

Supplemental Materials

Photo-Reactivity Assays

APF assay. APF (3' [p-aminophenyl] fluorescein) (Invitrogen, Grand Island, NY, USA) is an ROS indicator with great specificity for hydroxyl radicals (OH[•]) and resistance to light induced oxidation.¹ Studies on TiO₂/UVA photocatalytic processes have suggested that OH[•] is the major free radical product during the process,² and that the antibacterial properties of nano-TiO₂ are related to free and surface-bound hydroxy radicals.³ Therefore, production of OH[•] may be representative of the total photocatalytic ROS production. Stock suspensions of 200 mg/L for each of the samples (un-aged, and aged for either 45 min or 1,3,10, or 15 days) was prepared by dispersing the freeze dried ENM samples in PBS buffer (0.01 M, pH=7.4) by ultrasonication for 30 min. Working concentrations of 1, 5, and 10 mg/L were diluted from stock suspensions using PBS. Suspensions of Degussa P25 TiO₂ were prepared by the same method at identical concentrations for use as a positive control. For each sample, 2 μL of stock APF (5 mM) was added to 2 mL suspension to yield a final APF concentration of 5 μM. Each sample consisted of three wells with 300 μL mixed suspension in each well on a 96-well plate.

The plate was read for fluorescence at Ex/Em = 485/528 nm before and after exposure for 1 hour under simulated solar radiation (SSR). The increase in fluorescence is proportional to ROS generation (photo-reactivity). The simulated solar radiation is obtained through a Q-Sun 3000 Xenon Test Chamber (Q-Lab Corporation, Westlake, OH). The chamber uses xenon arc lamps that accurately simulate the full spectrum of sunlight, as described by Ma et al.⁴ The irradiance of SSR as measured by a radiometer was 51.9, 3.85, and 525 W/m² for UVA, UVB, and visible light, respectively.

TBARS. The thiobarbituric acid reactive substance (TBARS) assay was conducted as previously reported.⁵ The assay was selected for assessing nanoparticle bio-reactivity due to its use in air particulate pollution health effects research, where TBARS activity of air particulate matter was found to correlate with its acute pulmonary toxicity,⁶⁻⁸ and with one exception, was shown to be linearly related to the phototoxic potency of a variety of TiO₂ samples.⁵ Briefly, metals are reduced by ascorbate, and then reacted with hydrogen peroxide to produce hydroxide and OH[•]. OH[•] reacts with 2-deoxy-2-ribose to form malondialdehyde and thiobarbituric acid, which is a pink-colored adduct that is detected by absorbance at 532 nm. Degussa P25, untreated, and SPW treated Al(OH)₃-coated TiO₂ ENM were dispersed in saline, as previously described, and UV-TBARS activity determined as described by Sanders *et al.*⁵ Plates containing samples not exposed to UVA were covered with a lid and tin foil, while plates exposed to UVA radiation were left uncovered and exposed to 364 nm radiation (5.4 J/cm²). After irradiation, samples were centrifuged at 1300×g for 10 min, and the supernatant was mixed with 1 ml each of

thiobarbituric and trichloroacetic acid, heated at 100°C for 10 min and then cooled on ice. The absorbance was read at 532 nm using a UV/VIS spectrophotometer (Spectronic Model 601 UV/VIS spectrophotometer, Milton Roy, Rochester, NY).

Data were analyzed using mixed-effects models in SAS PROC MIXED (SAS v9.3; SAS Institute, Cary, NC) with restricted maximum likelihood estimation followed by the Tukey multiple-comparison test; p values were adjusted for multiplicity. Pairwise comparisons with p values <0.05 were considered to be significant.

Cells were grown in 1:1 Dulbecco's Modified Eagle's Medium and Ham's F-12 Nutrient Mixture (DMEM/F12; Invitrogen, Inc., Carlsbad, CA) containing 10% fetal bovine serum (FBS; Atlanta Biologicals, Lawrenceville, GA), 0.15% sodium bicarbonate (Fisher), 100 units/mL penicillin and 100 µg/mL streptomycin (Lonza, Walkersville, MD) at pH 7.4 in a humidified atmosphere of 5% CO₂ at 37°C. After reaching confluence (4 days), cells were passaged using trypsin/EDTA (0.05%/0.02%) in Hank's Buffered Saline Solution (HBSS; Life Technologies, Grand Island, NY) and were split by 1-mL aliquots into 24-well cell culture plates at a density of 1×10⁵ cells/mL. Each plate had wells designated for treatment with 1 mL of 3, 10, 30, or 100 µg/mL (8.59×10¹⁰, 2.86×10¹¹, 8.59×10¹¹, 2.86×10¹² particles/mL; 1.69×10¹⁴, 5.62×10¹⁴, 1.69×10¹⁵, 5.62×10¹⁵ nm²/mL) ENM suspensions, as well as a positive and negative control. After 24 hours of incubation, spent media was removed, and the designated treatment suspension was added to each well. For positive and negative controls, spent media was replaced with a 30-µg/mL (6.45×10¹¹ particles/mL; 1.53×10¹⁵ nm²/mL) suspension of Degussa P25 or fresh media, respectively. Suspensions of each sample (un-aged, 45 minute-aged, or 1, 3, 10 or 14 day-aged ENMs) were prepared immediately before treatment by sonication (Misonix Microson Ultrasonic Cell Disrupter XL, Farmingdale, NY; setting 4 for three 2 to 3 second bursts [4.5 Watts]) of the ENM powder in the cell culture media to yield a 1 mg/mL stock suspension. The treatment suspensions of 3, 10, 30, or 100 µg/mL were prepared by diluting the stock suspensions with the cell culture media. A stock suspension of uncoated TiO₂ (Degussa P25) was similarly prepared and diluted to 30 µg/mL for use as a positive control.⁵ Physical/chemical characterization of the positive control compound were described elsewhere.⁵

Cell-Culture Slides.

Cells (ARPE-19) were split into 4-chamber cell-culture slides (Lab-Tek® Chamber Slide System, Sigma Aldrich) at a concentration of 2×10⁴ cells/mL (500 µL per chamber). After the slides were incubated (5% CO₂, 37°C) for 24 hours, the cell transfection agent CellLight® Mitochondria-GFP, BacMam 2.0 (Life Technologies, Grand Island, NY; 10µL per chamber) was added. Slides were returned to the incubator for 6 hours, after which each chamber was treated with a 5-µL aliquot of 1 mg/mL ENM suspension (un-aged, or 1, 3, 10 or 14 day-aged), resulting in a 10 µg/mL ENM suspension. For each combination of transfection agents used, a chamber was left untreated for a negative control, and a positive control was created by treatment with a

5- μ L aliquot of 1 mg/mL Degussa P25 suspension. After 24 hours, slides were stained with CellMask™ Orange (CMO; Life Technologies, Grand Island, NY) and fixed with warm (37°C) paraformaldehyde solution (500 μ L per chamber, 4% paraformaldehyde in PBS). Cover slips were mounted using ProLong® Gold Antifade with DAPI (Life Technologies, Grand Island, NY).

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3. M. Cho, H. Chung, W. Choi and J. Yoon, *Water Research*, 2004, **38**, 1069-1077.
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Supplemental Table 1. Flow cytometry side scatter (SSC) and forward scatter (FSC) means reported as values relative to control for ARPE-19 cells exposed to P25, un-aged and $\text{Al}(\text{OH})_3 \cdot \text{TiO}_2$ ENM aged in SPW from 1 to 14 days.

Supplemental Figure 1. Scanning electron microscopy (SEM) images of original and SPW-aged ENM.

Supplemental Figure 2. TEM images of original and SPW-aged ENM

Supplemental Figure 3. EDS scans of original and SPW-aged ENM. Peaks to the left of each graph depict C, O, Al and Si peaks; peaks in the middle depict Ti peaks, and peaks on the right depict Cu peaks.

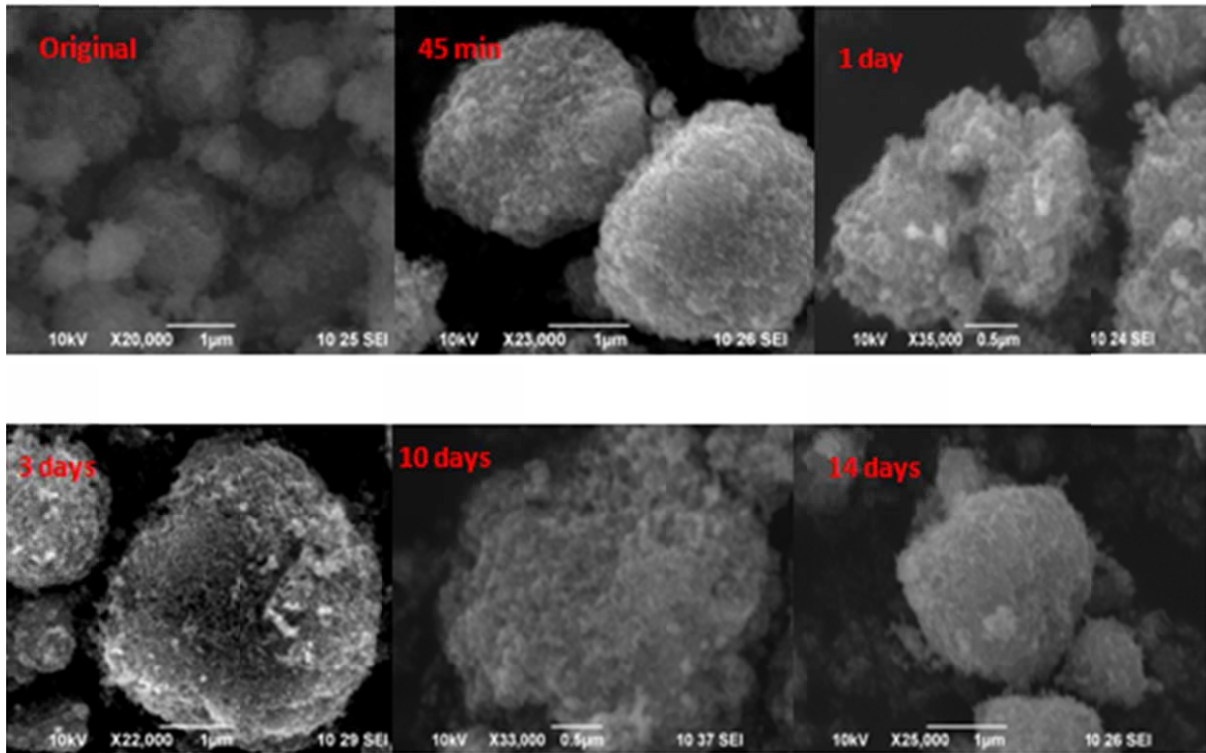
Supplemental Figure 4. Non-cellular assessment of ENM photocatalytic activity. $\text{Al}(\text{OH})_3 \cdot \text{TiO}_2$ ENM suspensions under simulated solar radiation for 3 assays, as described in the Materials and Methods: (A) Aminophenyl Fluorescein (APF) Assay, and (B) Thiobarbituric Acid Reactive Substance (TBARS) Assay. Degussa P25 TiO_2 , 31 nm, was used as positive control. In pairwise statistical comparisons, (*) indicates significantly different from un-aged, and (#) indicates 1 day

was significantly different from 3 days. “NS” signifies normal saline control. P25 displayed the greatest photo-reactivity in all three assays.

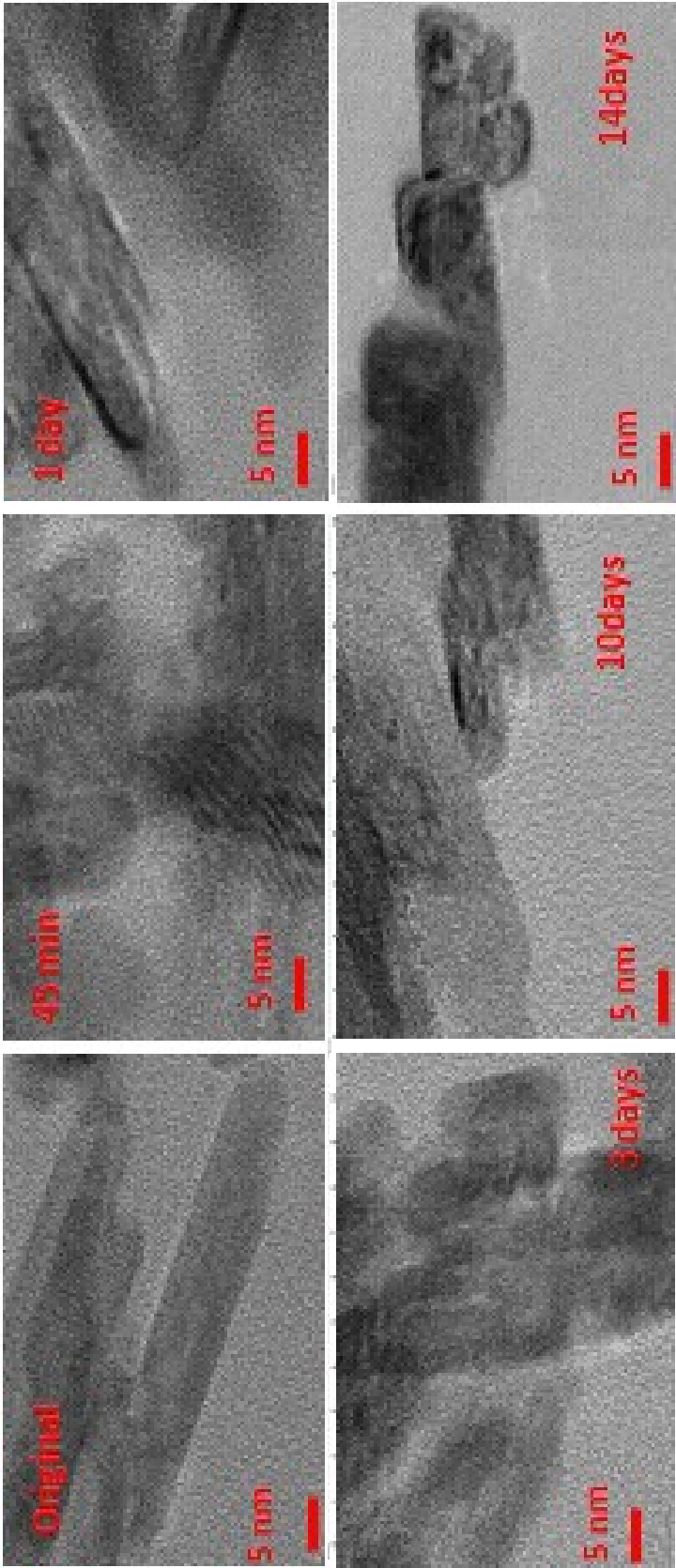
Supplemental Table 1

Nanoparticle Treatment	SSC	FSC
Control (untreated)	1.00	1.00
Al(OH) ₃ ·TiO ₂ (unaged)	3.29	1.00
Al(OH) ₃ ·TiO ₂ (1 day)	2.31	1.02
Al(OH) ₃ ·TiO ₂ (3 day)	2.78	1.00
Al(OH) ₃ ·TiO ₂ (10 day)	2.19	1.02
Al(OH) ₃ ·TiO ₂ (14 day)	2.84	1.02
P25 TiO ₂	6.66	0.89

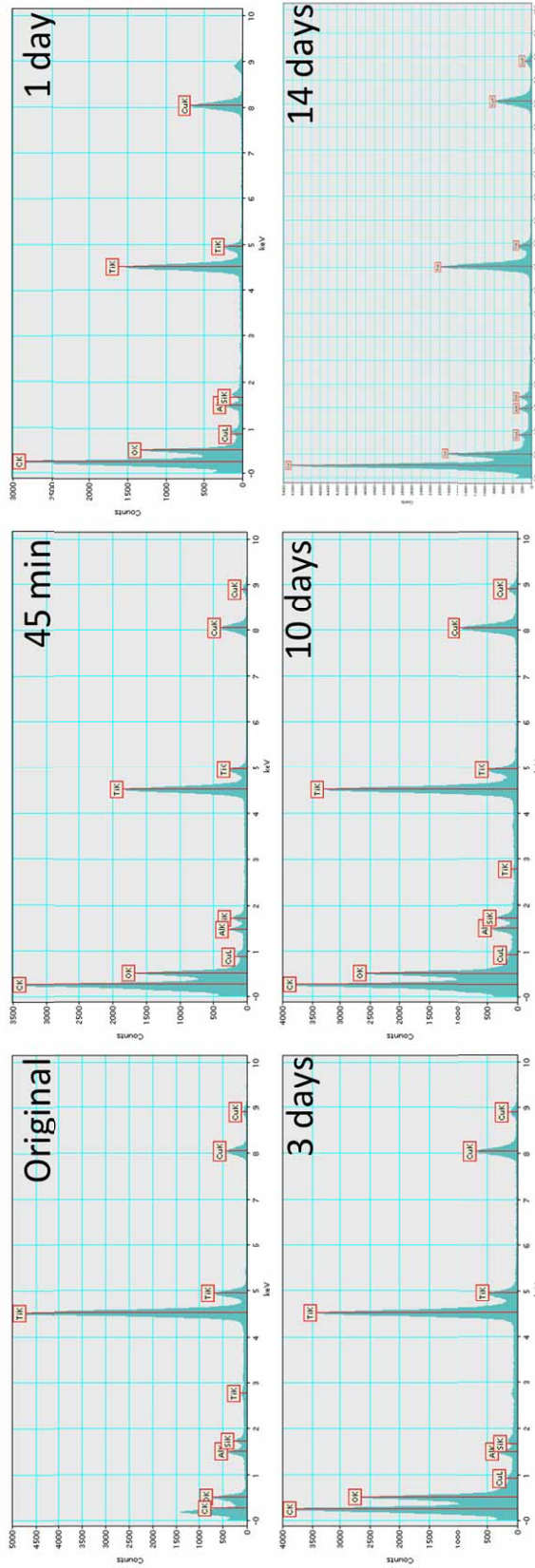
Supplemental Figure 1



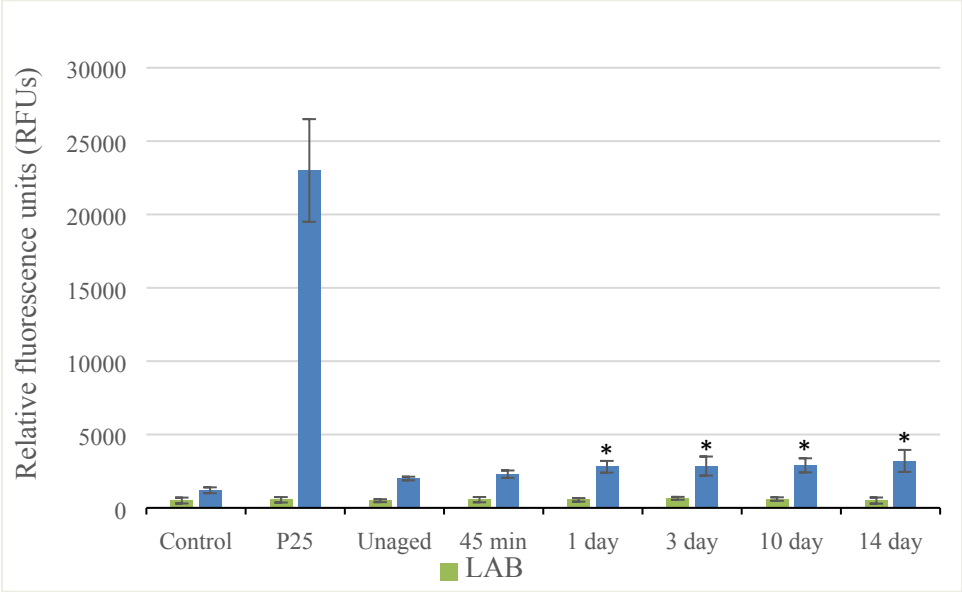
Supplemental Figure 2



Supplemental Figure 3



Supplemental Figure 4A



Supplemental Figure 4B

