Mapping the subcellular localization of Fe₃O₄@TiO₂ nanoparticles by X-ray Fluorescence Microscopy

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Abstract. The targeted delivery of Fe₃O₄@TiO₂ nanoparticles to cancer cells is an important step in their development as nanomedicines. We have synthesized nanoparticles that can bind the Epidermal Growth Factor Receptor, a cell surface protein that is overexpressed in many epithelial type cancers. In order to study the subcellular distribution of these nanoparticles, we have utilized the sub-micron resolution of X-ray Fluorescence Microscopy to map the location of Fe₃O₄@TiO₂ NPs and other trace metal elements within HeLa cervical cancer cells. Here we demonstrate how the higher resolution of the newly installed Bionanoprobe at the Advanced Photon Source at Argonne National Laboratory can greatly improve our ability to distinguish intracellular nanoparticles and their spatial relationship with subcellular compartments.

1. Introduction

Metal oxide nanoparticles (NPs) initially developed by chemists and material scientists, are now being investigated as nanomedicines. The key advantage of metal oxide nanoparticles over small molecule therapies lies in their peculiar surface chemistries and physical properties. For example, titanium dioxide (TiO₂) nanoparticles smaller than 20 nm can form exceptionally stable bonds with ortho-substituted enediol molecules such as dopamine or 3,4-dihydroxyphenylacetic acid (DOPAC) [1,2], while molecules with free hydroxyl groups can form a more labile corona on the NP surface [3]. This allows for the subsequent conjugation of functional molecules such as antibodies or peptides that can direct the delivery of these NPs. We have synthesized 6-7 nm Fe₃O₄@TiO₂ NPs [3] that can bind the Epidermal Growth Factor Receptor (EGFR) by surface modifying NPs with short EGFR-binding peptides (Yuan et al submitted). These peptides are derived from the B-loop region of Epidermal Growth Factor (EGF), the native ligand of EGFR, and have been previously shown to bind and activate EGFR in vitro [4]. Since EGFR is often overexpressed in epithelial type cancers and ligand bound EGFR can translocate to the nucleus [5], we wish to use these EGFR-binding B-loop NPs to achieve both cellular and subcellular targeted delivery.

In order to directly map the subcellular distribution of metal oxide NPs we must be able to image cells treated with NPs at sub-micron resolutions. For that purpose we decided to use X-ray fluorescence microscopy in order to simultaneously map (i) intracellular and NP associated iron (Fe)
and NP associated titanium (Ti) and (ii) different subcellular compartments by determining the distribution of intracellular trace metals and nanometal elements such as phosphorus (P), sulfur (S), and zinc (Zn) [6]. Using the X-ray fluorescence microprobe at the sector 2 beamline at the Advanced Photon Source (APS) at Argonne National Laboratory (ANL) we have mapped NPs inside cells in the past. We decided to do the same with cells treated with B-loop NPs and compare these elemental maps with the ones produced using a higher resolution instrument—the Bionanoprobe, which was installed at the Life Sciences Collaborative Access Team (LS-CAT) in the fall of 2011. This instrument can produce an X-ray beam spot size focused to 30 nm thus allowing detailed determination of the subcellular localization of metal oxide NPs within cells.

2. Materials and Methods

HeLa cervical carcinoma cells were grown at a density of 100,000 cells/mL overnight on 3.5 mm tissue culture dishes and then treated with 10 nM B-loop NPs for one hour at 4°C. The cells were then washed to remove loosely adhering NPs from the surface and chemically fixed in 4% formaldehyde in PEM buffer, detached from support using a cell scraper and resuspended in PBS. This suspension of cells was then mixed 1:1 with a molten low melting point 4% agarose in PBS, dehydrated in increasing concentrations of ethanol, paraffin embedded, and sectioned to a thickness of 7 μm.

Cell sections mounted on Si₃N₄ windows were raster scanned at the X-ray microprobe at sector 2-ID-E at the Advanced Photon Source at Argonne National Laboratory 10 keV hard X-rays produced by an undulator source were monochromatized through a single bounce Si<111> monochromator and focused by a Fresnel zone plate to a spot size of 0.7 μm x 0.5 μm. The sample was scanned with a step size of 200 nm and a dwell time of 2 seconds. The fluorescence spectrum at each scan position was acquired by an energy dispersive germanium detector and fitted using MAPS software [7] by comparing to sample spectra from NBS standards 1832 and 1833.

The same sample was then transferred to the BNP at 21-ID-D, which is also an undulator beamline, and scanned again as a comparison. A double crystal monochromator located five meters upstream of the BNP was used to generate 10 keV monochromatic radiation, which was then focused down on to the sample using a Fresnel zone plate with 70 nm outermost zone width, theoretically providing spatial resolution of 85.4 nm according to the Rayleigh criteria. The sample was scanned through the focused spot using 50 nm step size and 250 ms dwell time per step. At each scanning step, a full fluorescence spectrum was collected using a four-element silicon drift detector (Vortex-ME4®) at 90 degree of the incident beam and fitted using MAPS [7].

3. Results

HeLa cervical carcinoma cells were treated with 10 nM EGFR-binding B-loop NPs for 1 hour at 4°C. Under these conditions the NPs are able to bind to cell surface EGFR, but cannot be internalized due to the inhibition of energy-dependent receptor mediated endocytosis [8]. Since we have shown that even the uptake of bare NPs is energy-dependent [8] and the internalization of ligand bound EGFR is also a cell metabolism dependent process [9] we expect that with this experimental setup EGFR-binding NPs will be predominantly localized to the cell membrane.

XFM images of the intracellular distribution of trace metal elements show that the Fe and Ti from B-loop NPs co-localizes with S, an element that is distributed throughout the cell due to its incorporation in cellular proteins (Figure 1). Furthermore, the colocalization of S and Ti appears to be at the cell periphery implying that, at this treatment timepoint, the B-loop NPs are bound to cell surface receptors, but are not yet internalized. While the image from the X-ray fluorescence microprobe (Figure 1A) and Bionanoprobe (Figure 1B) show the same distribution pattern, the degree of detail discernable in the image obtained with the Bionanoprobe is significantly greater. The image in Figure 1A was done with a beam focused to a spot size of 0.7 μm x 0.5 μm while the image in Figure 1B was acquired with X-rays focused with the Fresnel zone plate setup of the Bionanoprobe instrument to a theoretical spot size of 85.4 nm according to the Rayleigh criteria. A tightly focused beam accompanied with the small step size (50 nm) in the image in Figure 1B allows identification of
additional Ti rich spots at the cell periphery and allows us to resolve the morphology of the Ti (NP) deposits. Importantly, the new Bionanoprobe instrument allows acquisition of images with samples tilted at different angles for a total of 180 degrees which will allow us to view this 7 μm thick cell slice tomographically and determine if the Ti hotspots that appear to be inside the cell are actually on the surface of cell membrane. With the thickness of 7 μm and the processing we used (agarose and paraffin embedding) preservation of cell morphology in three dimensions was achieved even though the image presented in Fig. 1 represents only a single 2D projection of cellular elemental content.

Figure 1. (A) XFM maps of S, Ti, Fe, and Zn distribution in B-loop NP treated HeLa cells scanned on the X-ray microprobe at sector 2-1D-E with a dwell time of 2 seconds and a step size of 200 nm. 3 element colocalization of Fe (red), Ti (green), Zn (blue) (B) The same HeLa cell scanned on the Bionanoprobe at sector 21-ID-D with a dwell time of 250 milliseconds and a step size of 50 nm. Images derived from the Bionanoprobe confirm that B-loop NPs are localized at or near the cell membrane at this treatment timepoint. Notably, in the images collected at higher resolution at the Bionanoprobe it is possible to distinguish additional and finer subcellular structures as well as individual clusters of NPs. The difference in the appearance of S and Zn is partly due to a difference in dwell times as well as to a difference in the angle (15 degrees) between the sample and the detector at the Bionanoprobe versus the microprobe leading to higher self-absorption of low Z elements.

4. Discussion
We have created EGFR targeted NPs by conjugating an EGFR-binding peptide to the surface TiO$_2$ molecules of Fe$_3$O$_4$@TiO$_2$ NPs. We have shown previously that these NPs colocalize with EGFR at the surface as well as within the nucleus of EGFR expressing HeLa cervical carcinoma cells at different treatment timepoints. Using the X-ray microprobe at sector 2-ID-E/D of the Advanced Photon Source at Argonne National Laboratory as well as the Bionanoprobe at sector 21-ID-D we wished to investigate the subcellular distribution of these NPs and showcase the differences between the two instruments. The difference in the degree of detail that can be visualized by the Bionanoprobe is impressive even though these images were not scanned at the highest resolution possible. In both cases, by mapping the distribution of intracellular elements such as S and Zn, we can distinguish the overall cell outline and cell nucleus, respectively. However, while the images derived from scans done at the microprobe show the distribution of NPs within cells, the higher resolution of the Bionanoprobe, especially when coupled with tomography, can be used to clarify the spatial relationship between internalized NPs and subcellular compartments. Reconstruction of 2-dimensional scans at different angles will allow for tomographic representation of the trace metal distribution within NP treated cells. This will greatly improve the ability to distinguish between NPs that are truly localized to the nucleus from cytoplasmic NPs that only appear to be in the nucleus in 2-dimensional projections.
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