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TiO₂ nanoparticles as a soft X-ray molecular probe†

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Abstract

This communication reports the development of a TiO₂–streptavidin nanoconjugate 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.

Soft X-ray tomography generates 3D images of whole, hydrated cells at a 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 per 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 or photooxidation of diaminobenzidine (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 two tags 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

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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. With the rapidly expanding field of nanoscience in biology, especially the successful application of semiconductor nanocrystals in biological imaging,⁴⁻⁷ additional nanoparticulate materials that exhibit strong absorption in the soft X-ray spectrum are being developed. One promising candidate is TiO₂. TiO₂ 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 a TiO₂-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 TiO₂ at an energy above, and then below, the Ti L_{2,3} X-ray absorption lines, one can easily distinguish the TiO₂ 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.

Triethylphosphine oxide (TOPO) capped TiO₂ particles (Fig. 1) were prepared using a high-temperature pyrolysis reaction.¹⁰ The TiO₂ nanocrystals were made water-soluble through ligand exchange of surface TOPO with polyacrylic acid (PAA).¹¹ Preparation of TiO₂-streptavidin nanoconjugates was recently demonstrated through the use of biotin as a bridging molecule between TiO₂ 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 TiO₂-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 (Fig. 2a). Conjugation of the TiO₂ 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 Fig. 2b. Next, transmission electron microscopy (TEM) was used to visualize the unlabeled MTs (Fig. 2c) as well as those tagged with the TiO₂-streptavidin (~6 nm particles) nanoconjugates (Fig. 2d). Images obtained from the TEM showed small electron-dense TiO₂ particles decorating the MT. To confirm that the electron-dense spots were indeed TiO₂, selected area electron diffraction (SAED) patterns were obtained, which established the presence of TiO₂ in anatase form (I41/amd; PDF#00-021-1272, Fig. 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 TiO₂ nanoparticles are sparsely distributed along the microtubules. Consequently there might be insufficient crystallites (TiO₂ nanoparticles) to form a continuous ring and only a finite number of orientations possible are observed.

Scanning transmission X-ray microscopy (STXM) was then implemented to examine the TiO₂-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 spectro-microscopy capability was used to obtain a stack scan (images at different energies) of the TiO₂ nanoparticles (Fig. 3a). The X-ray absorption spectrum of the TiO₂ nanoparticle indicated with the arrowhead showed the Ti L_{2,3}-edge X-ray absorption near edge spectra (XANES) (Fig. 3b). The Ti L_{2,3}-edges XANES features of individual nanoparticles are very similar to the spectra previously reported for ensembles of

TiO₂ nanoparticles.⁸ The Ti L_{2,3}-edge is caused by 2p to 3d transitions. Due to spin orbital splitting of the 2p orbital, two sets of L-edge features are observed, centered around 460 eV (L₃) and 465 eV (L₂), respectively. Multiplet structures in the L₃ and L₂ edges are further evidenced, due to the Coulombic and exchange interactions of the 2p–3d and 3d–3d orbitals.¹⁷ A Ti map image can also be obtained from STXM using the micrographs taken at two energies (450 eV and 466 eV) (Fig. 3a, Ti map). Each individual TiO₂ 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, TiO₂ 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 (Fig. 3c, left panel). The particles are clearly shown to be TiO₂ particles in the titanium map (Fig. 3c, center panel), while the carbon map image reveals the MT (Fig. 3c, right panel). The labeling pattern of the TiO₂ conjugate is similar to that seen with TEM (Fig. 2d), TiO₂ 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 4 nm monomers tightly bound to form a chain. But it is also possible that trace amounts of unconjugated streptavidin are competing with the TiO₂–streptavidin complex for binding to the biotin-tagged MT. Nevertheless the data shown here demonstrate the ability of the TiO₂ particles to specifically recognize the tubulin and demonstrate that these TiO₂ 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 TiO₂–streptavidin nanoconjugate 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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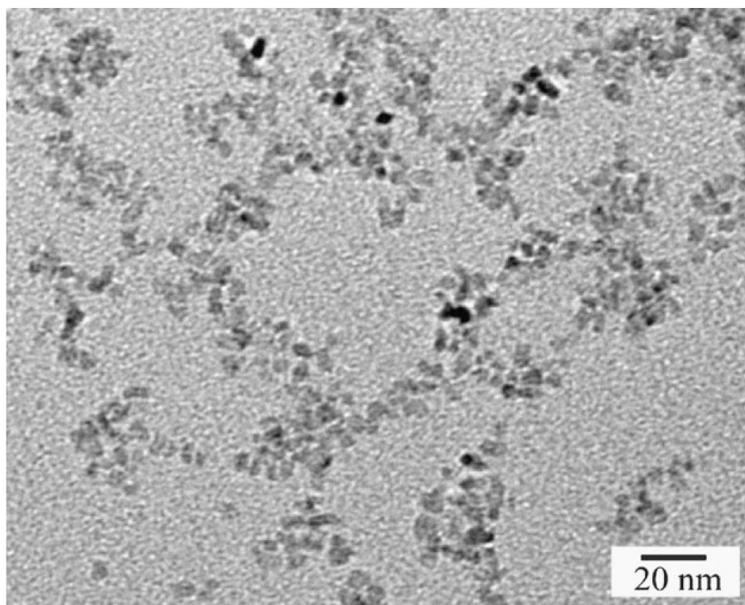


Fig. 1.
TEM image of PAA coated TiO₂ nanoparticles.

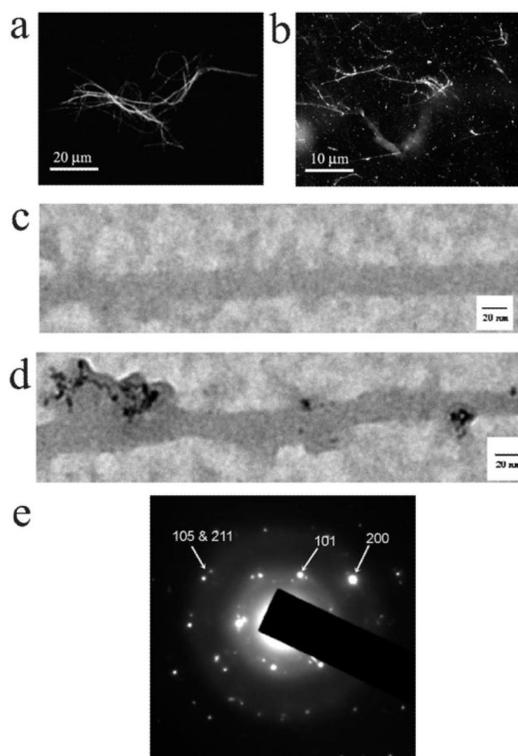


Fig. 2. Fluorescent image of streptavidin-Alexa (a) and TiO₂-streptavidin-Alexa (b) labelled microtubules. TEM image of unlabeled (c) and TiO₂-streptavidin-Alexa labelled (d) microtubules. (e) Selected area electron diffraction (SAED) pattern of TiO₂ (anatase) nanoparticles of the TiO₂-labeled MT sample.

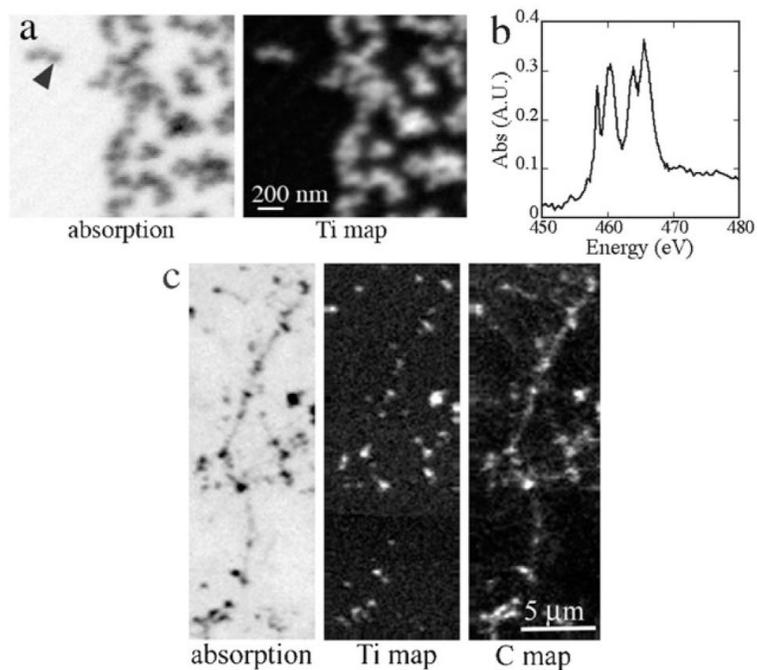
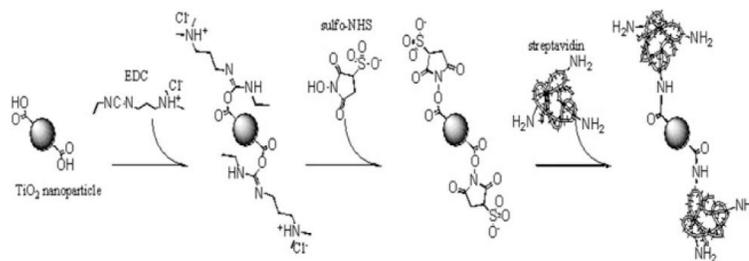


Fig. 3. (a) STXM images TiO₂ nanoparticles. (b) XANES spectrum of TiO₂ nanoparticles indicated with the arrowhead in (a). (c) STXM images of TiO₂ labeled microtubules. The absorption image shown was taken at 466 eV. The Ti map was obtained by subtracting the OD (optical density) image taken at 466 eV by the OD image taken at 450 eV. The C map was obtained by subtracting the OD image taken at 293 eV (above C/K-edge) by the OD image taken at 280 eV (below C/K-edge).



Scheme 1.
TiO₂-streptavidin conjugation.