Lack of Significant Dermal Penetration of Titanium Dioxide from Sunscreen Formulations Containing Nano- and Submicron-Size TiO₂ Particles

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Titanium dioxide (TiO₂) is included in some sunscreen formulations to physically block ultraviolet radiation. A dermal penetration study was conducted in minipigs with three TiO₂ 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/cm² skin (4 applications/day, 5 days/week, 4 weeks). Skin (multiple sites), lymph nodes, liver, spleen, and kidneys were removed, and the TiO₂ applications/day, 5 days/week, 4 weeks). Skin (multiple sites), treated with uncoated and coated nanoscale TiO₂. Using electron microscopy-energy dispersive x-ray analysis, all three types of TiO₂ particles were found in the stratum corneum and upper epidermis. These findings indicate that there is no significant penetration of TiO₂ nanoparticles through the intact normal epidermis.

Key Words: Sunscreen; TiO₂; 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 TiO₂ 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 TiO₂ 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 TiO₂ 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 \textit{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 \textit{et al.}, 1999; Hoffman \textit{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 (treated with 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 Tipeque 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 glucose, polyglyceryl-2 dipolyhydroxystearate, glycerin, cetearyl alcohol [Cognis Corporation, New Milford, CT], C\(_{12-15}\) alkyl benzoate [Finetex, Inc., Spencer, NC], octyl methoxycinnamate, and tocopherol 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 \textit{et al.}, 2008; Wokovich \textit{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 \textit{et al.}, 2008; Wokovich \textit{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 GMS 6320F (JEOL Ltd., Tokyo, Japan). All the sunscreen samples were prepared as described earlier (Tyner
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 TiO2 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/cm2 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 ~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 absence of blood flow from the femoral vessels and a mixture of nitric acid (HNO3) 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% HNO3 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 49Ti and 50Ti 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 Dunnnett’s method. The data were transformed (ln) when normalcy tests failed. Significance was set at p ≤ 0.05.

**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 (HNO3) 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% HNO3 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 49Ti and 50Ti isotope content.

**Transmission electron microscopy and scanning electron microscopenergy dispersive x-ray.** TEM and SEM were used to image the TiO2 particles in ~2–3 cm3 of pig abdominal skin tissue. Abdominal skin was chosen for the electron microscopic examination due to preliminary data showing TiO2 in the dermis from this tissue, and the epidermis in this area is very thin increasing the likelihood of epidermal penetration by the TiO2. 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 TiO2 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% OsO4, 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
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-TiO₂ and an increase in the right inguinal lymph nodes of minipigs treated with submicron TiO₂. 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-TiO₂ 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 TiO₂-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.

The titanium levels in the left, right, and middle lobes of the liver for the various treatment groups are shown in Figure 2. The titanium content was not significantly different between any of the TiO₂-treated groups when compared to the...
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.

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...
nanosized and submicron particles observed in dermis at comparable low levels. There was no obvious pathogenicity in cells around the observed isolated TiO₂ particles in the dermis. Calculations indicate that these few isolated particles represent a tiny fraction of the total amount of applied TiO₂ (~8 x 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 TiO₂ (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 TiO₂ nanoparticles were found in these samples.

TEM data (Figs. 6b and 7a) showed electron-dense spots (potential TiO₂ particles) primarily in the upper stratum corneum layers of all three skin samples treated with submicron-sized TiO₂ 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 TiO₂ 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 TiO₂ particles.

Electron-dense spots were also observed in skin samples treated with cream containing uncoated nanosize TiO₂ (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 TiO₂ 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 TiO₂ particles.

The TEM micrographs (Figs. 6d and 7c) of the skin samples exposed to the formulation containing coated nanosize TiO₂ particles contained far more TiO₂ particles than either of the samples treated with submicron-size or uncoated nanosize TiO₂ 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 TiO₂ in skin. As with the other cream-treated skin samples, the observed TiO₂ 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 TiO₂. 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 TiO₂ 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 TiO₂ showed the same overall trend as the longitudinal sections of the other skin samples, namely that the highest concentrations of TiO₂ particles were observed in the stratum corneum and only a few particles in the dermal layers. The few TiO₂ 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 TiO₂. In order to evaluate whether nanosize TiO₂ 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) TiO₂ for 2–6 weeks. TiO₂ was determined in the lower epidermis and dermis by ICP-MS. The authors found elevated but not significant (p = 0.14) levels of TiO₂ 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 TiO₂ levels. This work was one of the first reports to suggest TiO₂ might penetrate the skin.

Lansdown and Taylor (1997) applied suspensions of 20% “microfine” TiO₂ and ZnO to the shaved backs of New Zealand white rabbits. Surface coated TiO₂ or ZnO was removed by tape stripping and the skin removed after 24 or 48 h posttreatment. TiO₂ applied as a caster oil suspension penetrated the skin; TiO₂ applied as an aqueous suspension did not penetrate the skin. These results suggested that dermal penetration by micronized ZnO or TiO₂ was possible.

Bennat and Müller-Goymann (2000) examined the penetration of microfine TiO₂ into human skin in vitro and in vivo. The TiO₂ was suspended either as an aqueous or as an oil/water emulsion and applied at a dose of 2 mg/cm². 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.
strips and the TiO₂ content on the strip was determined. The authors concluded that TiO₂ 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 TiO₂ that penetrated into the follicle. Application of the TiO₂ as a 5% liposomal suspension resulted in consistent presence of TiO₂ in the epidermis, while application as an oil/water emulsion did not achieve the same level of penetration.

Lademann et al. (1999) applied a coated micronized TiO₂ sunscreen formulation to the skin and found that epidermal penetration could not be detected in intrafollicular areas of the skin; however, TiO₂ was detected in follicular-containing areas following tape stripping. Histological examination of the skin indicated TiO₂ in the open part of the follicle.

Schulz et al. (2002) used nanosize TiO₂ from several sources to determine the penetration of TiO₂ into human skin. TiO₂ that was hydrophobic (20 nm; coated with trimethyloctylsilane), amphiphilic (10–15 nm; coated with Al₂O₃ and SiO₂, 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
skin for TiO₂ particles using TEM and did not find evidence of skin penetration. Agglomerations of TiO₂ were detected on the surface of the skin.

While none of these studies were conclusive, they did raise concerns about the possibility that nanosize TiO₂ 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. TiO₂ 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 TiO₂ at ~5%. In addition, instead of monitoring TiO₂ 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 TiO₂ through intact pigskin, we used both ICP-MS and EM-EDX. Using the

lymphatics and liver as sentinel organs for TiO₂ 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 TiO₂ 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 TiO₂ detected in the skin based on EM results.

The levels of titanium found in the epidermis from control, submicron, uncoated nanosized, and coated nanosized TiO₂ 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 TiO₂ show electron-dense spots of the appropriate morphology to be TiO₂ 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 TiO₂. 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 TiO₂ particles primarily in the stratum corneum and upper follicular lumens in skin samples exposed to uncoated nano-TiO₂. These particles display a similar degree of agglomeration and similar morphology to those in the samples treated with submicron TiO₂ but are even smaller in size (around 30 nm in diameter). The TEM micrographs of the skin samples exposed to the coated nanosize TiO₂ cream showed that these samples contain significantly more TiO₂ nanoparticles than the skin samples treated with the creams containing submicron TiO₂ or uncoated nanosize TiO₂, 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 $8 \times 10^{-8}$ cm$^2$. Since 176 mg/cm$^2$ of total cream was applied containing 5% by weight TiO$_2$, $7.0 \times 10^{-7}$ 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 $1.4 \times 10^{-17}$ 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 $5.6 \times 10^{-14}$ mg. Therefore, each particle detected by electron microscopy would equate to $8 \times 10^{-6}$ 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 $8 \times 10^{-5}$ 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 $5.7 \times 10^{-6}$ percent of the total dose applied. Similarly, the detection of one submicron TiO$_2$ (50 × 207–nm rods) in the dermis would equate to $2.3 \times 10^{-5}$ 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 TiO$_2$ penetration.

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