Analytical Biochemistry 397 (2010) 67–72

Contents lists available at ScienceDirect

Analytical Biochemistry

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

Recovery of tubulin functions after freeze-drying in the presence of trehalose

Eduarda Dráberová [a], Vadym Sulimenko [a], Tetyana Sulimenko [a], Konrad J. Böhm [b], Pavel Dráber [a,*]

[a] Laboratory of Biology of Cytoskeleton, Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, CZ-142 20 Prague 4, Czech Republic
[b] Leibniz Institute for Age Research – Fritz Lipmann Institute, D-07745 Jena, Germany

ARTICLE INFO

Article history:
Received 13 July 2009
Received in revised form 22 September 2009
Accepted 8 October 2009
Available online 13 October 2009

Keywords:
Cytoskeleton
Tubulin
Stability
Freeze-drying
Bionanodevices

ABSTRACT

Microtubules represent cytoplasmic structures that are indispensable for the maintenance of cell morphology and motility generation. Due to their regular structural organization, microtubules have become of great interest for preparation of in vitro nanotransport systems. However, tubulin, the major building protein of microtubules, is a thermolabile protein and is usually stored at −80 °C to preserve its conformation and polymerization properties. Here we describe a novel method for freeze-drying of assembly-competent tubulin in the presence of a nonreducing sugar trehalose. Even after prolonged storage at ambient temperature, rehydrated tubulin is capable of binding antimitotic drugs and assembling to microtubules that bind microtubule-associated proteins in the usual way. Electron microscopy confirmed that rehydrated tubulin assembles into normal microtubules that are able to generate motility by interaction with the motor protein kinesin in a cell-free environment. Freeze-drying also preserved preformed microtubules. Rehydrated tubulin and microtubules can be used for preparation of diverse in vitro and in vivo assays as well as for preparation of bionanodevices.

Microtubules are dynamic polymers that are essential for many cellular functions such as cellular organization, intracellular transport, and cell division, to name just a few. They usually consist of 13 laterally associated protofilaments forming a cylinder whose external diameter is approximately 25 nm. Each protofilament is made up of α/β-tubulin dimers that are able to self-assemble in a head-to-tail fashion under the control of guanosine triphosphate (GTP).[1] Within cells, microtubules are anchored in microtubule-organizing centers (MTOCs) as centrosomes. The microtubule lattice is characterized by multiple protein–protein contacts of neighboring tubulins. Tubulin also binds divalent ions (e.g., Mg^{2+}, Ca^{2+}), antimitotic drugs (e.g., taxol, vinblastin, nocodazole), and a large number of microtubule-associated proteins (MAPs). Microtubules are known to interact with so-called motor proteins (e.g., kinesins, dyneins) that use adenosine triphosphate (ATP) hydrolysis to move along microtubule tracks [1]. Maintenance of proper three-dimensional structure of tubulin dimers is essential for assembling tubulin to microtubules, binding ligands, and associated proteins.

Stabilized microtubules are major components in experimental motility assays. In the gliding assay, surface-bound motors propel microtubules, whereas in the bead assay, motor protein-coated cargo moves along surface-adsorbed microtubules. Nanotransport systems based on microtubules, kinesins, and ATP are used in newly emerging biobased nanodevices [2]. Microtubules can be arranged into ordered arrays to build up artificial tracks along which different cargo materials can be transported by motor proteins [3–5]. Directed growth of microtubules can be facilitated by selective patterning of centrosome arrays on glass slides [6]. Alternatively, controlled directional microtubule gliding could be used for concentration of molecules and sensitive detection [7]. Standard batches of tubulin are necessary for efficient fabrication of such devices. Adequate techniques of long-term storage of microtubule-based systems before their activation are also highly desirable.

Tubulin is a thermolabile molecule that converts to a nonpolymerizing state within several hours on ice [8]. For this reason, one of the essential steps in preparation of tubulin for in vitro and in vivo studies is to ensure proper storage that preserves its polymerization properties. Tubulin is usually rapidly frozen in liquid nitrogen and stored in small aliquots at −80 °C or in liquid nitrogen. However, repeated freeze–thaw cycles completely deteriorate

* Corresponding author. Address: Laboratory of Biology of Cytoskeleton, Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, Videnska 1083, CZ-142 20 Prague 4, Czech Republic. Fax: +420 241 062 758.
E-mail address: paveldra@img.cas.cz (P. Dráber).

[1] *Abbreviations used:* GTP, guanosine triphosphate; MTOC, microtubule-organizing center; MAP, microtubule-associated protein; ATP, adenosine triphosphate; MTP-2, microtubule protein after two cycles of polymerization/depolymerization; DMSO, dimethyl sulfoxide; EGTA, ethyleneglycoltetraacetic acid; DTT, dithiothreitol; BCA, bicinchoninic acid; BSA, bovine serum albumin; IgM, immunoglobulin M; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Cy3, indocarbocyanate; TUB 2.1–Cy3, Cy3-conjugated TUB 2.1 antibody to β-tubulin; PBS, phosphate-buffered saline; SD, standard deviations; BRB80, 80 mM Pipes [pH 6.8], 1 mM EGTA, and 0.5 mM GTP.

0003-2697/$ - see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.ab.2009.10.016

the assembly properties of tubulin. Unfortunately, freeze-drying also destroys the capacity of tubulin to polymerize [9]. Therefore, techniques enabling safe long-term storage of active tubulin or microtubules at ambient temperature are required to improve manipulation, transport, and standardization of tubulin assays.

Previous studies have shown that sugars with known cryopreservative properties possess the capability to protect proteins under dehydration-induced stress [10,11]. In particular, the nonreducing disaccharide trehalose (α-D-glucopyranosyl-α-D-glucopyranoside) has a remarkable ability to preserve labile proteins during desiccation [12,13] and subsequent storage at higher temperature [14].

Here we report on the polymerization and binding characteristics of rehydrated tubulin freeze-dried in the presence of trehalose. Our data show that trehalose completely protected tubulin from its degradation during freeze-drying and long-term storage at ambient temperature. Rehydrated tubulin is fully assembly competent and binds antimitotic drugs and associated proteins, and microtubules have typical morphology. Thus, rehydrated tubulin can be used in diverse in vitro and in vivo assays based on microtubules.

Materials and methods

Protein preparation

Microtubule protein (MTP-2) was purified from porcine brain by two temperature-dependent cycles of assembly and disassembly [8]. A third polymerization step was performed in the presence of 500 mM Pipes and dimethyl sulfoxide (DMSO) to reduce the quantity of microtubule-associated proteins [15]. Subsequent purification of tubulin was performed by phosphocellulose chromatography [16]. The eluted tubulin in column buffer (100 mM Pipes [pH 6.9], 1 mM ethyleneglycoltetraacetic acid [EGTA], 1 mM $MgCl_2$, 1 mM dithiothreitol [DTT], and 0.5 mM GTP) was supplemented with GTP to 1 mM and polymerized by sodium glutamate added to a final concentration of 1.0 M. Pelleted microtubules were resuspended in BRB80 buffer (80 mM Pipes [pH 6.8], 1 mM EGTA, and 1 mM $MgCl_2$) supplemented with 0.1 mM GTP and depolymerized by cold. Tubulin concentration in supernatant was determined by measuring the absorbance at 280 nm using an extinction coefficient at 280 nm of 115,000 M^{-1} cm^{-1}. Recycled tubulin was more than 98% pure, and it was stored in liquid nitrogen. Thermostable MAPs containing MAP2 and tau proteins were isolated from MTP-2 as described previously [17]. Protein concentration was determined by means of a bicinchoninic acid (BCA) protein assay kit (Thermo Scientific, Rockford, IL, USA) using bovine serum albumin (BSA) as standard.

Freeze-drying and reconstitution

Purified tubulin or MTP-2 was freeze-dried (FreeZone 2.5, Labconco, Kansas City, MO, USA) for 15 h at −50 °C in the presence or absence of trehalose or sucrose (Sigma–Aldrich, Prague, Czech Republic) at a final concentration of 0.25 M. This concetration was previously found to be optimal for preserving binding activities of labile immunoglobulin M (IgM) monoclonal antibodies during freeze-drying [18]. The sugars were added into protein solution in a dry state. For convenient handling of small amounts of freeze-dried material, the samples were frozen as 20- to 60-μl aliquots in liquid nitrogen. Tubulin was freeze-dried at concentrations ranging from 50 to 400 μM. Freeze-dried preparations were stored in a dessicator at ambient temperature (~25 °C) for various time periods or at 50 °C for 1 week. Samples were rehydrated at 4 °C with distilled water to the original sample volume, incubated on ice for 20 min, diluted to the required concentration in BRB80, and centrifuged at 300,000*g* for 5 min. Tubulin concentration in supernatants was determined by measuring the absorbance at 280 nm. Taxol-stabilized microtubules were prepared by stepwise polymerization of purified tubulin at a concentration of 20 μM in BRB80 containing 1 mM DTT and 1 mM GTP. Tubulin was incubated at 37 °C with increasing concentrations of taxol from 2 to 200 μM as described previously (http://mitchison.med.harvard.edu/protocols.html). The reaction mixture at a tubulin concentration of 8 μM was then freeze-dried in the presence or absence of trehalose.

Turbidimetric measurement

The microtubule assembly was monitored by turbidimetry [19] at 350 nm and 37 °C using a recording spectrophotometer (UV-260, Shimadzu, Duisburg, Germany) with a temperature-controlled cuvette holder. The resulting assembly mixture contained tubulin in BRB80 supplemeted with GTP and glycerol at final concentrations 1 mM and 3.0 M, respectively. Tubulin was polymerized at concentrations ranging from 8 to 20 μM. In some cases, polymerization was performed in the presence of nocodazole (Sigma–Aldrich) at concentrations ranging from 0.5 to 10 μM or in the presence of 10 μM taxol (a gift from the National Cancer Institute, Bethesda, MD, USA). Assembly was also performed in the absence of glycerol. In that case, the assembly mixture contained 15 μM tubulin and 0.5 mg/ml of thermostable MAPs in BRB80 supplemented with 1 mM GTP. To distinguish between the turbidity caused by microtubule formation and that caused by nonmicrotubular aggregates, the turbidity drop after 15 min of incubation at 4 °C was recorded.

Colchicine binding assay

The binding of [^{3}H]colchicine to tubulin was determined by the modified DEAE–cellulose filter assay [20]. [^{3}H]Colchicine (75.5 Ci/mmol, Canberra Packard, Schwadorf, Austria) was diluted to 2.5 Ci/mmol with unlabeled colchicine (Sigma–Aldrich) [21]. The reaction mixture contained 5 or 1 μM tubulin in BRB80 with 1 M sucrose and 4.4 nM [^{3}H]colchicine. Test tubes were incubated at 37 °C for 3 h and then placed in icewater until filtered through four pieces of DE-81 filter disc (Whatmann) as described previously [20]. [^{3}H]Colchicine was determined with a Tri-Carb 2250TR Liquid Scintillation Analyzer (Packard Instrument, Meriden, CT, USA) using a BetaMax cocktail (ICN Biomedicals, Costa Mesa, CA, USA).

Sedimentation assay

Tubulin (20 μM) in BRB80 supplemented with 10 μM taxol and 1 mM GTP was incubated for 30 min at 37 °C. Taxol-stabilized microtubules (6 μM tubulin) were mixed with MAPs or BSA at a final concentration 0.13 mg/ml and incubated for 30 min at 37 °C. The incubation mixture was layered onto a cushion of 4 M glycerol in BRB80 containing 10 μM taxol and was pelleted at 43,000 rpm for 20 min at 25 °C in an MLS50 rotor (Beckman Coulter, Fullerton, CA, USA). The protein contents of the starting mixtures, supernatants, and pellets were determined on 7.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Pellets were resuspended in SDS sample buffer that equalled 0.5 volume of the supernatant.

Microtubule spin-down assay and immunofluorescence

Spin-down assay was performed as described previously [22]. Tubulin (18 μM) in BRB80 supplemented with 1 mM DTT was polymerized in the presence of 1 mM GTP for 20 min at 37 °C. After fixation with 1% glutaraldehyde, microtubules were centrifuged

through a 10% glycerol cushion on glass coverslips, postfixed in cold methanol, and used for immunostaining. Preparations after spin-down assay were stained for tubulin with indocarbocyanate (Cy3)-conjugated TUB 2.1 antibody to β-tubulin (TUB 2.1–Cy3, Sigma–Aldrich) diluted 1:600.

Freeze-dried taxol-stabilized microtubules were rehydrated, diluted in BRB80 with 10 μM taxol, and attached to glass coverslips pretreated with 0.2% poly(diallyldimethylammonium chloride) solution (Sigma–Aldrich) during a 15-min incubation at 25 °C. Samples were washed in BRB80 with 10 μM taxol, fixed with 3% paraformaldehyde in BRB80, and stained after washing in phosphate-buffered saline (PBS) with TUB 2.1–Cy3. They were examined with an Olympus A70 Provis fluorescence microscope.

Microtubule gliding assay

Microtubule gliding was examined in a narrow flow glass chamber as described previously [23]. The microtubules, prepared from 20 μM tubulin and stabilized by 10 μM taxol, and human neuron-specific kinesin KIF5A [24] were mixed in buffer (100 mM imidazole, 100 mM NaCl, 0.5 mM $MgCl_2$, 10 μM taxol, and 0.5 mM MgATP, pH 6.8), resulting in final concentrations of 0.4 μM tubulin and 1.4 μM kinesin, and were immediately transferred into the flow chamber. The gliding activity was monitored by video-enhanced differential interference contrast microscopy using an Axiophot microscope (Carl Zeiss, Göttingen, Germany) equipped with a Chalnicon 2400-01 video camera and the Argus 20 image processing system (Hamamatsu Photonics, Herrsching am Ammersee, Germany), tracking 25 microtubules per measurement.

Negative stain electron microscopy

The taxol microtubule suspension (20 μM tubulin) was dropped (5 μl) onto a Parafilm sheet. The microtubules were attached to formvar and carbon-coated 400-mesh copper grids (Sigma–Aldrich) by placing the grids onto the droplet surface. After 2 min, the grids were taken off and washed twice (5 s each) by placing them onto 20-μl droplets of warm BRB80 buffer containing 10 μM taxol. The microtubules were stained for 10 s by 0.5% uranyl acetate and were air-dried. Samples were evaluated by a Zeiss CEM 902 microscope (Carl Zeiss, Oberkochen, Germany).

Results

Assembly properties of rehydrated tubulin

Highly purified tubulin was either stored in liquid nitrogen or freeze-dried and kept under various storage conditions. Rehydrated tubulin freeze-dried in the absence of trehalose had very low capacity to form microtubules. On the other hand, the inclusion of 0.25 M trehalose protected tubulin capability to assemble into microtubules after rehydration. A typical example of turbidimetric measurement is shown in Fig. 1A. To verify that the increase in absorbance value reflects formation of microtubules rather than aggregated material, the polymerized reaction mixture was placed on ice for 15 min. Absorbance of tubulin freeze-dried in trehalose then returned to its original level. Subsequent increase in temperature to 37 °C led to repeated polymerization (not shown). Critical concentrations of tubulin stored in liquid nitrogen and tubulin freeze-dried with trehalose were 6.9 and 7.2 μM, respectively. Recovery of tubulin polymerization was observed in preparations freeze-dried at tubulin concentrations ranging from 50 to 400 μM. More than 96% of tubulin was soluble after rehydration and centrifugation of samples freeze-dried at such concentrations. Comparable solubility was retained in samples centrifuged after storage, at the same concentrations, in liquid nitrogen. However, tubulin concentration in samples used for freeze-drying or storage in liquid nitrogen affected the assembly properties (see Supplementary Fig. 1 in supplementary material). Samples stored at relatively high concentrations (400 and 200 μM) contained a higher proportion of active tubulin than those stored at tubulin concentrations of 100 and 50 μM. Therefore, 200 μM tubulin was routinely used for freeze-drying.

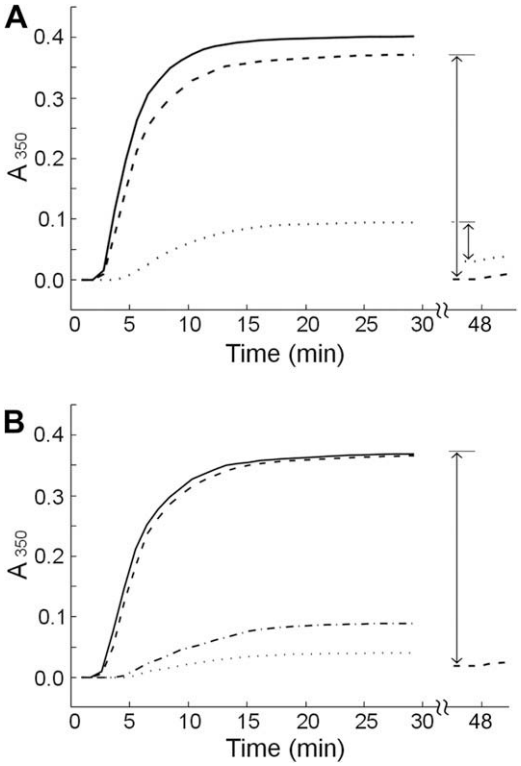

Fig. 1. Assembly properties of rehydrated tubulin in the presence of glycerol. (A) Comparison of tubulin assembly at 37 °C using tubulin stored in liquid nitrogen (solid line) and freeze-dried with trehalose (dashed line) or without trehalose (dotted line). A drop in the assembly extent at 4 °C is depicted. Assembly was carried out at a tubulin concentration of 15 μM in the presence of 3.0 M glycerol. Freeze-dried samples were stored for 3 months at 25 °C. (B) Comparison of tubulin assembly in samples stored at different temperatures. Shown are tubulin freeze-dried with trehalose and stored for 3 months at 25 °C (control) (solid line), and tubulin freeze-dried with trehalose (dashed line), with sucrose (dashed and dotted line), or without sugar (dotted line) and stored for 1 week at 50 °C. Assembly was at a tubulin concentration 15 μM.

A favorable effect of trehalose was clearly evident after 2 years of storage at ambient temperature. Repeated polymerization tests from the same batch of tubulin yielded similar results. Trehalose also preserved polymerization of tubulin that was freeze-dried in the MTP-2 form (not shown).

Trehalose had a unique protective effect on tubulin stored at elevated temperature. A typical comparison of tubulin polymerization in samples freeze-dried in the presence or absence of trehalose and stored for 1 week at 50 °C is shown in Fig. 1B. Tubulin assembly properties were preserved only when freeze-drying was carried out in the presence of trehalose. The nonreducing disaccharide sucrose did not protect at this temperature.

To rule out the possibility that rehydrated tubulin forms microtubules due to a strong stimulatory effect of glycerol on microtubule assembly, turbidimetric experiments were also performed in the presence of thermostable MAPs instead of glycerol. In the absence of glycerol, rehydrated tubulin assembled similarly as tubulin stored in liquid nitrogen (Fig. 2). Collectively, these data demonstrate that purified tubulin freeze-dried in the presence of

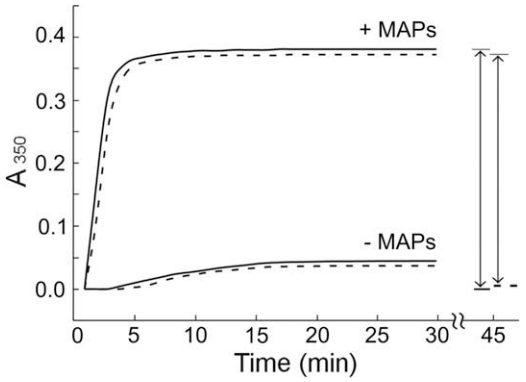

Fig. 2. Assembly properties of rehydrated tubulin in the presence of MAPs. Shown is a comparison of tubulin assembly at 37 °C using tubulin stored in liquid nitrogen (solid line) and freeze-dried with trehalose (dashed line) in the presence (+MAPs) or absence (−MAPs) of thermostable microtubule-associated proteins. A drop in the assembly extent at 4 °C is depicted. Assembly, without glycerol, was carried out at a tubulin concentration of 15 μM in the presence of 0.5 mg/ml MAPs. Freeze-dried samples were stored for 3 months at 25 °C.

trehalose is active and competent for microtubule assembly after prolonged storage at ambient temperature.

Binding of antimitotic drugs

To test the capacity of rehydrated tubulin to bind antimitotic drugs, we made use of nocodazole, which inhibits microtubule polymerization, and taxol, which decreases the critical concentration of tubulin and enhances tubulin assembly. Both drugs were used at the same concentration of 10 μM. Whereas nocodazole completely inhibited microtubule formation, taxol substantially enhanced polymerization both of reference tubulin stored in liquid nitrogen and of rehydrated tubulin (see Supplementary Fig. 2A). To define the concentration of nocodazole that inhibited microtubule assembly by 50% (IC_{50}), tubulin assembly was carried out in the presence of nocodazole at different concentrations (see Supplementary Fig. 2B). The IC_{50} values of tubulin stored in liquid nitrogen and tubulin freeze-dried with trehalose were 1.26 and 1.40 μM, respectively. A direct comparison of the ability of tubulin stored either in liquid nitrogen or in freeze-dried form to bind radioactively labeled colchicine was performed by means of the DEAE–cellulose filter assay. Whereas 4810 ± 212 dpm was detected in controls and 4424 ± 228 dpm was detected in rehydrated samples at a tubulin concentration of 5 μM, 969 ± 105 dpm was detected in controls and 1052 ± 88 dpm (means ± standard deviations [SD], $n = 3$) was detected in rehydrated samples at a tubulin concentration of 1 μM. Collectively, these data indicate that reconstituted tubulin retained its binding sites for antimitotic drugs.

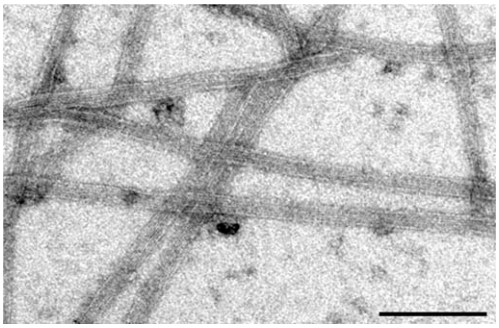

Fig. 3. Ultrastructure of taxol microtubules prepared from freeze-dried tubulin. Scale bar = 200 nm.

Taxol-stabilized microtubules were also examined by negative stain electron microscopy. A typical morphology of microtubules formed by protofilaments was disclosed (Fig. 3). There was no difference in morphology of microtubules assembled from rehydrated tubulin freeze-dried with trehalose or tubulin stored in liquid nitrogen.

Immunofluorescence visualization of microtubules

To find out whether or not trehalose can preserve preformed microtubules, taxol-stabilized microtubules were freeze-dried with or without trehalose. After 3 months of storage at ambient temperature, microtubules were rehydrated, attached to coverslips, fixed, and stained with anti-tubulin antibody. Fluorescence microscopy revealed microtubules in preparations containing trehalose (Fig. 4A), but microtubules were lacking in preparations without trehalose (Fig. 4B). Long microtubules were detected when tubulin freeze-dried with trehalose was rehydrated, polymerised, and spun down on coverslips after fixation in solution (Fig. 4C). On the other hand, only a few short microtubules were observed after tubulin freeze-drying without trehalose (Fig. 4D).

Binding MAPs and kinesin

To answer the question of whether microtubules prepared from tubulin freeze-dried with trehalose are competent in binding of MAPs, sedimentation assay with microtubules and thermostable porcine brain MAPs containing high-molecular-weight MAP2 and tau proteins was performed. Microtubules were prepared by taxol-driven polymerization of rehydrated tubulin and mixed with BSA (negative control) or MAPs. After sedimentation through glycerol cushion, BSA was found only in supernatant (Fig. 5, lane 2), whereas MAPs were concentrated in microtubule pellet (Fig. 5, lane 6). Association of MAPs with microtubules was specific as in the absence of microtubules; no MAPs were detected in pellet (Fig. 5, lane 9). Thus, binding sites on tubulin for thermostable MAPs are preserved in rehydrated tubulin.

To further test the capacity of microtubules prepared from freeze-dried tubulin to interact with tubulin binding proteins, we employed the microtubule gliding assay and compared the velocities during gliding of microtubules along immobilized kinesin molecules in the presence of ATP. No difference in velocities was observed between control tubulin stored in liquid nitrogen and tubulin freeze-dried with trehalose and stored for 3 months at 25 °C. Velocity in control sample was 0.87 ± 0.09 μm s^{-1}, whereas in rehydrated tubulin it was 0.90 ± 0.10 μm s^{-1} (means ± SD, $n = 25$). A sequence of still images demonstrating the gliding of microtubules prepared from such freeze-dried tubulin is shown in Supplementary Fig. 3. These data demonstrate that the surface of microtubules prepared from tubulin freeze-dried with trehalose is fully suitable for processive movement of motor protein kinesin.

Discussion

Several lines of evidence indicate that rehydrated tubulin freeze-dried with trehalose preserves its biological activities. First, tubulin remains competent for microtubule assembly. Second, tubulin binds low-molecular-weight ligands such as nocodazole and taxol. Third, microtubules prepared from rehydrated tubulin bind MAPs and motor protein kinesin. Fourth, typical morphology of microtubules observed in electron microscopy is retained. Finally, trehalose stabilizes not only tubulin but also preformed microtubules.

Unique thermostability conferred to biomolecules by trehalose has been demonstrated for enzymes, antibodies, liposomes, human

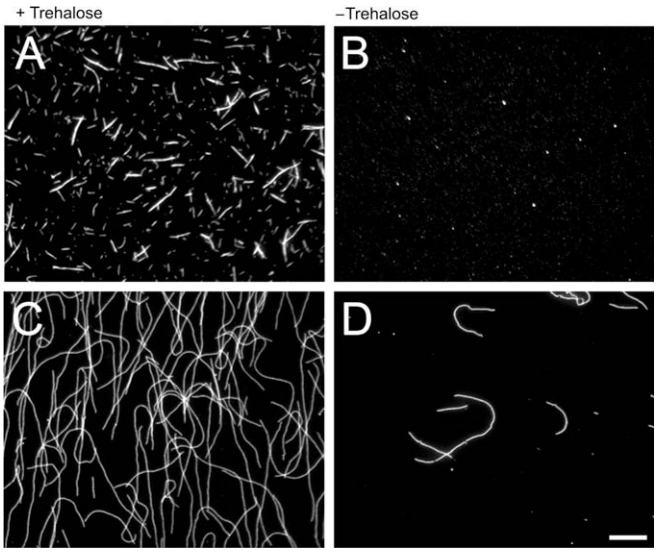

Fig. 4. Immunofluorescence analysis of microtubules. (A and B) Microtubules were prepared by taxol-driven polymerization and then freeze-dried at a tubulin concentration of 8 μM in the presence (A) or absence (B) of trehalose. Microtubules were rehydrated and attached to coverslips, and after fixation they were stained with anti-tubulin antibody. (C and D) Tubulin freeze-dried in the presence (C) or absence (D) of trehalose was rehydrated and incubated at a concentration of 18 μM with 1 mM GTP for 20 min at 37 °C. Microtubules were fixed in solution, centrifuged onto coverslips, and used for immunostaining. Scale bar = 20 μm in all panels.

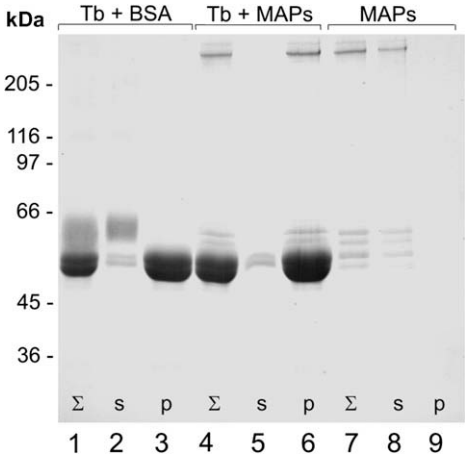

Fig. 5. Binding of MAPs to microtubules prepared from rehydrated tubulin freeze-dried with trehalose. Taxol microtubules were incubated with BSA (Tb + BSA, lanes 1–3) or thermostable MAPs (Tb + MAPs, lanes 4–6) and were pelleted through a 4-M glycerol cushion. MAPs without microtubules served as control (MAPs, lanes 7–9). Protein mixtures (Σ), supernatants (s), and pellets (p) were analyzed on 7.5% SDS–PAGE. Molecular mass markers (in kDa) are indicated on the left.

erythrocytes, and platelets, as reviewed in Ref. [11]. We showed previously that disaccharide sucrose (β-D-fructofuranosyl-α-D-glucopyranoside), which is often used as a cheap protectant in the course of the freeze-drying of proteins, also provided partial protection for freeze-dried labile monoclonal antibodies of the IgM class when stored at 50 °C [18]. However, 0.25 M sucrose failed to protect the assembly activity of tubulin when stored at higher temperature. Sucrose in the presence of chemically reactive amino groups of proteins splits into the reducing monosaccharides glucose and fructose. Thus, prolonged storage of dried proteins in reactive sugars can lead to chemical damage of the proteins [14]. The chemical stability and nonreducing nature of trehalose may be the most decisive feature in the stability of freeze-dried tubulin at high temperature. Maintenance of proper conformation of tubulin during freeze-drying in the presence of trehalose could be explained by the water replacement hypothesis assuming that the sugar molecules substitute for water from the protein hydration shell [25].

For preservation of biological activity of freeze-dried tubulin, it is important to recycle purified tubulin after phosphocellulose chromatography so as to remove assembly-incompetent tubulin. The solubility of such freeze-dried samples was very good, and aggregated tubulin represented less than 4% of total tubulin after rehydration and centrifugation of tubulin freeze-dried at a tubulin concentration of 200 μM. Tubulin can be freeze-dried in various formats, including multiwell plates for high-throughput screening for tubulin ligands such as antimitotic drugs. When rehydrated tubulin was applied at a subcritical concentration to detergent-extracted cell models, microtubules were nucleated from MTOCs (E. Dráberová et al., unpublished).

In the current study, we used tubulin and microtubule proteins prepared from porcine brain. However, assembly properties were also preserved in rehydrated mouse brain tubulin (not shown). We believe that tubulins isolated from various tissues of different species could be stabilized by the same procedure because of high phylogenetic conservation of tubulin.

Trehalose-protected tubulins are conveniently stored and transported at ambient temperature and could help to standardize bionanodevices based on both surface-adsorbed microtubules and gliding of microtubules along motor proteins. Alternatively, ordered microtubule assemblies with motor proteins could be freeze-dried in the presence of 0.25 M trehalose and stored for further application. Partial retention of motility of microtubules in gliding assay after rehydration of freeze-dried flow chamber containing microtubules and kinesin was demonstrated previously [26]. However, many microtubules were degraded after 14-day storage of flow chamber freeze-dried in the presence of 0.1 M trehalose [26]. But we showed previously that 0.1 M trehalose is not sufficient to protect the binding properties of freeze-dried antibodies [18]. We believe that higher trehalose concentration could prevent degradation of microtubules in freeze-dried flow chamber.

In conclusion, the presented results clearly demonstrate that inclusion of 0.25 M trehalose as stabilizer during freeze-drying of tubulin saves its biological activities and enables its long-term storage at ambient temperatures. The thermostability of such tubulin preparations facilitates their storage and transport. Trehalose-protected tubulin and microtubules could lead to easier standardization of assays and bionanodevices based on microtubules.

Acknowledgments

We thank Věra Richterová for help with documentation. Taxol was obtained from the Natural Products Branch Division of Cancer Treatment, National Cancer Institute, Bethesda, MD, USA. This work was supported in part by the Grant Agency of the Academy of Sciences of the Czech Republic (ASCR, grant KAN200520701), the Ministry of Education, Youth, and Sports of the Czech Republic (grant LC545), and Institutional Research Support (grant AVOZ 50520514).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ab.2009.10.016.

References

[1] T. Kreis, R. Vale, Guidebook to the Cytoskeletal and Motor Proteins, Oxford University Press, Oxford, UK, 1993.

[2] H. Hess, G.D. Bachand, V. Vogel, Powering nanodevices with biomolecular motors, Chemistry 10 (2004) 2110–2116.
[3] L. Limberis, R.J. Stewart, Toward kinesin-powered microdevices, Nanotechnology 11 (2000) 47–51.
[4] G.D. Bachand, S.B. Rivera, A.K. Boal, J. Gaudioso, J. Liu, B.C. Bunker, Assembly and transport of nanocrystal CdSe quantum dot nanocomposites using microtubules and kinesin motor proteins, Nano Lett. 4 (2004) 817–821.
[5] K.J. Böhm, R. Stracke, P. Muhlig, E. Unger, Motor protein-driven unidirectional transport of micrometer-sized cargoes across isopolar microtubule arrays, Nanotechnology 12 (2001) 238–244.
[6] W. Shang, J.S. Dordick, R.E. Palazzo, R.W. Siegel, Direct patterning of centrosome arrays as templates for the assembly of microtubules, Biotechnol. Bioeng. 94 (2006) 1012–1016.
[7] M.G.L. van den Heuvel, C. Dekker, Motor proteins at work for nanotechnology, Science 317 (2007) 333–336.
[8] M.L. Shelanski, F. Gaskin, C.R. Cantor, Microtubule assembly in the absence of added nucleotides, Proc. Natl. Acad. Sci. USA 70 (1973) 765–768.
[9] D. Soifer, A.H. Laszlo, J.M. Scotto, Enzymatic activity in tubulin preparations: I. Intrinsic protein kinase activity in lyophilized preparations of tubulin from porcine brain, Biochim. Biophys. Acta 271 (1972) 182–192.
[10] J.H. Crowe, J.F. Carpenter, L.M. Crowe, T.J. Anchordoguy, Are freezing and dehydration similar stress vectors? A comparison of modes of interaction of stabilizing solutes with biomolecules, Cryobiology 27 (1990) 219–231.
[11] J.H. Crowe, L.M. Crowe, A.E. Oliver, N. Tsvetkova, W. Wolkers, F. Tablin, The trehalose myth revisited: Introduction to a symposium on stabilization of cells in the dry state, Cryobiology 43 (2001) 89–105.
[12] J.F. Carpenter, J.H. Crowe, Modes of stabilization of a protein by organic solutes during desiccation, Cryobiology 25 (1988) 459–470.
[13] E. Israeli, B.T. Shaffer, B. Lighthart, Protection of freeze-dried *Escherichia coli* by trehalose upon exposure to environmental conditions, Cryobiology 30 (1993) 519–523.
[14] B. Roser, Trehalose drying: A novel replacement for freeze-drying, Biopharmacy 5 (1991) 47–52.
[15] E.M. Mandelkow, M. Herrman, V. Ruhl, Tubulin domains probed by limited proteolysis and subunit-specific antibodies, J. Mol. Biol. 185 (1985) 311–327.
[16] M.D. Weingarten, A.H. Lockwood, S.Y. Hwo, M.W. Kirschner, A protein factor essential for microtubule assembly, Proc. Natl. Acad. Sci. USA 72 (1975) 1858–1868.
[17] A. Fellous, J. Francon, A.M. Lennon, J. Nunez, Microtubule assembly in vitro: Purification of assembly-promoting factors, Eur. J. Biochem. 78 (1977) 167–174.
[18] P. Dráber, E. Dráberová, M. Nováková, Stability of monoclonal IgM antibodies freeze-dried in the presence of trehalose, J. Immunol. Methods 181 (1995) 37–43.
[19] F. Gaskin, C.R. Cantor, M.L. Shelanski, Turbidimetric studies of in vitro assembly and disassembly of porcine neurotubules, J. Mol. Biol. 89 (1974) 737–755.
[20] C. Tse, R.A. Doherty, An improved DEAE–cellulose filter assay for colchicine binding protein (during mouse brain development), J. Neurochem. 35 (1980) 767–774.
[21] G.G. Borisy, A rapid method for quantitative determination of microtubule protein using DEAE–cellulose filters, Anal. Biochem. 50 (1972) 373–385.
[22] L. Evans, T. Mitchison, M. Kirschner, Influence of the centrosome on the structure of nucleated microtubules, J. Cell Biol. 100 (1985) 1185–1191.
[23] K.J. Böhm, P. Steinmetzer, A. Daniel, M. Baum, W. Vater, E. Unger, Kinesin-driven microtubule motility in the presence of alkaline–earth metal ions: Indication for a calcium ion-dependent motility, Cell Motil. Cytoskel. 37 (1997) 226–231.
[24] N. Kalchishkova, K.J. Böhm, The role of kinesin neck linker and neck in velocity regulation, J. Mol. Biol. 382 (2008) 127–135.
[25] J.F. Carpenter, J.H. Crowe, An infrared spectroscopic study of the interactions of carbohydrates with dried proteins, Biochemistry 28 (1989) 3916–3922.
[26] R. Seetharam, Y. Wada, S. Ramachandran, H. Hess, P. Satir, Long-term storage of bionanodevices by freezing and lyophilization, Lab Chip 6 (2006) 1239–1242.