimers by a factor of about 2000–5000. To get visible effects the ionic strength had still to be reduced by some orders of magnitude. But, unfortunately we were not able to decrease the ionic strength of the buffer to much lower values as the microtubules disassembled rapidly in these cases.

The isoelectric points of alpha and beta tubulin are found at pH 5.45–5.65 and pH 5.30–5.45, respectively [21,22]. Approaching the buffer pH from 6.8 to these values should theoretically result in a decrease of electrophoretic mobility of the microtubules. However, our pH dependency with suspended microtubules did not reveal significant differences down to pH 5.1 (Fig. 4A). Only at pH 4.5 the mobility became significantly lower. It has to be pointed out that down to pH 4.5, i.e., below the isoelectric points of alpha and beta tubulin monomers, the microtubules migrated to the anode, still indicating a negative net charge. This observation leads to some interesting considerations: The determination of the isoelectric point of a protein is usually performed under denaturing conditions. The question arises what isoelectric points have the tubulin monomers in the native state. In addition, it is not clear how tubulin dimerization and microtubule assembly affect the isoelectric points. Our experiments indicate that the isoelectric point of a microtubule (determined to be at about pH 4.2) is remarkably lower than that of the tubulin monomers.

It has also to be taken into account that taxol is used in this study to stabilize the microtubules. Stabilization is an essential precondition to dilute the microtubule suspension to such a degree which allows the observation of single microtubules. It is known that taxol binding causes a hydrophilic cleft in beta tubulin to be converted into a hydrophilic surface [23]. Such structural changes can be assumed to be accompanied by changes in the electrostatic properties of tubulin and/or the microtubules.

One of the motivations of the present study was to align microtubules in electric fields. However, using highly diluted suspensions of taxol-stabilized microtubules we could not observe a preferential orientation of the microtubule axis in the field or any other direction. To explain possible mechanisms of microtubule orientation in electric fields some considerations on tubulin dipole moments were made: The y- and the z-components of the tubulin dimer dipole moment (see Table 1) are defined to be oriented perpendicular to the microtubule surface. Due to the circular arrangement of the tubulin dimers forming the microtubule wall, the dipole moment components directed in y- and z-directions compensate each other, especially when the microtubules have an even protofilament number, as it was the case in this study. Thus, only the x-components can contribute to the dipole moment of the whole microtubule. To estimate the relation between the energy of the dipole-field interaction and the thermal energy, the potential energy of a 5-μm long microtubule dipole in an electric field of $E = 10\,\mathrm{V/cm}$ for $p_x = 337\,\mathrm{Debye}$ (see Table 1) was calculated by the formula $W_{\mathrm{dipole}} = Ep_{\mathrm{micr}}$ (dipole moment vertical to field direction) to be $W_{\mathrm{dipole}} = 8.3 \times 10^{-21}\,\mathrm{W\,s}$. The thermal energy is on the order of magnitude of $W_{\mathrm{thermal}} = kT$ ($k = 1.38 \times 10^{-23}\,\mathrm{W\,s/K}$ is the Boltzmann constant and T the absolute temperature). With $T = 298\,\mathrm{K}$ the thermal energy results in $W_{\mathrm{thermal}} = 4.1 \times 10^{-21}\,\mathrm{W\,s}$. The energy of the dipole-field interaction is only by a factor of about 2 higher than the thermal energy, which is obviously not sufficient for a detectable alignment in electric fields of moderate strength as our experimental results showed. However, it has to be mentioned that in cases where thermal effects were widely excluded an orientation of microtubules was observed [24,25]. Furthermore, the group of Vassilev [26–28] demonstrated that pulsed electric fields were able to align microtubules during the phase of their formation from tubulin dimers. To understand the complex behaviour of microtubules in electric fields not only was the dipole moment calculated on the basis of the tubulin sequence but also some other components have to be considered to contribute to the dipole moment formation in and around a microtubule:

(a) the dipole moment obtained as a result of net charge neutralization by counter charges in neighbouring monomers.
(b) the induced dipole moment arising from the motion of mobile electrons (or protons) between equivalent locations inside the protein, against the opposite charge background. Assuming 1–2 mobile elementary charges per protein multiplied by a charge distance of 4 nm, an induced dipole moment per dimer of $p \sim 200$–$400\,\mathrm{Debye}$ was estimated.
(c) the dipole moment of the highly negatively charged C-terminus tail. But, the contribution of the C-terminus tail to the dipole moment has so far neither been imaged nor calculated.

Considering the discrepancy between our results and those of other authors (see [25–28]), it has to be taken into account that the microtubules used in these studies were formed in the presence of microtubule-associated proteins which are known to bind to the microtubule surface at the highly negatively charged C-terminus. The interaction of microtubule-associated protein with tubulin dimers should also result in changes of charge distribution in the microtubules.

Based on our measurements performed on suspended microtubules, an electric force of 0.2 pN was calculated for a 5-μm long microtubule at 10 V/cm. The force exerted by only one kinesin molecule is about 4–8 pN [17,29–31], which is 20–40 times greater than the value exerted by the electric field. While the electric field acts constantly (dc) on the microtubule, motor proteins provide bursts of activity only when their motor domain-containing heads perform work. Assuming for the sake of simplicity that the motor domains are active only over 1–5% of the time of the mechanochemical cycle [32], several motor molecules acting asynchronously could provide roughly the same time-averaged force as the electric field effect on the microtubule.

In the case of gliding microtubules thermal effects also do not play a significant role because they are bound to the substrate by kinesin molecules. Under our standard conditions, more than one kinesin molecule perform work on a microtubule so that we are sure that the relative influence of the electric field is even weaker as mentioned above. There are two phenomena which have to be considered when the microtubules are bound to the glass substrate. First, the influence of the electroosmotic flow has to be discussed. The glass surface is negatively charged and in the electric field positive ions enriched near the surface move to the negative electrode causing a fluid flow in the same direction. The microtubules glide on the surface where this fluid flow is strongest. Therefore, the electroosmotic flow counteracts the influence of the field on microtubules. Second, the degree to which the field influences microtubules depends on the strength of the kinesin-mediated binding to the glass surface, which can vary due to several factors. Both these effects could contribute to explain why the orientation of the microtubules in the field is not complete and that the results differed slightly from one experiment to the other. Regardless of these effects, also in the gliding assay the microtubules exhibited at pH 6.8 a definite tendency to move to the positive electrode. To explain the influence of the electric field on microtubules gliding across kinesin-coated glass surfaces the following mechanism is proposed: Normally a gliding microtubule is bound by some kinesin molecules along its length, but the leading end is bound more weakly because it must find new binding sites on the surface coated by statisti-

cally distributed kinesin molecules [33]. The electric force can act efficiently only on this weakly bound leading end, turning it gradually in the direction to the positive electrode. Orientation of the whole microtubule in field direction seems to be a stable state; when the leading part deviates from this direction it will be turned back by the electric force. According to this hypothesis, the electric field might exert a steering function in kinesin-based microtubule-gliding.

Conclusions

Using video contrast microscopy to follow the migration of single microtubules in constant electric fields, for the first time the electrophoretic mobility of microtubules and the average net charge of the tubulin dimer in a microtubule at physiological pH and ionic strength were calculated. We demonstrated that under these conditions most of the negative charges determined theoretically on the basis of the crystallographic data of tubulin were compensated, possibly by binding of buffer cations. In addition, it was shown that the isoelectric point of a taxol-stabilized microtubule is significantly lower than that of the alpha and beta tubulin monomers. Finally, due to the negative net charge of microtubules at pH 6.8, constant electric fields might be used to steer kinesin-mediated microtubule gliding into a desired direction.

Acknowledgments

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