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Modifications of the bacterial reverse mutation test reveals mutagenicity of TiO_2 nanoparticles and byproducts from a sunscreen TiO_2-based nanocomposite

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HIGHLIGHTS

- The Ames test is not suitable for nanoparticle (NP) genotoxicity assessment.
- The Ames test medium prevents electrostatic interactions between bacteria and NPs.
- The Ames test medium strongly promotes the aggregation of NPs.
- Simple pre-exposure step in an adequate medium improve the accuracy of the test.
- Modified Ames test showed mutagenicity of NP-TiO_2 and NP-TiO_2-based nanocomposite.

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ABSTRACT

The bacterial reverse mutation test, recommended by the Organization for Economic Co-operation and Development (OECD) to determine genotoxicity of chemical compounds, has been recently used by several authors to investigate nanoparticles. Surprisingly, test results have been negative, whereas in vitro mammalian cell tests often give positive genotoxic responses. In the present study, we used the fluctuation test procedure with the *Salmonella typhimurium* strains TA97a, TA98, TA100 and TA102 to determine the mutagenic potential of TiO_2 nanoparticles (NP-TiO_2) and showed that, when it is used conventionally, this test is not suitable for nanoparticle genotoxicity assessment. Indeed, the medium used during exposure prevents electrostatic interactions between bacterial cells and nanoparticles, leading to false-negative responses. We showed that a simple pre-exposure of bacteria to NP-TiO_2 in a low ionic strength solution (NaCl 10 mM) at a pH below the nanoparticle isoelectric points (pH 5.5) can strongly improve the accuracy of the test. Thus, based on these improvements, we have demonstrated the genotoxicity of the engineered NP-TiO_2 tested and a NP-TiO_2 byproduct from a sunscreen nanocomposite. It was also shown that strain TA102 is more sensitive than the other strains, suggesting an oxidative stress-mediated mechanism of genotoxicity.

1. Introduction

Due to the increasing industrialization of many countries and resulting technological advances, environmental pollution has become a serious health issue. Many efforts have been made to protect the environment and human health. A priority area concerns the development of in vitro assays to evaluate the toxicological effects of environmental chemicals and then build prioritization models of in vivo toxicity. Over the years, multiple bioassays have been developed utilizing many organisms. Microbial tests have several advantages over other bioassays, including rapid response times due to the much shorter microbial life cycles, reproducibility of test conditions, amenability to genetic manipulations, increased sensitivity and reduced cost (Davoren, 2005). In addition, microorganisms possess the majority of the same biochemical pathways present in higher organisms, they exhibit significant membrane structure organization and generally elicit toxic responses to many chemicals through mechanisms similar to that of higher organisms (Qureshi et al., 1984).

The bacterial reverse mutation assay is recommended by national and international environmental protection agencies for substance evaluations (e.g. Organization for Economic Co-operation and Development (OECD test guideline 471); and The International Conference on Harmonization (ICH)) (Mortelmans

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and Zeiger, 2000). This test has also been recently approved as one of the two assays recommended by The Committee on Mutagenicity of Chemicals in Food, Consumer Products and the Environment (COM) (Kirkland et al., 2011). The bacterial reverse mutation test uses several strains of *Salmonella typhimurium* with mutated histidine synthesis genes. The test principle is based on the fact that reverse mutations caused by exposure to mutagenic compounds can reactivate the ability of mutated bacterial strains to synthesis histidine, thereby allowing them to grow in the absence of this essential amino acid. Several procedures for performing the bacterial reverse mutation test have been described. Among those commonly used are the plate incorporation method, commonly named the Ames test (Ames et al., 1972, 1973a,b), the preincubation method (Maron and Ames, 1983; Aeschbacher et al., 1987), the fluctuation method (Green et al., 1976; Hubbard et al., 1984; McPherson and Nestmann, 1990), and the suspension method (Thompson and Melampy, 1981). Over the years, many validation studies have been performed to determine the sensitivity and correlation of this test with animal carcinogenicity studies. It has been established that there is a high predictivity of a positive mutagenic response in the test for rodent carcinogenicity ranging from 77% to 90% (McCann et al., 1975; Tennant et al., 1987; Zeiger, 1998). To date, there are thousands of research and testing laboratories throughout the world using this assay to screen potentially mutagenic drugs and chemicals. Many companies and regulatory agencies use the results from this assay as part of their short-term toxicological testing programs to determine chemical safety (Felton and Wu, 2003).

Nanotechnology is a relatively new branch of science, heralded as a technological revolution (The White House, 2000). Engineered nanoparticles have rapidly moved from the laboratory to industry and are currently being used in many consumer products. Currently, there are over 1000 products in the consumer marketplace that include nanomaterials (Woodrow Wilson Database: http://www.nanotechproject.org), which is projected to substantially increase in the near future. This phenomenon has aroused great concern about potential human health effects and, on a larger scale, environmental effects (Nel et al., 2006), giving birth to a new biological field known as "Nanotoxicology". The Royal Society and Royal Academy of Engineering first raised this concern in 2003 (The Royal Society and the Royal Academy of Engineering, 2003; The Royal Society, 2004), paving the way for a rapid increase in investigational studies on nanoparticle toxicity; in particular, genotoxicity studies, as many nanoparticles were found to cause chromosomal aberrations, DNA strand breaks, oxidative DNA damage, and subsequent genetic mutations (Singh et al., 2009). In commonly used in vitro (chromosomal aberrations, comet assay, micronucleus) and in vivo mammalian test cell systems, nanoparticles have been largely found to promote positive genotoxic responses, while negative responses have been generally obtained for these nanoparticles with the bacterial reverse mutation test (Doak et al., 2012). It was reported that within 19 published studies, where this test was used for the genotoxicological analysis of nanoparticles, 17 showed negative mutagenicity. The two remaining studies only revealed weak mutagenic effects. Therefore, these studies seemed to have indicated that although the Ames test is excellent for testing chemical mutagenic activity, it does not appear to be suitable for nanoparticles. This might be related to the degree of nanoparticle uptake by bacterial cells, which is likely to be less than in mammalian cells (Singh et al., 2009; Doak et al., 2012). Indeed, bacteria cannot perform endocytosis and their cell wall forms a barrier against simple diffusion of nanoparticles. This lack of uptake could potentially lead to false negative results.

Based on our previous work (Pagnout et al., 2012), we think that another plausible hypothesis that leads to false negative results is the lack of interactions between nanoparticles and bacterial cells due to the use of an inappropriate medium during the exposure. We also think that performing the bacterial reverse mutation test by the fluctuation method instead of the plate incorporation method, with a pre-exposure step in a low-ionic strength solution at a pH value below the nanoparticle isoelectric points (NaCl 10 mM, pH 5.5), could improve these interactions and make the test more accurate for the assessment of the nanoparticle genotoxicity. As a consequence, in the present study, we assessed the mutagenic potential of two engineered TiO_2 nanoparticles and a TiO_2-byproduct derived from a nanocomposite material commonly used in sunscreens with the conventional fluctuation test and with a modified version of this test according to the modification mentioned above. TiO_2 nanoparticles (NP-TiO_2) were used as a model in this study for the following reasons: (i) these nanoparticles are widely used in consumer products (e.g. paints, plastics, paper, ceramics, cosmetics, and sunscreens) with expanded applications over the last decade (Colvin, 2003; Gleiche et al., 2006); (ii) in 2006, TiO_2 was reclassified from *Unclassifiable as to carcinogenicity in humans* (Group 3 carcinogen) to *Possibly carcinogenic to humans* (Group 2B carcinogen) based on sufficient evidence using experimental animals (Ng et al., 2010); (iii) NP-TiO_2 was recently listed by the OECD as one of the priority nanomaterials for immediate testing (OECD, 2008); (iv) NP-TiO_2 nanoparticles are minimally water-soluble and their potential carcinogenic effects cannot be attributed to the release of titanium ions in the medium; and (v) several studies showed no mutagenicity (Warheit et al., 2007; Pan et al., 2010) or very weak mutagenicity (Kumar et al., 2011) caused by NP-TiO_2 with the bacterial reverse mutation test (plate incorporation procedure), whereas NP-TiO_2 has been found to have positive genotoxic responses in other in vitro cellular test systems (Balasubramanyam et al., 2009; Di Virgilio et al., 2010; Osman et al., 2010; Shi et al., 2010).

2. Materials and methods

2.1. Evaluated nanomaterials

TiO_2 nanopowder AEROXIDE® P25 (TiO_2-P25) was provided by Evonik Degussa GmbH (Frankfurt, Germany, Stock # 4168050298). These nanoparticles are described by the supplier as having a primary size of 25 nm with a specific surface area (SSA) of 50 ± 15 m^2/g and a ratio of anatase/rutile forms of 80/20. The TiO_2-P25 stock suspension was prepared by dispersing 100 mg of NP-TiO_2 in 10 mL of sterile ultrapure water (milli-Q water, 18.2 MΩ cm). The resultant suspension was then probe-sonicated (Sonics Vibra-cell 750 W, Sonics & Materials, Inc., Newton, CT, USA; frequency 20 kHz, 3 mm micro tip, amplitude 40%) for 30 min at 4 °C to homogenize and break the larger agglomerates apart (Pagnout et al., 2012).

The second type of NP-TiO_2 used in this study (TiO_2-NA) was provided as a 15% (w/v) stable suspension in acidified water produced by Nanostructured & Amorphous Materials, Inc. (Houston, TX, USA – Stock # 7012WJWR). These nanoparticles are described by the supplier as being 100% anatase, with a primary size ranging from 5 to 30 nm, a SSA of 200–220 m^2/g, and a purity >99.5%. The stock suspension was prepared to 10 g/L by dilution in sterile ultrapure water.

The third nanomaterial used in this study was a byproduct obtained after alteration of a TiO_2-based nanocomposite, namely T-Lite™ SF (BASF, Germany) (TiO_2-TLB), which is commonly used in sunscreens as a UV blocker. The T-Lite nanocomposite consisted of a TiO_2 rutile core (5–10 nm cross-section per 50–200 nm length) arranged together in large clusters, which had an average size of 200 nm. These clusters were embedded in an amorphous layer of aluminum oxide [$Al(OH)_3$] and polydimethylsiloxane (PDMS) (Labille et al., 2010; Auffan et al., 2010). The byproduct, resulting from an accelerated ageing process, was provided by Jérôme Labille (CEREGE Laboratory, Aix-en-Provence, France). Briefly, the alteration process consisted of mixing 100 mg of TiO_2-TLB in 250 mL of ultrapure water. The mixture was magnetically stirred at 690 rpm under a white light (400 W Philips® 114 Master HPI-T Plus) for 48 h. After alteration, this mixture was settled for 48 h and the supernatant containing stable altered TiO_2 nanocomposites (byproducts) was obtained (Labille et al., 2010; Auffan et al., 2010). As previously described by Bigorgne et al. (2011), the quantity of TiO_2 byproducts was measured by filtering an aliquot of suspension (20 mL) through a 25 nm membrane filter and drying filter at 105 °C for 24 h. The concentration of TiO_2 byproducts obtained was adjusted to 100 mg/L with ultrapure water.