 Structural Changes in Tubulin Sheets Caused by Immobilization on Solid Supports

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Supporting Information

ABSTRACT: In the presence of zinc, the protein tubulin assembles into two-dimensional sheets that are a useful model system for the study of both tubulin and microtubule structure. Tubulin sheets present an ideal protein structure for study with atomic force microscopy because they contain a two-dimensional crystalline protein lattice and retain many of the structural features of tubulin and microtubules. However, high-resolution imaging requires nonperturbative immobilization onto an appropriate imaging substrate. In this report, several substrates commonly used for scanning probe microscopy are evaluated for their ability to effectively immobilize tubulin sheets: mica, gold, highly ordered pyrolytic graphite, and carbon-coated electron microscopy grids. We hypothesize that the different intermolecular interactions presented by these substrates will affect the morphology of adsorbed tubulin sheets as well as the amount of other contaminating adsorbates. Tubulin sheets were successfully imaged on all of these substrates and structural characterization is reported. The most consistent results were obtained on carbon-coated electron microscopy grids, which preserved fine structural features of the sheets and had the least amount of contamination from the adsorption of unpolymerized tubulin. Images of tubulin sheets obtained with atomic force microscopy also compare favorably with published electron micrographs of sheets produced using similar procedures. This work demonstrates the importance of assessing substrate effects when studying two-dimensional protein crystals and identifies suitable substrates for immobilizing tubulin sheets.

INTRODUCTION

Tubulin is an important cytoskeletal protein that is the primary component of microtubules, essential intracellular structures responsible for a wide variety of functions in eukaryotic cells. As such, tubulin plays a role in intracellular transport, mitosis and meiosis, and as one of the key components of the cytoskeleton. Microtubules are the most common and important form of polymeric tubulin, in which the protein is arranged into rodlike protofilaments, which are associated laterally to form a hollow tube. However, in vitro tubulin has demonstrated the unique ability to polymerize into a wider variety of lesser known morphologies, including rings, ribbons, macrotubules, and sheets.\(^2\)\(^{-}\)\(^{15}\) Many of these polymeric structures have been important in developing an understanding of the tubulin and microtubule structure, for example, identifying factors influencing the straight versus bent conformation of protofilaments\(^3\)\(^{-}\)\(^{4}\) or the solution of the tubulin crystal structure.\(^16\) The present work focuses on immobilization of these two-dimensional (2D) crystals on solid supports for study with scanning probe microscopy and the resultant structural changes induced by substrate effects.

The assembly of tubulin into 2D crystals induced by the presence of zinc was first reported in 1976\(^6\) and has been the subject of numerous studies over the past 40 years.\(^7\)\(^{-}\)\(^{16}\) Although they have no known function in vivo, tubulin sheets have been essential in the study of microtubules. Because there are only minor differences in the tubulin structure in sheets compared to microtubules and because the protofilaments are nearly identical in both structures, sheets constitute a convenient and appealing system that has been exploited to study both dimeric and polymeric tubulin. The most successful experimental application for tubulin sheets was their use for electron crystallography which resulted in the determination of the atomic-resolution structure of tubulin.\(^16\)\(^,\)\(^{17}\) This molecular structure was subsequently combined with high-resolution cryoelectron micrographs of whole microtubules to construct an atomic resolution model of the microtubule\(^18\) which has proven invaluable in developing the current understanding of the microtubule structure and assembly. More recently, tubulin sheets have been used to study the motility of motor proteins,\(^19\) as an important control in the study of tubulin acetylation\(^20\) and as nanoscale templates for patterned deposition of nanoparticles.\(^21\)\(^,\)\(^{22}\)

Because tubulin sheets are such an attractive experimental system, we are investigating their use as model structures in the study of tubulin and polymeric tubulin with atomic force

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microscopy (AFM). AFM is an effective approach for the study of tubulin, yet has focused primarily on microtubules, for both structural and mechanical characterization. However, the cylindrical nature of the microtubule complicates AFM measurements, as the perturbative nature of AFM imaging tends to destroy immobilized microtubules, high-resolution imaging on the tightly curved surface of the microtubule is extremely challenging, and interpreting mechanical measurements requires the use of sophisticated modeling strategies such as finite element modeling. Although other non-microtubule tubulin polymers have been studied with AFM, we know of only a small number of reports of tubulin sheets studied with AFM. Thus, AFM represents a novel approach that may yield new information about the structure, mechanics, and function of tubulin.

Tubulin sheets are a nearly ideal system for AFM because of their crystallinity and ability to lie uniformly on an imaging substrate, both of which greatly facilitate imaging with scanning probe microscopies. Also, the tubulin and protofilament substructure ordinarily present in microtubules are intact, allowing the use of sheets as model substrates for structural and physical characterization of protofilaments. The smoothness of the sheets may ultimately allow for higher resolution AFM imaging than is usually possible on the highly curved surface of microtubules. 2D protein crystals in general have provided some of the best and highest resolution AFM images of proteins because they enable detailed analysis of images through, for example, image averaging or Fourier analysis. AFM has been used to study substrate-supported transmembrane proteins in buffer, 2D streptavidin crystals that selectively immobilize individual protein complexes, and ligand-induced gating of ion channels. Often in these studies, buffer pH and ionic strength are used to tune interactions of the 2D crystals with the substrate surface, thus allowing optimization of both immobilization and image quality.

Zn-induced tubulin sheets present a similarly uniform 2D protein crystal surface that is a very suitable surface for high-resolution imaging with AFM. However, they provide a unique challenge because their morphology depends on both buffer pH and ionic strength. Specifically, low pH is required to preferentially form sheets instead of tubes, and large, uniform sheets may require high salt concentration. With this consideration, conditions favorable for sheet assembly in solution do not necessarily correspond to conditions favorable for sheet immobilization on solid substrates.

Thus, the goal of the current work is to study the adsorption and immobilization of tubulin sheets for AFM imaging and to examine structural changes induced by substrate binding. The ideal substrate would exhibit the following characteristics: relatively smooth over large, micrometer-sized areas; strong but nonperturbative physical adsorption of the protein sheets; and preferential adsorption of sheets over other contaminants from solution, including unpolymerized tubulin. Toward this end, three substrates commonly used for scanning probe imaging were investigated: mica, gold, and highly ordered pyrolytic graphite (HOPG). Additionally, to allow direct comparison with previous electron microscopy (EM) work, we examined tubulin sheets immobilized on carbon-based EM grids. Below, we describe the characteristics of these substrates, practical limitations for their use, and the structure of adsorbed sheets following immobilization. This work provides a basis for the development of new methods of structural and mechanical interrogation of tubulin sheets affording greater flexibility in the types of studies of tubulin that can be accomplished with AFM.

## RESULTS AND DISCUSSION

For a direct comparison of sheet immobilization on the four substrates used in this study, Figure 1 presents representative 3 μm images of tubulin sheets on mica, gold, HOPG, and a carbon EM grid. Additionally, the relative roughness of each substrate can be assessed more quantitatively using the 2D line profiles depicted in Figure 2A–D, which were taken from the images in Figure 1A–D at the positions indicated by the horizontal line in each image. In these data, tubulin sheet growth solutions were stabilized with paclitaxel post-assembly and then deposited on the respective substrates. While tubulin sheets were observed on all substrates, differences between the morphologies of both the sheets and the underlying substrate are evident.

The mica substrate (Figures 1A and 2A) presents a very smooth, uniform, and easily prepared substrate that is often ideal as a substrate for imaging proteins. However, in this work, it was difficult to adsorb selectively only tubulin sheets on mica, as conditions favorable for growth and immobilization of tubulin sheets often resulted in a nearly uniform coating of small tubulin polymers or unpolymerized tubulin. As depicted in Figure 1A, the mica is covered with a pitted layer, and the locations of a few of the pits are indicated in Figure 1A with arrows. Scratching experiments (Supporting Information Figure S1) revealed that this layer is about 5 nm thick, consistent with a single layer of tubulin. The pits may arise from an incomplete coating of tubulin or partial dewetting of the protein layer, as described below. The measured heights of the tubulin sheets are ~6–7 nm above the adsorbed layer.
The gold surface (Figures 1B and 2B) has a granular structure typical of gold evaporated on silicon. The roughness is evident in the 2D line profile (Figure 2B) and on the surface of the sheets, indicating that they are flexible enough to adopt the structure of the underlying substrate. Imaging of the tubulin sheets on gold was the most inconsistent of all of the substrates examined.

To image tubulin sheets, a 40-fold dilution of the sheet growth solution was required, while on the other substrates, acceptable imaging conditions could be obtained with minimal or no dilution of the sheet growth solution. Attempts to image sheets without dilution resulted in a surface covered by large aggregates on the surface.

On HOPG (Figure 1C), a networked or web-like surface coating can be observed, which is characteristic of a hydrophobic surface interacting with proteins. An analogous morphology has been observed previously for collagen on HOPG,39 collagen on polystyrene,40 and for albumin on polystyrene.41 These network-like protein films are thought to arise from the hydrophobic interaction between the substrate and protein, which causes strong adsorption of the protein and then allows for subsequent dewetting of the proteinaceous layer during drying steps. As depicted in Figure 2C, the network layer is ∼2–4 nm in thickness, while the sheets are ∼5–7 nm in height above this layer. Evidence of possible damage to a sheet caused by dewetting can also be observed as holes developing in one of the sheets in Figure 3.

The most consistent and reproducible results were obtained on the carbon EM grid (Figures 1D and 2D). Following exposure to the sheet growth solution, the EM grid appears to have minimal adsorption of the unpolymerized protein on the substrate surface. The measured thickness of the sheets adsorbed on the EM grid is similar to that on the mica and HOPG, ∼5–7 nm for a single sheet. Compared to the other substrates, fine, delicate features of the sheets are most evident on the EM grid. There are many small, thin fragments and the sheets themselves have thin, ribbon-like protrusions, presumably single or groups of protofilaments. In contrast, on the other three substrates, the edges of the sheets are typically blunt, without protruding protofilaments. The existence of the more delicate structures observed on the EM grid implies that these sheets are most representative of those in the deposition solution and that there are less substrate-induced structural changes on the EM grid than on the mica, gold, or HOPG.

In both Figure 1C,D, the underlying substrates do not appear flat but rather with broad (hundreds of nanometers wide) protrusions and depressions. This was a feature that we occasionally observed on the carbon transmission EM (TEM) grids, which we attribute to deformation in the thin carbon film. In general, over the range of tens of micrometer, the TEM grids did not present a flat surface, with significant distortions around the copper mesh that supported the carbon film. However, in local areas centered between the mesh, appropriately flat areas could be found for AFM imaging. On HOPG, the broad protrusions observed in Figure 1C may be caused by terracing/steps in the underlying substrate. The presence of multiple steps/terraces was a feature frequently observed on bare substrates.

Higher resolution imaging of the EM grid (Figure 3) provides a better picture of the relative amount of nonspecific protein adsorption typical on this substrate. While the surface is not completely devoid of the unpolymerized protein, the amount of surface coating is much less than was observed on the mica and is distinct from that which was observed on the HOPG. The 2D line profile provided in Figure 3A shows this adsorbed protein to be ∼2 nm in thickness and reveals that the surface of the sheet has a roughness similar to the underlying substrate. This is analogous to that which was observed on the gold surface, indicating that the sheets are flexible enough to adopt the topography of the surface on which they are adsorbed. Also evident are some small tubulin aggregates on the sheet surface.

On the basis of the data presented above, the dominant surface–protein interaction not only influences the characteristics of the adsorbed protein layer but also affects the morphology of the sheets immobilized on the surface. The substrates tested here represent ends of a range between hydrophilic (mica and gold) and more hydrophobic (HOPG and carbon EM grid). When cleaved, the layered structure of mica exposes an atomically smooth surface which, when placed in water at neutral pH, has a net negative charge. This surface is very amenable to electrostatic interaction with charged proteins44 such that in our case, the entire mica surface is coated with a disordered layer of the protein. Conversely,
HOPG presents a hydrophobic surface where the dominant protein-to-surface interaction is via dispersion interactions, which leads to orientations of adsorbed proteins at the surface distinct from that of mica and precludes substantial hydration of the protein at the surface. This is further exacerbated during drying steps where the proteinaceous layer is dehydrated and dewets into the observed network pattern. The relative wettability/hydrophobicity of the substrates was assessed using static contact angle measurements (values reported in Supporting Information, Table 1). On the basis of macroscopic contact angles of water droplets on the surface, the carbon EM substrate is more hydrophobic than HOPG. The EM grid substrate has a chemical composition that is distinct from HOPG, as it consists of a carbon thin film that presents a relatively smooth, but probably amorphous, surface made up of a mixture of microdomains of diamond-like and graphitic carbon. At the scale of single protein adsorption, there are a wider variety of functional groups which allow for more stable adsorption of tubulin and much less dewetting of the adsorbed protein upon drying of the samples.

Paclitaxel is a drug that is known for its ability to induce polymerization of tubulin and stabilize tubulin polymers such as microtubules and tubulin sheets and is commonly used in vitro to produce tubulin polymers that are stable at room temperature. To evaluate the effects of paclitaxel stabilization on sheet growth and structure, some sheets were examined where paclitaxel was added prior to incubation and growth. Figure 4A,B depicts these solutions deposited on mica. The sheets are narrower and more multilayered than those stabilized using paclitaxel addition post-assembly, and several of the structures in Figure 4A have multiple layers that are aligned in the direction of the major axis of the polymer. The most likely explanation for this structure is that some sheets have rolled up into large tubes or "macrotubules" similar to those reported previously from sheet assembly at high pH. A closer examination of the proteinaceous layer coating the mica (Figure 4B) also shows what appear to be small, polymeric assemblies of tubulin, most on the order of 50–100 nm in length. The presence of numerous small polymers is consistent with the rapid nucleation of tubulin polymers induced by paclitaxel.

Also, consistent with our observations of sheets on gold (Figure 1B) and the EM grid (Figures 1D and 3), the surface structure of the sheet or macrotubule has adopted a roughness similar to the underlying substrate.

In addition to our primary goal of assessing the functionality of the various substrates, we also compared AFM data with previously published EM data. For direct comparison with reported electron micrographs, sheets were produced under conditions optimized for EM. Deposition of these aqueous samples onto mica substrates most often resulted in large aggregates of hundreds of nanometer in apparent height and width. These aggregates were present even in the best samples (Figure 5A) and when sheets were present. However, sheets can be observed in relatively low density on the substrate between aggregates (Figure 5A,B). The sizes of these sheets were up to several hundred nanometers in width, which can be compared with sheets prepared with a similar protocol for EM, which were reported to be up to several micrometers in width. Compared to our standard sheet growth protocol, the mica surface was much cleaner when we used these growth conditions. This is not surprising, given that the longer growth times used in this method may simply produce higher yield of polymerized tubulin such that the concentration of free tubulin is too low to noticeably contaminate the mica surface and the higher ionic strength of the growth solution reduces electrostatic interactions between the protein and substrate, correspondingly reducing the adsorption of free protein.

When determining the best conditions for assembly of tubulin sheets for this work, we developed a procedure that, in our lab, produced the most consistent and interpretable AFM data and a procedure that is well within the range of growth conditions previously reported. A survey of the literature reporting assembly of zinc-induced tubulin sheets reveals a wide range of polymerization conditions. This includes the use of piperoxane-N,N'-bis(2-ethanesulfonic acid) or 2-((N-morpholino)ethanesulfonic acid buffer, with concentrations ranging from 0.05 to 100 mM, tubulin concentrations ranging from 0.6 to 10 mg/mL, pH ranging from 5 to 6.8, temperature ranging from room temperature to 37 °C, and growth times from minutes to 24 h. On the basis of the wide range of conditions that have been used historically, we infer that the mere presence of zinc-induced sheets in solution depends only on the presence of zinc but that the yield, number, quality, shape, and uniformity of the sheets depend on growth conditions such that careful control over growth conditions is necessary to produce the largest crystalline sheets.

Unpolymerized tubulin or small tubulin oligomers were consistently present in most of the samples we analyzed and resulted in adsorbed tubulin layers along with our adsorbed sheets. A simple model of our surfaces with sheets adsorbed is presented in Figure 6, showing the sheets adsorbed over a nearly uniform layer of tubulin that was observed on mica, gold, and the TEM grid (Figure 6B) or the sheets adsorbed...
over a dewetted layer of the protein that was observed on graphite (Figure 6A).

Figures 1, 4, and 5 can be used to directly compare some of the growth methods that we tested. Our standard protocol (Figure 1) yielded sheets that were typically rectangular in shape, containing a long axis with relatively straight edges. This type of structure is consistent with previous EM work that used similar growth conditions (see for example, Figure 2 in ref 2), with the long axis corresponding to the orientation of protofilaments in the sheet. In contrast, when sheets were polymerized using long growth times and higher ionic strength, the overall shape of the sheets was less uniform and often did not contain one obvious long axis (Figure 5). This is consistent with morphologies reported previously (for example, see Figure 2 in ref 15).

CONCLUSIONS

We have examined sheets of the protein tubulin immobilized on a variety of substrates and have examined a range of experimental conditions for producing these sheets to study the effect of adsorption substrate and growth conditions on the structure of the sheets. The best substrate for visualization of the sheets with AFM was found to be carbon EM grids, which show low levels of contamination and preserve the sheets with AFM was found to be carbon EM grids, which show low levels of contamination and preserve the structure of the sheets. The best substrate for visualization of the eexperimental conditions for producing these sheets to study the overall shape of the sheets was less uniform and often did not contain one obvious long axis (Figure 5). This is consistent with morphologies reported previously (for example, see Figure 2 in ref 15).

METHODS

Purified, lyophilized tubulin was purchased from Cytoskeleton, Inc. AFM tips were silicon microlevers (NCH, Nanoworld Innovative Technologies) with nominal spring constants of 42 N/m. The substrates used in these experiments were mica (grade V-1, Electron Microscopy Sciences), carbon-coated TEM grids consisting of a copper grid coated with amorphous carbon (Cu-400CK, Pacific Grid Tech), gold-on-silicon (AU.1000, Platypus Technologies), and HOPG (SPI-2 grade, Structure Probe, Inc.). All other reagents were purchased from Sigma-Aldrich.

Prior to use, tubulin was dissolved in BRB80 buffer (80 mM K-Pipes, 1 mM ethylene glycol tetraacetic acid (EGTA), 1 mM MgCl₂, pH 6.85) at 10 mg/mL and used immediately or snap-frozen and stored at −80 °C. For growth of sheets, a wide range of solution growth conditions were tested, including concentrations of Zn²⁺ ranging from 0.1 to 5 mM, tubulin from 0.4 to 4 mg/mL, NaCl from 0 to 200 mM, pH from 4.7 to 6.0, and incubation temperature from 25 to 37 °C. The most consistent results for sheet growth were obtained by diluting tubulin to a final concentration of 2.5 mg/mL into sheet growth buffer [50 mM MES, 1 mM EGTA, 0.8 mM MgCl₂, 0.8 mM ZnCl₂, and 1 mg/mL guanosine 5’-triphosphate (GTP), pH 5.0], followed by incubation at 33 °C for 30 min. Tubulin sheets were stabilized with paclitaxel (0.025 mM), added immediately after sheet growth. In some cases, paclitaxel was added to the mixture prior to incubation to assess its effects on sheet growth. Samples were diluted to concentrations appropriate for imaging in BRB80 containing 0.025 mM paclitaxel.

Some previous work has demonstrated that long growth times (up to 24 h) and high ionic strength buffer (up to 200 mM) may be necessary to produce the highest quality, large tubulin sheets. In accord with this work, several experiments were attempted where tubulin sheets were polymerized using 4 mg/mL tubulin in buffer containing 80 mM MES, 1.25 mM MgSO₄, 1.25 mM ZnSO₄, 3 mM GTP, pH 5.3, with 100–200 mM NaCl, and incubated for 24 h at 32 °C.

Gold substrates were prepared by rinsing with ethanol and acetone followed by a 30 min exposure in a custom-built UV-ozone chamber containing an ozone-producing UV grid lamp (BHK, Inc, Ontario, CA). Mica and HOPG were freshly cleaved before every use. Carbon EM substrates were used as received. All substrates were affixed to a metal AFM specimen disc using a minimal amount of Krazy Glue. Aqueous solutions containing sheets were deposited on the substrates for 1–2 min and then blown dry with nitrogen gas. AFM imaging was conducted with a Veeco Multimode Nanoscope IIIa operating in tapping mode. To assess uniformity of the samples, typically ~10 images were collected on each substrate at different locations. Each successful imaging condition was repeated at least three times to confirm reproducibility of the samples. Representative images are presented herein.

On two samples, scratching experiments were conducted to assess the thickness of adsorbed layers of the protein. The protein was removed from a small area of the surface by imaging in contact mode at a relatively high force, corresponding to a deflection setpoint of 2 V read by the AFM photodetector with a cantilever with a nominal spring constant of 42 N/m (RTESP-300, Bruker AFM Probes). The depth of the resulting hole was then assessed by reimaging the larger surrounding area in tapping mode.

Initial characterization of the substrates prior to protein deposition was done using contact angle measurements and AFM characterization of the freshly prepared substrates. Static contact angles of ~2 μL of drops of water were measured using a rame-hart model 190 contact angle goniometer and DROPimage CA software. Surface roughness was assessed from AFM images of small (4–25 μm²) areas of each surface by calculating $R_s$ (average roughness) and $R_{rms}$ (rms roughness).
using the NanoScope AFM Analysis software. Results of these surface characterizations are reported in the Supporting Information.

**ASSOCIATED CONTENT**

1. Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.8b02475.

Data describing results of scratching experiments; contact angle measurements; and surface roughness measurements (PDF)

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Notes

The authors declare no competing financial interest.

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