TiO_2 nanoparticles as a soft X-ray molecular probe†

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Received (in Cambridge, UK) 25th January 2008, Accepted 25th February 2008
First published as an Advance Article on the web 26th March 2008
DOI: 10.1039/b801392f

This communication reports the development of a TiO_2–streptavidin nanoconjugate as a new biological label for X-ray bioimaging 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 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 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 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 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 a 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.

Trioctylphosphine oxide (TOPO) capped TiO_2 particles (Fig. 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–streptavidin nanoconjugates 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 (Fig. 2a). Conjugation of the TiO_2 nanoparticles to the streptavidin-

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† Electronic supplementary information (ESI) available: Materials and methods. See DOI: 10.1039/b801392f

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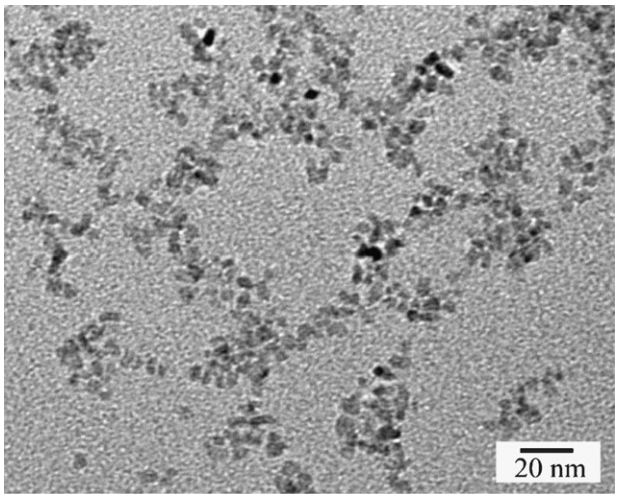
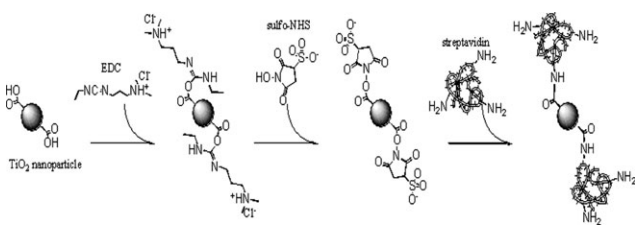


Fig. 1 TEM image of PAA coated TiO_2 nanoparticles.

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 TiO_2–streptavidin ($\sim$6 nm particles) nanoconjugates (Fig. 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 (*I*41/*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 TiO_2 nanoparticles are sparsely distributed along the microtubules. Consequently there might be insufficient crystallites (TiO_2 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 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 (Fig. 3a). The X-ray absorption spectrum of the TiO_2 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 TiO_2



Scheme 1 TiO_2–streptavidin conjugation.

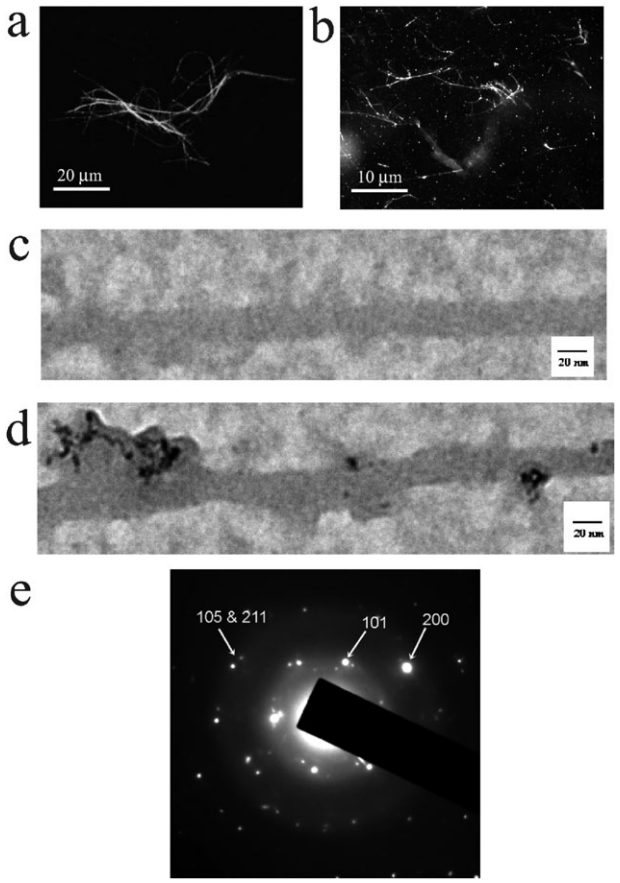


Fig. 2 Fluorescent image of streptavidin-Alexa (a) and TiO_2–streptavidin-Alexa (b) labelled microtubules. TEM image of unlabeled (c) and TiO_2–streptavidin-Alexa labelled (d) microtubules. (e) Selected area electron diffraction (SAED) pattern of TiO_2 (anatase) nanoparticles of the TiO_2-labeled MT sample.

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 (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 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 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 (Fig. 3c, left panel). The particles are clearly shown to be TiO_2 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 TiO_2 conjugate is similar to that seen with TEM (Fig. 2d), TiO_2 conjugate labeling of the MTs is punctate, rather than continuous as is seen with fluorescence microscopy. This pattern of labeling is commonly

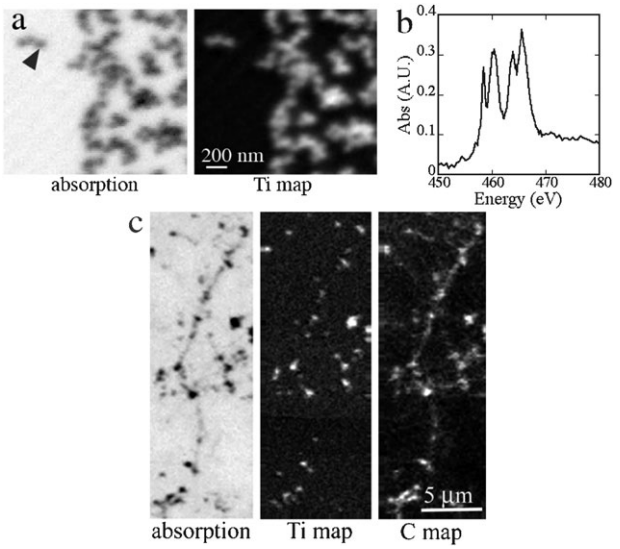


Fig. 3 (a) STXM images TiO_2 nanoparticles. (b) XANES spectrum of TiO_2 nanoparticles indicated with the arrowhead in (a). (c) STXM images of TiO_2 labeled mictrotubules. The absorption image shown was taken at 466 eV. The Ti map was obtained by subtracting the OD (optical density) image taken at 466 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).

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 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 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.

This work was supported by the National Institutes of Health: the National Center for Research Resources (P41 RR019664) and National Institutes of General Medicine (GM 70445 and GM 63948); and the US Department of Energy, Office of Biological and Environmental Research (DE-AC02-05CH11231). Y. Yin thanks the University of California, Riverside for start-up funds and the Regent's Faculty Fellowship. Portions of this work were performed at the Molecular Foundry, Lawrence Berkeley National Laboratory, which is supported by the Office of Science, Office of Basic Energy Sciences, of the US Department of Energy under Contract No. DE-AC02—05CH11231.

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