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Lack of Significant Dermal Penetration of Titanium Dioxide from Sunscreen Formulations Containing Nano- and Submicron-Size TiO_2 Particles

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Titanium dioxide (TiO_2) is included in some sunscreen formulations to physically block ultraviolet radiation. A dermal penetration study was conducted in minipigs with three TiO_2 particles (uncoated submicron sized, uncoated nano-sized, and dimethicone/methicone copolymer-coated nanosized) applied 5% by weight in a sunscreen. These and control formulations were topically applied to minipigs at 2 mg cream/cm^2 skin (4 applications/day, 5 days/week, 4 weeks). Skin (multiple sites), lymph nodes, liver, spleen, and kidneys were removed, and the TiO_2 content was determined (as titanium) using inductively coupled plasma mass spectroscopy. Titanium levels in lymph nodes and liver from treated animals were not increased over the values in control animals. The epidermis from minipigs treated with sunscreens containing TiO_2 showed elevated titanium. Increased titanium was detected in abdominal and neck dermis of minipigs treated with uncoated and coated nanoscale TiO_2. Using electron microscopy-energy dispersive x-ray analysis, all three types of TiO_2 particles were found in the stratum corneum and upper follicular lumens in all treated skin samples (more particles visible with coated nanoscale TiO_2). Isolated titanium particles were also present at various locations in the dermis of animals treated with all three types of TiO_2-containing sunscreens; however, there was no pattern of distribution or pathology suggesting the particles could be the result of contamination. At most, the few isolated particles represent a tiny fraction of the total amount of applied TiO_2. These findings indicate that there is no significant penetration of TiO_2 nanoparticles through the intact normal epidermis.

Key Words: **Sunscreen; TiO_2; Nano; skin.**

Nanotechnology is the manipulation of matter on a size scale between 1 and 100 nm (National Nanotechnology Initiative, 2009). Nanosize materials are being developed for a wide range of materials, which will be included in a large variety of consumer products (Woodrow Wilson International Center for Scholars, 2008). Since many of the properties of nanosized materials differ from those of larger particles, there have been numerous publications detailing concerns regarding the safe use and potential environmental impact of such materials (Cross *et al.*, 2007; Lansdown and Taylor, 1997; Nohynek *et al.*, 2007; Pflucker *et al.*, 1999; Ryman-Rasmussen *et al.*, 2006; Warheit *et al.*, 2007).

Consumers in many countries are encouraged to use sunscreens to avoid the harmful effects of ultraviolet radiation in sunlight. Sunscreens contain organic molecules that absorb both ultraviolet B (280–320 nm) and ultraviolet A (320–400 nm) radiation. In addition, many sunscreens contain physical blocking agents such as TiO_2 and zinc oxide (ZnO) to physically reflect (i.e., scatter) the ultraviolet radiation or trap the ultraviolet photon in the crystal matrix. In an effort to decrease the opacity of sunscreens containing TiO_2 and ZnO, manufacturers have decreased the size of the particles from micron to submicron (i.e., nanosize) particles. The inclusion of nanosize materials in sunscreens has prompted safety concerns regarding the possible penetration of nanomaterials into and through the skin (Brand *et al.*, 2007; Dunford *et al.*, 1997; Gamer *et al.*, 2006). In 2006, the Food and Drug Administration (FDA) received a citizen petition (International Center for Technology Assessment [ICTA], 2006) regarding the use of nanosize materials in certain FDA-regulated products, including sunscreens. The petition requested that FDA require manufacturers to declare all currently available sunscreen drug products containing engineered nanoparticles of ZnO and/or TiO_2 as an imminent hazard to public

health. The petition also requested FDA to order entities using nanoparticles in sunscreens regulated by FDA to cease manufacture until broader FDA nanotechnology regulations are developed and implemented. TiO_2, at concentrations up to 25% by weight, is generally recognized as safe and effective as an ingredient in over-the-counter (OTC) sunscreens (21 CFR 352.10) if it meets United States Pharmacopeia (USP) monograph guidelines. Currently, the USP monograph for TiO_2 does not specify a standard for particle size. The ICTA petition requested that manufacturers be required to recall all publicly available sunscreen drug products containing engineered nanoparticles of TiO_2 and/or ZnO and submit new drug applications for the products.

TiO_2 exists in several crystalline polymorphic forms (Mogyorósi *et al*., 2003): anatase, rutile, or brookite. The TiO_2 crystals reflect light based on Mie's law, where the wavelengths of light that are reflected are a function of the size of the particle surface. Micron-sized particles reflect visible and ultraviolet light; however, as the particle size decreases into the nanometer range, visible light is no longer reflected, while shorter wavelength ultraviolet light is still reflected. This property of passing visible light while blocking or reflecting ultraviolet light is a highly desired property of a sunscreen. In an effort to avoid the generation of reactive oxygen species when irradiated with ultraviolet light (Blake *et al*., 1999; Hoffman *et al*., 1995; Mills and Le Hunte, 1997), some manufacturers have coated TiO_2 nanoparticles with non-semiconductor materials such as silica or zirconium. Other manufacturers have coated the particles with hydrophobic materials.

For this study, sunscreens were prepared including three different titanium dioxide materials. A coated nanosized rutile titanium dioxide product that is commercially available for use in sunscreens was chosen for one formulation. An uncoated nanosized 80% anatase and 20% rutile titanium dioxide was chosen for the second formulation. A submicron rutile titanium dioxide was chosen for the third titanium-containing formulation.

One of the critical questions regarding the safety of a topically applied material is whether the material will penetrate the epidermal barrier of the skin or follicular lumen and distribute to the dermis and other organs. We have shown that polyethylene glycol–coated quantum dot nanomaterials distribute to the lymph nodes, liver, and kidney following injection into the dermis, thereby suggesting that these organs can be used as sentinel organs to monitor penetration of nanosize materials through the skin and evaluate biodistribution (Gopee *et al*., 2007). In an effort to determine if topically applied nanosize TiO_2 will penetrate the skin, we used minipigs as a surrogate for human skin due to their anatomical similarities and use as a model for drug penetration of human skin (Monteiro-Riviere, 1991; Sekkat *et al*., 2002; Williams, 2003; Xia *et al*., 2010) and monitored sentinel organ titanium levels following topical application of the creams (i.e., sunscreen formulations) with TiO_2 for 22 days over a period of 1 month. Upon necropsy, various tissues were removed and analyzed by inductively coupled plasma mass spectroscopy (ICP-MS) for titanium content. Additionally, skin samples were subjected to transmission electron microscope (TEM) and scanning electron microscope-energy dispersive x-ray (SEM-EDX) analysis, in order to better assess the localization of titanium particles and to evaluate if the treatment regimen employed in this study resulted in dermal penetration of TiO_2 nanoparticles in pigs.

MATERIALS AND METHODS

Sunscreen preparation. Four formulations were generated for this study, three with different TiO_2 particles and one without added TiO_2 (i.e., control). Uncoated nano titanium dioxide (Degussa Aeroxide P25, a mixture of anatase and rutile and known to be photocatalytic; "uncoated nano," obtained from electron microscope (EM) Sullivan Associates, Paoli, PA), coated (aluminum hydroxide/dimethicone copolymer) nano titanium dioxide (BASF T-Lite SF obtained from BASF, Shreveport, LA; rutile; "coated nano"), and uncoated submicron titanium dioxide (treated with aluminum hydroxide, Ishihara Tipaque CR-50 obtained from Ishihara Corporation, San Francisco, CA; rutile; "submicron") were utilized to formulate sunscreen products. The base formulation consisted of the following ingredients: phase A (dibutyl adipate, cocoglycerides, sodium cetearyl sulfate, lauryl glucoside, polyglyceryl-2 dipolyhydroxystearate, glycerin, cetearyl alcohol [Cognis Corporation, New Milford, CT], $C_{12–15}$ alkyl benzoate [Finetex, Inc., Spencer, NC], octyl methoxycinnamate, and tocopheryl acetate [BASF]), phase B (titanium dioxide), phase C (glycerin, allantoin, xanthan gum [Government Scientific Source, MP Biomedicals Inc., Solon, OH], disodium EDTA [BASF], magnesium aluminum silicate [R.T. Vanderbilt Company, Inc, Norwalk, CT], and water), and phase D (phenoxyethanol, methylparaben, ethylparaben, butylparaben, propylparaben, and isobutylparaben [Clariant Corporation, Charlotte, NC]). For the blank sunscreen, no TiO_2 (phase B) was added; for all other formulations, the specified titanium dioxide was added at a concentration of 5% by weight.

Sunscreen preparation occurred in four phases:

(1) Phase A was heated to 80°C, and phase C was heated to 80°C.

(2) Phase B was added to phase A, and phase A + B was homogenized (Tyner *et al*., 2008; Wokovich *et al*., 2009). For the blank sunscreen, the homogenization was carried out in the absence of phase B.

(3) Phase C was added to phase A + B, and phase A + B + C was homogenized.

(4) Phase A + B + C was cooled to about 40°C, phase D was added and phase A + B + C + D was homogenized (Tyner *et al*., 2008; Wokovich *et al*., 2009).

The concentration of titanium in the formulations was determined using ICP-MS with a Fisons (Thermo) PQ3 instrument using dissolution and analysis methods described below for tissue analysis. Samples of sunscreen were removed to determine the homogeneity of the distribution and average concentration of TiO_2 in the sunscreen (Table 1). All sunscreens, including the blank sunscreen, contained 1.50% magnesium aluminum silicate, which contained ~3% titanium dioxide by weight, resulting in a background level of ~0.33 mg titanium/g sunscreen. The levels of TiO_2 in the formulations were 5.6% for submicron TiO_2, 6.1% for uncoated nano-TiO_2, and 4.7% for coated nano-TiO_2. The relative SD for homogeneity in each case was less than 5%, indicating the mixtures were homogeneous.

Characterization of sunscreens. The titanium dioxide raw materials and the four sunscreen formulations were characterized by scanning electron microscopy (SEM) using a JEOL model GSM 6320F (JEOL Ltd., Tokyo, Japan). All the sunscreen samples were prepared as described earlier (Tyner

TABLE 1
Concentration of Titanium and Size of TiO_2 in Creams

Test article	Titanium (mg/g)	Percent of target (target = 5% by weight)	Percent homogeneity (relative SD)	Particle size of TiO_2 in raw material and formulation by SEM
Blank cream	0.334 ± 0.021	< 0.05	4.45	Not applicable
Cream containing submicron TiO_2	33.689 ± 1.239	112.3 ± 4.1% (5.6% TiO_2 by weight)	2.84	300–500 nm
Cream containing uncoated TiO_2	36.628 ± 2.481	122.1 ± 8.3% (6.1% TiO_2 by weight)	4.42	30–50 nm
Cream containing coated TiO_2	28.489 ± 1.765	95 ± 5.9% (4.7% TiO_2 by weight)	1.44	20–30 nm in diameter and about 50–150 nm in length

et al., 2008; Wokovich *et al*., 2009). No solvent was used for the SEM sample preparation. Results are shown in Table 1. The formulation process did not alter the size of the titanium dioxide particles (Wokovich *et al*., 2009).

Animals. Female Yucatan minipigs (~4 months of age; $n = 12$) were obtained from Sinclair Research Center (Auxvasse, MO). The minipigs were acclimated for 1 week prior to use. The minipigs were randomly assigned three per group to the four treatment groups. Housing and treatment of the minipigs conformed to the Institutional Animal Care and Use Committee guidelines at an American Association for Laboratory Animal Science accredited facility (National Center for Toxicological Research).

Treatment. Topical application of cream preparations with and without TiO_2 was carried out four times daily, 5 days a week, for a total of 22 days. Each cream was applied uniformly at a dose of 2 mg/cm^2 beginning at the neck and covered the entire dorsal surface and ventral abdomen ending at the base of the tail. Each minipig received a total of 176 mg/cm^2 cream over the 22 treatment days, resulting in a group average of ~1.32 l of cream per animal. One day after the final application, the minipigs were deeply anesthetized using a mixture of ketamine and xylazine and 50 ml of blood removed via the ear vein for analytical chemistry. Euthanasia was completed by exsanguination via the femoral artery and death was confirmed based on the absence of a heartbeat and respiration. Metal-free (i.e., titanium-free) necropsy was conducted using tungsten carbide, Teflon-coated, and plastic instruments to prevent titanium cross-contamination between tissues. Instruments used during necropsy had been shown not to leach titanium when either in contact with fluids or when wiped excessively (ICP-MS determination; results not shown). Tissues dissected at necropsy were divided into weighed samples for ICP-MS analysis of titanium (stored at −20°C), samples for histopathology (placed in 10% neutral-buffered formalin [NBF]), samples for potential gene expression (snap frozen in liquid nitrogen), and samples for electron microscopy. Sections of skin (5 × 10 cm) were cleaned of residual sunscreen using cotton balls and a 2% soap solution prior to dissection. To avoid inadvertently dragging TiO_2 from the epidermis into the dermis with instruments, we initiated cuttings from the dermis side proceeding into the epidermis and blades were changed between each sectioning. Skin sections for EM analysis were placed in chilled freshly prepared Karnovsky's fixative, fixed for 48 h, and then transferred to 10% NBF. Tissues collected included skin from five different skin sites (neck, dorsolateral back, ventral abdomen, left and right inguinal regions, and left and right axillary regions), lymph nodes (left and right inguinal, prescapular, and submandibular nodes), liver (left, right, and middle lobes), kidneys (cortical and medullary regions of the left and right kidneys), gall bladder, heart (left and right ventricular walls and intraventricular septum), lungs (cranial, middle, and caudal lobes), brain (cerebrum, thalamus, midbrain, and cerebellum), and feces (sample from upper colon). Epidermis was separated from dermis prior to ICP-MS analysis using the dry heat method described by Frank *et al*. (1995). According to this method, skin is heated between two blocks at 63°C for 2 min and the two layers are separated using titanium-free instruments.

Inductively coupled plasma mass spectrometry. ICP-MS was performed utilizing a Fisons (Thermo) PQ3. Prior to ICP-MS analyses, weighed tissue samples (or 100-µl blood) were transferred to 25- or 125-ml advanced composite vessels and a mixture of nitric acid (HNO_3) and hydrofluoric acid (HF; 4:1, vol/vol) was added. Microwave dissolution was performed for 35 min at up to 300 W power, 200°C, and 220 PSI utilizing CEM MDS2000 or MARS microwave systems. Following treatment, samples were diluted with 2% HNO_3 and an yttrium internal standard was added. National Institute of Standards and Technology-traceable titanium and yttrium standards were utilized throughout. Sample titanium content was quantified using the internal standard and averaging the ^{49}Ti and ^{50}Ti isotope content.

Statistical analysis of the titanium levels determined via ICP-MS was performed using SigmaStat (Jandel Scientific, San Rafael, CA). The levels of titanium in the tissues were compared to the levels in the control tissues by one-way ANOVA using Dunnett's method. The data were transformed (ln) when normalcy tests failed. Significance was set at $p \leq 0.05$.

Transmission electron microscopy and scanning electron microscope-energy dispersive x-ray. TEM and SEM were used to image the TiO_2 particles in ~2–3 cm^2 of pig abdominal skin tissue. Abdominal skin was chosen for the electron microscopic examination due to preliminary data showing TiO_2 in the dermis from this tissue, and the epidermis in this area is very thin increasing the likelihood of epidermal penetration by the TiO_2. In total, more than 2500 TEM images and 400 SEM images were taken of tissue sections. These images included both interfollicular skin areas and hair follicle lumens. TEM thin sections were cut in a fashion that minimized transfer of particles from the skin surface to deeper layers. Certain tissues were sectioned both longitudinally and at cross sections to allow visualization of the penetration of particles into skin layers.

Energy dispersive x-ray (EDX) spectroscopy was employed to confirm the identity of the TiO_2 particles via their elemental spectra. SEM-EDX maps of tissue areas of interest were created to localize the distribution of titanium and oxygen with respect to tissue features. More than 400 EDX spectra and 200 SEM-EDX elemental maps of the pigskin tissue sections were acquired.

Prior to EM analysis, skin samples were transferred to EM fixative (4% formaldehyde and 2% glutaraldehyde in 0.1M cacodylate buffer) for 2 h at room temperature, followed by three 10-min 0.1M cacodylate buffer rinses, stained for 1 h in 1.0% OsO_4, followed by two cacodylate buffer and one 0.1 N acetate buffer rinses. The samples were then en bloc stained in 0.5% uranyl acetate (UA) for 1 h, followed by two 10-min 0.1 N acetate buffer rinses. Samples were gradually dehydrated by washing with increasing concentrations of ethyl alcohol, followed by three 100% alcohol and three propylene oxide (PO) rinses. Epon-812-based epoxy resin (components are not list here) was mixed in a vial with an equal quantity of PO and the sample was added. The vial was spun at low speed overnight in a tissue rotator. Epoxy Resin-812-based resin components were mixed in a plastic disposable beaker, placed in a vacuum chamber connected to a rotary pump to degas for at least half an hour, and then transferred to polyethylene-embedding capsules. The sample was

removed from the resin/PO mixture, immersed in a bath of 100% resin, placed into the prepared embedding capsule, and put in the oven for 48 h to harden. The resin block was trimmed with a razor blade to expose an area of tissue ~1 mm^2 and then placed on the microtome for thin sectioning.

All four groups of epoxy resin–embedded skin samples were thin sectioned with a diamond knife approximately perpendicular to the skin surface (i.e., with a longitudinal cut along the hair follicle). All longitudinal sections were 90 nm thick. The thin sections were mounted on copper finder grids (special labels on the grid help locate specific survey areas) and stained with a mixture of 2.0 ml of 70% alcohol and 4.0 ml of 0.5% UA for 2 min. After rinsing five times with H_2O, the grids were stained with 3.0% lead citrate for 90 s. The grids were rinsed five times with double-distilled H_2O (ddH_2O) and allowed to air dry. The grids were then placed into a vacuum evaporator where a thin layer of carbon film was evaporated onto the sections to avoid heat buildup and dissipate any charge accumulation on the sample surface that may occur while imaging.

Various regions of the epidermis (stratum corneum, stratum granulosum, stratum spinosum, and stratum basale) as well as dermis present in each section were surveyed. Interfollicular areas and follicular lumens in all samples were carefully examined using TEM and SEM. Elemental analysis was performed using an EDX spectrometer. Two sets of TEM montage images of follicles were captured for each particle species. A number of TEM images at high and low magnifications near follicular lumens were also obtained for comparison. SEM and EDX analysis was performed on likely TiO_2 particles within each skin sample. The TEM measurement was performed on a Hitachi 7600 (Hitachi High Technologies America, Inc., Pleasanton, CA) with an operating voltage of 80 kV. SEM-EDX measurement was performed on a Compact EDX Detector Unit (EDAX, Mahwah, NJ) incorporated into a Hitachi S3000 SEM system. SEM images, EDX spectra, and x-ray mappings were obtained using a modified STEM adaptor (Electron Microscopy Sciences, Hatfield, PA). The same survey areas visualized by TEM were located in the SEM using the finder grids. The EDX spectra were acquired with an acceleration voltage of 15 or 20 kV (dependent on required resolution) and the signal was collected for 45 s. Elemental x-ray spectral mappings were obtained with an operating voltage of 20 kV and collected for one and one-half hours each.

RESULTS

The application of the formulations to the skin of the minipigs for ~1 month had no apparent adverse effects on the animal skin (e.g., irritation).

Relatively low and constant levels of titanium were detected via ICP-MS in the skin from minipigs treated with the control cream. We measured 34.64 ± 10.39 μg/g Ti in the epidermis and 1.003 ± 0.048 μg/g in the dermis. The level of titanium in the combined lymph nodes was 1.226 ± 0.221 μg/g and 2.267 ± 0.129 μg/g was found in the liver.

The levels of titanium in the lymph nodes from treated minipigs are shown in Figure 1. There was an increase in titanium in the left inguinal lymph nodes following treatment with uncoated nano-TiO_2 and an increase in the right inguinal lymph nodes of minipigs treated with submicron TiO_2. When the data were combined for left and right inguinal lymph nodes for the same minipig, there were statistically significant increases in titanium from 0.758 μg/g in the control group to 1.133 and 1.232 μg/g in the submicron and uncoated nano-TiO_2 treatment groups, respectively. This pattern of increased titanium was not evident in the other lymph nodes that were analyzed (Fig. 1). There were no statistical differences in the titanium levels in the prescapular or submandibular lymph nodes taken from any of the TiO_2-treated minipigs. When all the lymph node data were combined (Fig. 1), there were no statistical differences in the lymph node titanium concentrations between the treatment groups.

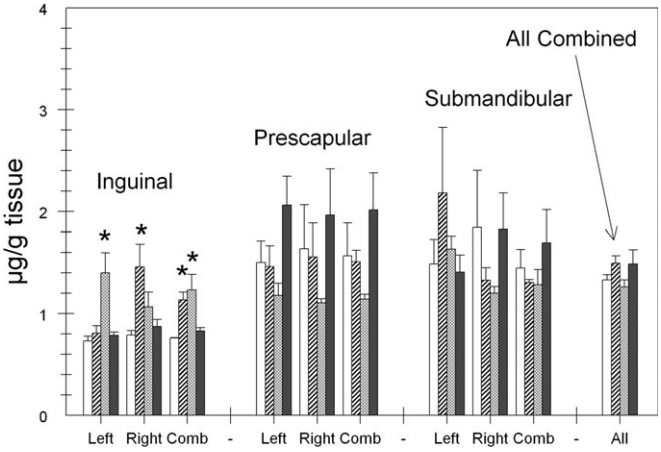

FIG. 1. Levels of titanium in left, right, and combined (Comb) inguinal, prescapular, and submandibular lymph nodes taken from minipigs treated with sunscreen formulations evaluated via ICP-MS: control, no TiO_2 (open bar); submicron-sized TiO_2 (gray bar); uncoated nanosized TiO_2 (hashed bar); and coated nanosized TiO_2 (filled bar). The ''all combined'' group represents the average of all lymph nodes for animals in the indicated treatment groups. Data are presented as mean ± SE for each group (three minipigs per group). *statistically significant difference from the control value for each group ($p \leq 0.05$).

The titanium levels in the left, right, and middle lobes of the minipig livers for the various treatment groups are shown in Figure 2. The titanium content was not significantly different between any of the TiO_2-treated groups when compared to the

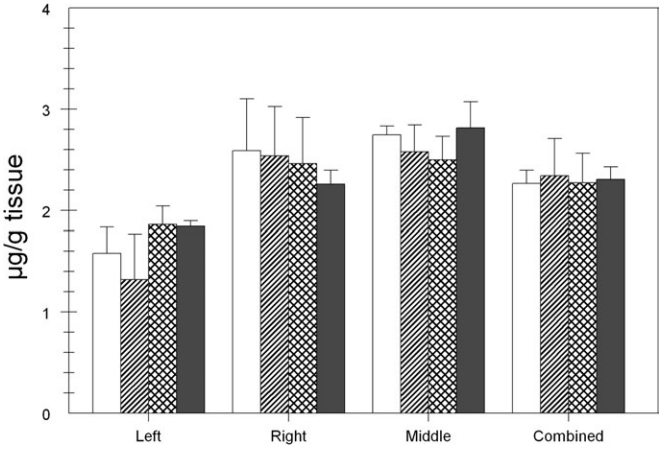

FIG. 2. Levels of titanium in left, right, and middle lobes of the liver taken from minipigs treated with sunscreen formulations containing the following TiO_2 (from left to right): control, no TiO_2 (open bar); submicron (grey bar); uncoated nano (hashed bar); and coated nano (filled bar). The ''combined'' group represents the average value for liver samples for each animal in the indicated treatment group. Data are presented as mean ± SE for three minipigs per group.

levels in the tissues from control-treated minipigs. This was true for the individual lobes of the liver and when the data were combined to give an average liver titanium content.

Sections of skin from each of the minipigs were separated into the epidermis and dermis using a dry heat technique to prevent migration of the TiO_2 in the tissues. Titanium levels in the epidermis of minipigs for each treatment group are shown in Figure 3. Significant increases over control levels are seen for all groups. This is not surprising since the cleaning method for the skin would not remove the stratum corneum where significant levels of TiO_2 are likely to be trapped as a result of the topical treatment.

The titanium levels in the dermis for each of the groups are shown in Figure 4. Increased titanium was detected in the abdominal dermis from minipigs treated with uncoated and coated nanosize TiO_2, in the neck dermis from the uncoated and coated nanosize TiO_2, and in the left inguinal dermis from the submicron TiO_2–treated minipigs. When the dermal titanium values are combined, there was a significant increase in titanium in all TiO_2 treatment groups when compared to the control values. While the dry heat method results in the separation and removal of almost all of the epidermis, some portions of the epidermis remained attached to the dermis, as shown in Figure 5. Since the levels of titanium in the epidermis are significantly higher than those detected in the dermis (e.g., ~11 mg/g epidermis and ~26 μg/g dermis with submicron TiO_2 on abdominal sample), we could not rule out that the elevated levels of titanium in the dermis were due to contaminating epidermis.

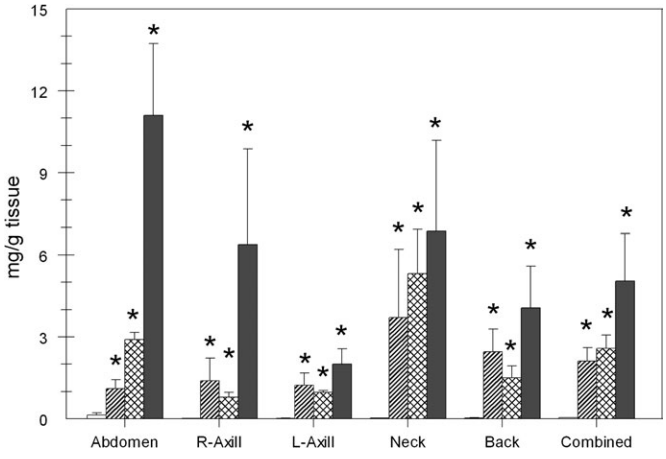

FIG. 3. Level of titanium in epidermis isolated from skin taken from minipigs treated with sunscreen formulations containing the following TiO_2 (from left to right): control, no TiO_2 (open bar); submicron (grey bar); uncoated nano (hashed bar); and coated nano (filled bar). The skin was taken from the abdomen, right and left axillary (axil) areas, neck, and back, and the dermis separated from the epidermis with a dry heat method. The ''combined'' group represents the average value for all epidermal samples for each animal. Data are presented as mean ± SE for three minipigs per group. *statistical significance ($p \leq 0.05$) from the control values for each grouping.

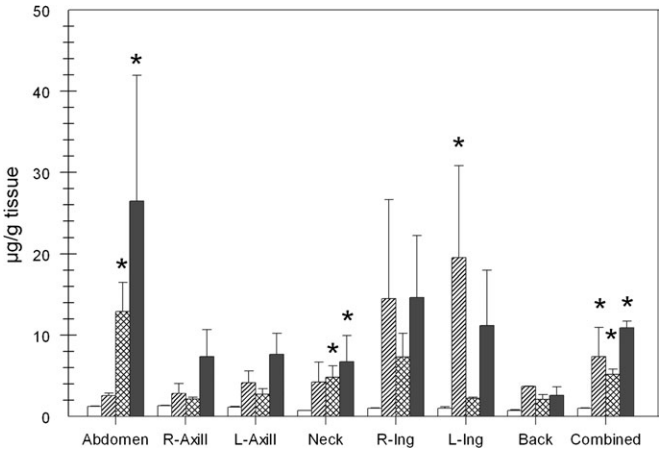

FIG. 4. Level of titanium in dermis isolated from skin taken from minipigs treated with sunscreen formulations containing the following TiO_2 (from left to right): control, no TiO_2 (open bar); submicron (grey bar); uncoated nano (hashed bar); and coated nano (filled bar). The skin was taken from the abdomen, right and left axillary (axil) and inguinal (Ing) areas, neck, and back, and the dermis separated from the epidermis with a dry heat method. The ''combined'' group represents the average value for all epidermal samples for each animal. Data are presented as mean ± SE for three minipigs per group. *statistical significance ($p \leq 0.05$) from the control values for each grouping.

In order to evaluate if the elevated titanium levels in the dermis were due to remnants of epidermis, penetration of TiO_2 into the dermis, or accumulation of TiO_2 in the lower aspects of hair follicles, the skin samples from treated and control animals were analyzed by electron microscopy, followed by EDX spectroscopy. Though TEM is not able to provide a quantitative estimation of total titanium distribution in the skin due to its limited survey area, it was obvious that the TiO_2 particles (electron-dense particles) were primarily detected in stratum corneum. Fewer than 10 isolated individual TiO_2 particles were detected and confirmed with EDX in the dermis layer and hair follicles. No pattern of distribution of these particles was observed, indicating that their presences could be the result of contamination during tissue sectioning rather than penetration. There was no observable trend with particle size, with both

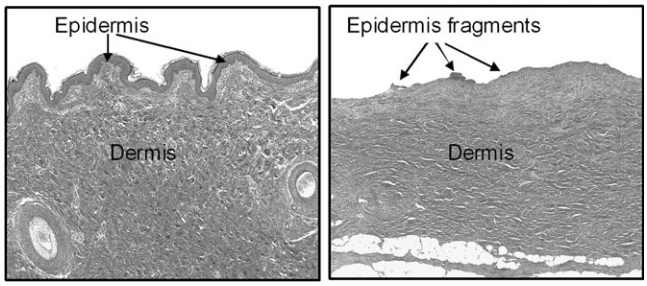

FIG. 5. Photomicrograph of a section of minipig skin before (left panel) and after (right panel) separation of the epidermis from the dermis using the dry heat method. Sections were stained with hematoxylin and eosin. Fragments of the epidermis that were not removed with the dry heat method are identified in right panel.

nanosized and submicron particles observed in dermis at comparable low levels. There was no obvious pathogenicity in cells around the observed isolated TiO_2 particles in the dermis. Calculations indicate that these few isolated particles represent a tiny fraction of the total amount of applied TiO_2 (~8×10^{-5} percent for 10 particles; see Discussion section). The results of this analysis are shown in Figures 6–8. For clarity, each treatment is described below in a separate paragraph; however, in order to allow for a better visual comparison between the treatments, all the treatments are combined in one figure composed of multiple panels.

Electron-dense spots were found in the skin samples exposed to control cream (see Fig. 6a); however, EDX analysis showed that these spots were not TiO_2 (see EDX spectra below Fig. 6a). Based on morphology, size, and elemental composition, we believe these particles are melanosomes. Melanosomes are more often observed in the stratum spinosum, but some can be shed off into the stratum corneum. No TiO_2 nanoparticles were found in these samples.

TEM data (Figs. 6b and 7a) showed electron-dense spots (potential TiO_2 particles) primarily in the upper stratum corneum layers of all three skin samples treated with submicron-sized TiO_2 particles. These particles appeared slightly aggregated and were well below micron sized (the average particle diameter was ~207 ± 53 nm, determined by measuring 15 particles chosen at random in each skin sample). A few electron-dense individual particles were also detected in the upper follicular lumens. These particles had sizes and shapes similar to those observed in the stratum corneum in nonfollicular areas. Scattered TiO_2 particles were found in the lower follicular lumens. EDX analysis (Fig. 6b) shows the titanium Kα peak at 4.510 keV confirming these electron-dense spots to be TiO_2 particles.

Electron-dense spots were also observed in skin samples treated with cream containing uncoated nanosize TiO_2 (Figs. 6c and 7b). These particles displayed a similar degree of agglomeration and similar morphology to the particles observed in the samples treated with submicron-sized TiO_2 but were much smaller in size. (The average particle size was ~30 ± 8 nm in diameter. This number was reached by measuring 13 particles chosen at random in each skin sample.) EDX analysis (Fig. 6c) confirmed these electron-dense spots to be TiO_2 particles.

The TEM micrographs (Figs. 6d and 7c) of the skin samples exposed to the formulation containing coated nanosize TiO_2 particles contained far more TiO_2 particles than either of the samples treated with submicron-size or uncoated nanosize TiO_2 creams; however, since only certain sections of the skin samples were imaged, this is a qualitative finding and does not necessarily reflect larger total amounts of TiO_2 in skin. As with the other cream-treated skin samples, the observed TiO_2 particles were primarily detected in the upper stratum corneum layers. These particles appeared highly aggregated, forming fibril-like structures ~57 ± 18 nm in length and 15 ± 5 nm in width (Fig. 8). EDX analysis confirmed these highly aggregated particles as TiO_2. A few particles were located in TEM images of the lower epidermis, the dermis, and in hair follicle lumens; however, EDX was not able to identify these particles as TiO_2 since any titanium signal was below the detection threshold.

As shown in Figure 9, the particle distribution and particle concentration level in the various cross sections of a skin sample exposed to submicron TiO_2 showed the same overall trend as the longitudinal sections of the other skin samples, namely that the highest concentrations of TiO_2 particles were observed in the stratum corneum and only a few particles in the dermal layers. The few TiO_2 particles observed in the dermal layers may have been the result of cross-contamination during specimen handling. These particles appeared isolated and randomly distributed. There was no evidence of penetration via expected routes such as follicular lumens or that the particle concentration decreased with depth in skin.

DISCUSSION

Concern has been expressed in the published literature that nanosized particles have unknown toxicological profiles differing from those of larger sized particles, and because of their small size, they may penetrate the skin and pose safety concerns. This matter has gained more attention due to the large number of sunscreens available on the market claiming to contain nanosize TiO_2. In order to evaluate whether nanosize TiO_2 can penetrate intact skin, several studies have been conducted and published. Tan *et al.* (1996) excised skin from human volunteers following 2- to 6-week applications of a sunscreen containing 8% microfine (i.e., nanosized) TiO_2 for 2–6 weeks. TiO_2 was determined in the lower epidermis and dermis by ICP-MS. The authors found elevated but not significant ($p = 0.14$) levels of TiO_2 in the skin of the test patients when compared to skin from cadavers; however, if one extreme value is removed from the cadaver group, a significant difference was detected ($p = 0.006$) with an ~50% increase in skin TiO_2 levels. This work was one of the first reports to suggest TiO_2 might penetrate the skin.

Lansdown and Taylor (1997) applied suspensions of 20% "microfine" TiO_2 and ZnO to the shaved backs of New Zealand white rabbits. Surface coated TiO_2 or ZnO was removed by tape stripping and the skin removed after 24 or 48 h posttreatment. TiO_2 applied as a caster oil suspension penetrated the skin; TiO_2 applied as an aqueous suspension did not penetrate the skin. These results suggested that dermal penetration by micronized ZnO or TiO_2 was possible.

Bennat and Müller-Goymann (2000) examined the penetration of microfine TiO_2 into human skin *in vitro* and *in vivo*. The TiO_2 was suspended either as an aqueous or as an oil/water emulsion and applied at a dose of 2 mg/cm^2. The stratum corneum of the epidermis was removed 24 h later using tape

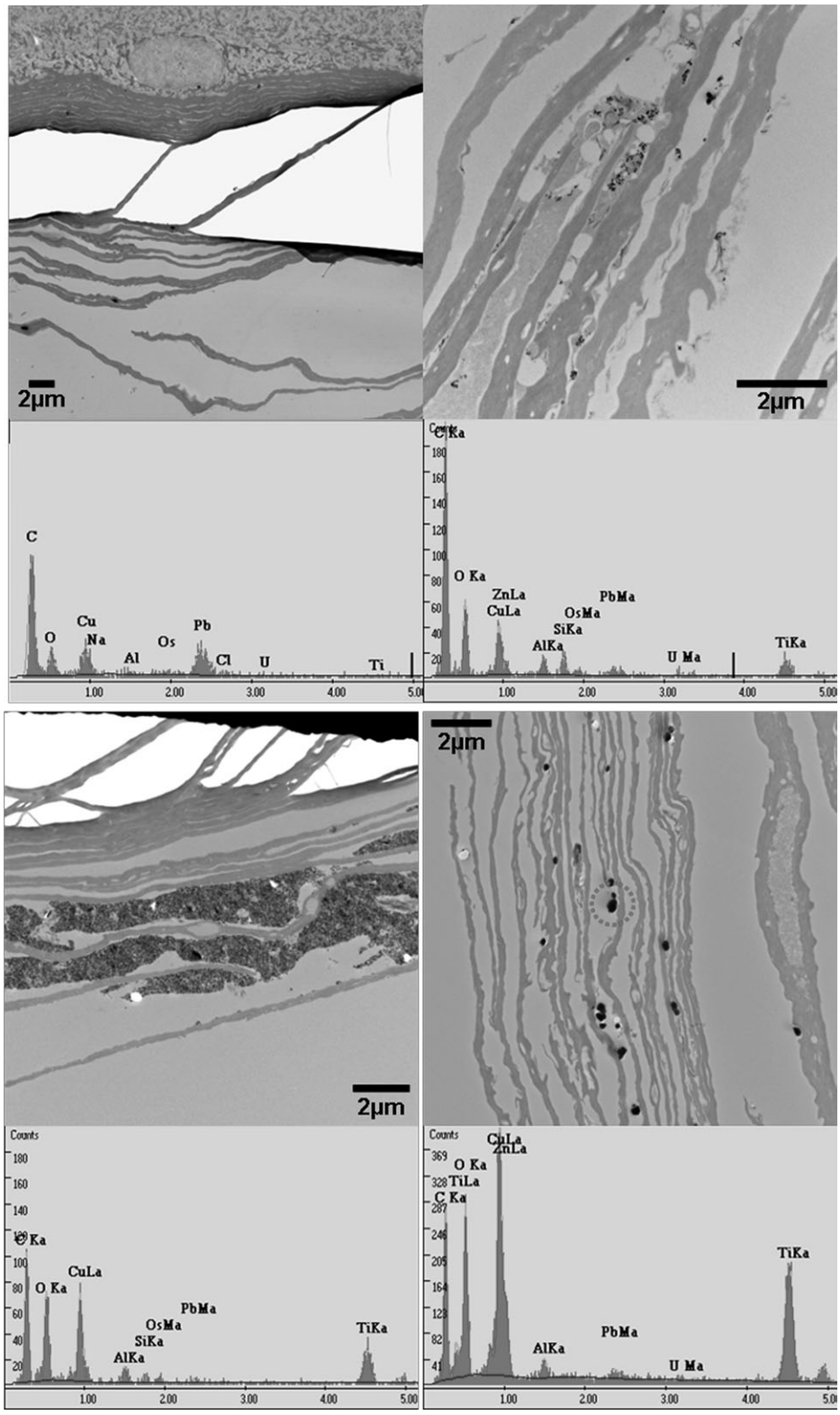

FIG. 6. Representative TEM micrographs and corresponding SEM-EDX spectra (below micrograph) of minipig skin stratum corneum. (upper left) Control (no TiO_2); (upper right) Sub-micron-sized TiO_2; (lower left) Uncoated nanosized TiO_2; (lower right) Coated nanosized TiO_2. The areas of stratum corneum of skin were surveyed and EDX spectra were acquired at the circled area (lower right micrograph, dotted circle) in the TEM micrographs, respectively. The acquisition time for the EDX spectrum associated with lower right image is much shorter than the acquisition times for the other EDX spectra, reflecting the higher density of particles in the coated nano TiO_2 treated sample. A higher magnification TEM image of the circled area in lower right panel is shown in Figure 8.

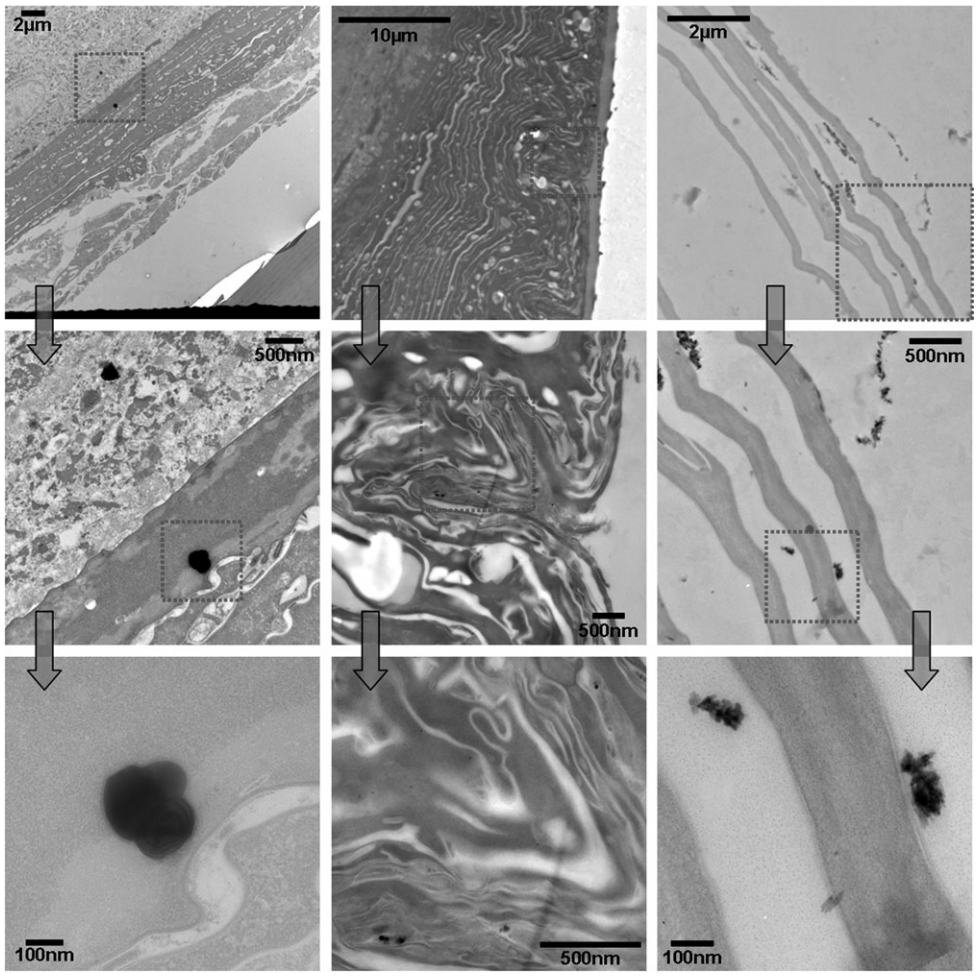

FIG. 7. Representative TEM micrographs of minipig skin in areas containing hair follicles. (left column of photomicrographs) Sub-micron-sized TiO_2; (middle column of photomicrographs) Uncoated nano-sized TiO_2; (right column of photomicrographs) Coated nano-sized TiO_2 in hair follicle areas. The images in the lower photomicrographs are higher magnification images of the corresponding upper panels.

strips and the TiO_2 content on the strip was determined. The authors concluded that TiO_2 penetrated the upper layers of the epidermis when applied as a 40% suspension in oil, with less penetration from a water suspension. This penetration was enhanced ~10-fold when the material was applied on hairy skin, suggesting that either the hair follicles could be a route of penetration or in this study, tape stripping was inadequate in removing the TiO_2 that penetrated into the follicle. Application of the TiO_2 as a 5% liposomal suspension resulted in consistent presence of TiO_2 in the epidermis, while application as an oil/water emulsion did not achieve the same level of penetration.

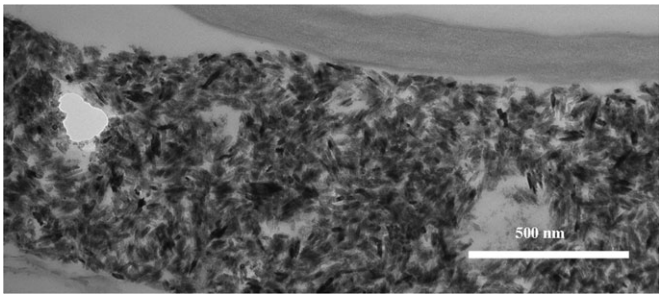

FIG. 8. ×15,000 magnification TEM image of tissue in Figure 6d (minipig skin treated with coated nanosized TiO_2). Hundreds of particles are visible in this small region of stratum corneum tissue, illustrating the higher density of particles in the coated nano-TiO_2–treated skin samples.

Lademann *et al.* (1999) applied a coated micronized TiO_2 sunscreen formulation to the skin and found that epidermal penetration could not be detected in intrafollicular areas of the skin; however, TiO_2 was detected in follicular-containing areas following tape stripping. Histological examination of the skin indicated TiO_2 in the open part of the follicle.

Schulz *et al.* (2002) used nanosize TiO_2 from several sources to determine the penetration of TiO_2 into human skin. TiO_2 that was hydrophobic (20 nm; coated with trimethyloctylsilane), amphilic (10–15 nm; coated with Al_2O_3 and SiO_2, aggregating to 100-nm needles), and hydrophilic (100-nm needles) were applied as 4% emulsions in a complex cream to human skin and the skin biopsied after 36 h. The authors examined the

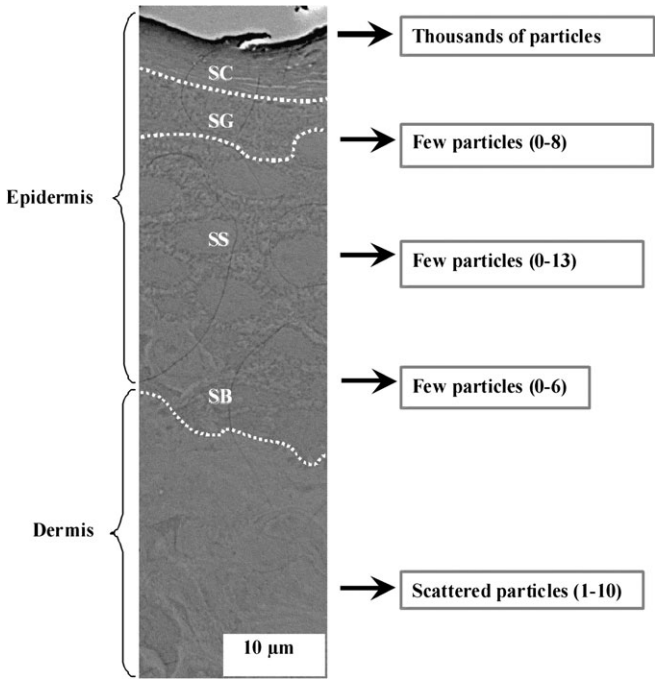

FIG. 9. Representation of TiO_2 particle distribution in different layers of minipig abdominal skin (longitudinal slice of skin) exposed to submicron TiO_2 showing the results from the cross section analysis of each skin layer. Numbers in parentheses are estimates of the numbers of TiO_2 particles observed in each layer. Abbreviations are: SC, stratum corneum; SS, stratum spinosum; SG, stratum granulosum; and SB, stratum basale.

skin for TiO_2 particles using TEM and did not find evidence of skin penetration. Agglomerations of TiO_2 were detected on the surface of the skin.

While none of these studies were conclusive, they did raise concerns about the possibility that nanosize TiO_2 can penetrate skin. The ambiguity in the literature, combined with the public perception of a health concern (demonstrated in a citizen petition submitted to FDA), led us to conduct the present study. Because the pig and minipig have skin that more closely resembles human skin, as compared to other species, in regards to epidermal cellular thickness, overall physiology, and hair follicle density, we chose this animal model to conduct our study (Prunieras, 1993; Sekkat and Guy, 2001; Tiemessen, 1993; Wester and Maibach, 1999).

In order to have our study provide the highest relevance to the sunscreen-using public, the doses and vehicle used in this study were chosen to closely resemble the material currently used in marketed products. The formulations used in this study were very similar to those used in a "typical" sunscreen. TiO_2 may be included in sunscreens as an ingredient as long as it does not exceed 25% by weight (21 CFR 352.10). We chose to include TiO_2 at ~5%. In addition, instead of monitoring TiO_2 levels following a single administration of sunscreen, we chose to mimic the continuous use of sunscreen over the course of 22 days.

To optimize assessment of penetration by TiO_2 through intact pigskin, we used both ICP-MS and EM-EDX. Using the lymphatics and liver as sentinel organs for TiO_2 penetration, we did not detect by ICP-MS consistent increases in these organs following 22 days of sunscreen application. The sentinel organ approach has been previously demonstrated to detect the distribution of PEG-coated quantum dots following intradermal administration (Gopee *et al.*, 2007) and dermal penetration following disruption of the skin (Gopee *et al.*, 2009). In this study, we show that titanium levels in lymph nodes and liver are not elevated as would be expected if TiO_2 had penetrated the epidermis.

The use of EM was important to demonstrate whether the titanium found in dermis was due to contamination from epidermis or actual penetration. However, to put our findings into context, the following calculations were done, as a means to estimate the dose of TiO_2 detected in the skin based on EM results.

The levels of titanium found in the epidermis from control, submicron, uncoated nanosized, and coated nanosized TiO_2 was 0.035, 2.11, 2.57, 5.05 mg/g tissue, respectively, whereas the levels of titanium found in the dermis were 1.003, 7.34, 5.17, 10.92 µg/g. These results indicate that the amount of titanium found in the epidermis was 35-, 287-, 498-, and 463-fold higher than the levels detected in the dermis. Although the dry heat method for separating the epidermis from dermis was very good, it is possible that, as shown in Figure 5, a small piece of epidermis could occasionally remain on the dermis, following separation. If the epidermis left behind on the dermis was ~0.2–0.3% of the original epidermis, this could account for the level of titanium that was detected in the dermis. To address this concern, we used TEM-EDX, which could resolve whether the titanium detected in the dermis was due to contamination from residual epidermis.

TEM images of the skin exposed to submicron TiO_2 show electron-dense spots of the appropriate morphology to be TiO_2 particles primarily in the upper stratum corneum layers of all three skin samples. These particles appear slightly aggregated and are smaller than expected (the average particle diameter is ~210 nm). EDX analysis confirmed that these particles were TiO_2. Only a few particles (< 50) were observed in the lower epidermis layer or viable dermis layer, suggesting that minimal nanoparticle penetration occurs through intact epidermis. Scattered electron-dense individual particles were also detected in the upper follicular lumens and in the lower follicular lumens. These particles have similar sizes and shapes as those observed in the stratum corneum at nonfollicular areas.

We also found TiO_2 particles primarily in the stratum corneum and upper follicular lumens in skin samples exposed to uncoated nano-TiO_2. These particles display a similar degree of agglomeration and similar morphology to those in the samples treated with submicron TiO_2 but are even smaller in size (around 30 nm in diameter). The TEM micrographs of the skin samples exposed to the coated nanosize TiO_2 cream showed that these samples contain significantly more TiO_2 nanoparticles than the skin samples treated with the creams containing submicron TiO_2 or uncoated nanosize TiO_2, though this qualitative finding does not necessarily reflect a difference

in the total amount of TiO_2 in the skin. Thousands of these particles were detected in the upper stratum corneum layers and appear highly aggregated (the aggregations reaching several microns in size), forming fibril-like structures with the individual particles being, on average, 57 nm in length and 15 nm in width (as seen in Fig. 7c).

All three types of TiO_2 particles were most concentrated in the stratum corneum layer, and these particles were highly aggregated in between the layers of keratin, which compose the stratum corneum. In lower skin layers, only a very few isolated TiO_2 particles were observed. These few particles appeared isolated and randomly distributed. There was no evidence of penetration via expected routes such as follicular lumens or evidence that the particle concentration decreased with depth in skin. The presence of these particles in the lower skin layers may have been the result of cross-contamination during tissue sectioning.

Although a very small number of scattered isolated TiO_2 particles were seen in the dermis of pigs treated with sunscreen formulations containing nanoparticles, these are possibly the result of contamination of samples during sample handling. Even if these particles did reach the dermis via penetration, they represent a very small fraction of the applied TiO_2. The electron microscopy results allowed us to consider a quantitative approach to determine the level of TiO_2 that were confirmed to be present in the electron micrographs. If one considers the area covered by the electron micrographs that were examined, the corresponding surface area equates to 8×10^{-8} cm^2. Since 176 mg/cm^2 of total cream was applied containing 5% by weight TiO_2, 7.0×10^{-7} mg TiO_2 was applied to the skin above the EM viewing area.

If we assume that the P25 nano-TiO_2 had a nominal primary particle size of 30 nm, the volume of that sphere would be 1.4×10^{-17} cm^3, and if the specific density is 4 g/cm^3, the weight of each uncoated nano-TiO_2 of primary particle size would be 5.6×10^{-14} mg. Therefore, each particle detected by electron microscopy would equate to 8×10^{-6} percent of the total dose that was applied. As a result, and assuming the TiO_2 particles of uncoated nano-TiO_2 that were detected were primary particles, the detection of 10 confirmed particles of uncoated nano-TiO_2 would equate to 8×10^{-5} percent of the total applied dose. Using a similar analysis as described above for the coated nanoscale TiO_2 (15 × 57–nm rods), the detection of one particle in the dermis would equate to 5.7×10^{-6} percent of the total dose applied. Similarly, the detection of one submicron TiO_2 (50 × 207–nm rods) in the dermis would equate to 2.3×10^{-4} percent of the applied dose. This evaluation of the doses that were detected is based on several assumptions; however, it provides a reasonable measure of the order of magnitude of dose that is detected by electron microscopy. This quantitative analysis of the skin penetration of nanoscale uncoated TiO_2 showed that our sensitive electron microscopic methods detected a measurable but insignificant amount of the TiO_2 applied to the skin of pigs, regardless of the type or particle size used in the study.

Recently Wu *et al*. (2009) have shown that dermal application of TiO_2 (4 or 60 nm) to pig ears for a period of 30 days did not result in penetration of nanoparticles beyond the deep epidermal layers. The results of Wu *et al*. (2009) are in agreement with the results of the present study, where we did not find any significant dermal penetration of nanoparticles of TiO_2 in pigs treated for 1 month with sunscreens formulated with various forms of TiO_2, including coated nano, uncoated nano, and submicron-sized particles of TiO_2; however, the study by Wu *et al*. (2009) did report dermal penetration of TiO_2 nanoparticles in hairless mice, with subsequent appearance of lesions in multiple organs. The relevance of the finding in mice to our present study in minipigs and to human exposure is unclear. Hairless mouse skin does contain abnormal hair follicles, and mouse stratum corneum has a higher lipid content than human stratum corneum, which in part probably explains why some drugs penetrate mouse skin differently than human skin.

Overall, our study demonstrates that nanosized or submicron-sized TiO_2 suspended in the specific formulation used in this study do not penetrate the intact epidermis to any significant extent. While this study does not address any issues within the epidermis where concentrated TiO_2 could have an effect on the immune system (e.g., contact dermatitis) or cause toxicity following irradiation with ultraviolet light (e.g., photoactivation, phototoxicity), the study does show that the dermis and other organs may not be at risk from topically applied sunscreens containing nanosize TiO_2. Furthermore, no obvious structural abnormalities were observed in the skin cells, which can be attributed to the presence or absence of TiO_2 particles. This study supports the conclusion that nanosized TiO_2 included in a formulation similar to currently marketed sunscreens does not significantly penetrate intact normal pigskin to any significant degree and therefore is unlikely to significantly penetrate human skin. We believe that the inclusion of nanosized titanium dioxide in OTC sunscreens does not pose a significant health threat because nanosized particles do not appear to significantly penetrate intact skin; however, since some studies have shown that compromised skin allows particle penetration through the skin (Gopee *et al*., 2009, and references therein), we cannot at this time rule out the possibility that damaged skin could be a risk factor for TiO2 penetration.

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those of the authors and should not be interpreted as the official opinion or policy of the U.S. Food & Drug Administration, Department of Health and Human Services, or any other agency or component of the U.S. government. The mention of trades names, commercial products, or organizations is for clarification of the methods used and should not be interpreted as an endorsement of a product or manufacturer.

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