Imaging of self-assembled tubulin polymorphs used as metallization templates

W Habicht¹, S Behrens¹, KJ Böhm² and E Dinjus¹

¹ Institute for Technical Chemistry (ITC-CPV), Forschungszentrum Karlsruhe, PO-Box 3640, D-76021 Karlsruhe, Germany
² Leibniz Institute for Age Research - Fritz Lipmann Institute e.V., Beutenbergstraße 11, D-07745 Jena, Germany

Abstract. Tubulin, a protein isolated from eukaryotic cells, is able to self-assemble in vitro under well-defined chemical conditions into highly ordered polymorphic suprastructures such as tubules, rings or sheets. Here we report about the imaging and the morphological appearance of such tubulin assemblies utilized as templates for the deposition of metal nanoparticles or continuous metallization. The structural imaging and analyzing tools like field emission scanning electron microscopy (FESEM) with respect to different electron detectors (Inlens-SE, BSE, TE) are addressed. An EDX-unit is applied for the verification of the deposited metals. Atomic force microscopy (AFM) in tapping-mode (TM) is adopted for 3D-rendering and morphological measurements (height). Tip-surface interactions, the influence of fixation and cantilever-types are considered. Transmission electron microscopy (TEM) is applied to visualize deposited nanoparticles.

1. Introduction
Controlled metallization of self-assembled biomacromolecules offers a promising tool for developing microelectronic devices and has attracted much interest for alternative techniques in the synthesis of nanostructures. One strategy to control metal deposition into particle arrays or into a continuous metal coverage is the use of bioorganic templates with ability for self-assembly. Here we focus on structures derived from the tubulin αβ-dimers, forming a variety of polymorphs, among them tubules, rings, and sheets.¹ The tubulin heterodimers are of about 8 nm length and a diameter of 4 – 5 nm. Due to their specific molecular recognition capabilities, these tubulin building blocks are able to self-assemble under appropriate chemical conditions. The heteroatoms of the amino acid side chains located at the tubulin surface provide active binding sites for the nucleation of noble metal ions (e.g. Ag⁺, Pd₂⁺) and thus enable the template-directed metallization of the biomolecules.²,³

Among the tubulin polymorphs microtubules (MTs) are the most prominent ones occurring in vivo, having typical dimensions of about 25 nm in diameter and up to several micrometers in length. They are highly dynamic structures of a hollow cylindrical filamentous appearance formed by a parallel alignment of 13 - 14 protofilaments of alternating α- and β- tubulin monomers. Their high geometrical aspect ratio makes them applicable as a biomolecular template to form nanowires e.g. as interconnects.
in nanoelectronic devices. Ring-like tubulin assemblies are formed in vitro in the presence of Ca\textsuperscript{2+}-ions, causing the protofilaments to spiral up into a ring-shaped appearance.\textsuperscript{4} Followed by a subsequent metallization step, such nanorings form potentially stable vortex states or information storage devices.\textsuperscript{5}

Zn\textsuperscript{2+}-induced two-dimensional tubulin sheets are derived by an alternating antiparallel alignment of protofilaments into a single layer during the tubulin assembly process.\textsuperscript{4,6,7}

2. Experimental

Microtubule self-assembly was performed in vitro from purified tubulin (PC-tub). They were assembled in a buffer solution (20 mM PIPES, 80 mM NaNO\textsubscript{3}, 0.5 mM Mg(NO\textsubscript{3})\textsubscript{2}, 1 mM EGTA; pH 6.8) by adding guanosine-5\textsuperscript{'}-triphosphate (GTP) and taxol at 37 °C. Rings were assembled from tubulin (1 mg mL\textsuperscript{-1}) in glycerine (0.5 M) in the presence of Ca\textsuperscript{2+} ions by adding GTP at 37 °C. Prior to metallization, the assembled MTs and rings were fixed by glutaric dialdehyde (GA, 0.1 or 1.0 wt.\%).\textsuperscript{1,2-4,8}

Sheets were formed, e.g., in a buffer solution (100 mM MES, 250 mM Mg(NO\textsubscript{3})\textsubscript{2}; pH 6.4) by adding Zn\textsuperscript{2+} ions, GTP and taxol (after 60 min.). They were fixed with 0.1 wt.% GA. Taxol was added to suppress tubulin dynamics. GA was added to the solution to covalently cross-link the protein molecules. These procedures stabilized the protein structures against thermal and mechanical impacts and to metal-ion induced depolymerization.\textsuperscript{9,10} The synthesis of Pd- and Ag-nanoparticles and the preparation of Ag-nanowires is described in detail in the corresponding references.\textsuperscript{2-4}

Silicon substrate and TEM-grid preparation: The surface of the SiO\textsubscript{x} wafer was cleaned with piranha solution. A 100 µL droplet of the sample solution was pipetted onto the surface and blow-dried with air. The TEM-grids were prepared by pipetting a 20 µl droplet onto a carbon-filmed TEM-grid and the excess liquid removed.

Standard electron microscopy was performed using an FESEM (DSM 982 Gemini, Zeiss) with a high-brightness secondary electron (inlens-SE) detector in normal and in the low-voltage (LV) mode. An EDX-unit (Link Isis 300, Oxford Corp.) was operated to verify the metal deposition.

An atomic force microscope (AFM, Nanoscope IIIa Veeco/Digital Instruments Inc.) was operated in tapping-mode under ambient conditions in air. “Soft” silicon cantilevers (Olympus AC240, spring constant F = 1.8 N/m ± 20\%, tip radius = 8 nm), were used at resonance frequencies of ≈ 70 kHz. The amplitude set-point and the settings of the feedback loop were adjusted to keep the amount of force applied to the surface as low as possible. This is achieved by increasing the set-point A\textsubscript{SP} close to the free air oscillation amplitude A\textsubscript{0} of the cantilever and keeping the ratio A\textsubscript{SP}/A\textsubscript{0} between a value of 0.9 – 1, the range of “soft” tapping where the tip-specimen force is minimized. The scan rates varied between 0.7 – 1 Hz. The AFM images were processed by aid of WSxM®; http://www.nanotec.es.

3. Results and discussion

3.1. Native and Ag-metallized microtubules

An example of native bundles of straight aligned MTs is shown in Figure 1a. A dedicated cross-
sectional profile is depicted accordingly in Figure 1b. The MTs were deposited from solution on a thermally treated silicon wafer piece (SiO\textsubscript{x}) as described above. TMAFM measurements achieved a typical average height of 9.2 ± 0.9 nm. Outlined in Figure 2a are several MT strings, partially Ag-decorated. A relatively broad distribution of the measured heights and widths is observed for the metallized regions, related to the amount of Ag deposited onto the protein. Therefore, the metallized parts of the tubule exhibit a mean height value of 20.7 ± 3.6 nm and a width of 80.7 ± 11.2 nm (without deconvolution of tip radius), respectively (Figure 2b). The fabrication of silver nanowires obtained by hydroquinone reduction of AgNO\textsubscript{3} led to a quasi-continuous coverage of the MTs, which was confirmed by EDX measurements. The FESEM image (Figure 3) shows Ag-metallized MTs prepared on a TEM-grid and visualized by transmitted electrons (STEM-in-SEM mode). Figure 4 depicts partially Ag-metallized bundles of MTs prepared on a silicon wafer piece. In contrary to the non-metallized native MTs (figure 1a) curling and bending of the strings due to the metallization procedure was observed. The EDX line-scan shown in the inset frame of figure 4 revealed a successful Ag deposition, but the width exceeds several times the diameter of a single filament, indicating some uncontrolled metallization. The silicon-supported specimen was imaged by the high-brightness inlens-SE detector. Beam energy of 10 keV was applied to compromise between contrast and the X-ray excitation requirements. The flattening of the MTs observed by AFM compared to the nominal diameter of 25 nm is most probable caused by dehydratation, especially at lower GA concentrations. Deformation is influenced additionally by adsorption related forces between protein and substrate, the pH value etc.

3.2. Native and Ag-metallized, ring-like tubulin polymorphs

Ring-like tubulin polymorphs were produced by addition of Ca\textsuperscript{2+} ions to the assembly solution. In tubulin rings the MTs protofilaments are curved and arranged from inside out to outside in.\textsuperscript{[11]} A representative scheme of their spiral-up of protofilaments into a circular appearance is given in Figure 5. They usually arrange in three turn spirals. Derived from various height determinations by TMAFM, a pile-up of two to four spirals in axial direction is suggested.\textsuperscript{[4,11]} An inlens-SE image (Figure 6) of
TEM-grid prepared tubulin assemblies, fixed with 0.1 wt.% GA, and negatively stained with uranylacetate (0.1 wt.%) revealed the ring-like structure of the polymers. Height determinations by AFM in air under ambient conditions depend strongly on the GA-concentration used. Adjusting the cantilever’s amplitude set-point to either high or low values, i.e. operating in the soft or hard tapping regime, is of minor impact to the measured heights. The apparent heights of the native rings prepared with 1 wt.% GA were higher (10.5 ± 1.6 nm) than that with 0.1 wt.% GA (7.2 ± 1.2 nm) concentration. Anyway, the height depression of the protein measured as a function of increasing tip-sample force was found to exceed no more than 5%. The difference in height between the native rings and the metallized ones is roughly 5 nm depending on the applied metallization conditions. The measured width of the native ring-shaped structures is 71.2 nm ± 7.5 nm. In Figure 7a, native ring-like assemblies with a representative cross-section (Figure 7b) is outlined. An example of protein rings decorated with Ag-nanoparticles is depicted in Figure 8a, and a representative profile in Figure 8b. The successful deposition of Ag-nanoparticles was confirmed by TEM studies which elucidated also their spiral-like alignment on the protein surface. For the Ag-metallized rings a mean height of 12.1 ± 1.9 nm of was determined.

3.3. Native and Pd-decorated tubulin sheets
The assembly of tubulin sheets was performed in the presence of Zn\(^{2+}\) ions. Such sheets were formed by a parallel monolayer alignment of protofilaments, but with alternating polarity and inside-outside aspects. Consequently, the thickness of the sheets should be expected in the diameter range of a single protofilament (4 - 5 nm). An LV-SEM image of native sheets is presented in Figure 9a. The 2D-structures are clearly mapped by the inlens-SE detector at 3 keV primary beam energy. At this voltage charging is reduced to a minimum for the oxidized silicon target, and this corresponds to the upper crossover energy (E2) for SiO\(_2\). The AFM image (Figure 9b) revealed the flat sheet-like structures, partially folded twice and threefold. The different sheet heights are depicted in the line profile in
Figure 9c and are in accordance with the diameter of a single protofilament. Tubulin sheets decorated with Pd-nanoparticles acquired by TEM are displayed in Figure 10.

4. Summary

Three representative tubulin polymorphs have been investigated by atomic force and electron microscopic methods. Both were essential tools to explore such structures in the lower nanosize range. Metallization has been confirmed by X-ray detection. The operation of different electron detectors complemented one another and enabled a standard FESEM to a versatile instrument for many applications. If superior resolution is required in case of nanoparticles detection, only TEM provided sufficient results. TMAFM is unique in achieving structural (3D) rendering and to gain additional vertical measurement data. It is superior where SEM methods lack in contrast, even at specimen on bulk support of low conductivity.

References

[1] Unger E, Böhm KJ and Vater W Electron Microsc. Rev. 1990; 3: 355
[2] Behrens S, Rahn K, Habicht W, Böhm KJ, Rösner H, Dinjus E and Unger E Adv. Mater. 2002; 14, 22: 1621
[3] Behrens S, Wu J, Habicht W and Unger E Chem. Mater. 2004; 16: 3085
[4] Behrens S, Habicht W, Wagner K and Unger E Adv. Mater. 2006; 18: 284
[5] Zhu FQ, Fan D, Zhu X, Zhu JG, Cammarata RC and Chien C-L Adv. Mater. 2004; 16, 23-24: 2155
[6] Lowe J and Amos LA The EMBO Journal 1999; 18, 9: 2364
[7] Melki R and Carlier M-F Biochemistry 1993; 32: 3405
[8] Vater W, Böhm KJ and Unger E Cell Motility and the Cytoskeleton 1997; 36: 76
[9] Kis A, Kasas S, Babic B, Kulik AJ, Benoit W, Briggs GAD, Schoenberger C, Catsicas S and Forro L 2002 Phys. Rev. Lett. 89 248101
[10] Boal AK, Rivera SB, Miller NE, Bachand GD and Bunker BC 2004 Materials Research Society Symp. Proc. Vol. 826E pp V3.1.1-3.1.6
[11] Erickson PE and Stoffler D The Journal of Cell Biology 1996, 135: 5
[12] Joy DC and Joy CS Micron 1996, 27, 3-4: 247
[13] Zorbas V, Ortiz-Acevedo A, Dalton AB, Yoshida MM, Dieckmann GR, Draper RK, Baughman RH, Yacaman MJ, Musselman IH. J. Am. Chem. Soc. 2004; 126: 7222
[14] Poenitzsch VZ and Musselman IH Microsc. Microanal. 2006, 12: 221