TiO_2 Nanoparticles as a Soft X-ray Molecular Probe [**]

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With the emergence of soft x-ray techniques for imaging cells, there is a pressing need to develop protein localization probes that can be unambiguously identified within the region of x-ray spectrum used for imaging. TiO_2 nanocrystal colloids, which have a strong absorption cross-section within the "water-window" region of x-rays, are ideally suited as soft x-ray microscopy probes. To demonstrate their efficacy, TiO_2- streptavidin nanoconjugates were prepared and subsequently labeled microtubules polymerized from biotinylated tubulin. The microtubules were imaged using scanning transmission x-ray microscopy (STXM), and the TiO_2 nanoparticle tags were specifically identified using x-ray absorption near edge spectroscopy (XANES). These experiments demonstrate that TiO_2 nanoparticles are potential probes for protein localization analyses using soft x-ray microscopy.

Soft x-ray tomography generates 3D images of whole, hydrated cells at resolution better than 50 nm [1, 2]. High-contrast images of cellular structures are obtained because organic material absorbs roughly an order of magnitude more than water at this energy (517 eV). Minimal cell processing is required, as cells need only be frozen, and data collection is rapid (3-5 min/tomographic data set). X-ray tomography is, therefore, an appealing imaging technique for those experiments that require better resolution than is possible with light microscopy. With light microscopy, fluorescent tags are routinely used to label molecules. For x-ray microscopy, we need probes that use the inherent x-ray properties and can specifically label proteins within the cellular environment. Since x-ray transmission is sensitive to absorbance and density differences in the specimen, as is transmission electron microscopy (TEM), probes used in TEM should also work for x-ray microscopy. Current approaches for localization used with TEM include labeling with Au nanoparticles, heavy metals or photooxidation of diaminobenzidene

(DAB). Similar approaches have proven viable for soft x-ray microscopy, as demonstrated by the use of gold nanoparticles conjugated to antibodies to localize proteins in whole cells [3].

Co-localization studies with light microscopy are routinely done using probes that fluoresce at two different wavelengths. For similar studies with x-ray microscopy, we need a second tag that can be unambiguously identified, which means the probes need to have different absorption properties, such as an x-ray edge absorption. The dense DAB reaction product and the Au nanoparticles are directly visible in the soft x-ray microscope. However, the contrast mechanism of both probes is based on absorption density of the matter. No x-ray edge absorption is available for either of them within the range of the operation energy. Therefore, indistinguishable x-ray absorption properties of these probes negate the possibility to utilize them in double labeling experiments with soft x-ray microscopy. However, with the rapidly expanding field of nanoscience in biology, especially the successful application of semiconductor nanocrystals in biological imaging [4, 5 6, 7], additional nanoparticular materials that exhibit strong absorption in the soft x-ray spectrum are being developed. One promising candidate is TiO_2. TiO_2 nanoparticles have been widely used in other industries due to their photocatalytic activity and UV light absorption properties [8]. They are also highly biocompatible. A recent study demonstrated the use of TiO_2-oligonucleotide nanocomposite as a unique light inducible nucleic acid endonuclease [9]. Most importantly for soft x-ray imaging, Ti $L_{2,3}$-edge absorption lines (between 470 eV to 450 eV) fall directly within the 'water window' [O (K:543 eV) ~ C (K:284 eV)] region of the x-ray spectrum, where cellular structures generate high-contrast images. By imaging a specimen labeled with a functionalized form of TiO_2 at an energy above, and then below, the Ti $L_{2,3}$ x-ray absorption lines, one can easily distinguish the TiO_2 label from a second label (e.g. nanogold). The use of two spectrally separated probes will significantly increase the protein labeling capabilities of soft x-ray microscopy.

Triocytlphosphine oxide (TOPO) capped TiO_2 particles (**Figure 1**) were prepared using a high-temperature pyrolysis reaction [10]. The TiO_2 nanocrystals were made water-soluble through ligand exchange of surface TOPO with polyacrylic acid (PAA) [11]. Preparation of TiO_2-strepavidin nano-conjugates was recently demonstrated through the use of biotin as a bridging molecule between TiO_2

and streptavidin [12]. In this paper, a different conjugation approach was implemented using 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) as shown in **Scheme 1** [13].

A simple *in vitro* system was applied to demonstrate the biolabeling ability of the TiO_2-streptavidin conjugates. Biotinylated tubulin polymerizes *in vitro* under ideal conditions and forms characteristic filamentous structures, known as microtubules (MTs). This can be seen using streptavidin-Alexa Fluor 488 visualized with fluorescence microscopy (**Figure 2a**). Conjugation of the TiO_2 nanoparticles to the streptavidin-Alexa Fluor 488 does not interfere with the biotin-streptavidin interaction, as shown by the comparable labeling pattern with this conjugate seen in **Figure 2b.** Next, transmission electron microscopy (TEM) was used to visualize the unlabeled MTs (**Figure 2c**) as well as those tagged with the TiO_2-streptavidin (~ 6 nm particles) nanoconjugates (**Figure 2d**). Images obtained from the TEM showed small electron-dense TiO_2 particles decorating the MT. To confirm that the electron-dense spots were indeed TiO_2, selected area electron diffraction (SAED) patterns were obtained, which established the presence of TiO_2 in anatase form (anatase; I41/amd; PDF#00-021-1272, **Figure 2e**). Typically, with a reasonable amount of polycrystalline sample, a continuum corresponding to all the possible nanoparticle orientations (resembling a ring pattern) is observed [14, 15]. In the sample under investigation, however, the TiO_2 nanoparticles are sparsely distributed along the microtubules. Consequently there might be insufficient crystallites (TiO_2 nanoparticles) to form a ring and only a small, finite number of orientations possible. thus generating the pattern observed.

Scanning transmission x-ray microscopy (STXM) was then implemented to examine the TiO_2-conjugates and labeled MTs. In addition to imaging the sample at a fixed energy, STXM can provide an energy scan by focusing the beam on a sample spot and changing the photon energy [16]. This spectromicroscopy capability was used to obtain a stack scan (images at different energies) of the TiO_2 nanoparticles (**Figure 3a**). The x-ray absorption spectrum of the TiO_2 nanoparticle highlighted in red showed the Ti $L_{2,3}$-edge x-ray absorption near edge spectra (XANES) (**Figure 3b**). The Ti $L_{2,3}$-edges XANES features of individual nanoparticles are very similar to the spectra previously reported for ensembles of TiO_2 nanoparticles [8]. The Ti $L_{2,3}$-edge is caused by $2p$ to $3d$ transitions. Due to spin orbital

splitting of the 2*p* orbital, two sets of L-edge features are observed, centered around 460 eV (L_3) and 465 eV (L_2), respectively. Multiplet structures in the L_3 and L_2 edges are further evidenced, due to the Coulombic and exchange interactions of the 2*p*-3*d* and 3*d*-3*d* orbitals [17]. A Ti map image can also be obtained from STXM using the micrographs taken at two energies (450 eV and 466 eV) (**Figure 3a,** Ti map). Each individual TiO_2 nanoparticle shows distinct contrast, which indicates that the nanoparticles are effective probes for biological soft x-ray microscopy.

To demonstrate the ability to examine labeled proteins with x-ray microscopy, TiO_2 nanoparticles were conjugated to the streptavidin complex used previously (**Scheme 1**). These conjugates were then added to biotinylated MTs and examined using STXM. The absorption image shows strongly absorbing particles decorating the dense filamentous microtubule (**Figure 3c**, left panel). The particles are clearly shown to be TiO_2 particles in the titanium map (**Figure 3c**, center panel), while the carbon map image reveals the MT (**Figure 3c,** right panel). The labeling pattern of the TiO_2 conjugate is similar to that seen with TEM (**Figure 2b**), TiO_2 conjugate labeling of the MTs is punctate, rather than continuous as is seen with fluorescence microscopy. This pattern of labeling is commonly seen with immunogold electron microscopy [18], due to the steric hindrance between the bulky conjugates and the target molecules, which are 4nm monomers tightly bound to form a chain. But it is also possible that trace amounts of unconjugated streptavidin are competing with the TiO_2-streptavidin complex for binding to the biotin-tagged MT. Nevertheless the data shown here demonstrate the ability of the TiO_2 particles to specifically recognize the tubulin and demonstrate that these TiO_2 nanoconjugates can be detected with x-ray microscopy. Further refinements of the labeling procedures are in progress.

New biological imaging techniques and appropriate molecular probes are vital steps in gaining a better understanding of cellular processes. Soft x-ray microscopy is a new imaging technique optimal for imaging whole cells and obtaining 3D quantitative information at better than 50 nm resolution. Gold tags have already been used to localize a single molecular species, but additional probes are required to simultaneously examine two molecules. In this paper, we report the development of a TiO_2-streptavidin nano-conjugate as a new biological label for x-ray bio-imaging applications. This new probe, used in

conjunction with the nanogold probe, will make it possible to obtain quantitative, high-resolution information about the location of proteins using x-ray microscopy.

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Scheme Title:

Scheme 1. TiO_2-strepavidin conjugation using EDC as cross-linking agent.

Figure Captions:

Figure 1. TEM image of PAA coated TiO_2 nanoparticles.

Figure 2. TEM image of unlabeled (a) and TiO_2-streptavidin labeled (b) microtubules. (c) Selected area electron diffraction (SAED) pattern of TiO_2 (anatase) nanoparticles of the TiO_2-labeled MT sample.

Figure 3. a) STXM images TiO_2 nanoparticles. b) XANES spectrum of TiO_2 nanoparticles indicated with the arrowhead in (a). c) STXM images of TiO_2 labeled mictrotubules. The absorption image shown was taken at 466 eV. The titanium map was obtained by subtracting the O.D. (optical density) image taken at 466 eV by the O.D. image taken at 450 eV. The carbon map was obtained by subtracting the O.D. image taken at 293 eV (above carbon K-edge) by the O.D. image taken at 280 eV (below carbon K-edge).

Scheme 1

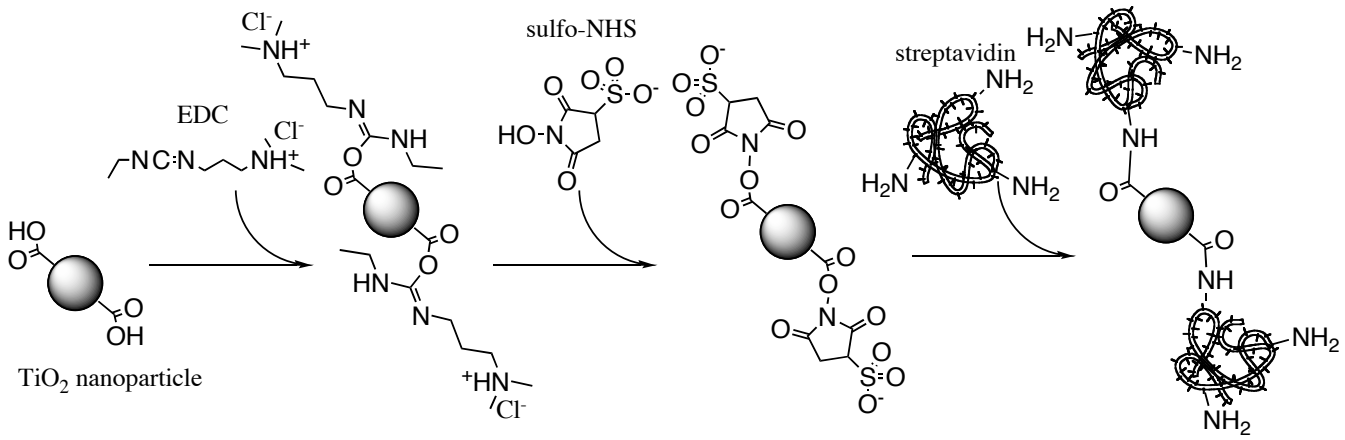


Figure 1

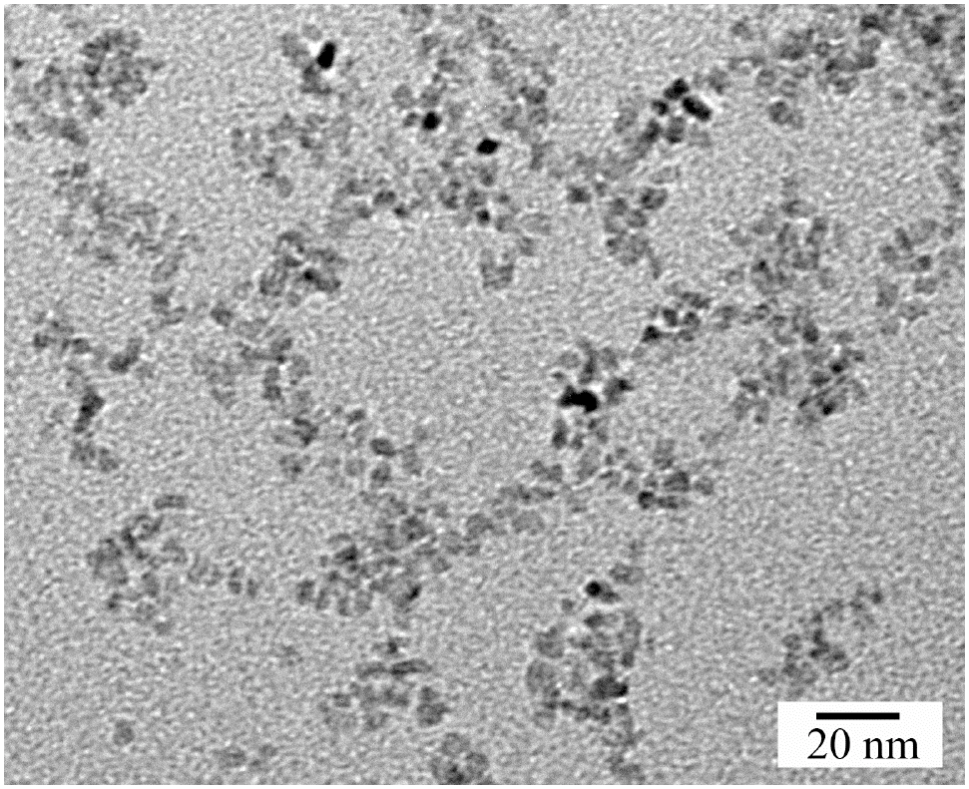


Figure 2

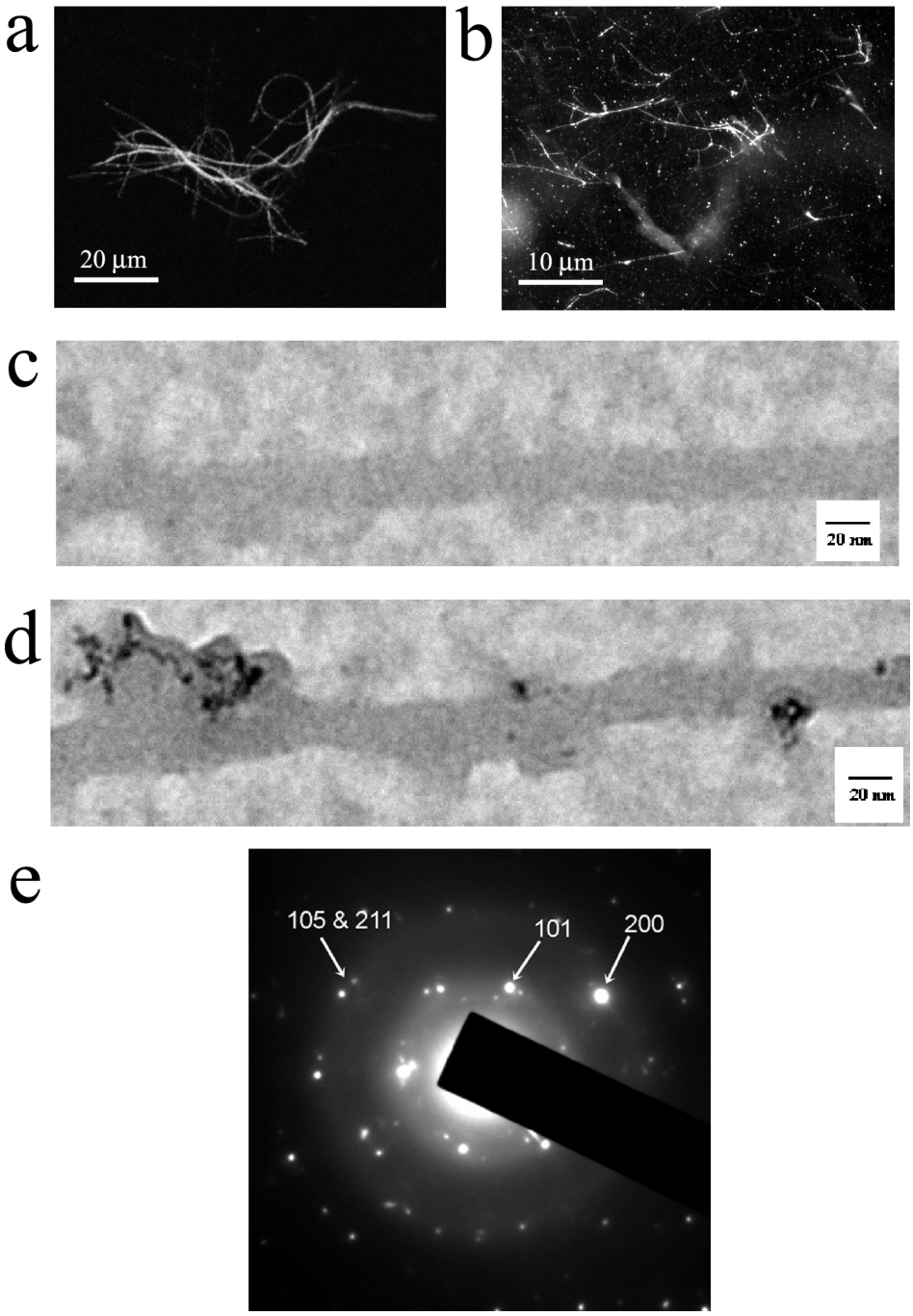
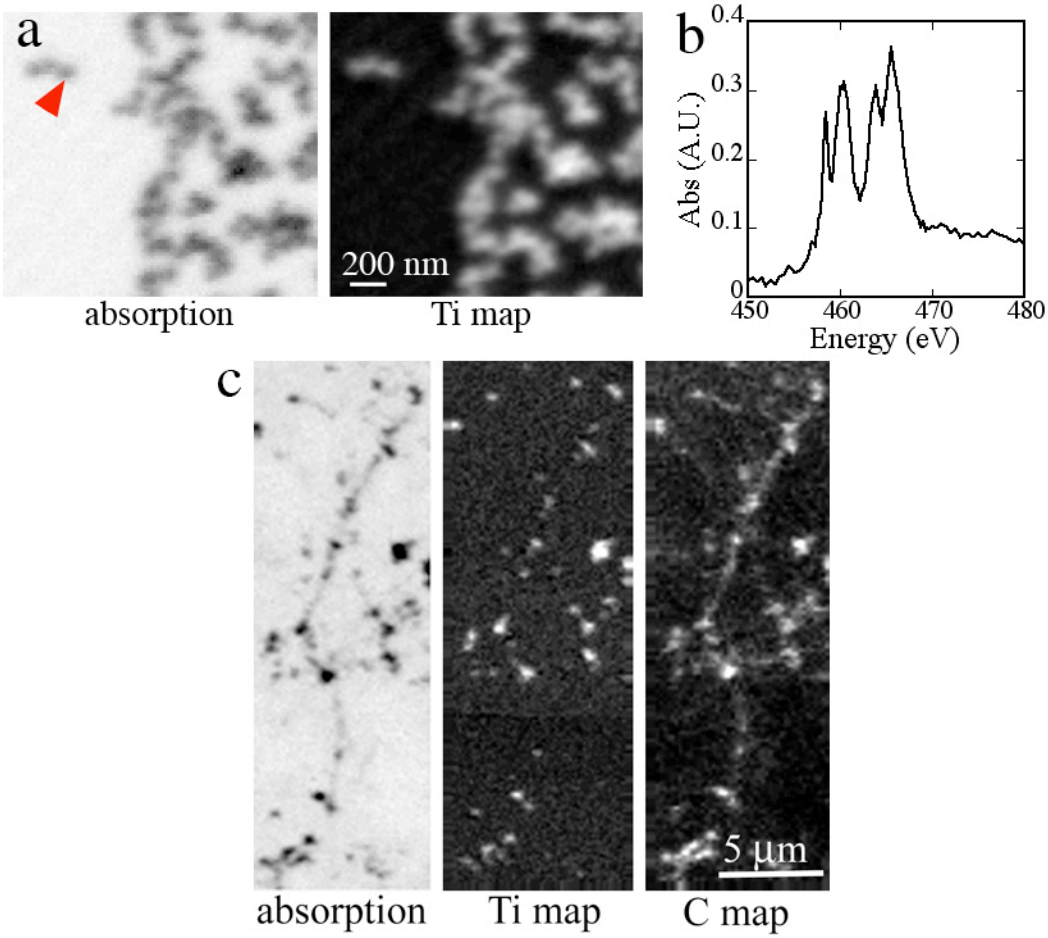


Figure 3


a
200 nm
absorption
Ti map
b
Abs (A.U.)
0.4
0.3
0.2
0.1
0
450
460
470
480
Energy (eV)
c
5 µm
absorption
Ti map
C map

Supporting Information

Materials and Methods

(1) 6-nm TiO_2 nanoparticle preparation

The 6-nm TiO_2 particles (Figure S1) were prepared using a high-temperature pyrolysis reaction [1]. Triocytlphosphine oxide (TOPO, 5.0 g, 13.1 mmol)) was degassed at 150 °C for 10 min under vacuum to remove trace water and oxygen, followed by additional heating at 200 °C for 10 min under argon atmosphere. Titanium (IV) tetrabutoxide (0.681 mL, 2.0 mmol) were then added and heated at 200 °C for another 10 min. The temperature was increased to 320 °C with subsequent rapid injection of 0.275 mL (2.5 mmol) $TiCl_4$. This solution then underwent a condensation reaction at this temperature for 2 h with vigorous stirring. Finally, the solution was cooled down to 60°C and addition of excess acetone precipitated the TiO_2 nanocrystals. The precipitate was harvested by centrifugation and washed with acetone several times to remove excess TOPO. The TiO_2 nanocrystals were made water-soluble through ligand exchange of surface TOPO with polyacrylic acid (PAA) [2]. A solution of 0.1 g PAA in 8.0 ml diethylene glycol (DEG) was heated to 110 °C with vigorous stirring under a nitrogen atmosphere. TiO_2 nanocrystals (10.0 mg) in 1.0 ml toluene were injected into the PAA solution and heated to 240 °C for ~5 minutes and the milky solution turned clear. The solution was kept at 240 °C for another 6 hours, then cooled down to room temperature with subsequent addition of dilute HCl to precipitate the nanoparticles. The white TiO_2 powder was then washed three times with water, and finally dispersed in aqueous solution at pH = 6-8.

(2) 6-nm TiO_2-streptavidin conjugate preparation

The TiO_2-streptavidin conjugation was accomplished using a protocol which allows for sequential coupling without exposing the streptavidin to EDC, in essence negating the effect of EDC on the carboxyl groups of streptavidin. First, 10 μl of 0.1 M EDC and 15 μl of 0.1 M *N*-

hydroxysulfosuccinimide (sulfo-NHS) were added to 1 mL of 5% PAA coated TiO_2 in water. The pH was then adjusted to 6.0 and the reaction stirred at room temperature for 30 minutes. The reaction solution was then eluted down a nap-5 column using a borate buffer at pH 8.0 to remove the derivatized TiO_2 from excess EDC and sulfo-NHS. 30 μl of streptavidin-Alexa Fluor 488 purchased from Invitrogen (2mg/ml) were then added to the derivatized TiO_2 and stirred for 2 hours at room temperature after which the reaction solution was centrifuged for 5 min in order to remove any streptavidin precipitate.

(3) TiO_2 labeling of microtubules

Microtubules (MTs) were polymerized by incubating 5mg/ml tubulin tagged with biotin in G-PEM buffer (80 mM PIPES, pH 6.8, 1 mM $MgCl_2$, 1 mM EGTA, 1 mM GTP), containing 30% glycerol, at 37 °C for 40 minutes. Paclitaxel was then added to a final concentration of 10 μM to stabilize the MT. TEM grids were incubated in 1% poly-L-lysine solution and allowed to sit for 15 minutes at room temperature, after which excess poly-L-lysine was removed with filter paper. The MT solution was diluted with G-PEM/paclitaxel solution to 1mg/ml and incubated with an equal amount of solution of TiO_2-streptavidin (prepared as above) for 45 min. Approximately 2-4 μl of MT solution was spread over the entire surface of the coated TEM grids and incubated at room temperature for 10 minutes, after which excess solution was removed using filter paper. For light microscopy, the grids were viewed right away. For TEM and x-ray microscopy, the MT were fixed with 2% glutaraldehyde for 10 min, then washed with distilled water three times and dried overnight.

(4) Microscopy measurement

Fluorescent light microscopy was done using a Zeiss AxioVert 200M fluorescence inverted microscope with a 40X 1.2NA water immersion lens and an AxioCam MRm CCD camera. TEM micrographs were obtained using a Tecnai G2 20 microscope operating at 200 kV with a LaB6 filament and S-TWIN objective lens. Selected area electron diffraction (SAED) was obtained on a JEOL 2010

TEM operating at 200 keV. Scanning transmission X-ray microscopy (STXM) was performed at beamline 5.3.2 at Advanced Light Source, Lawrence Berkeley National Laboratory.[3] A Fresnel zone plate with an outermost zone width of 25nm was used to focus the x-ray onto the sample.

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