Effective dispersal of titanium dioxide nanoparticles for toxicity testing

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ABSTRACT — Currently, protocols for the dispersal of titanium dioxide (TiO2) nanoparticles are not standardized and often yield non-uniform particles and/or insufficient dispersal in liquid medium. Our study aimed to improve dispersal so that TiO2 nanoparticles are of uniform size, making nanotoxicity testing more reliable. Various combinations of vehicles, sonication durations, and sonication volumes were assessed for optimizing preparations of TiO2 nanoparticles. We tested each of five vehicles: ultrapure water (UPW), 0.2% disodium hydrogen phosphate (DSP), Dulbecco’s phosphate-buffered saline (PBS), 0.9% saline (S), or S containing 0.05% Tween 80 (ST). We also assessed two sonication durations and three sonication volumes. Each suspension underwent ultrasonication and centrifugation; the supernatants were then analyzed. Particle size was measured by dynamic light scattering. P25 nanoparticles (~100 nm; the type of TiO2 nanoparticles used in our study) in UPW and 0.2% DSP were effectively dispersed; however, those in PBS, S, or ST were not. Relevant duration time and volume for sonication were examined with 0.2% DSP. A sonication time of 30 min and volume of 10 mL for each vial were determined to be optimal sonication conditions as determined with our dispersal assay. Under these optimal conditions, P25 nanoparticles sonicated/centrifuged in UPW or 0.2% DSP remained dispersed and exhibited long-term stability (90 days). We thus have developed a reliable procedure for preparing TiO2 nanoparticles in liquid-phase dispersions for toxicity testing.

Key words: Nanoparticle, Polydispersity index, Titanium dioxide, Z-average

INTRODUCTION

Nanomaterials are defined as natural, incidental, or manufactured materials containing particles in an unbound state, i.e., not in an aggregate or agglomerate, in which more than 50% of the particles in the number-size-distribution in one or more external dimensions is less than 100 nm (European Commission 2011/696/EU). Nanoparticles (NPs) are particles of less than 100 nm in diameter, and titanium dioxide (TiO2) NPs have been widely manufactured for consumer, medical, and industrial products for a variety of applications (Tsuzuki, 2009; Shi et al., 2013). The effects of TiO2 NPs on experimental animals have been reviewed in detail by Ema et al. (2010) and Iavicoli et al. (2012). Despite the publication of many TiO2-specific reproductive and developmental toxicity studies, conditions for the handling, measuring, and tracking of NPs differ (Takeda et al., 2009; Yamashita et al., 2011; Gao et al., 2013; Zhao et al., 2013; Jia et al., 2014). The differences in particle preparation (including their dispersal during application) and measurement are challenges in the field and have led to data that are incongruous at times.

The potential adverse health effects of TiO2 NPs remain unclear (Oberdörster et al., 2005). Although many different NP preparation protocols have been reported (Bihari et al., 2008; Wang et al., 2008; Kobayashi et al., 2009; Takeda et al., 2009; Kim et al., 2010; Zhang et al., 2010; Wu et al., 2014), most of the resulting NPs tend to aggregate to form larger particles owing to their very large reactive-surface areas. As the size of a particle decreases, its relative surface area increases. Toxicologically, both particle size and surface area are critical characteristics of NPs (Sager et al., 2007); moreover, because of the reactive nature of NPs and their tendency to aggregate, it is necessary to prepare NPs that remain stable and
to find conditions that prevent agglomeration and aggregation over time. Indeed, choosing an appropriate preparation protocol is key to achieving reliable data during toxicity studies.

TiO$_2$ NPs naturally exhibit three different crystalline structures, namely anatase, rutile, and brookite. P25 NPs, which are the TiO$_2$ NPs used in our present study, are commercially available and were chosen owing to their broad applications in industry, and indeed much research has been published concerning the potential biological effects of P25 NPs. The purpose of this study was to develop a method to optimize the preparation of TiO$_2$ NPs for use in liquid-phase dispersions for toxicity testing. To further improve on published preparation methods, various combinations of vehicles, sonication durations, and volumes were tested along with a time-course for NP stability.

**MATERIALS AND METHODS**

**Chemicals**

P25 TiO$_2$ nanopowder, which is a mixture of anatase and rutile phases in an approximate 8:2 ratio (Lot#: MKBJ961V, purity > 99.5%, primary size ~21 nm, specific surface area ~50 m$^2$/g), Dulbecco’s phosphate-buffered saline (PBS), and Tween 80 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium chloride was purchased from Wako Pure Chemical Industries (Osaka, Japan). Ultrapure water (UPW; Milli-Q, Merck KGaA, Darmstadt, Germany) was autoclaved and filtered through a cellulose acetate membrane (0.20 µm pore size, Osaka, Japan). TiO$_2$ UPW and filtered through the aforementioned cellulose membrane. Disodium hydrogen phosphate (DSP) and sodium chloride were dissolved in autoclaved UPW and filtered through the aforementioned cellulose acetate membrane (0.20 µm pore size, Osaka, Japan). Disodium hydrogen phosphate (DSP) and sodium chloride were dissolved in autoclaved UPW and filtered through the aforementioned cellulose acetate membrane. TiO$_2$ NPs were suspended in a solution in a 100-mL vial. Each suspension was vortexed vigorously and ultrasonicated for 30 min. The suspension from each vial was transferred to a 10-mL centrifuge tube (Eiken Chemical Co. Ltd., Tokyo, Japan) and centrifuged at 1,000 × g for 60 min at 25ºC. Then, 5 mL of the supernatant was removed from the top of the tube. Particle size was assessed by DLS.

**Ultrasonication of TiO$_2$**

NP samples were subjected to ultrasonication with an ultrasonic cleaner (Branson 5510J-DTH, 180 W, 40 kHz, Branson Ultrasonic Corporation, Danbury, CT, USA). The water level of the bath was adjusted to the level of suspension surface in 100-mL glass vials (Nichiden-Rika Glass, Hyogo, Japan) to achieve maximum dispersal.

**Determining the size and concentration of TiO$_2$ NPs**

The average size of the TiO$_2$ NPs (Z-average and polydispersity index; PDI) in each final preparation (supernatant) was determined by dynamic light scattering (DLS) using a Zetasizer Nano ZS (Malvern, Malvern Hills, UK). Cumulant analysis is a standard method of analyzing the autocorrelation function generated by a DLS experiment. Given that it is a moment expansion, it can produce several values; however, only the first two terms are used in practice, namely a mean value for the particle size (Z-average) and a width parameter known as the PDI. The Z-average is an intensity-based calculated value (International Standard ISO13321, 1996; International Standard ISO22412, 2008). TiO$_2$ supernatant (1 mL) in a 5-mL glass vial was heated and the liquid evaporated to dryness at 200ºC for 120 min. The TiO$_2$ concentration in the supernatant was then back-calculated on the basis of the mass of the remaining solid (Endoh, 2011). TiO$_2$ particles prepared in 0.2% DSP solution were measured by DLS as reported elsewhere (Kobayashi et al., 2009; Naya et al., 2012). The DLS technique is considered a sensitive and valuable method for the measurement and analysis of NP (Brar and Verma, 2011).

**Experiment 1**

The sample preparation procedure for each of experiments 1–4 is illustrated in Fig. 1. To examine the conditions needed to achieve appropriate TiO$_2$ dispersal in solution, the five vehicles described above were compared. P25 was suspended in a solution in a 100-mL glass vial (Nichiden-Rika Glass) at a concentration of 0.2 g/10 mL. Each suspension was vortexed vigorously and ultrasonicated for 30 min. The suspension from each vial was transferred to a 10-mL centrifuge tube (Eiken Chemical Co. Ltd., Tokyo, Japan) and centrifuged at 1,000 × g for 60 min at 25ºC. Then, 5 mL of the supernatant was removed from the top of the tube. Particle size was assessed by DLS.

**Experiment 2**

P25 (2 g) was suspended by vortexing in 100 mL of 0.2% DSP (stock solution). To examine the effects of sonication time on TiO$_2$ particle size, 50 mL of this suspension was dispensed into a 100-mL vial. Each suspension was vortexed and ultrasonicated for 30 or 120 min. The suspension (50 mL) was transferred to a 50-mL centrifuge tube (Corning Life Sciences, Tewksbury, MA, USA) and centrifuged at 1,000 × g for 60 min at 25ºC. The top-most 25 mL of the supernatant (i.e., half the volume) was carefully collected, and then the particle size was assessed by DLS.
Experiment 3
P25 (2 g) was suspended by vortexing in 100 mL of 0.2% DSP (stock solution). To examine the effects of sonication volume on TiO₂ particle size, three different volumes of a P25 suspension (10, 30, 50 mL) were dispensed in individual 100-mL vials. Each suspension was vortexed and ultrasonicated for 30 min. The 10-, 30-, or 50-mL suspensions from each vial were each transferred to a 50-mL centrifuge tube and centrifuged at 1,000 × g for 60 min at 25°C; the top-most 5, 15, or 25 mL of the supernatant (i.e., half the volume), respectively, was carefully collected, and the particle size was assessed by DLS.

Experiment 4
P25 (2 g) was suspended by vortexing in 100 mL UPW or 0.2% DSP (to yield two separate stock solutions). To examine the long-term stability of particle size, 10 mL of P25 suspension from each stock solution was ultrasonicated and centrifuged at 1,000 × g for 60 min at 25°C; the top-most 5, 15, or 25 mL of the supernatant (i.e., half the volume), respectively, was carefully collected, and the particle size was assessed by DLS.
was dispensed in individual 100-mL vials. A total of ten 100-mL vials containing 10 mL of P25 suspension were prepared with each of the 0.2% DSP and UPW stocks. All samples were ultrasonicated for 30 min, and five 100-mL vials containing 10 mL of P25 suspension for each of the 0.2% DSP and UPW stocks were randomly selected for centrifugation. Then, the suspensions of each set of five 10-mL samples were each transferred to a 10-mL centrifuge tube (Eiken Chemical Co., Ltd.) and centrifuged at 1,000 × g for 60 min at 25°C. After centrifugation, the top-most 5 mL of each supernatant (i.e., half the volume) was carefully collected and pooled in a new 50-mL centrifuge tube and then used for analysis. The remaining five 100-mL vials for each group were not centrifuged, but the suspensions were carefully collected and pooled in a new 50-mL centrifuge tube and used for analysis. The pooled supernatants (estimated volume ~25 mL) and suspensions (estimated volume ~50 mL) were stored at room temperature throughout the experiment. Particle size was determined by DLS. To examine the long-term stability of NPs in the P25 supernatant, the Z-average, PDI, and concentrations of P25 were measured for up to 90 days in both 0.2% DSP and UPW.

RESULTS

We used various types and combinations of vehicles, sonication durations, and volumes to carry out a time-course study of P25 stability. In experiment 1, P25 NP suspensions prepared using UPW and 0.2% DSP were both well dispersed. The Z-average was ~80 nm, and the PDI value was ~0.15 for each group (Fig. 2). In contrast, the P25 supernatants prepared using PBS, S, or ST were not well dispersed for NPs (Z-average: PBS, ~2,000 nm; S, ~1,200 nm; ST, ~1,100 nm). Therefore, in subsequent experiments, only UPW and 0.2% DSP were used as candidate vehicles. In experiment 2, the appropriate sonication duration time was evaluated using 0.2% DSP as a more effective vehicle for dispersion on the basis of the results of experiment 1. The results of the particle size measurement were similar for the 30- and 120-min sonication groups (Fig. 3). In experiment 3, sonication volume was optimized. The Z-average increased in a volume-dependent manner for each of the groups, and the PDI values were similar among groups (Fig. 4). Hence, a sonication time of 30 min and sonication volume of 10 mL for each vial were found as optimal sonication conditions. In experiment 4, the long-term stability of particle size was examined for 90 days. The diameters of dispersed particles collected from supernatants (with centrifugation) were consistently < 100 nm (~78 nm), the PDI was ~0.15, and the concentrations were stable for up to 90 days after preparation with 0.2% DSP (Fig. 5, upper panels). The average diameter of NPs in the suspensions prepared using 0.2% DSP was ~150 nm and the PDI was ~0.2, and the concentrations were stable throughout the study (Fig. 5,
Effective method for dispersing nanosized TiO₂

The inconsistent application of the various NP sample-preparation protocols among researchers, combined with the lack of accepted protocol standardization, has likely contributed to the observed variability in results between toxicity studies. Moreover, the dispersal of NPs in aqueous media is still not fully standardized (Hartmann et al., 2017). The discrepancies between our results and those of other researchers may reflect differences in NP preparation conditions, e.g., sonication power and/or sonication volume, although the exact basis of the differing results remains unclear. For in vivo and in vitro studies, the addition of a dispersant (e.g., serum) to the vehicle can result in an underestimation of the acute pro-inflammatory response to TiO₂ NPs (Vranic et al., 2017). Therefore, in general, a dispersant is not added to the vehicle because it may interfere with the assessment of particle size. In our present experiments, we did not assess the morphology of the prepared P25 NPs, i.e., in solution, with or without centrifugation. DLS was used to obtain an accurate estimate of TiO₂ NP stability, but DLS measures broad distributions of sizes and the aggregation of particles.

In experiment 4, we examined the effects of ultrasonication on long-term stability. In the samples from the non-centrifuged groups using 0.2% DSP or UPW as the vehicle, the PDI values and the concentrations of NPs were relatively consistent, but the size of the prepared particles (Z-average) was relatively large, i.e., ~150–160 nm, and this size range is not appropriate for nanotoxicology studies. We conclude that ultrasonication is a necessary step during the preparation of NPs, i.e., to ensure that the particles behave as nano-sized aggregates and exhibit long-term stability regardless of the vehicle used for dispersion. Notably, analysis of the NP microstructure, e.g., using transmission electron microscopy, can help determine whether NPs have been adequately dispersed. Therefore, transmission electron microscopy can be used to determine whether the crystalline structure or morphology of NPs is influenced by any particular sonication process i.e., whether sonication promotes the formation of an amorphous phase or induces morphological changes in P25 NPs.

Although the procedure we describe here was adequate for the dispersal of the P25 NPs that we produced, further tests will be required to determine whether this dispersal procedure is applicable to the dispersal of NPs produced using other methods. DSP is a good phosphate-based vehicle that is used as a negative control for pulmonary toxicity studies in vivo (Kobayashi et al., 2009; Naya et al., 2012). However, details of the effects of DSP on other organs are unknown. Although we found that P25 NP suspensions prepared using UPW and 0.2% DSP were both effectively dispersed, the toxicological effects of these vehicles should be more fully investigated both in vivo and in vitro. Therefore, any practical protocol for preparing NPs should be thoroughly investigated and should reflect, as close as possible, realistic exposure conditions (Vranic et al., 2017).

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Fig. 5. Changes in Z-average, PDI, and concentration of P25 nanoparticles dispersed in 0.2% DSP with centrifugation (upper) or without centrifugation (lower). Each point represents the mean ± S.D. (n = 3–4). NP: nanoparticle.

Fig. 6. Changes in Z-average, PDI, and concentration of P25 nanoparticles dispersed in UPW with centrifugation (upper) or without centrifugation (lower). Each point represents the mean ± S.D. (n = 3–4). NP: nanoparticle.
on ultrafine particles of metal oxides) of NIOSH, Japan.

Conflict of interest---- The authors declare that there is no conflict of interest.

REFERENCES


