

## Nano-cerium-element-doped titanium dioxide induces apoptosis of Bel 7402 human hepatoma cells in the presence of visible light

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### Abstract

**AIM:** To investigate the apoptotic effect of photoexcited titanium dioxide (TiO<sub>2</sub>) nanoparticles in the presence of visible light on human hepatoma cell line (Bel 7402) and to study the underlying mechanism.

**METHODS:** Cerium-element-doped titanium dioxide nanoparticles were prepared by impregnation method. Bel 7402 human hepatoma cells were cultured in RPMI 1640 medium in a humidified incubator with 50 mL/L CO<sub>2</sub> at 37°C. A 15 W fluorescent lamp with continuous wavelength light was used as light source in the photocatalytic test. Fluorescence morphology and agarose gel electrophoresis pattern were performed to analyze apoptotic cells.

**RESULTS:** The Ce (IV)-doped TiO<sub>2</sub> nanoparticles displayed their superiority. The adsorption edge shifted to the 400-450 nm region. With visible light illuminated for 10 min, 10 μg/cm<sup>3</sup> Ce (IV)-doped TiO<sub>2</sub> induced micronuclei and significant apoptosis in 4 and 24 h, respectively. Highest 33258 staining of the fixed cells revealed typical apoptotic structures (apoptotic bodies), agarose gel electrophoresis showed typical DNA ladder pattern in treated cells but not in untreated ones.

**CONCLUSION:** Ce (IV) doped TiO<sub>2</sub> nanoparticles can induce apoptosis of Bel 7402 human hepatoma cells in the presence of visible light.

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**Key words:** Nano titanium dioxide; Cerium; Photocatalysis; Apoptosis; Hepatoma cells

### INTRODUCTION

Application of titanium dioxide (TiO<sub>2</sub>) photocatalysis has received increasing attention since the first report of microbiocidal effects by Matsunaga *et al*<sup>[1]</sup> in 1985. Recent studies have investigated application of TiO<sub>2</sub> in life science, especially in the field of cancer treatment<sup>[2-4]</sup>. In the presence of ultraviolet (UV) light with a wavelength less than 400 nm, nano TiO<sub>2</sub> can induce cancer cell apoptosis and be used as a photosensitizer in photodynamic therapy for endobronchial and esophageal cancers<sup>[5]</sup>.

However, TiO<sub>2</sub> displays its high photoactivity only when it is irradiated by ultraviolet light due to its wide band gap (3.2 eV for anatase). Doping with impurities has been widely used to modify the properties of TiO<sub>2</sub> by introducing new states in its electronic structure<sup>[6,7]</sup>. Because of the unique 4f electron configuration, lanthanide metal ions are ideal dopants to modify the electronic structure of TiO<sub>2</sub><sup>[8-10]</sup>. For example, cerium element doping could introduce new energy level into band gap of nano TiO<sub>2</sub>, making it possible for light with a wavelength over 400 nm (generally named visible light) enough to excite electron jumping to conduct band from valence band<sup>[10]</sup>.

As hole and electron separate successfully on the surface of nanoparticles, they could react with absorbed water or other adsorbates to produce reactive oxygen specimens such as OH· and H<sub>2</sub>O<sub>2</sub><sup>[6]</sup>. These reactive radicals can destroy cell membrane structure<sup>[11,12]</sup> and induce micronuclei (MN)<sup>[13]</sup>, even cause DNA damage<sup>[12,14]</sup>.

Apoptotic cells can be characterized by a number of morphologic, molecular, and biochemical features, including shrinkage of cells, blebbing of cells and nuclear membranes, compaction and condensation of chromatin toward the nuclear periphery, and fragmentation of DNA into oligonucleosomes<sup>[15]</sup>. Detection of more than one of these parameters is essential for the clear characterization of apoptotic cells.

In this communication, we reported the occurrence of oxidant-mediated DNA damage resulting in apoptosis and

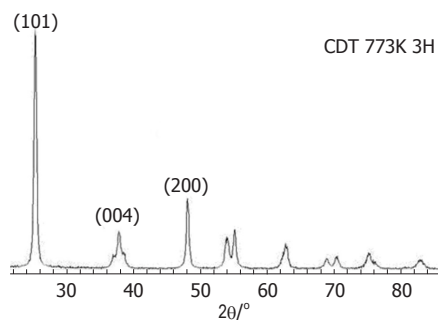


Figure 1 XRD spectra of CDT calcined at 500°C for 3 h.

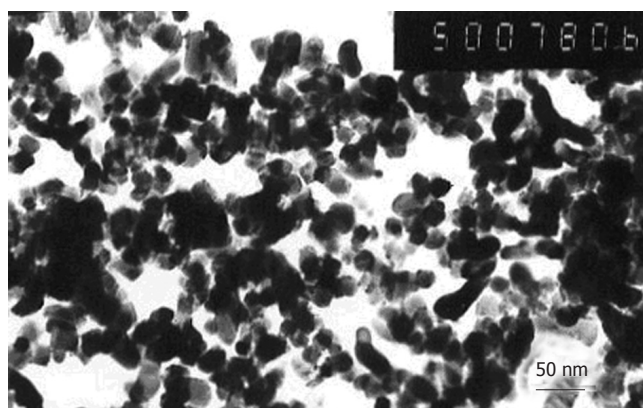


Figure 2 TEM of CDT.

induction of micronuclei (MN) by Ce-doped TiO<sub>2</sub> nanoparticle (CDT) in the presence of visible light in Bel 7402 human hepatoma cells.

## MATERIALS AND METHODS

### Preparation and characteristics of CDT

CDT samples were prepared by impregnation technique<sup>[6]</sup>. Cerium sulphate [Ce(SO<sub>4</sub>)<sub>2</sub>] aqueous solution was slowly dropped into nano-anatase TiO<sub>2</sub> (13 nm) gel by vigorous stirring, and water-bathed for 2 h at 40°C, then mixture was filtered, dried at 120°C for 2 h and finally calcined at 500°C for 3 h. CDT was suspended in physiologic saline, sterilized and diluted with RPMI 1640 medium to appropriate concentration when exposed to cells.

The characteristics of CDT were determined with UV-Vis spectrometer (SPECORD 2000, German) by X-ray diffraction (XRD, DX-2000), and transmission electron microscopy (TEM, JEOL JEM-2011).

### Cell culture and treatment

The present study was carried out using Bel 7402 human hepatoma cell line, which was cultured with RPMI 1640 (Gibco) containing 10% (v/v) fetal calf serum (FCS), 50 U/mL penicillin, 50 µg/mL streptomycin, and 1% (v/v) 0.2 mol/L L-glutamine. Cells were maintained at 37°C in a humidified atmosphere containing 50 mL/L CO<sub>2</sub>.

After attached to culture plate, Bel 7402 cells were exposed to CDT for 4 h in incubator, then washed three times softly with PBS and irradiated for 10 min with the 15 W fluorescent lamp at a vertical height of 30 cm.

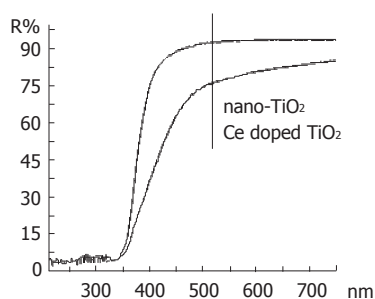


Figure 3 UV-Vis reflectance spectra of pure nano-TiO<sub>2</sub> and CDT.

### Morphological studies

Variations in cell morphology were analyzed by fluorescence microscopy. Cells attached to coverslips were fixed in cold methanol for 5 min, air dried, stained with Hoechst-33258 (2.5 mg/mL in milli-Q water, 2 min). Microscopic observations were carried out under Olympus BX61 microscope, and photographs were taken with Olympus DP50 digital camera.

### Analysis of DNA fragmentation

Fragmentation of DNA was analyzed by agarose gel electrophoresis during apoptosis. Bel 7402 cells were treated with 10 µg/cm<sup>3</sup> CDT for 4 h, then irradiated for 10 min with fluorescent light, and incubated for 4 h or overnight.

DNA was extracted with phenol/chloroform and precipitated in ethanol. The extracted DNA was separated by agarose gel electrophoresis (1.5% agarose gel containing 0.4 µg/mL ethidium bromide, 100 V, 1 h) and visualized under UV light.

## RESULTS

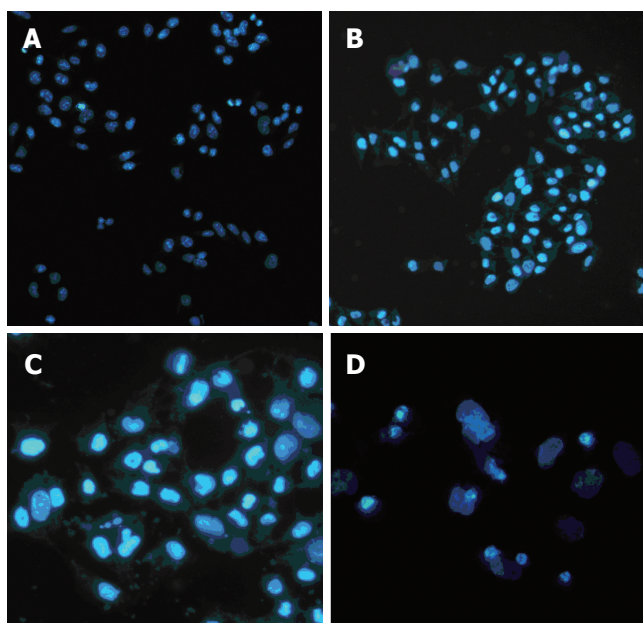
### Phase formation and microstructure of CDT

Figure 1 shows XRD patterns of CDT. The presence of peaks ( $2\theta = 25.4, 37.8$  and  $48.1$ ) could be regarded as an attributive indicator of anatase TiO<sub>2</sub><sup>[10]</sup>. CDT could maintain anatase crystalline structure at 500°C, and no other crystal phases were observed. According to Scherrer formula,  $d = \lambda/\beta \cos\theta$ , particle size was about 21 nm.

Morphologies of CDT were studied under transmission electron microscope (Figure 2). Most particles were in sphere shape, with a size of about 20-30 nm, coincided with that of XRD. Diffuse reflectance of UV-vis adsorption spectra of undoped TiO<sub>2</sub> and CDT calcined at 500°C was observed (Figure 3). The adsorption edge of CDT shifted to a longer wavelength region (named red shift) than that of undoped TiO<sub>2</sub>.

### Fluorescence morphology

Bel 7402 cells were stained with Hoechst 33258 (Figure 4). Neither CDT had effect on cells in the absence of light (Figure 4A), nor light (Figure 4B). Formation of MN could be found 4 h after cells were exposed to CDT (10 µg/cm<sup>3</sup>, with visible light illuminated for 10 min) (Figure 4C). Treated cells appeared typical changes in apoptosis after incubated overnight (Figure 4D). Many apoptotic bodies containing nuclear fragments were found in CDT-treated cells. Normal cells were stained uniformly,



**Figure 4** Fluorescence morphology of Bel 7402 cells treated with Ce (IV) doped nanometer titanium dioxide and stained with Hoechst 33258. **A:** CDT 10 µg/cm<sup>3</sup>, without light irradiation, × 200; **B:** Light irradiation, × 200; **C:** CDT (10 µg/cm<sup>3</sup> illuminated for 10 min and incubated for 4 h), × 400; **D:** Cells exposed to CDT (10 µg/cm<sup>3</sup> illuminated for 10 min and incubated overnight), × 400.

apoptotic cells appeared a brighter fluorescence. At the same time, cytoplasmic shrinkage was also observed.

### DNA electrophoresis

Fragmentation of CDT-treated DNA (at the concentration of 5 and 10 µg/cm<sup>3</sup>, irradiated for 10 min, incubated overnight) was found after agarose gel electrophoresis (Figure 5). In contrast, DNA in untreated cells did not show such a DNA ladder pattern in electrophoresis. There was no conspicuous difference in the effect of both concentrations.

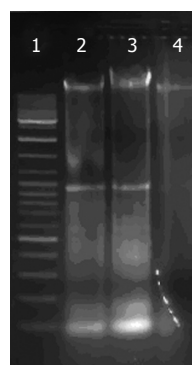
## DISCUSSION

In this experiment, CDTs were easy to attach to cellular membranes and aggregated. They were also easy to enter cytoplasm *via* phagocytosis (data not shown).

Nano TiO<sub>2</sub> showed no cytotoxicity to cells in the absence of UV light<sup>[16]</sup>, neither did UV light alone<sup>[17]</sup>. Visible light could only elevate culture temperature to 36°C, since it was lower than the culture temperature of cells, thermal death did not occur<sup>[18]</sup>.

However, cell viability decreased as the concentration of UV-irradiated TiO<sub>2</sub> nanoparticles in cells increased. Furthermore, cell morphology changed with increasing nanoparticle concentration, giving rise to shrinkage and fragmentation of cells<sup>[4]</sup>.

DNA was damaged when exposed to UV and nano TiO<sub>2</sub>. Strand breaks in DNA were assayed on agarose gels by transformation of a supercoiled plasmid into the relaxed or linear form<sup>[14]</sup>. Nakagawa *et al.*<sup>[12]</sup> studied the effects of four sizes of UV-irradiated TiO<sub>2</sub> particles on a mouse lymphoma cell line. DNA tail length was measured by SCG assay. The results showed that UV-irradiated samples could increase DNA damage and decrease cell survival.



**Figure 5** Agarose gel electrophoresis of DNA from Bel 7402 cells irradiated with visible light for 10 min. Lane 2: 10 µg/cm<sup>3</sup>; lane 3: 5 µg/cm<sup>3</sup>; lane 4: Bel 7402 cells in the presence of light as control; lane 1: DNA marker (100 base pairs).

These observations have led the researchers to believe that the mechanism of cell death caused by photoexcited TiO<sub>2</sub> nanoparticles is through reactive oxygen species (ROS).

Uchino *et al.*<sup>[17]</sup> studied the relationship between the amount of radicals produced in UV irradiated TiO<sub>2</sub> particles and cytotoxicity. The viability of Chinese hamster ovary (CHO) cells with internalized TiO<sub>2</sub> particles decreased significantly after UV irradiation. Although the intensity of UV light did not influence cytotoxicity, the anatase fraction in TiO<sub>2</sub> particles had a significant effect on cytotoxicity. In addition, cell viability was proportional to the formation of DMPO-OH radical adducts. The Electron spin resonance (ESR) results confirmed the presence of DMPO-OH radical adducts, consistent with the formation of OH· radicals. The optimum crystal size for OH· radical formation was 30 nm for anatase. Most anatase samples produced more hydroxyl radicals than rutile or amorphous TiO<sub>2</sub>. These findings indicate that when anatase forms TiO<sub>2</sub> with UV exposure, hydroxyl radicals have cytotoxic effects<sup>[17]</sup>.

Molecular scavengers of both hydrogen peroxide and hydroxyl radicals, catalase and cysteine could effectively diminish cell death when added to cell samples. These findings provide evidence that hydroxyl radicals and hydrogen peroxide play a role in cell death<sup>[14,17,19]</sup>.

Cerium element doping can reduce band gap energy by introducing new energy into the surface band gap of nano TiO<sub>2</sub><sup>[10]</sup>. The adsorption edge of nano TiO<sub>2</sub> could shift to the long-wavelength region, and the energy of light at a length of 400-450 nm is enough to excite electron jump from valence band to conduct band by Ce (IV) doping. At the same time, Ce (IV) can separate e<sup>-</sup>/h<sup>+</sup> pair by grasped electron, then liberate h<sup>+</sup> to reacting H<sub>2</sub>O to generate ·OH and H<sub>2</sub>O<sub>2</sub>, leading to accumulation of ROS on cell membranes and in cytoplasm.

MN is frequently used to detect genetic damage induced by environmental mutagens, and/or carcinogens. In general, MN may arise from the breakage of chromosomes or the loss of an entire chromosome<sup>[13]</sup>. Our results show that CDT at a noncytotoxic dose can significantly increase the frequency of MN after UV irradiation. The formation of MN may be due to ROS and/or the presence of these particles around the mitotic apparatus.

Apoptosis is a unique type of programmed cell death, and oxidant-mediated DNA damage is one of the key factors for its induction<sup>[20]</sup>.

In the present study, Bel 7402 cells treated with photo-

excited CDT were severely damaged, with cells contracted and a lot of cell fragments simultaneously observed under inverted phase-contrast microscope.

Degradation of internucleosomal DNA segments as a consequence of activation of endogenous endonucleases is considered a characteristic end point of apoptosis and can be analyzed by gel electrophoresis<sup>[21]</sup>. In the present study, DNA agarose gel electrophoresis depicted typical inter-nucleosomal fragmentation in DNA of CDT-treated cells.

Cell damage induced by nanoparticles occurs in two stages. The first stage is oxidative damage by the photo-excited TiO<sub>2</sub> nanoparticles as they come in contact with cell membranes, resulting in permeabilization of the cell membranes but not producing a significant decrease in cell viability. The decrease in cell viability, eventually cell death, occurs as a result of leaking intracellular components, or CDT trafficking into the damaged cells and directly attacking nuclei and other intracellular components.

In conclusion, nano CDT induces apoptosis in the presence of visible light. However, the precise mechanism of MN formation and induction of apoptosis by ultra fine particles and fibers is still unknown. Further insight is required into intracellular signaling and the role of reactive oxygen species.

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