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DNA Damaging Potential of Photoactivated P25 Titanium Dioxide Nanoparticles

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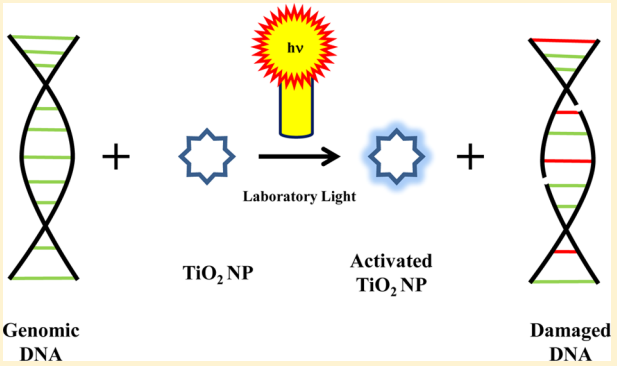
Supporting Information

ABSTRACT: Titanium dioxide nanoparticles (TiO_2 NPs) are found in numerous commercial and personal care products. Thus, it is necessary to understand and characterize their potential environmental health and safety risks. It is well-known that photoactivated TiO_2 NPs in aerated aqueous solutions can generate highly reactive hydroxyl radicals ($^{\bullet}OH$), which can damage DNA. Surprisingly, recent *in vitro* studies utilizing the comet assay have shown that nonphotoactivated TiO_2 NPs kept in the dark can also induce DNA damage. In this work, we utilize stable isotope-dilution gas chromatography/tandem mass spectrometry to quantitatively characterize the levels and types of oxidatively generated base lesions in genomic DNA exposed to NIST Standard Reference Material TiO_2 NPs (Degussa P25) under precisely controlled illumination conditions. We show that DNA samples incubated in the dark for 24 h with TiO_2 NPs (0.5−50 μg/mL) do not lead to the formation of base lesions. However, when the same DNA is exposed to either visible light from 400 to 800 nm (energy dose of ∼14.5 kJ/m^2) for 24 h or UVA light at 370 nm for 30 min (energy dose of ∼10 kJ/m^2), there is a significant formation of lesions at the 50 μg/mL dose for the visible light exposure and a significant formation of lesions at the 5 and 50 μg/mL doses for the UVA light exposure. These findings suggest that commercial P25 TiO_2 NPs do not have an inherent capacity to oxidatively damage DNA bases in the absence of sufficient photoactivation; however, TiO_2 NPs exposed to electromagnetic radiation within the visible portion of the light spectrum can induce the formation of DNA lesions. On the basis of these findings, comet assay processing of cells exposed to TiO_2 should be performed in the dark to minimize potential artifacts from laboratory light.



INTRODUCTION

Engineered titanium dioxide nanoparticles (TiO_2 NPs) are found in numerous commercial and personal care products.[1] Many of the personal care products, such as over-the-counter sunscreen creams and/or aerosolized sunscreen sprays, are used by large segments of the population (adults and children). Characteristic exposure routes for TiO_2 NP-containing personal care products can include either skin adsorption or lung inhalation, depending on the product formulation. Thus, it is necessary to understand and characterize the potential environmental and biological effects posed by these NPs.

It is well-known that photoactivated TiO_2 NPs can generate highly reactive hydroxyl radicals ($^{\bullet}OH$) in aerated aqueous solutions and superoxide radical anions ($O_2^{\bullet-}$) in non-aqueous media.[2] The mechanisms for the formation of $^{\bullet}OH$ from photoactivated TiO_2 NPs in solution have been investigated and documented extensively[2−5] and will not be reiterated here. However, once formed, $^{\bullet}OH$ can interact with and damage various biological molecules, such as DNA, at diffusion-limited rates, resulting in the formation of oxidatively induced DNA damage (e.g., strand breaks, DNA base and sugar lesions, and abasic sites).[6] The single cell gel electrophoresis assay (alkaline comet assay) is commonly used to measure and characterize NP-induced DNA damage (single-strand breaks, SSBs).[4] When the comet assay specifically incorporates the use of DNA glycosylases or DNA endonucleases such as *Escherichia coli* formamidopyrimdine DNA glycosylase (Fpg) or *E. coli* endonuclease (III) (EndoIII) into the assay procedure, the observed SSBs predominantly correspond to the presence of oxidatively modified purine or pyrimidine DNA base lesions, respectively.

Many *in vitro* studies have shown, using the comet assay, that photoactivated TiO_2 NPs can indeed induce oxidative damage to DNA in the form of SSBs.[7−9] Surprisingly, numerous recent *in vitro* studies, using the comet assay, have shown that purportedly nonphotoactivated TiO_2 NPs (NPs exposed to cells in the dark or to cells under ambient (visible) laboratory

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light) can also induce significant levels of SSBs.[10−15] Other recent *in vitro* studies report no statistically significant formation or accumulation of SSBs when exposures occur in the dark or under normal ambient laboratory lighting conditions.[16,17] Thus, the inherent capacity of TiO_2 NPs to induce oxidative damage to DNA without photoactivation is unclear. Importantly, the capacity of using the comet assay to characterize and accurately measure the purported DNA damage from TiO_2 NPs has also been seriously questioned as a result of false-positive results,[18] a finding similar to an earlier study suggesting an artifact in the comet assay with germanium nanoparticles.[19] In the Rajapakse et al. study,[18] the authors concluded that many of the false-positive comet assay results were due to the TiO_2 NPs directly interacting *post festum* with the DNA to produce exaggerated comet tails. Overall, determining the DNA damaging potential of photoactivated TiO_2 NPs has been complicated by the large variety of cellular exposure models utilized in previous studies, insufficient physicochemical characterization of the tested NPs, discrepancies in NP dispersion procedures, and uncontrolled or unreported lighting conditions utilized during the testing procedures.

In this fundamental, molecular level investigation, we utilize high-resolution, stable isotope-dilution gas chromatography/tandem mass spectrometry (GC/MS/MS) to quantitatively characterize the levels and discriminate among the types of oxidatively generated DNA base lesions. Advantages of the isotope-dilution mass spectrometry approaches have been recently described at length[4] and have been successfully used in several recent nanogenotoxicity studies by our group.[20−24] Genomic DNA samples were analyzed using GC/MS/MS after direct exposure to well-characterized National Institute of Standards and Technology (NIST) Standard Reference Material (SRM) TiO_2 NPs (SRM 1898, which was made using Degussa P25) under three controlled illumination conditions: darkness, visible light, and ultraviolet A (UVA) radiation. While other studies have been performed using plasmid DNA and/or calf thymus DNA (ct-DNA) exposed to illuminated TiO_2 NPs,[8,9,25−28] this is the only study, to our knowledge, that clearly identifies and quantifies specific mutagenic and cytotoxic DNA base lesions resulting from exposure to varying TiO_2 NP concentrations and precisely controlled light irradiation.

MATERIALS AND METHODS

Reagents and Consumables. Nitrilotriacetic acid disodium salt (NTA), iron chloride ($FeCl_3$), 30% (volume fraction) hydrogen peroxide (H_2O_2), hydrochloric acid (99.99% purity), ct-DNA (sodium salt), sodium phosphate monobasic, and sodium phosphate dibasic were obtained from Sigma-Aldrich (St. Louis, MO). Internal standards 4,6-diamino-5-formamidopyrimidine-$^{13}C,^{15}N_2$ (Fapy adenine-$^{13}C,^{15}N_2$), 2,6-diamino-4-hydroxy-5-formamidopyrimidine-$^{13}C,^{15}N_2$ (Fapy guanine-$^{13}C,^{15}N_2$), 8-hydroxyadenine-$^{15}N_5$ (8-OH-adenine-$^{13}C,^{15}N_2$), 5-hydroxy-5-methylhydantoin-$^{13}C,^{15}N_2$ (5-OH-5-MeHyd-$^{13}C,^{15}N_2$), and 8-hydroxy-2′-deoxyguanosine-$^{15}N_5$ were purchased from Cambridge Isotope Laboratories (Andover, MA). 8-Hydroxyguanine-$^{15}N_5$ (8-OH-guanine-$^{15}N_5$) was obtained by hydrolysis of 8-hydroxy-2′-deoxyguanosine-$^{15}N_5$ with 60% formic acid at 140 °C for 30 min followed by lyophilization. Subsequently, 8-OH-guanine-$^{15}N_5$ was dissolved in 10 mM NaOH before use. *E. coli* formamidopyrimdine DNA glycosylase (Fpg) and *E. coli* endonuclease (III) (EndoIII) were purchased from Trevigen (Gaithersburg, MD). NIST SRM 1898 (P25 TiO_2 NP) was obtained from the NIST Standard Reference Materials Program. Chelex 100 resin was purchased from BioRad Laboratories (Hercules, CA). Plastic UV-transparent spectrophotometric cuvettes were purchased from Sigma (St. Louis, MO). Microcon Ultracel YM-30 centrifugal filters (30 kDa molecular weight cutoff) were obtained from Millipore (Billerica, MA). Distilled and deionized water (ddH_2O), obtained from a Waters Milli-Q system, was used to prepare all aqueous solutions.

Nanoparticle Characterization. The TiO_2 NP powder (SRM 1898) used in this study was formed from AEROXIDE TiO_2 P25 powder obtained from Evonik Degussa GmbH, Germany. SRM 1898 has been extensively characterized physically and chemically[29,30] and has been established, for example, to have a specific surface area (multipoint BET) of 55.55 ± 0.70 m^2/g, a relative phase fraction (X-ray diffraction) of 0.76 ± 0.03 (anatase) and 0.24 ± 0.03 (rutile), and a particle suspension size in PBS (laser diffraction spectroscopy) of 75 ± 4 nm. The errors in the specific surface area and relative phase fraction measurements represent expanded uncertainties about the mean values, and the error in the particle size measurement represents the approximate 95% confidence interval about the mean value.

Preparation of Nanoparticle Dispersions. A stock solution (10 mg/mL) of TiO_2 NPs in ddH_2O was prepared using a standardized sonication method previously described.[30] NP suspensions were prepared by sonication using a Branson 450 analogue sonicator (Branson Ultrasonics Corp., Danbury, CT) for 15 min in 80% pulsation mode and with a sonication power of ≈50 W. This approach follows a standardized dispersion protocol using a calibrated ultrasonicator.[30] The particle size distribution was analyzed initially and every time a DNA damage experiment was initiated using laser diffraction spectrometry (LDS) (Partica LA-950 V2; Horiba Instruments Inc., Irvine, CA).

Preparation of ct-DNA Stock Solutions and Positive Controls. Calf thymus DNA stock solutions (500 mg/L) were prepared by dissolving the appropriate amount of ct-DNA in ddH_2O. Transition metals and other impurities were removed by treating the ct-DNA solutions with Chelex resin combined with extensive dialysis (3500 Da MWCO membranes) against ddH_2O at 4 °C.[31] Calf thymus DNA stock solutions were stored at 4 °C until needed. Positive control ct-DNA (Fenton chemistry damaged) was prepared using NTA, $FeCl_3$, and H_2O_2 as previously described.[31] The positive control ct-DNA samples were prepared in 100 μg aliquots, dried (SpeedVac), and stored at 4 °C until needed. Positive controls were resolubilized with ddH_2O (gentle shaking at 4 °C for 24 h) and run with every set of samples to verify sample preparation procedures and instrument performance.

Protocol for Dark Exposure Studies. A 250 mg/L TiO_2 NP suspension was prepared by volumetrically diluting the 10 mg/mL TiO_2 NP stock solution with ddH_2O. A 100 mmol/L phosphate buffer solution (pH 7.4) was prepared by appropriately combining monobasic and dibasic phosphate salts in ddH_2O. After utilizing Chelex resin to remove transition metal impurities, the pH of the phosphate buffer was adjusted using HCl. The following step was conducted in a darkened laboratory with no exterior sunlight. Appropriate volumes of the 100 mmol/L phosphate buffer solution, the 500 mg/L ct-DNA stock solution, and the 250 mg/L TiO_2 NP solution were added into UV opaque amber 2 mL microfuge vials (Eppendorf) to make 1 mL samples containing 5 mmol/L phosphate buffer, 250 μg ct-DNA, and increasing TiO_2 NP concentrations (0.5, 5, and 50 mg/L). Negative controls without TiO_2 NPs were prepared similarly. Five or six samples were prepared per condition. All of the samples were incubated at 37 °C on a rotating (100 rpm) shaker (C24 Incubator Shaker, New Brunswick Scientific) for 3 or 24 h. After the exposure period, the samples were first centrifuged at 15 700*g* for 1 h at 4 °C to pellet the TiO_2 NPs. Preliminary experiments based on digestion of test samples with hydrofluoric acid and analysis with inductively coupled plasma−optical emission mass spectrometry (ICP−OES) indicated that 97.8 ± 1.4% of the TiO_2 NPs were removed by centrifugation under these conditions (see Supporting Information for additional details). This high TiO_2 NP removal efficiency indicated that any remaining TiO_2 NPs in the sample were unlikely to cause additional damage to the DNA during subsequent experimental steps. Next, 500 μL of the sample supernatant (containing the ct-DNA) was transferred into a 30 kDa centrifugal filter unit and centrifuged at 15 700*g* for at least 15 min at 20 °C.

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Another 400 μL of ddH_2O was then added to the top of the filtration membrane and washed through by centrifuging at 15 770g for at least an additional 15 min at 20 °C. To resolubilize the DNA for quantification, another 400 μL of ddH_2O was added to the top of the filtration membrane, and the filter was capped and stored at 4 °C for ≥24 h. After this period, the samples were reverse-eluted by centrifugation at 3000g for 5 min at 20 °C into clean 1.5 mL clear Eppendorf tubes and stored at 4 °C for ≤24 h. The DNA concentration in each sample was determined by spectrophotometric absorbance at 260 nm (absorbance of 1 = 50 μg of double-stranded DNA/mL). Fifty micrograms of DNA was added into clean Eppendorf tubes along with all of the stable isotopically labeled DNA lesion analogues (excluding 8-OH-guanine) and dried under vacuum. All samples were stored with desiccant at 4 °C until enzymatic digestion. Prior to enzymatic digestion and GC/MS/MS analysis (below), isotopically labeled 8-OH-guanine was added to all of the samples. All samples were stored with desiccant at 4 °C until enzymatic digestion. Even though the determination of oxidatively induced DNA damage via GC/MS procedures has been designed and optimized to minimize artifactual formation of 8-OH-guanine,[32] an additional experiment was performed to confirm the lack of artifactual 8-OH-guanine formation. We prepared an additional set of exposure samples incubated for 24 h in the dark with and without TiO_2 NPs and analyzed these samples for 8-OH-deoxyguanosine levels by LC/MS/MS[22] (see Supporting Information for LC/MS/MS method details) and for 8-OH-guanine levels by GC/MS/MS.

Protocol for Visible Light Exposure Studies. This study was designed to simulate lighting conditions in a laboratory under normal operating conditions. Illumination experiments were conducted at 37 °C by exposing DNA samples to a table top fluorescent lamp positioned ∼1 ft. (0.3 m) above the sample holder (a beaker). Exposures were performed using only the visible portion of the electromagnetic radiation spectrum (400−800 nm, Figure 1). Samples were contained in plastic UV transparent cuvettes freely floating in a water bath, equilibrated at 37 °C. Light exposure was monitored using an optical fiber inserted into one of the cuvettes and connected to an Ocean Optics Chem2000 fiber optic spectrometer. The spectrometer detector was calibrated for the absolute photon flux by exposing the optical fiber end to the opening in the integrating sphere of the NIST calibrated halogen lamp (Supporting Information, Figure S1). Importantly, light flux was measured continuously during sample exposure, and the total photon flux reaching the samples inside the cuvettes was calculated using a calibration curve. Following setup of the exposure system, a complete set of TiO_2 NP/ct-DNA samples was prepared as described previously for the dark exposure studies except that precautions were not taken to limit laboratory light during sample preparation. Then, the samples were exposed to visible radiation for 24 h to yield a total dose of 14.5 kJ/m^2. After the exposure period, the samples were centrifuged at 15 700g for 1 h at 4 °C to pellet the TiO_2 NPs and processed as described previously for the dark studies. Each of the 50 μg ct-DNA exposure samples was stored with desiccant at 4 °C until enzymatic digestion.

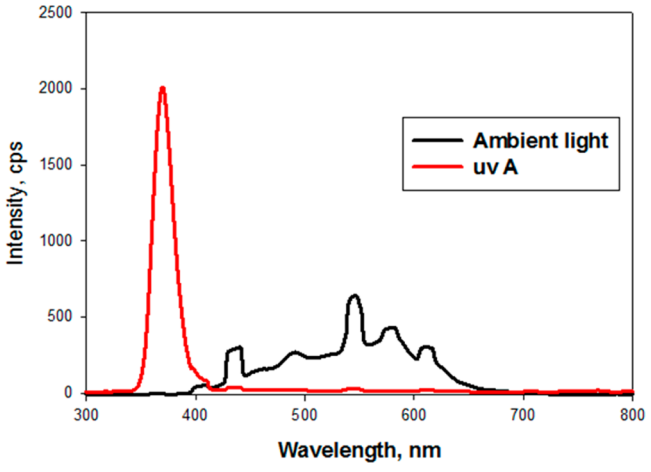

Figure 1. Spectral intensities of ambient light and UVA light irradiation utilized in the study.

Protocol for UVA Light Exposure Studies. This study was designed to simulate lighting conditions representative of irradiant energy levels in typical sunlight. A UVA wavelength (370 nm, Figure 1) was selected that was less than the band gap (∼400 nm) for TiO_2.[2] Illumination experiments were conducted at 37 °C by exposing DNA samples to black UV lamp light inside a UV reactor (Rayonet, Southern New England Ultraviolet Company). A lamp spectral profile is shown in Figure 1. Samples were contained in plastic disposable UV transparent cuvettes freely floating in water, equilibrated at 37 °C. The light flux (10.0 kJ/m^2) through the duration of the sample exposures was carefully controlled and quantified as described above. Following setup of the exposure system, a complete set of TiO_2 NP/ct-DNA samples was prepared as described previously for the dark exposure studies. Then, the samples were exposed to UVA radiation for 30 min. After the exposure period, the samples were centrifuged at 15 700g for 1 h at 4 °C to pellet the TiO_2 NPs and processed as described previously for the dark studies. Each of the 50 μg ct-DNA exposure samples was stored with desiccant at 4 °C until enzymatic digestion.

Enzymatic Digestion and GC/MS/MS Analysis of Exposure Samples. All samples were enzymatically digested and analyzed via GC/MS/MS methodology based on procedures described in previous studies.[20,21,23] Full details of the methodology are available in the Supporting Information. Briefly, the samples were dissolved in an aqueous buffer and then treated with *E. coli* Fpg and EndoIII DNA deglycosylases. The reaction was stopped by addition of ice-cold ethanol, and the excised DNA lesions (Figure 2) were isolated from undamaged DNA by centrifugation. The samples were trimethylsilylated and analyzed using isotope-dilution GC/MS/MS in the multiple reaction monitoring (MRM) mode.

Electron Paramagnetic Spectroscopy (EPR) Spin-Trapping Experiments. TiO_2 NP samples at a concentration of 50 mg/L were prepared in amber microfuge vials with ct-DNA and phosphate buffer identical to the previously described DNA damage experiments, except that 3-amino-2,2,5,5-tetramethyl-1-pyrrolidinyloxy (3AP) spintrap (final concentration = 500 μmol/L) was also included. A Bruker Elexsys E500 spectrometer equipped with an X-band microwave bridge was used to perform EPR spectroscopy. The concentration of 3AP, as well as EPR instrument parameters, was optimized to obtain the appropriate signal intensity and sensitivity for the TiO_2 NP incubation samples. The following instrument parameters were utilized: microwave power = 2 mW (10 was too high, so it was changed to 2 mW), modulation frequency = 100 kHz, and modulation amplitude = 0.1 mT. The TiO_2 NP samples were incubated in a water bath at 37 °C for at least 1 h before each EPR measurement to mimic the conditions utilized in the DNA damage assays. The incubation/spin trap samples were measured for at least 30 min. Two light conditions were tested: dark exposures with no light added and a laboratory light condition where a special filter was added to the EPR instrument to remove UV light. A 500W xenon arc lamp (Oriel model 66021) equipped with a 90 degree deflecting mirror (Newport model 66246) served as a visible light filter. The arc lamp was positioned 0.6 m from the perforated EPR sample compartment, and the light intensity and distribution that reached the samples was calibrated using the system described in the Protocol for Visible Light Exposure Studies (also see Supporting Information, Figure S1).

Statistical Analyses. GraphPad Prism 5.0 software was utilized for statistical analyses. Significant differences were determined by one-way analysis of variance (ANOVA) with posthoc Dunnett's multiple comparison test (α = 0.05) between the control samples and the experimental samples in terms of the measured lesion levels. The final data was log-transformed before statistical significance testing because homogeneity of variance (Bartlett's test) was not satisfied in the nontransformed data. The positive control lesion data were included in the ANOVA analyses.

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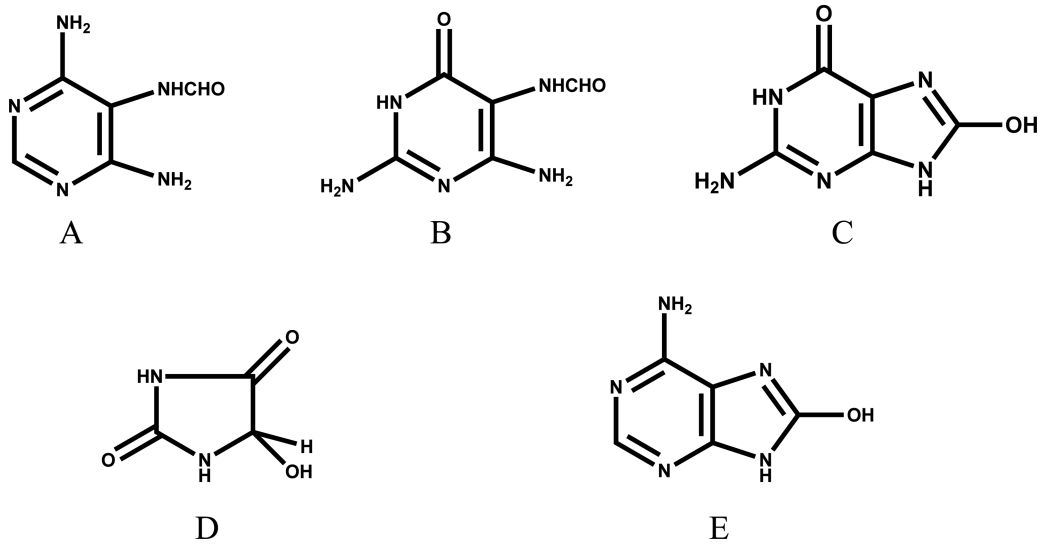

Figure 2. Oxidatively modified DNA base lesions investigated in this study: (A) 4,6 diamino-5-formamidopyrimidine (Fapy Adenine), (B) 2,6-diamino-4-hydroxy-5-formamidopyrimidine (Fapy Guanine), (C) 8-hydroxyguanine (8-OH-Guanine), (D) 5-hydroxy-5-methylhydyantoin (5-OH-5-MeHyd), and (E) 8-hydroxyadenine (8-OH-Adenine).

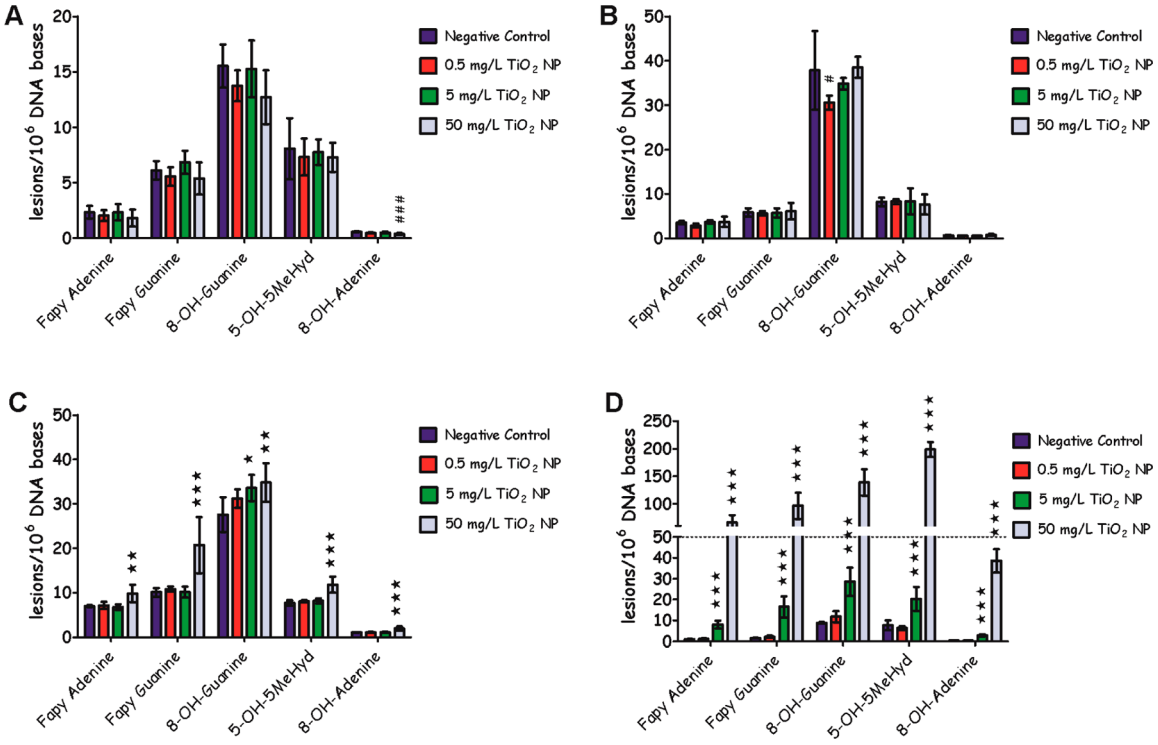

Figure 3. DNA base damage profile for ct-DNA exposed to increasing concentrations of P25 TiO_2 NPs (0.5−50 mg/L) after exposure (A) in the dark for 3 h, (B) in the dark for 24 h exposure, (C) using ambient lighting conditions (24 h; 14.5 kJ/m^2, 400−800 nm), and (D) using UVA irradiation conditions (30 min exposure; 10.0 kJ/m^2, 370 nm). Number signs (#) indicate statistically significant decreased lesion levels, and asterisks indicate significantly increased lesion level results compared to that of the control samples using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. One, two, and three asterisks or number signs indicate $p < 0.05$, 0.01, and 0.001, respectively. All data points represent the mean of 5 to 6 independent measurements. Uncertainties are standard deviations.

RESULTS

Characterization of NP Dispersions. TiO_2 NP dispersions were tested within a week of every experiment using LDS to ensure that the distribution had not changed. The mean, median, D10, and D90 particle sizes from eight different measurements during the time course of the experiments were 74.9 ± 16.0, 70.8 ± 2.6, 59.8 ± 1.3, and 83.0 ± 3.9 nm,, respectively (n = 8; uncertainties represent standard deviations except for the mean in which the error was propagated for the instrument uncertainty and the uncertainty from the replicates). The resulting data indicated that the NP dispersions were stable.

Dark Exposure Study. The DNA damage profiles for the 3 and 24 h TiO_2 NP exposures in the dark are shown in Figure 3A,B, respectively; Figure S2A,B includes the data for the 3 and

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24 h exposures, respectively, with positive control samples. For both exposure periods, there was no statistically significant formation of oxidatively induced DNA damage in comparison to that of the negative control levels over the tested NP exposure range. LC/MS/MS and GC/MS/MS analyses were also performed on an additional set of samples exposed to TiO_2 NPs for 24 h to confirm the lack of guanine oxidation during the GC/MS/MS analysis procedure. Statistically significant increases in the levels of 8-OH-deoxyguanosine or 8-OH-guanine were not observed after LC/MS/MS or GC/MS/MS analysis (see Figure S3), respectively, and artifactual formation of 8-OH-guanine was not observed.

Visible Light Exposure Study. The DNA damage profile for the 24 h TiO_2 NP exposure under ambient light conditions is shown without and with the positive control samples in Figures 3C and S2C, respectively. There was a statistically significant increase in oxidatively induced DNA damage, in comparison to that for the negative control levels, for all five monitored lesions at the highest NP exposure (50 mg/L). For the 8-OH-guanine lesion, there was a distinctive, linear dose–response effect that includes a statistically significant increase in the lesion at the 5 mg/L NP exposure.

UVA Light Exposure Study. The DNA damage profile for the 30 min TiO_2 NP exposure under UVA light conditions is shown with and without the positive control samples in 3D and S2D, respectively. There was a dramatic, statistically significant increase in oxidatively induced DNA damage, in comparison to the negative control levels, for all five monitored lesions at both the 5 and 50 mg/L NP exposure levels. Additionally, for all lesions, there was an order of magnitude increase or greater in the absolute number of lesions generated at the 50 mg/L NP exposure level. As observed in the ambient light exposure study, the 8-OH-guanine lesion shows a linearly increasing response in terms of NP dose.

EPR Spin-Trapping Study. When TiO_2 NP/ct-DNA samples (50 mg/L TiO_2 NP) were incubated in the dark, there was a slight (~5%) increase, in comparison to $t = 0$, in the level of detected free radicals over time (Figure 4). However, when the same samples were incubated under visible light conditions, there was a distinct (~20%) increase, in comparison to $t = 0$, in the level of detected free radicals over time (Figure 4). Using linear least-squares regression analysis, the dark and visible light EPR best fit lines (slopes) were significantly differently at the 95% confidence level. This finding indicated that the level of free radicals detected under visible light conditions was significantly enhanced in comparison to the level of free radicals detected under the dark conditions in the test samples.

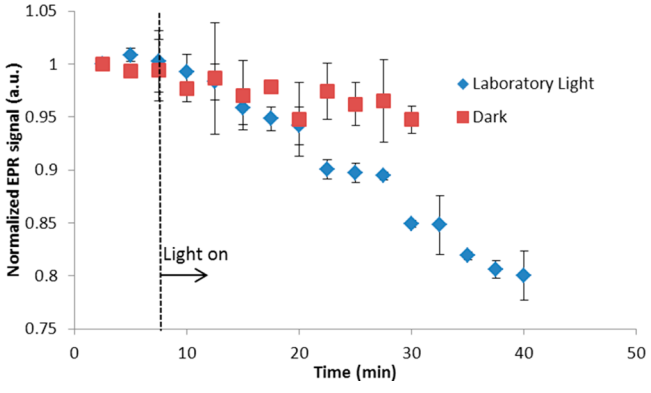

Figure 4. EPR spin trapping spectroscopic analysis of 50 mg/L TiO_2 NP/ct-DNA samples under dark and visible light exposure conditions. Data represent means and standard deviations of duplicate measurements.

DISCUSSION

DNA oxidation can result in damage to either the 2′-deoxyriboses and/or to the nucleobases in duplex DNA. If the oxidizing species is $^{\bullet}OH$, then both direct SSBs and oxidatively modified DNA bases can be generated. The formation and biological effects of these lesions has been widely investigated.[33] However, not all lesions possess equivalent mutagenic or cytotoxic potential; thus, it is important to identify, quantify, and characterize the likely spectrum of lesions generated under a given exposure scenario. For example, Fapy adenine and Fapy guanine are both purine-derived, imidazole ring-opened compounds that are major promutagenic lesions.[34] Fapy adenine induces A → C transversion mutations and possibly A → T transversion mutations, while Fapy guanine induces G → T transversion mutations. The guanine nucleobase is readily attacked by $^{\bullet}OH$ to produce abundant levels of 8-OH-guanine, a lesion that is commonly recognized as both an *in vitro* and *in vivo* biomarker of oxidative stress. 8-OH-guanine is also a mutagenic lesion that induces G → T transversion mutations.[35] The formation and accumulation of 5-OH-5-MeHyd within cells represents a potential lethality; this cytotoxic lesion is a major oxidation decomposition product of thymine and 5-methylcytosine in DNA and acts as a potential block to DNA polymerases.[36,37] Finally, when $^{\bullet}OH$ attacks the adenine nucleobase in DNA, the weakly mutagenic 8-OH-adenine lesion forms. Accumulation of 8-OH-adenine induces A → C transversion and A → G transition mutations.[38−40]

In the present study, we have attempted to characterize and understand the lesion generation capacity of commercial P25 TiO_2 NPs under carefully controlled photoactivation conditions. The debate over the type of reactive oxygen species (ROS), if any, generated from both nonphotoactivated and photoactivated TiO_2 NPs in aqueous solution is ongoing. Nevertheless, recent and consistent evidence, based on the use of EPR[2,41] and fluorescence[42] spectroscopy, demonstrate that $^{\bullet}OH$ is not produced by TiO_2 NPs at detectable levels when the NPs are incubated in aqueous solutions in the dark. However, when these same aqueous NP solutions are exposed to UVA radiation exceeding the TiO_2 band gap (~400 nm), $^{\bullet}OH$ is the only ROS formed at significant levels.[2,41,42] It is known that TiO_2 in dry powder form has a bare surface that can support the catalytic reduction of oxygen to $O_2^{\bullet-}$, as well as other radical reactions, that are independent of dark or UVA exposure.[3,41] The resulting ROS that are formed due to surface catalysis are of such low relative concentration in comparison to those formed from UV irradiation that when the TiO_2 NPs are introduced into an aqueous environment the potential reactivity from these surface ROS becomes negligible. Using acellular, aqueous conditions, we have clearly shown that TiO_2 NPs incubated with DNA in the dark do not lead to statistically significant accumulation (in comparison to that of control samples) of ROS-generated DNA base lesions over a 24 h exposure period (Figures 3A,B and S2A,B). The measured background level of 8-OH-guanine (~35 lesions/10^6 DNA bases) in the control samples for the 24 h incubation study was increased by more than 130% (Figure 3B) in comparison to the

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background level of 8-OH-guanine (~15 lesions/10^6 DNA bases) in the 3 h incubation study (Figure 3A). However, this effect was due to a combination of the increased time and oxygen exposure factors during the 24 h incubation period and not due to specific NP interactions with DNA. The guanine base has the lowest reduction potential of the four DNA bases, and guanine is known to readily oxidize to 8-OH-guanine in DNA samples maintained in oxygenated environments.[43]

The majority of the TiO_2 NP/UVA radiation exposure studies reporting DNA damage in the literature utilized radiation doses between 2.5 and ≥275 kJ/m^2.[7,10,25,27,44] Thus, we chose to investigate the lower portion of the tested dose range (≤15 kJ/m^2) for both the visible and UVA exposure protocols in the present study in order to focus on the potential DNA damage response, if any, resulting from relatively low energy doses. In sharp contrast to the findings for the TiO_2 NP/DNA samples under dark exposure conditions, TiO_2 NP/DNA samples exposed to a low dose (14.5 kJ/m^2) of visible light radiation generated statistically significant ($p < 0.01$) increases in lesion levels (in comparison to control levels) for all five lesions at the highest (50 mg/L) NP dose (Figure 3C and S2C). In addition, for the 8-OH-guanine lesion, there was a clear dose−response increase in the level of this lesion, which correlated with increasing NP doses. This observation is logical based on the putative visible light photoactivation of TiO_2 NPs[45] to produce •OH and the subsequent rapid attack and easy oxidation of guanine in DNA by •OH. However, when comparing the relative DNA lesion increases across all of the detected lesions at the 50 mg/L NP dose, 8-OH-guanine increased the least (26%) and Fapy guanine increased the most (105%), in comparison to control lesion levels. The potential biological relevance of this result (4-fold difference between 8-OH-guanine and Fapy guanine relative accumulation) cannot be understood without evaluating the kinetics of lesion formation under the experimental exposure conditions in an actual cell-based model.

We also determined the spectrum of base lesions resulting from a dose of UVA light (10 kJ/m^2) using UVA radiation at 370 nm, a wavelength that is less than the band gap excitation wavelength range (λ = 385−410 nm) for P25 TiO_2 NPs.[45] As expected, these conditions resulted in the formation of large, statistically significant ($p < 0.001$) DNA lesion levels in all of the TiO_2 NP/DNA samples at both the 5 and 50 mg/L NP exposure doses (Figures 3D and S2D).

The 3AP spin probe is a nonspecific nitroxide reagent commonly used for trapping free radicals in EPR spectroscopy experiments. The reagent does not inherently possess specificity for oxygen-, carbon-, or nitrogen-centered radical species. Thus, on the basis of the present EPR results (Figure 4), it cannot be definitively concluded that •OH was the radical species generating the DNA damage profiles shown in Figure 3C,D. However, both the observed pattern and levels of lesions produced during the visible light and UVA exposures are strongly indicative of •OH attack on DNA.[31] More importantly, even though a low amount of free radicals species are found under dark exposure conditions, these radicals do not appear to be capable of promoting oxidatively induced damage to DNA. However, once visible light is introduced into the TiO_2 NP/ct-DNA sample, the level of free radical species generated is high enough that significant oxidatively induced DNA damage is observed.

Our overall results suggest that ordinary room light, i.e., visible light from laboratory fluorescent light bulbs, has the capacity to photoactivate P25 TiO_2 NPs and induce extensive DNA damage at the molecular level when TiO_2 is present at higher concentrations. Indeed, mixed anatase/rutile crystal phase NPs like P25 have previously been shown to be photoactive in the visible region of the radiation spectrum.[45] Under visible light radiation, the rutile portion of the mixed-crystal P25 lattice (band gap of 3.0 eV and excitation wavelengths that extend into the visible region (410 nm)) acts like an antennae to trap electrons and slow electron−hole recombination. These trapped electrons are then capable of being transferred to the anatase phase, thus maintaining charge separation. In contrast, pure rutile NPs cannot trap electrons and thus are not photoactive in the visible region of the spectrum. Our visible light studies might partly explain some of the controversial comet assay-based *in vitro* results showing the induction and formation of SSBs in cellular samples that were either intentionally kept in the dark or exposed only to ambient (visible) light.[11,13] For example, Gurr et al. utilized the Fpg-modified comet assay to show that BEAS-2B cells exposed to either pure rutile TiO_2 NPs or to a 50:50 (volume fraction) mixture of anatase/rutile TiO_2 NPs (10 mg/L) in the dark could accumulate statistically higher levels of SSBs than that of untreated control cells.[13] The measured Fpg-induced SSBs directly correlate to the presence of oxidatively induced DNA base lesions (8-OH-guanine, Fapy adenine, etc.) in the cells. Our GC/MS/MS and EPR data suggest that these lesions are not formed to any significant extent in the dark. Thus, it is feasible that the cells in the Gurr study were either exposed to visible light and/or that a NP-induced inflammatory response and increase in ROS level was responsible for the reported DNA damage. Recently, Gerloff et al. showed Fpg-modified comet assay results for Caco-2 cells exposed to P25 TiO_2 NPs (20 μg/cm^2) under dark conditions.[11] The authors found that there was a slight (nonsignificant) increase in the level of detected SSBs for TiO_2 NP exposed cells when the comet assay samples were processed in the dark. However, the increased level of SSBs for cells exposed to TiO_2 NPs under identical culture conditions became statistically significant when the comet assay was conducted under normal laboratory lighting conditions. These results directly support our DNA base lesion results for both the dark and visible light exposure conditions. In our dark exposure studies (Figure 3A,B), we observed an increase in the background levels for 8-OH-guanine, depending on the length of the sample incubation period (3 or 24 h), but there was not a significant difference in the lesion levels for samples incubated with or without TiO_2. In contrast, the measured 8-OH-guanine level became statistically significantly increased for samples with TiO_2 after visible light radiation (Figure 3C).

Furthermore, our data clearly shows that 8-OH-guanine is not the only biologically relevant lesion formed due to visible or UVA light induced TiO_2 NP photoactivation. Previous *in vivo* and *in vitro* TiO_2 NP exposure studies[27,46,47] have measured only 8-OH-guanine or 8-OH-deoxyguanine to the potential exclusion of other lesions that could evince equal or greater mutagenicity or cytotoxicity depending on exposure conditions. In our exposure models, the relative formation and accumulation of 8-OH-guanine in the NP samples was lower than the formation and accumulation of the other four lesions. The accumulation of any of the measured lesions in cells can result in SSBs in the DNA molecule based solely on base excision repair processes; thus, it is important to identify and quantitatively measure more than 8-OH-guanine alone.

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Previously, it was reported that photoirradiated TiO_2 NPs could lead to site-specific guanine and thymine cleavages in ct-DNA due to the generation and accumulation of oxidatively modified guanine and thymine bases.[25] The authors hypothesized that guanine and thymine lesions could be formed in ct-DNA at significant levels. We have now demonstrated, at the molecular level, that photoactivated P25 TiO_2 NPs can induce a wide spectrum of measurable and discrete thymine, guanine, and adenine base lesions.

In closing, our experiments demonstrate that P25 TiO_2 NPs incubated with duplex DNA in the dark do not lead to the formation of DNA base lesions. However, when the same samples are exposed to either visible light from 400 to 800 nm (energy dose = 14.5 kJ/m^2) or UVA light at 370 nm (energy dose = 10 kJ/m^2), a wide spectrum of potentially cytotoxic and/or mutagenic DNA base lesions are generated at statistically significant levels, dependent on the NP concentration in solution. The presented data suggests that commercial P25 TiO_2 NPs do not have an inherent capacity to oxidatively damage DNA bases in the absence of sufficient photoactivation. However, radiation within the visible portion of the electromagnetic radiation spectrum has the potential to induce the formation of DNA base lesions and thus DNA SSBs. The observed DNA damage appears to be mediated and promoted by TiO_2 NP induced $^{\bullet}OH$ formation. Future studies using the comet assay to assess SSBs after exposing cells to TiO_2 NPs should conduct the processing steps in the dark to minimize potential artifacts from laboratory light and should report the conditions used during the processing steps. Understanding potential artifacts in nanotoxicology experiments is critical for providing reliable data and accurate risk assessment.[48]

■ ASSOCIATED CONTENT

Supporting Information

Spectral profile of the NIST calibrated halogen lamp, a DNA base damage profiles with positive control samples included for the TiO_2 NP exposure samples, profile of DNA nucleoside damage (8-OH-deoxyguanosine) and DNA base damage (8-OH-guanine) after 24 h dark exposure using LC/MS/MS and GC/MS/MS, respectively, methods for verification of removal of TiO_2 NPs by centrifugation, detailed description of the DNA enzymatic digestion procedure and GC/MS/MS methodology utilized in the present study, and detailed description of the isotope-dilution LC/MS/MS methodology utilized for the determination of 8-OH-deoxyguanosine levels. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

Certain commercial equipment, instruments, and materials are identified in this article to specify an experimental procedure as completely as possible. In no case does the identification of particular equipment or materials imply a recommendation or endorsement by the National Institute of Standards and Technology nor does it imply that the materials, instruments, or equipment are necessarily the best available for the purpose.

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