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# Surface-modified $TiO_2$ nanoparticles with ascorbic acid: Antioxidant properties and efficiency against DNA damage *in vitro*

Vladan Bajić[a], Biljana Spremo-Potparević[b], Lada Živković[b], Andrea Čabarkapa[b], Jelena Kotur-Stevuljević[c], Esma Isenović[a], Dušan Sredojević[a,d], Ivana Vukoje[a], Vesna Lazić[a], S. Phillip Ahrenkiel[e], Jovan M. Nedeljković[a,*]

[a] *Institute of Nuclear Sciences Vinča, University of Belgrade, P.O. Box 522, Serbia*
[b] *Department of Physiology, Faculty of Pharmacy, University of Belgrade, Serbia*
[c] *Department of Biochemistry, Faculty of Pharmacy, University of Belgrade, Serbia*
[d] *Texas A&M University at Qatar, P.O. Box 23874, Doha, Qatar*
[e] *South Dakota School of Mines and Technology, 501 E. Saint Joseph Street, Rapid City, SD 57701, USA*

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ABSTRACT

The antigenotoxic and antioxidative properties of surface-modified $TiO_2$ nanoparticles (NPs) with ascorbic acid (AA) were compared with those of constituents (free AA and bare $TiO_2$ NPs). Colloids consisting of the $TiO_2$ NPs with anatase crystal structure were prepared by acidic hydrolysis of $TiCl_4$. The synthesized $TiO_2$ NPs were characterized using transmission electron microscopy and X-ray diffraction analysis. The charge transfer (CT) complex formation between surface Ti atoms and AA is indicated by immediate appearance of red color. Composition and stability constants of CT complex were determined using Job's method and Banesi-Hildebrand analysis, respectively. The surface structure of CT complex was determined from infra-red spectra of free and bound AA to the surface Ti atoms. The experimental data were supported with quantum chemical calculations based on density functional theory (DFT). The antigenotoxic potential of CT complex was evaluated in leukocytes of whole blood cells *in vitro* by comet assay method. For evaluation of antioxidant properties, total antioxidant status (TAS) and total oxidant status (TOS) were determined in human serum pool *in vitro*.

The presented results indicate that bare $TiO_2$ NPs have more pronounced antigenotoxic effects in comparison with either surface-modified $TiO_2$ NPs with AA or free AA. No significant differences between the antigenotoxic and antioxidative properties of free and bound AA on the $TiO_2$ NPs were noticed in the investigated concentration range. It seems that surface-modified $TiO_2$ NPs with AA and/or similar compounds can be used to maintain its beneficial activities.



## 1. Introduction

The $TiO_2$ has been extensively used in many industrially relevant processes ranging from environmental applications to clean energy, and from paints to cosmetics and medicine [1–3]. In particular, $TiO_2$ finds application in medicine as a photosensitizer for photodynamic and photothermal therapy of cancer, as well as for drug delivery [4,5]. Wide use of bulk $TiO_2$ is based on its exceptional chemical stability and low cost, as well as inertness and biocompatibility. However, when particles are in a nanosize regime, issues concerning their toxicity have been raised [5,6]. The need to assess the genotoxic *versus* antigenotoxic properties of nanomaterials comes from their ability to penetrate tissues and cells.

The genotoxicity of $TiO_2$ NPs is still a matter of debate in literature. On the one hand, it was shown that the $TiO_2$ NPs may induce oxidative stress through the production of reactive oxidative species (ROS) including active forms of oxygen and nitrogen. Of course, ROS can cause mutagenic and carcinogenic changes in the cell nuclei, and because of that $TiO_2$ was included on the IARC list of potential carcinogenic substances (group 2B) [7–14]. On the other hand, a number of studies have shown no genotoxic effects of $TiO_2$ NP in *in vitro* comet assay (single-cell gel electrophoresis), bacterial and mammalian cell mutation tests, chromosomal aberration assays, and *in vivo* micronucleus assays [15–21]. The discrepancies between these results may be due to variation in size distribu-

* Corresponding author.
*E-mail address:* jovned@vin.bg.ac.rs (J.M. Nedeljković).

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tions of $TiO_2$ particles, diversity of preparation methods, surface properties, dosage, administration route, etc. [19,21–23].

It is well-known that due to large curvature $TiO_2$ particles with sizes smaller than 20 nm have under-coordinated surface structure with square pyramidal geometry instead of octahedral one [24,25]. Consequently, surface Ti atoms are very reactive and their binding to electron-donating ligands leads to the formation of charge transfer (CT) complexes accompanied with a red absorption shift. So far, the CT complex formation has been reported for colloidal $TiO_2$ NPs surface-modified with either catecholate- or salicylate-type of ligands [26–31]. Also, the appearance of absorption in the visible spectral region has been observed upon surface modification of colloidal $TiO_2$ NPs with ascorbic acid (AA) [25,32]. Primary motive of research in this area has been enhancement of variety of light-driven processes by extension of $TiO_2$ absorption into more practical spectral range [33–36]. However, electron donating ligands have pronounced radical scavenging ability, and, to the best of our knowledge, antioxidant and antigenotoxic properties of free and bound ligand molecules were never compared.

The main focus of this study is to evaluate antioxidant and antigenotoxic properties of CT complex between surface Ti atoms that belong to the colloidal $TiO_2$ NPs and standard antioxidant AA. Thorough microstructural and optical characterization of surface-modified $TiO_2$ NPs with AA was performed, including transmission electron microscopy (TEM), X-ray diffraction analysis (XRD), and spectroscopy. Coordination of AA to the surface of $TiO_2$ NPs was studied using Fourier transformation infra-red spectroscopy (FTIR) with the support of quantum chemical calculations based on density functional theory (DFT). Having in mind that surface complex is a new chemical entity whose antioxidant and antigenotoxic properties has not been examined before, the data for unmodified/bare $TiO_2$ NPs, free AA, CT complex between surface Ti atoms and AA, and CT complex in coexistence with free AA were tested in a wide concentration range. The level of DNA damage in leukocytes of whole blood cells *in vitro* was evaluated by the comet assay method, while for the evaluation of antioxidant properties, total oxidative status (TOS), total antioxidant status (TAS), and total sulfhydryl groups content (tSHG) were determined in the human serum pool.

## 2. Experimental section

### 2.1. Synthesis and characterization of surface-modified $TiO_2$ NPs with AA

All chemicals used were of the highest purity available and were used without additional purification (Aldrich, Fluka). Milli-Q deionized water (resistivity 18.2 $M\Omega\,cm^{-1}$) was used as a solvent. The $TiO_2$ colloids were prepared by dropwise addition of titanium(IV) chloride to cooled water as described elsewhere [37]. The pH of the solution was between 0 and 1, depending on the $TiCl_4$ concentration. Slow growth of the particles was achieved by using dialysis at 4 °C against water until the pH 3.5 was reached. The concentration of $TiO_2$ (0.17 M) was determined from the concentration of the peroxide complex obtained after dissolving the colloid in concentrated $H_2SO_4$ [38].

The CT complex formation between $TiO_2$ NPs and AA is indicated by the immediate appearance of red color upon addition of ligand to the colloid. For uniform $TiO_2$ particles, the concentration of surface Ti atoms ($[Ti_{surf}]$) in molar units can be calculated using following equation [39]:

$$\left[Ti_{surf}\right] = 12.5 \times [TiO_2]/D \quad (1)$$

where $[TiO_2]$ is the molar concentration of $TiO_2$ colloid and $D$ is the diameter of the particle in angstroms. The $TiO_2$ colloids with sub-monolayer concentration of AA were marked as $TiO_2$/S-AA, while colloids where concentration of AA exceeds monolayer concentration were marked as $TiO_2$/E-AA.

As a consequence of enhanced particle–particle interaction upon surface modification that eliminates the surface charge, precipitation or "gelling" of the solution may occur. To avoid these problems pH of the solution was adjusted to 2 by diluting $TiO_2$ or modifier stock solutions with 0.01 M aqueous solution of HCl. According to p*K*a values known, carboxylic group is more than 90% in protonated form at pH 2. For the determination of CT complex binding constants, the absorption spectra were recorded at room temperature in cells with 1 cm optical path length using Thermo Scientific Evolution 600 UV/Vis spectrophotometer. In the application of continual variations method (Job's method) [40] for the spectrophotometric determination of the complex composition, the solutions were prepared by mixing some different volumes of equimolar solutions of 2 mM $Ti_{surf}$ (7.2 mM $TiO_2$) and 2 mM AA. A series of solutions was prepared in which the sum of the total concentration of $Ti_{surf}$ and AA was constant (2 mM), but their proportions were continuously varied: volumes of $TiO_2$ solution used was varied from 1 to 9 mL and those of modifiers' solutions from 9 to 1 mL with the total volume being always 10 mL.

Transmission electron microscopy (TEM) was performed using a JEOL JEM-2100 $LaB_6$ instrument operated at 200 kV. TEM images were acquired with a Gatan Orius CCD camera at 2 × binning. X-ray diffraction (XRD) powder patterns were recorded using Rigaku SmartLab instrument under the Cu $K\alpha_{1,2}$ radiation. The intensity of diffraction was measured with continuous scanning at 2°/min. The data were collected at 0.02° intervals. Infrared spectroscopy measurements of free and bound AA to the surface of $TiO_2$ NPs were performed using a Thermo Nicolet 6700 FTIR spectrometer at spectral resolution of 8 $cm^{-1}$ in the region of 4000–400 $cm^{-1}$. All samples for FTIR measurements were 8 wt.-% of the sample in KBr matrix.

### 2.2. Numerical calculations

All DFT calculations were carried out with the Gaussian 09 program package [41]. To investigate the electronic structure and optical properties of CT complex, formed upon the adsorption of AA on the $TiO_2$ surface, the hexa-cluster $(TiO_2)_6$ was used as a model system for calculations, its geometry taken from the existing literature [42]. The ground-state geometry optimization of three different geometries of AA/$H_2(TiO_2)_6$ complex were performed at a gas phase by using HSE06 range-separated hybrid functional [43–45] with 6-31G** basis set. Frequency calculations were performed at the same level of theory in order to demonstrate that optimized geometries represent stationary points (with no imaginary frequencies). In order to calculate the vertical excitation energies ($E_{vert}$), the nature of transitions, as well as the oscillator strengths (f), the same hybrid functional was chosen within the framework of TD-DFT.

### 2.3. Genotoxic and antigenotoxic potential of bare $TiO_2$ NPs, free AA, $TiO_2$/S-AA and $TiO_2$/E-AA in vitro

Whole blood samples with heparin were obtained from three healthy volunteers (two men and one woman). All subjects who participated in the study were 20–25 years old. They were non-smokers, and negated any use of alcohol, medicals, supplements or receiving any therapy at the time of the study. Collected whole blood (1 mL per subject) was immediately used for *in vitro* comet assay analyses to evaluate genotoxic and antigenotoxic properties. *In vitro* genotoxicity research is the first line of safety evaluations in testing of novel compounds, which permit a simpler, more convenient, and more detailed analysis before their further applications could be assesed.