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Assessing the effects of surface-bound humic acid on the phototoxicity of anatase and rutile TiO_2 nanoparticles *in vitro*

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ABSTRACT

In this study, the cytotoxicity of two different crystal phases of TiO_2 nanoparticles, with surface modification by humic acid (HA), to *Escherichia coli*, was assessed. The physicochemical properties of TiO_2 nanoparticles were thoroughly characterized. Three different initial concentrations, namely 50, 100, and 200 ppm, of HA were used for synthesis of HA coated TiO_2 nanoparticles (denoted as A/RHA50, A/RHA100, and A/RHA200, respectively). Results indicate that rutile (LC_{50} (concentration that causes 50% mortality compared the control group) = 6.5) was more toxic than anatase (LC_{50} = 278.8) under simulated sunlight (SSL) irradiation, possibly due to an extremely narrow band gap. It is noted that HA coating increased the toxicity of anatase, but decreased that of rutile. Additionally, AHA50 and RHA50 had the biggest differences compared to uncoated anatase and rutile with LC_{50} of 201.9 and 21.6, respectively. We then investigated the formation of reactive oxygen species (ROS) by TiO_2 nanoparticles in terms of hydroxyl radicals ($^{\cdot}OH$) and superoxide anions (O_2^-). Data suggested that O_2^- was the main ROS that accounted for the higher toxicity of rutile upon SSL irradiation. We also observed that HA coating decreased the generation of $^{\cdot}OH$ and O_2^- on rutile, but increased O_2^- formation on anatase. Results from TEM analysis also indicated that HA coated rutile tended to be attached to the surface of *E. coli* more than anatase.

Introduction

Titanium dioxide (TiO_2) nanoparticles are the most widely used photocatalyst for environmental remediation (Chen and Mao, 2007; Kwon et al., 2008), particularly in natural aquatic environments. However, recent studies have raised the concerns over the potential health risks to humans and environments caused by nano TiO_2 throughout its life cycle (Boxall et al., 2007; Sharma, 2009; He et al., 2014b). The behavior and fate of TiO_2 nanoparticles can be altered by suspended solids and dissolved organic matter (DOM), once they are released into aquatic environments. In addition, the lack of knowledge of nano-bio-eco interactions could limit the use of TiO_2 nanoparticles for field applications. Therefore, it is imperative that their

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physicochemical properties be assessed by conducting feasibility studies before we employ such nanotechnology for environmental remediation.

In general, unmodified TiO_2 nanoparticles can only be excited by UV light, owing to their large band gap (theoretically 3.0 eV for rutile and 3.2 eV for anatase). However, TiO_2 nanoparticles can be sensitized through specific photosensitizers, for instance, dyes (Persson et al., 2000; De Angelis et al., 2007). Recently, humic acid (HA) has also been suggested to be capable of serving as a photosensitizer in HA/TiO_2/visible light system (Selli et al., 1999; Cho and Choi, 2002; Ryu and Choi, 2004). The supplementation with HA essentially expands the applicability of TiO_2 as a photocatalyst into visible light region. In addition, TiO_2 nanoparticles on most occasions, tend to aggregate in aqueous solutions and exist as aggregates, normally over 1 μm. Appreciably, surface coating can largely improve the stability and dispersibility of TiO_2 nanoparticles in aqueous solutions. Thus, lately, the physicochemical properties of HA coated TiO_2 nanoparticles have been studied and reported (Yang and Xing, 2009; Chen et al., 2012). Besides the expanded spectrum of light excitation, HA coated TiO_2 nanoparticles may also differ from the uncoated TiO_2 nanoparticles in the presence of free HA (Lin et al., 2012). It was reported that HA coating could reduce the adhesion of TiO_2 nanoparticles to algal cells, decrease the formation of reactive oxygen species (ROS), and consequently alleviate the algal toxicity (Lin et al., 2012). However, Yang et al. (2013) reported that oxidative deoxyribonucleic acid (DNA) damage and toxicity to zebrafish (*Danio rerio*) were increased by the supplement of HA to TiO_2 nanoparticles in the absence of light irradiation (Yang et al., 2013). Thus, it is necessary to investigate the alteration of physicochemical properties of TiO_2 nanoparticles coated with HA and to assess the effects on the subsequent nanotoxicity in the presence of sunlight or only visible light.

Furthermore, the effect of crystallinity has also been suggested to be attributed to the different toxicological profiles of TiO_2 nanoparticles. It is generally recognized that anatase is more active and toxic than rutile under UV irradiation. According to Luttrell et al. (2014), this is owing to the larger band gap of anatase. Under UV irradiation, anatase TiO_2 nanoparticles could generate higher amounts of ROS intracellularly and extracellularly than the rutile phase (Chen et al., 2007; Guichard et al., 2012). However, this could be altered or even reversed under visible light irradiation, or in the absence of light, as substantiated by the reports (Sayes et al., 2006; Lipovsky et al., 2012; Numano et al., 2014). Notably, ROS formation in water suspensions of TiO_2 was much higher in rutile than anatase after visible light illumination (400–800 nm, 40 mW/cm^2) (Lipovsky et al., 2012). They suggested that the difference between anatase and rutile under visible illumination might be owing to a difference in their band-gap energies (E_g), in which E_g (anatase) = 3.2 eV (387 nm), and E_g (rutile) = 3 eV (415 nm). On the basis of the above consideration, it is important to investigate how the photoactivity and toxicity differ with crystallinity under sunlight irradiation.

In this study, we synthesized HA coated TiO_2 nanoparticles in both rutile and anatase phases. We investigated their toxicity to *Escherichia coli* (*E. coli*) under simulated sunlight (SSL) irradiation. To the best of our knowledge, no study has been reported to have specifically investigated the effect of surface-bound HA on the physicochemical properties and toxicity of TiO_2 nanoparticles to living organisms.

1. Materials and methods

1.1. Materials

TiO_2 nanoparticles (Sample A and Sample B) were purchased from US Research Nanomaterials, Inc. (US Research Nanomaterials, Inc., Houston, TX, USA). All organic solvents and the humic acid (>99%) used in this study were purchased from Sigma Aldrich (Sigma-Aldrich Co., St. Louis, MO, USA). All solutions were prepared using nanopure water (Thermo Scientific™ NERL™ Reagent Grade Water, Nerl Diagnostics LLC, East Providence, RI, USA). Bacteria *E. coli* (ATCC#25254) was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA).

1.2. Preparation of HA coated TiO_2

The steps of synthesis of HA coated TiO_2 followed the previous description of (Yang and Xing, 2009) with slight modifications. Briefly, 1 g of TiO_2 (Sample A or Sample B) was added into 100 mL of HA solution to reach the different final concentrations of 50, 100, and 200 ppm. After stirring for 2 day at 180 r/min, the mixture was then centrifuged at 5000 ×*g* for 30 min and washed three times with nanopure water to eliminate any unbounded HA residues. The pellet was collected after removing the supernatant, and freeze-dried. Lyophilization was then conducted under vacuum at 0.014 mbar for 48 hr with a Labconco Freezone Plus 2.5 L Benchtop Cascade Freeze Dry Systems (Labconco, USA) equipped with a Welch 8912Z-02 Vacuum (Welch 8912Z-02, Gardner Denver Welch Vacuum Technology Inc., USA). Samples A and B were identified by X-ray diffraction (XRD) as anatase TiO_2, and rutile TiO_2, respectively. AHA50, AHA100, and AHA200 were the products from 50, 100, and 200 ppm HA coated with Sample A (anatase TiO_2) respectively. Correspondingly, RHA50, RHA100, and RHA200 were 50, 100, and 200 ppm HA coated with Sample B (rutile TiO_2), respectively.

1.3. Characterization

The characterization of HA coated TiO_2 nanoparticles was conducted with XRD, transmission electron microscopy (TEM), scanning electron microscopy (SEM), energy dispersive X-ray spectroscopy (EDX), Fourier transform infrared spectroscopy (FTIR), UV-Vis spectroscopy, dynamic light scattering (DLS), and phase analysis light scattering (PALS). The content of coated HA was determined with total organic carbon (TOC) analysis.

XRD patterns were obtained using a Rigaku D/MAX-Ultima-III diffractometer (Rigaku D/MAX-Ultima-III diffractometer, Rigaku, Japan) at room temperature with Cu Kα radiation at a tube current of 44 mA and an acceleration voltage of 40 kV. The scan ranges were 2–40° and 2–75° at a step interval of 0.1° and a scanning rate of 0.05°/min. Primary nanoparticle size was determined using a Jeol, JEM 1011 electron microscope working at 100 kV (JEM 1011, Joel USA, Inc., USA) equipped with a Gatan camera model 785. Morphology of TiO_2 and variation of growth for synthesized HA coated TiO_2 were