Interrogation of EGFR Targeted Uptake of TiO$_2$ Nanoconjugates by X-ray Fluorescence Microscopy

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Abstract

We are developing TiO$_2$ nanoconjugates that can be used as therapeutic and diagnostic agents. Nanoscale TiO$_2$ can be surface conjugated with various molecules and has the unique ability to induce the production of reactive oxygen species after radiation activation. One way to improve the potential clinical usefulness of TiO$_2$ nanoparticles is to control their delivery to malignant cells by targeting them to cancer cell specific antigens. Epidermal Growth Factor Receptor is one potential target that is enriched in epithelial cancers and is rapidly internalized after ligand binding. Hence, we have synthesized TiO$_2$ nanoparticles and functionalized them with a short EGFR binding peptide to create EGFR-targeted NCs. X-ray Fluorescence Microscopy was used to image nanoconjugates within EGFR positive HeLa cells. Further labeling of fixed cells with antibodies against EGFR and Protein A nanogold showed that TiO$_2$ nanoconjugates can colocalize with receptors at the cell’s plasma membrane. Interestingly, with increased incubation times, EGFR targeted nanoconjugates could also be found colocalized with EGFR within the cell nucleus. This suggests that EGFR-targeted nanoconjugates can bind the receptor at the cell membrane, which leads to the internalization of NC-receptor complexes and the subsequent transport of nanoconjugates into the nucleus.

Keywords

nanoconjugates; TiO$_2$; peptides; EGFR

INTRODUCTION

TiO$_2$ nanoparticles (NPs) are a promising vehicle for the delivery of therapeutic and diagnostic agents. The unique surface chemistry of particles smaller than 20 nm allows for the conjugation of drugs, imaging contrast agents, and fluorescent dyes to create biologically active nanoconjugates (NCs).$^1, 2, 3, 4$ Moreover, the semiconductor and photocatalytic properties of TiO$_2$ make it a potent source of electrons and electropositive holes as well as reactive oxygen species (ROS)—all of which can react with cellular DNA.$^1, 5, 6, 7$ However, in order for NCs to be useful cytotoxic agents they must first be internalized and retained by cells. Furthermore, the subcellular localization of NCs is another important factor in determining their biological function.$^1, 2$ Therefore, the ability to control the uptake of NCs and target them towards specific cells and subcellular compartments would be very useful.
To achieve this goal, we have created TiO$_2$ NCs that can bind Epidermal Growth Factor Receptor (EGFR). This cell surface receptor is overexpressed by cancer cells of epithelial origin, is rapidly endocytosed upon ligand binding, and can be transported into the nucleus.

EGFR is an essential receptor tyrosine kinase (RTK) that controls many essential cell functions including cell differentiation, growth, proliferation, and migration. Hence, it also has a central role in tumorigenesis and is often overexpressed or overactive in epithelial cancers of the head and neck, colon, cervix, ovaries, lungs, and brain. Targeted therapies using monoclonal antibodies that recognize the extracellular domain of EGFR or tyrosine kinase inhibitors (TKIs) that block EGFR’s kinase activity have become a mainstay of cancer chemotherapy.

The three natural ligands that can specifically bind to EGFR are EGF, transforming growth factor-α (TGF-α), and amphiregulin. Structurally, these ligands share a 40 amino acid long EGF motif that contains six conserved cysteine residues (Figure 1). In the native form, these cysteine residues form intermolecular disulfide bonds that divide the EGF domain into three loop regions: A-loop (amino acids 1–20), B-loop (amino acids 14–31), and C-loop (amino acids 32–53). A study on isolated fragments corresponding to the three loop regions found that only peptides containing residues corresponding to the B-loop region (amino acids 14–31 or 20–31) are able to compete with full length EGF for binding to EGFR. More recently, an eleven amino acid long fragment corresponding to B-loop residues 20–31 has been used to improve the delivery of the chemotherapeutic agent doxorubicin to EGFR positive cancer cells.\cite{7,8}

Another attractive feature of targeting EGFR is that once bound and activated the receptor is able to translocate into the nucleus where it can act as a transcriptional co-factor and directly influence the expression of genes involved in cancer progression such as cyclin D1 (CCND1) and inducible nitric oxide synthase (iNOS). The cytoplasmic domain of EGFR contains a putative arginine rich nuclear localization signal that can bind the nuclear transport protein importin-β1.\cite{11} The interaction of EGFR and importin-β1 is further enhanced by ligand binding, which leads to a concomitant increase in nuclear EGFR. Ligand induced receptor activation and internalization also appears to be necessary as treatment of cells with PD158780, an inhibitor of EGFR’s tyrosine kinase activity, decreases nuclear EGFR levels. Similarly, cells that express a dominant negative dynamin mutant also show decreased nuclear EGFR, presumably due to the loss of clathrin mediated uptake of ligand bound receptor.

**METHODS**

Utilizing the reactive surface chemistry of TiO$_2$ NPs, an eleven amino acid peptide fragment corresponding to amino acids 20 through 31 of the B-loop of EGF (MYIEALDKYAC) was attached to 6 to 8 nm NPs via a 3,4-dihydroxyphenylacetic acid (DOPAC) linker (Figure 1). In order to assess the ability of these NCs to bind cell surface EGFR, EGFR-targeted NCs or bare NPs were then used to treat serum starved HeLa cells grown on silicon nitride windows (Silson) for 5 minutes or 25 minutes in serum free Eagle’s Minimum Essential Media (ATCC). After a quick wash with 0.2 M glycine and 1X PBS to remove excess NPs or NCs,
the cells were then fixed with 4% formaldehyde in PEM buffer and permeabilized with 0.5% saponin in PEM buffer in preparation for immunolabeling with a rabbit polyclonal primary antibody against EGFR (Abcam). These cells were also incubated with antibody binding Protein-A nanogold (5nm gold) so that EGFR-primary antibody complexes can be detected by X-ray Fluorescence Microscopy (XFM). The samples were then raster scanned at a resolution of 0.5 microns at the beamline at Sector 2-ID-E of the Advanced Photon Source at Argonne National Laboratories. XFM images were then analyzed using MAPS software to visualize the intracellular distribution of S, Ti, Zn, and Au.

RESULTS

XFM images of the intracellular distribution of Ti and EGFR specific Au signal revealed that while bare NPs remained predominantly outside of the cell (Figure 2A), EGFR-targeted NCs appeared to be in close proximity to cell surface distributed EGFR (Figure 2B). Furthermore, while the receptor specific Au signal remains diffuse in NP treated HeLa cells, the Au signal corresponding to EGFR distribution appears to cluster near Ti in NC treated cells. This is reminiscent of what occurs when EGFR binds its natural ligands, because ligand bound EGFR forms dimers that cluster within clathrin coated pits. This clustering is one of the first steps in the clathrin mediated internalization of ligand-receptor complexes from the cell surface.

Interestingly, in cells that were incubated with NCs for longer periods of time (25 min.), the Ti from NCs can be seen colocalized with EGFR specific Au signal within the cell nucleus, which is demarcated by areas of high Zn signal (Figure 2C). This suggests that NCs that are able to bind EGFR at the cell surface are then not only internalized but also carried into the nucleus along with EGFR. In fact, previous reports have shown that ligand bound EGFR begins to appear within the nucleus within 20 minutes of EGF stimulation and reaches its peak nuclear levels 30 minutes post-stimulation.

DISCUSSION

Functionalizing TiO$_2$ NPs with an EGFR binding peptide could allow for targeted delivery of therapeutic and diagnostic NCs to epithelial cancer cells. This is because binding EGFR can induce rapid receptor mediated internalization of NC-EGFR complexes. Indeed, our preliminary results suggest that EGFR-targeted NCs can associate with receptors at the cell surface and induce clustering of receptors, a necessary first step in clathrin mediated endocytosis. Furthermore, ligand bound EGFR is capable of nuclear translocation and EGFR-targeted NCs could perhaps be transported into the nucleus in association with the receptor. High resolution XFM images reveal that after 25 minutes of treatment NCs can be found in close association with EGFR within the cell nucleus. Nuclear localization of TiO$_2$ NCs would greatly improve the therapeutic efficiency of photodynamic therapy, as any ROS produced through light activation of TiO$_2$ will be in close proximity to the genetic material of malignant cells.

Whether NC-EGFR complexes are taken up predominantly via the clathrin pathway of endocytosis remains to be further characterized. Previous research has shown that ligand
bound EGFR can also be internalized through the slower caveolin mediated pathway of endocytosis. Another question that remains to be answered is if the nuclear localization of EGFR-targeted NC will lead to a concomitant increase in the cytotoxic effect of TiO$_2$ NCs following light activation.

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FIGURE 1.
Synthesis of EGFR-targeted TiO$_2$ NCs. An 11 amino acid fragment (red) of the B-loop region of EGF was conjugated to DOPAC and then used to surface modify 6–8nm TiO$_2$ NPs to create NCs capable of binding cell membrane EGFR.
FIGURE 2.
XFM maps of the distribution of S, Ti, Au, and Zn within HeLa cells treated with TiO$_2$ NPs or NCs. (a) Ti signal is predominantly extracellular in HeLa cells incubated with bare NPs for 5 min. (b) 5 min. incubation of HeLa cells with EGFR-targeted TiO$_2$ NCs to colocalization and clustering of Ti and EGFR specific Au signal at the cell surface. (c) In HeLa cells incubated with NCs for 25 min., NC associated Ti signal and EGFR associated Au signal colocalize within the Zn rich area of the cell nucleus.