Detection of TiO_2-DNA Nanocomposites in Mammalian Cells Using X-Ray Fluorescence Microanalysis

T. Paunesku, [1] N. Stojićević, [1] S. Vogt, [2] D. Legnini, [2] J. Maser, [2] B. Lai, [2] T. Rajh, [3] M. Thurnauer, [3] G. Woloschak [1,4]

[1]Department of Radiology, [4]Department of Cellular and molecular Biology, Northwestern University, 303 E. Chicago Ave., Chicago, IL 60611, U.S.A.; [2]X-ray Operations and Research Division, [3]Chemistry Division, Argonne National Laboratory, 9700 South Cass Avenue, Argonne, IL 60439, U.S.A.

Introduction

Bio-nanocomposites are nanometer-sized particles created by the conjugation of inorganic with "traditionally" biological molecules. Recent years have seen a proliferation of hybrid nanotechnology, and several recent review articles [1, 2, 3] list different nanocomposites currently in development and emphasize the dichotomy between their potential uses. For example Penn [2] separates nanocomposites into 4 groups: quantitation tags; substrates; nanoparticles that leverage signal transduction; and functional nanoparticles.

We are developing for intracellular use a bio-nanocomposite that has a potential for functional uses inside cells and *in vitro*. These nanocomposites are composed of metal oxide (TiO_2) nanoparticles (4.5 nm in size, surface coated with glycidyl isopropyl ether) and DNA oligonucleotides bound via dopamine to the nanoparticles. Within the nanocomposites DNA oligonucleotides retain base-pairing specificity, while the TiO_2 nanoparticles exhibit a characteristic photoreactivity. In particular, TiO_2 nanocomposites exhibit semiconducting properties through both constituents [4, 5, 6]—excitation of TiO_2 (exposure to electromagnetic radiation of energy above 3.2 eV) results in charge separation ultimately resulting in irreversible trapping of the electropositive holes in the sugar molecules of the DNA phosphodiester backbone leading to the cleavage of the DNA [7]. This endonuclease activity is therefore: (a) excitable by a factor not naturally encountered by the cells *in vivo* (electromagnetic radiation of energy higher than 3.2 eV); and (b) highly sequence specific—to the degree that it can be directed toward a single target gene in a whole genome (due to the high specificity of long oligonucleotide base-pairing). Due to the fact that electropositive holes can travel some distance from the nanoparticle and cause DNA scission at a relatively distant site [according to Lewis *et al*. [8] the charge can travel up to 200 nucleotides (nt) distances] this endonuclease activity is not identical to the behavior of protein endonucleases. Nevertheless, since 200 nt is well below the size of genes, this cleavage is definitively gene specific. Therefore, the complete titanium dioxide-oligonucleotide nanocomposites act as inducible gene-specific endonucleases with allele-differentiating sequence specificity.

As first steps towards use of these nanocomposites in vivo, we intoduced them into cultured mammalian cells.

Methods and Materials

TiO_2 nanocomposites preparation: All oligonucleotides with 5' carboxy deoxy thymidine nucleotide were synthesized by Midland Scientific. TiO_2 nanoparticles were prepared as previously described [9]. All the chemicals were reagent grade and used without further purification (Aldrich or Baker). In order to bind TiO_2 nanoparticles, oligonucleotides were dopamine end-labeled. When dopamine is added into TiO_2 colloidal solutions at $8 < pH < 2.5$, the formation of the charge transfer complex between dopamine and TiO_2 is instantaneous, and the resulting complex—nanocomposite is extremely stable.

Cells, transfections.: Cell lines used were HeLa-HIVcat [10], EL4 (ATCC), PC12 (ATCC), MCF7 (ATCC) and HL60 (ATCC), grown in Dulbecco's minimal essential medium (or F12K medium) supplemented with 10% fetal calf serum (or combination of fetal calf serum and horse serum 1:2), antibiotics (Penicillin and Streptomycin) and antimycotics, in humidified 5% CO_2 environment.

Cells were grown to log phase and harvested on the day of transfection, or plated in the appropriate density the day before the transfection. Nanocomposites were introduced into the cells in one of the two following ways: (1) complexed with the SuperFect Reagent (Qiagen); or (2) by electroporation using either a BTX machine or the Mammozapper™ following manufacturers instructions. Transfections were incubated 2 to 16 hours, cells were washed for 2-24 hours, collected by centrifugation, and cell pellets (10^6 - 10^7 cells) were resuspended in 40 µl of Phosphate Buffered Saline and spotted onto formvar membrane coated gold Oxford EM grids (Electron Microscopy Sciences), or fixed in 2.5% glutaraldehyde and spotted onto formvar membrane coated gold Oxford EM grids. After a brief drying, cells were washed in 100% ethanol for 10 minutes. Isolated nuclei spotted onto grids were isolated by ultracentrifugation through a 2.2 M sucrose cushion [11] and then handled the same as complete cells.

Elemental mapping using a hard X-ray fluorescence microprobe: The most expedient way to detect and quantify titanium inside the cells is to monitor titanium specific K_α X-ray fluorescence. The grids were mounted on a standardized kinematic specimen holder and placed in the specimen chamber of the 2-ID-E X-ray fluorescence microprobe at the 2-ID-E beamline at the XOR-CAT at the Advanced Photon Source at Argonne National Laboratory. The specimen chamber is filled with helium to optimize the detection of X-ray fluorescence of lighter elements, and to minimize the X-ray fluorescence background from argon gas in the air. A tunable, monochromatic X-ray beam from an undulator X-ray source was focused in a 0.5 µm x 0.3 µm spot using a Fresnel zone plate. Incident photon energy of 10keV was chosen, which allows excitation of K_α X-ray fluorescence in elements with Z=30 (Zn) and below. An energy dispersive Ge detector collected X-ray fluorescence radiation emitted by the specimen. The specimen was raster-scanned in x and y, and full fluorescence spectra were collected at each x/y position. This allowed for later data manipulation and optimized extraction of elemental concentrations. These procedures are well established and are continuously improved [7, 12, 13, 14].

Results

Using standard transfection methods we were able to introduce oligonucleotide-TiO_2 nanocomposites into mammalian cells *in vitro* (Fig. 1). The location of titanium in the cells was mapped by detecting Ti K_α X-ray fluorescence.

A total of 514 HL60 cells from 24 different samples transfected with seven different nanocomposites were inspected for the presence of titanium signal. Depending on the type of the experiment, 20 to 50% of the cells accepted and retained titanium nanoparticles. The addition of "free" oligonucleotides generally increased the success of titanium nanocomposite transfection and retention (Table 1.).

Ti Signal	"Free" Oligonucleotide Added		
	None	Heterologous DNA	Identical DNA
Present [%]	29	49	49
Absent [%]	71	51	51

Table 1. Percentage of cells displaying Ti signal upon transfection.

Chi square analysis (with 2 degrees of freedom) of the contingency table of these data showed, at a confidence level greater than 0.995, that there is an association of transfection success and addition of "free" oligonucleotides to transfection mixtures. The sequence of the "free" oligonucleotides (either identical or heterologous when compared to the sequence of oligonucleotide bound to Ti nanoparticle) did not modulate this association.

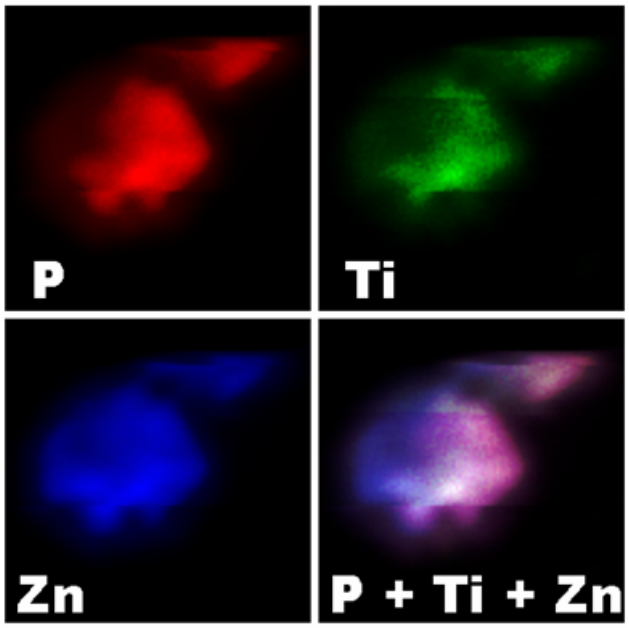

Figure 1. Kα X-ray fluorescence signals of phosphorus, zinc, and titanium, and overlapped with each other, showing the highest titanium signal density in a circular subregion of the nucleus resembling a nucleolus.

The area covered by this scan was 21 x 21 μm with a single nucleus isolated from HL60 cells transfected with ribosomal DNA (nucleolus specific) nanocomposite, and a "free" ribosomal oligonucleotide. By its size and shape this nuclear subregion closely resembles the nucleolus—subregion of the interphase nucleus where ribosomal DNA is located, and would therefore be the most likely nuclear location for retention of an nucleolus specific nanocomposite. Presumably, such retention would be dependent on hybridization/annealing of oligonucleotide from the nanocomposite with the genomic ribosomal DNA located inside nucleolus.

High resolution images of different transfection combinations show that transfection with "free" TiO_2 leads to the appearance of high intensity Ti signal outside of the cell, in comparison to Ti signal following transfection with a nanocomposite, where Ti signal is inside the cells and of comparatively lower intensity.

A high resolution image of a nucleus isolated from cells transfected with a nanocomposite complementary to the ribosomal (nucleolar) genomic DNA shows a localized titanium signal possibly indicating nucleolar localization, is shown in Figure 1. Scans showing Ti presence in six of thirteen sampled nuclei demonstrated that the TiO_2-DNA nanocomposites, once introduced into mammalian cells, reach the nucleus.

Discussion

The data we have generated so far demonstrate that TiO_2–oligonucleotide nanocomposites can be introduced successfully into mammalian cells and be retained in a specific subcellular compartment such as nucleolus. Possible future applications of TiO_2–oligonucleotide nanocomposites include gene surgery, development of nano-tools for intracellular manipulations, nano-footprints for intracellular studies of protein/DNA binding patterns, platforms for intracellular structural studies, development of nano-sized diagnostic devices, and highly localized intracellular delivery of pro-drugs and their activation. While both oligonucleotides therapies and titanium dioxide nanoparticles have been known for a long while, in the context of nanocomposites these components acquire a new function—they can be used as inducible gene specific endonucleases with allele-differentiating sequence specificity, they can be used to remove or inactivate certain functional DNA sequences from the cells, while leaving the remainder of the cellular genetic material intact.

Ackgnowledgenents

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