Australian Government
Department of Health and Ageing
Therapeutic Goods Administration

A review of the scientific literature on the safety of nanoparticulate titanium dioxide or zinc oxide in sunscreens

Summary

The potential for titanium dioxide (TiO2) and zinc oxide (ZnO) nanoparticles in sunscreens to cause adverse effects depend primarily upon the ability of the nanoparticles to reach viable skin cells.

To date, the current weight of evidence suggests that TiO2 and ZnO nanoparticles do not reach viable skin cells, rather, they remain on the surface of the skin and in the outer layer (stratum corneum) of the skin that is composed of non-viable, keratinized cells.

There is evidence from isolated (*in vitro*) cell experiments that ZnO and TiO2 may induce free radical formation in the presence of light and this free-radical generation may cause cell damage (photo-genotoxicity with ZnO). However, recent work suggests that the photo-genotoxicity seen in these studies (with ZnO) may be due to an UV-induced experimental artifact in an *in vitro* assay, rather than the presence of the nanoparticles (40).

Background

Zinc oxide and titanium dioxide have been used as sunscreens for many years. They are particularly valuable because of their ability to filter UVA as well as UVB radiation, giving broader protection than other sunscreening agents. One disadvantage of zinc oxide and titanium dioxide is that they are visible, giving the skin a white colour. This effect can be reduced by decreasing the particle size of the material. When used in 'nanoparticle' form (less than 100 nanometers, with a nanometer being one millionth of a millimeter), they can't be seen on the skin but still retain the sunscreening properties of the coarser material. Nano-sized Titanium dioxide particles have been used in sunscreens since at least 1990 and nano-sized zinc oxide since 1999.

In January 2006 the TGA conducted a review of the scientific literature in relation to the use of nanoparticulate zinc oxide and titanium dioxide in sunscreens. That review concluded that:-

There is evidence from isolated cell experiments that zinc oxide and titanium dioxide can induce free radical formation in the presence of light and that this may damage these cells (photo-mutagenicity with zinc oxide). However, this would only be of concern in people using sunscreens if the zinc oxide and titanium dioxide penetrated into viable skin cells. The weight of current evidence is that they remain on the surface of the skin and in the outer dead layer (stratum corneum) of the skin.

The Medicines Evaluation Committee endorsed this conclusion at its meeting on 2 February 2006.

Since that time, the TGA has been monitoring the emerging scientific literature in this area and working cooperatively with international agencies to monitor these issues to ensure that appropriate action is undertaken if any unacceptable risks are identified. The following report is an update (May 2009) of the January 2006 review. The TGA will continue to assess the literature and provide annual updates to this review.

An on-line search on the safety of nanoparticulate TiO2 and ZnO in sunscreens was undertaken on the following databases: Medline, Embase, Biosis, Cabi and a Dialog search on a large number of medical and pharmaceutical databases as well as Google. The search strategy used was:

> *(Nanoparticle OR nanoparticles OR nanoparticulate OR nanoscale OR nanosize OR nanomaterials) AND (zinc oxide OR titanium dioxide) AND (sunscreen OR sunblock OR sun block OR sun screen OR UV blockers OR physical sunscreen) AND (safety OR toxicology OR toxic OR safe)*

LITERATURE OVERVIEW

The studies reviewed and summarised in the following section represent available published articles on issues relating to the use of nanoparticulate TiO2 and ZnO as UV filters in sunscreens; some related studies have been identified but were not in the public domain and not able to be accessed (or summarised). A number of the studies presented did not directly address the main issues identified below; type of toxicity of TiO2 or ZnO and ability of these inorganic materials to penetrate the skin to reach viable cells. Study numbers in the brackets below identify studies that provide relevant information on these issues.

Hazard - toxicity

Toxicity (pathological and functional changes) that could occur following exposure to inorganic TiO2 and ZnO (UV filters) has been linked to free radical generation (9, 13, 17, 19, 20 and 31). In these studies, there is evidence that TiO2 and ZnO can induce the production of free radicals (likely hydroxyl radicals through oxidation) and cause adverse effects in isolated cell experiments (*in vitro*). A recent study (35) examining *in vivo* and *in vitro* toxicity of ultrafine TiO2 concluded that ultrafine TiO2 exhibited low hazard potential in mammalian and aquatic species/cell lines following acute exposure.

There were three unpublished studies (10, 11 and 12) investigating photo-mutagenicity (possibly linked to free radical formation) that had been discussed by the SCCNFP (European group: Scientific Committee Cosmetic Non-Food Products) in their review of micronised ZnO. The SCCNFP conclusions for these studies were recorded in the preceding documents (10, 11 and 12). The overall conclusion by the SCCNFP was that, "micronised material (ZnO) has been found to be clastogenic, possibly aneugenic and inducing DNA damage in cultured mammalian cells *in vitro*, under the influence of UV light".

Recent work (29, 40) suggests that possible photo-genotoxicity associated with ZnO maybe influenced by UV radiation induced increases in background sensitivity of cell populations. Levels of photo-genotoxicity (micronucleus assay) following exposure to ZnO were

relatively small (2-4 fold increase) compared with known photo-clastogenic agents (8-MOP; >15000 fold increase). A further article discussed by the SCCNFP looked at photo-mutagenicity of micronised ZnO in cultured bacteria (Ames assay) and noted micronised ZnO was negative for photo-mutagenic activity.

Photo-toxicity was discussed by the SCCNFP regarding intact skin of human volunteers, with no evidence of any reactions in 2 photo-irritation studies (n=40) and 2 photosensitisation studies (n=55). Toxicokinetic assessment (*in vivo*) using human volunteers with healthy and diseased skin (psoriasis) found no evidence of an increase in systemic Zn levels after dermal application of ZnO. An *in vitro* assay using human skin (with stratum corneum stripped away) indicated 0.34% absorption of an applied ZnO dose based on recovery from receptor fluid. It is worth noting that HaCaT cells (human keratinocytes – living cell of epidermis) have been tested, with no evidence of any DNA damage for ZnO in the presence or absence of UV irradiation.

On balance, the evidence implicating nanoparticulate ZnO as a photo-genotoxic agent has been generated from *in vitro* studies, which has been questioned by some researchers (40). Evidence of a photo-genotoxicity effect *in vivo* appears to be limited or absent (29, 40). TiO2 has been documented as not being a genotoxic agent (40). Also, *in vivo* and *in vitro* studies (paragraph above) in humans or cells derived from humans did not identify toxic effects with ZnO. TiO2 was found to be of low hazard based on a series of toxicity studies (35). The hazard associated with the use of these substances (TiO2 & ZnO) appears to be low.

Treatment of nano-TiO2 and ZnO with coating agents (eg. inert oxides of silica) has been used as an approach to address the concern of ROS generation and associated hazard with promising results. Tsuji et al (Tox. Sci.; 2006, 89(1), 42-50) mentioned the usefulness of this process in modifying ROS generation from TiO2 (anatase) following UV irradiation.

Any potential hazard that is based on nano-sized particles may be reduced if these particles aggregate and form particles above the nano-scale that then do not dissociate into smaller (nano-sized) particles. It has been mentioned that free nanoparticles (or in suspension) tend to agglomerate to form an aggregate that no longer conforms to the nano-scale definition (SCCP, June 2007).

The complex nature of interactions of agents (nano-particles/quantum dots) with cell cultures was highlighted in an *in vitro* study (39), where difficulties (many variables) with interpretation of results from these types of studies were identified. This study did not look at the toxicity or dermal penetration of either TiO2 or ZnO. Furthermore, a recent workshop in Canberra, Australian in May (4^{th}-6^{th}) 2009 on nanotechnology highlighted the limitations of *in vitro* studies based on methodological inconsistencies.

If TiO_2 and ZnO, in the presence of sunlight, triggered the generation of free radicals as seen in cell cultures (*in vitro*) the potential toxicity associated with this event would be negated if it did not take place in viable cells. The available evidence suggests that the likelihood of penetration beyond the stratum corneum into viable cells is extremely low.

Dermal penetration - exposure

Dermal penetration (studies 1, 6, 7, 16, 18, 23, 24-28, 34, 36, 37, 38 and 40) of nanoparticulate TiO2 and/or ZnO through the outer layer of the skin was investigated in 16

studies, with 15 of the 16 indicating an inability of TiO2 and ZnO to reach viable cells. The only study to suggest TiO2 (not shown statistically) penetrated beyond the stratum corneum (18) had several limitations that put the validity of this study in doubt (ZnO not examined in this study).

Evidence that nanoparticles or nano-materials penetrate into skin has been presented. A recent study (30) examined nanoparticle fluosphere penetration, which showed 40 nm particles may penetrate hair follicles to locate in Langerhan cells (surrounding follicle) in the dermis. This was only achieved by tape-stripping debris from the opening to follicles, as well as removing a significant proportion of the protective stratum corneum and subjecting the human skin samples (*in vitro*) to prolonged exposure (16 hours) with the test material in a humidified chamber. Another study (32) showed nanoparticle fluospheres (quantum dots of 14 and 18nm) did not penetrate normal, flexed or stripped skin, while minimal penetration was observed in abraded skin. These studies (30 and 32) did not look at TiO2 or ZnO, which may have significantly different skin absorption properties/potential to the material (fluospheres) tested.

The articles discussed in this review are not an exhaustive list of those available on this topic, but generally (additional articles are sourced) represent articles linked to the search criteria stated at the beginning of this review. The TGA will continue to gather relevant information and will update this review at regular intervals.

ARTICLES REVIEWED

Reference 1

Lademann J, Weigmann H, Rickmeyer C, Barthelmes H, Schaefer H, Mueller G, Sterry W: **Penetration of titanium dioxide microparticles in a sunscreen formulation into the horny layer and the follicular orifice.** *Skin Pharmacol Appl Skin Physiol* 1999, 12:247-256.

Summary and comment

In this study, the dermal penetration of coated microparticles of TiO2 was studied. This study aimed at explaining the findings of Tan *et al* (18), who found TiO2 in the epidermis and dermis of TiO2 treated human skin samples taken during surgery. It was stated that it was of great importance to determine whether TiO2 was present within dermal tissue or whether it might be localised in orifices of the hair follicles that reach down into the dermis.

The distribution of the microparticles in the horny layer was analysed using tape stripping to remove layers of skin and spectroscopic measurements of particle positioning. Biopsies were conducted to further investigate the distribution of the microparticles into deeper layers. Human volunteers had 2 mg/m^2 of the test substance applied to an area of 160 cm^2 of the left forearm; the right forearm (control) had vehicle only (-TiO2) applied to it in a similar manner. The application rate was 5 times/day on days 1, 2 and 3, and once on day 4.

Results showed that the deeper layers of non-follicular stratum corneum were free of TiO2. TiO2 concentration decreased rapidly with increasing depth of the stratum corneum. The corneocytes were free of microparticles in the lower half of the horny layer. The only structures containing microparticles of TiO2 were the pilosebaceous orifices. Potential penetration to deeper sites (studied using biopsy) was assessed, with TiO2 only found in the

open part of the follicle. The detected TiO2 concentration inside these follicles was two orders of magnitude smaller than in the upper part of the horny layer. It was also noted that only some follicles contained TiO2 particles.

Penetration of microparticles of TiO2 into viable skin tissue could not be detected. The biopsy failed to detect TiO2 in the epidermal tissue outside of the follicles.

The potential for TiO2 to move out of the follicles and into the viable tissue was not discussed. However, the follicle is lined by a cellular inner and outer root sheath of epidermal origin and is invested with a fibrous sheath derived from the dermis. Penetration of TiO2 through this sheath would probably be unlikely, since no TiO2 was found in either the epidermal or dermal tissue surrounding the follicle.

Reference 2

Kreilgaard M: **Influence of microemulsions on cutaneous drug delivery.** *Adv Drug Deliv Rev* 2002, 54:S77-S98.

Summary and comment

This is a review article examining the use of microemulsions (vehicle) for cutaneous drug delivery (through the skin), with focus on the influence of composition and structure of vehicles (microemulsions) on drug delivery. Microemulsions aggregates were described as typically <150 nm, which is above the dimension (<100 nm) used to describe/define nanoparticles. Microemulsions can be formed with numerous different aqueous, surfactant and oil constituents. The article does not mention TiO2 or ZnO, which are actives (UV filters) formulated in sunscreens to stay on the skin surface to limit UV exposure of the skin.

Reference 3

Tinkle SS, Antonini JM, Rich BA, Roberts JR, Salmen R, DePree K, Adkins EJ: **Skin as a route of exposure and sensitization in chronic beryllium disease.** *Environ Health Perspect* 2003, 111:1202-1208.

Summary and comment

This study investigated the possibility that the skin was a route of exposure and sensitisation in chronic beryllium disease. Exposure to beryllium causes an incurable occupational lung disease, chronic beryllium disease (CBD), in approx. 3-5% exposed workers. CBD is a progressive granulomatous disease characterised by an MHC lymphocytosis. Industrial hygiene studies demonstrated that disease prevalence correlates with beryllium ultrafine particle counts, not with beryllium mass measurements (2 articles cited). This study examined the potential sensitisation activity of beryllium (500-1000 nm) applied topically to the ear of mice in the local lymph node assay (LLNA). In a separate *in vitro* assay using human skin, penetration of fluoro-spheres (0.5, 1, 2 & 4 μm dia. fluorescein isothiocynate conjugated dextran beads) in the layers of the skin was assessed. The data indicated that fluoro-spheres (≤ 1μm) did pass into the stratum corneum into the epidermis (location of immune reactive structures) of human skin when flexed *in vitro*; no fluoro-sphere penetration was seen in skin

that was not flexed. This study showed that topically applied beryllium could trigger an increased ear thickness in a murine (LLNA) model, which is consistent with the development of a cell-mediated immune response. There have been no reports of titanium or zinc triggering a similar response. This study did not use beryllium in the skin penetration section of the study; however, it did suggest that dermal absorption could be influenced by size of particle and flexing the skin.

Reference 4

Menon GK, Elias PM: **Morphologic basis for a pore-pathway in mammalian stratum corneum.** *Skin Pharmacol* 1997, 10:235-246.

Summary and comment

This study examined the morphologic basis for a pore-pathway (localisation) in mammalian stratum corneum. Using mice, tracer agents (ferritin HRP; lanthanum salts; sucrose; FITC-dextran; no mention of size of particle) were applied to skin (*in vivo*) and their movement through the skin was followed. This study used exaggerated conditions treating the skin with high energy sound (sonophoresis) and absorption enhancers to assist the passage of the tracers into the skin. It was shown that tracers invariably localised to discrete lacunar (space between cellular elements of epidermis) domains as a result of alignment following sonophoresis or the use of absorption enhancers. With the use of sonophoresis or absorption enhancers it appears the alignment of lacunae creates structural continuity and enables substances to pass through; lacunar domains remained discontinuous under normal basal conditions (limited movement of substances through skin). This study did not study the movement of nanoparticles of Ti or Zn through skin. It did look at possible pathways for the movement of substances through skin under extremely exaggerated conditions.

Reference 5

Andersson KG, Fogh CL, Byrne MA, Roed J, Goddard AJ, Hotchkiss SA: **Radiation dose implications of airborne contaminant deposition to humans.** *Health Phys* 2002, 82:226-232.

Summary and comment

The primary focus of this article was assessing the adverse effects of a surface layer of radioactive material (radio-pollutants) on the skin and clothes following accidental airborne nuclear releases. It was concluded that the dose to skin from β-emitters and the whole-body dose from γ-emitters on body surfaces were found to give potentially significant contributions to systemic exposure. In addition, it was suggested that skin penetration of some contaminants could lead to significant internal doses. Ti and Zn were not mentioned in this article. It was noted that physical clearance of skin contaminants was inversely proportional to particle size (not nano-scale particles); smaller particles had longer half-lives. It was noted that shedding of the stratum corneum would influence the clearance of skin contaminants. Also, a variable between animal models and humans, which could significantly affect skin permeability, is the number of hair follicles per unit of skin area. Some degree of skin permeability has been associated with movement of substances into hair follicles and into

lower layers of skin bypassing the stratum corneum. Penetration of iodine (elemental and radioactive) was the main concern following exposure of the skin to fallout contaminants.

Reference 6

Lademann J, Otberg N, Richter H, Weigmann HJ, Lindemann U, Schaefer H, Sterry W: **Investigation of follicular penetration of topically applied substances.** *Skin Pharmacol Appl Skin Physiol* 2001, 14:17-22.

Summary and comment

This article investigated the influence of specific follicle properties, sebum production and hair growth on the follicular penetration of topically applied substances. This article did not include Zn, and did not examine particle size in the study protocol. In the introduction the authors discussed microparticulate TiO2 and its ability to penetrate into the stratum corneum during long-term application. Small amounts of TiO2 were found in deeper parts of the stratum corneum in the follicle orifices, while the surrounding corneocyte aggregates were free of TiO2 in these parts of the horny layer. TiO2 microparticles penetrated into the acroinfundibulum of follicles without reaching the layer of viable cells. The microparticles were not found in every hair follicle, but only in 1 in10.

Three different penetration pathways were identified; the intercellular, the transcellular and the follicular penetration routes. It did show that the penetration process of topically applied substances depends on the phase of the hair growth cycle. The follicles are active when hair growth and/or sebum production are detected. The follicles are inactive when no hair growth and no sebum production can be measured.

Reference 7

Schulz J, Hohenberg H, Pflucker F, Gartner E, Will T, Pfeiffer S, Wepf R, Wendel V, Gers-Barlag H, Wittern KP: **Distribution of sunscreens on skin.** *Adv Drug Deliv Rev* November 2002, 54(1):157-163.

Summary and comment

The distribution of micronised TiO2 (sunscreens in general) on the skin was investigated. The SPF (efficacy) of sunscreens depends on the distribution of the sunscreen on the skin. Three different products containing nanoparticles (over size range of 10-100 nm) of TiO2 (application rate 4 mg/cm^2 for each product) were used in this study. These products were formulated differently using different coatings (trimethyloctylsilane, Al2O3 and SiO2) that changed the properties of the material (hydrophobic, amphiphilic). It was thought the distribution of the active TiO2 on/in the skin may vary as a result of the difference in base phases. This was an *in vivo* study using human volunteers (sunscreens applied to forearm). Punch biopsies were conducted on each subject, with the distribution of TiO2 assessed in each skin sample/section.

The sunscreen products were found to provide protection against sunburn. It was stated that micronised TiO2 was solely deposited on the outermost surface of the stratum corneum and could not be detected in deeper skin layers (epidermis and dermis). The surface

characteristics, particle size and shape of the micronised TiO2 did not affect its absorption through the stratum corneum. The coatings characteristics did not affect the absorption of TiO2 through the skin.

The authors concluded that sunscreens using TiO2 nanoparticles were both effective and safe, and provide topical protection against the sun in humans. However, these conclusions were based on limited exposure and subject numbers.

Reference 8

Nanoderm Project: **Quality of skin as a barrier to ultra-fine particles.** *LIFE QUALITY* January 2003.

Summary and comment

Unavailable for assessment.

Reference 9

Serpone N, Salinaro A, Emeline A: **Deleterious effects of sunscreen titanium dioxide nanoparticles on DNA: efforts to limit DNA damage by particle surface modification.** *Proc. SPIE* June 2001, 4258:86-98.

Summary and comment

This article (review) described deleterious effects of micronised TiO2 on DNA. Details of study methodology were not included so an analysis of the studies (*in vitro*) could not be carried out. The authors indicated that they had tested for the formation of the hydroxyl (●OH) radicals produced on irradiation of TiO2 extracted from sunscreens. They verified TiO2 as an initiator of harmful reactions inducing DNA damage (strand breaks) through the generation of free radicals by photo-catalytic reactions. The data indicated that the results were generated in both *in vitro* and *in vivo* studies using human cells.

The authors then described how they have focused on producing the most photo-catalytically inactive TiO2 specimens for possible use in sunscreens, while retaining the spectroscopic features of TiO2 that make it an excellent UVA/UVB blocker. They did not describe the process to alter TiO2 reactivity (appears to relate to surface activity properties of TiO2), but they stated that they had generated some promising TiO2 species that retained the photo-protection properties, but had greatly reduced photo-catalytic activity. They emphasised the need for more investigation into TiO2 prior to acknowledging that TiO2 was definitely safe for use in sunscreens.

Reference 10

RCC-CCR project, Czich A: **In vitro test on induction of chromosome aberrations in V79 cells with HR 99/104702 (a), 00/T00017 (b), 00/106407 (c), after simultaneous irradiation**

with artificial UV irradiation.

Summary and comment

Unpublished, currently unable to assess.

The Scientific Committee on Cosmetics and Non-Food Products (SCCNFP) in the EU evaluated this study and their conclusions are as follows; "based on the structural chromosome aberrations observed in the absence and in the presence of UV light in both experiments, the test agent HR00/106407 (ZnO) dissolved in culture medium at different concentrations, displayed positive effects in cultured Chinese Hamster V79 cells under the conditions of the study. It should be noted that the observed effects were induced with low doses. This micronised material has clastogenic activity on mammalian cells cultured *in vitro*; it is also photoclastogenic in the same V79 cell system".

Reference 11

Guidance document elaborated by the COLIPA task force, **Photomutagenicity** (1995).

Summary and comment

Unpublished, currently unable to assess. The SCCNFP evaluated this study and their conclusions are as follows; "based on the structural and/or numerical chromosome aberrations observed in the absence and in the presence of UV light, the test agent HR96/104702 (ZnO) dissolved in 10% emulsion in 3% Tego Care 450 at different concentrations showed positive effects in cultured Chinese Hamster Ovary (CHO) cells under the conditions of the study. Micronised material has clastogenic activity on mammalian cells cultured *in vitro*; it has been also shown, that the test agent displays aneugenic activity; it is also photoclastogenic and possibly photoaneugenic in the same cell system".

Reference 12

Bayer report, Brendler-Schwaab S, HR 00/106407: **Photo-comet assay in vitro.** Unpublished report no. T 8069508.

Summary and comment

Unpublished, currently unable to assess. The SCCNFP evaluated this study and their conclusions are as follows; "based on the mean tail length (DNA damage), the test agent HR00/106407 dissolved in deionised water is considered photogenotoxic in cultured Chinese Hamster V79 cells under the conditions of the photocomet assay *in vitro*. It is worth noting that HaCaT cells (human keratinocytes – living cells of epidermis) were also tested in this assay, with no evidence of any DNA damage in the presence or absence of UV irradiation.

Reference 13

Dunford R, Salinaro A, Cai L, Serpone N, Horikoshi, Hidaka H, Knowland J: **Chemical oxidation and DNA damage catalysed by inorganic sunscreen ingredients.** *FEBS Lett* 1997, 418:87-90.

Summary and comment

There were 3 parts to this study. Firstly, this study examined chemical oxidation of TiO2 extracted from OTC sunscreen products using an organic substrate (phenol) under conditions of irradiation (310-400nm). Secondly, TiO2 (in water at 0.025% w/v) was mixed with an equal volume of plasmid DNA in buffer and irradiated. Direct strand breaks were assayed from the conversion of supercoiled plasmid to the relaxed form. Thirdly, an assay described as *in vivo* (but actually cultured human fibroblast cells; so *in vitro*) irradiation of DNA was conducted; the potential for DNA damage following exposure to TiO2 (0.0125% w/v) was assessed in this assay. Included in parts of the analysis were anatase and rutile forms of TiO2 and ZnO.

TiO2 (both anatase and rutile) and ZnO caused strand breaks in plasmid DNA. Furthermore, the extracted TiO2 caused DNA damage in cultured human fibroblasts. There was some evidence that hydroxyl radicals may have played a part in the observed DNA effects, since damage was suppressed by DMSO and mannitol (free radical quenchers). Oxidation of phenol was accelerated by the presence of both forms TiO2 (to varying degrees) and ZnO.

It was concluded that there appears to be possible health hazards with TiO2 if it can enter viable/living cells after penetrating the stratum corneum. The authors of this study did indicate that absorption of TiO2 has yet to be adequately demonstrated.

The authors described the systems studied as *in vitro* and *in vivo*, but none are actually conducted *in vivo* since they used measurements of chemical reactivity (oxidation, phenol disappearance), suspensions of plasmids and cultured human fibroblast cells. Also, whether this study dealt with nanoparticles is unsure, because the TiO2 used in this study was extracted from marketed sunscreen products with no reference to size of particle extracted.

Reference 14

Oberdoerster G: **Toxicology of ultrafine particles: in vivo studies.** *Phil Trans R Soc Lond* 2000, 358:2719-2740.

Summary and comment

This article primarily described work examining the toxicology of ultra-fine (UF) particles following exposure to airborne material.

The introduction described how UF particles (<100nm) can be encountered in ambient air and at the work place. In general, background levels in urban atmosphere are $1\text{-}4x10^{4}cm^{-3}$ (mass concentration < $2\mu g.m^{-3}$). Workplace concentrations can be appreciably higher coming from metal and polymer fumes, which can lead to acute inflammatory responses in the lung following inhalation. It was suggested studying the UF material generated at the workplace, although not representative of the composition of atmospheric air, can aid in the understanding of the toxicity of UF particles.

Inhalation studies in rats using UF polytetrafluoroethylene (PTFE) showed high levels of pulmonary toxicity, toxicity decreased following agglomeration to larger particles, repeated pre-exposure for very short periods had a protective effect (reduced toxicity) and PTFE moved into epithelial, interstitial and endothelial sites.

Further studies were carried out using UF carbonaceous material (likely to be found in urban atmosphere) in rats at an inhalation exposure of 100 $\mu g.m^{-3}$. Varying conditions were included in the study protocols. It was shown that UF carbon:- could induce slight inflammatory responses; endotoxin priming and concurrent exposure to ozone heightened the inflammatory responsiveness to UF carbon; older lungs (22 months vs 10 weeks old rats) were more susceptible to UF particle induced oxidative stress.

Data for intra-tracheally instilled TiO2 was described, where a comparison between UF (av. size 20nm) and fine (av. size 250nm) TiO2 found the same mass dose of UF particles had a significantly greater (36 fold) inflammatory potential than fine particles. The value of 36 fold was derived form a 3.6 times greater deposition efficiency into the alveolar and a 10 fold larger particle surface area per given mass. Inflammation was assessed using measurements of cellular and biochemical markers in lung lavage. It was suggested that the increased surface area of UF particles was an important factor in the heightened responsiveness to UF particles.

The author described, "TiO2 particles as rather benign in nature and have been used in the past in a number of studies as control particles of low toxic potency against which effects of other particles types have been compared". The inflammatory response seen with UF TiO2 was suggested as likely to be similar to the oxidative stress mechanism seen with the greater pulmonary intoxicant PTFE.

Overall, this study did not examine dermal application of UF TiO2 and has little relevance to the use of UF TiO2 in sunscreens. It did indicate that UF TiO2 was likely to cause a greater pulmonary inflammatory response when compared to fine TiO2. This finding would be considered to have relevance in the industrial workplace situation.

Reference 15

Oberdoster G: **Extrapulmonary translocation of ultrafine carbon particles following whole-body inhalation exposure of rats**. *Journal of Toxicology and Environmental Health*, Part A, 65:1531, 2002

Summary and comment

This study was described as a pilot study to examine whether ultrafine (UF) elemental carbon particles translocate to the liver and other extrapulmonary organs following inhalation as singlet particles (20-29nm) in rats. UF ^{13}C particles were presented as an aerosol, which was introduced into a whole-body inhalation chamber for an exposure period of 6 hours at concentrations of 80 and 180$\mu g.m^{-3}$.

Normalised to exposure concentration, the added ^{13}C particles/gram of lung in the post-exposure period was approximately 9 ng/g organ/ $\mu g/m^3$. Significant amounts of ^{13}C had

accumulated in the liver by 30 minutes post-inhalation, but only at the higher concentration of UF labelled particles. At 18 and 24 hours post-exposure, the ^{13}C in livers of all exposed rats was approximately 5 times greater than the ^{13}C content of the lung. No significant increase in ^{13}C was detected in other organs examined at the end of exposure. It was concluded that UF carbon particles could translocate from the lung to the liver by 1 day after inhalation exposure. Translocation appeared to involve absorption from the lung and possibly the GIT (licking of fur to remove aerosol). Furthermore, it was suggested that translocation to blood and extrapulmonary tissues may well be different for UF carbon compared with metallic (eg. Ti & Zn) UF particles.

Overall, this study did not examine dermal application of UF TiO2 and has little relevance to the use of UF TiO2 in sunscreens.

Reference 16

Pfluecker F, Hohenberg H, Hoelzle E, Will T, Pfeiffer S, Wepf R, Diembeck W, Gers-Barlag H: **The outermost stratum corneum layer is an effective barrier against dermal uptake of topically applied micronized titanium dioxide.** *International Journal of Cosmetic Science* 1999, 21(6):399-411.

Summary and comment

Dermal uptake properties of micronised TiO2 were studied *in vitro* using a Franz-type diffusion cell on excised porcine skin for 24 hours (limited exposure since single application). Localisation of TiO2 in the skin was determined using transmission electron microscopy (TEM). Tape stripping of the stratum corneum (SC) was also used to confirm the distribution.

TiO2 was found exclusively on the outermost SC layer. Surfaces deposits (using TEM) featured clearly distinguishable agglomerates, as well as single particles with a characteristic cubic shape and a primary particle size of about 20-50nm. Micrographs initially showed an even distribution of TiO2 on the skin surface, however, skin stripping showed TiO2 was localised in the furrows and not on the partially removed ridges of the skin surface. Tape stripping initially removed TiO2 and SC layers only from the ridges and not from the deeper furrows. TiO2 was found only in trace amounts in the upper part of the follicle without any evidence of uptake into follicular epithelium. It was suggested that this shows the follicles are not a relevant route of penetration for TiO2.

Reference 17

Cai R, Hashimoto K, Itoh K, Kubota Y and Fujishima A: **Photo-killing of malignant cells with ultrafine TiO2 powder**. *Bull Chem Soc Jpn 1991, 64:1268-1273.*

Summary and comment

This study examined the potential cytotoxicity (in presence and absence of photo-irradiation) of ultrafine (UF) TiO2 powder in HeLa cells, the mechanism for the cytotoxicity and the distribution of the TiO2 powder. The potential use of TiO2 in photokilling of malignant cells (photodynamic therapy) was discussed.

Cultured HeLa cells were exposed (incubation) to UF TiO2 (average size 30nm) in the presence and absence of electromagnetic radiation (varying wavelengths). Appropriate controls (minus irradiation/TiO2) were included in the study. Distribution of TiO2 through treated cells was determined using TEM.

During 10 minute exposure periods to UV radiation in the presence of TiO2 (100µg/mL) HeLa cells were completely killed. HeLa cells exposed to TiO2 (up to 360 µg/mL), but not irradiated, showed slight cytotoxicity. It was suggested that cytotoxicity was caused by possibly two mechanisms, the formation of reactive radicals or direct oxidation by photo-generated holes in TiO2.

Distribution analysis of TiO2 in the cultured cells showed incorporation into cell membrane and some particles also in the cytoplasm; there was no evidence of penetration into the nucleus.

The data presented provided evidence of cytotoxicity (by oxidative stress) of rapidly dividing tumour cells in culture with TiO2 following irradiation. This study used cultured viable HeLa tumour cells, which were in intimate contact with TiO2 in the culture. There was no protective barrier (stratum corneum/epidermis) that is present when TiO2 containing products are applied to the skin.

Reference 18

Tan MH, Commens CA, Burnett L, Snitch PJ: **A pilot study on the percutaneous absorption of microfine titanium dioxide from sunscreens.** *Aust J Dermatol* 1996, 37:185-187.

Summary and comment

This study was described as a pilot investigation into the potential percutaneous absorption of microfine TiO2 from sunscreens. A group of patients (n=13; samples taken n=16) having surgery for skin lesions had agreed to have surplus skin removed following its treatment with a sunscreen containing 8% microfine TiO2. The subjects had a mean age of 71 years (4 female, 9 males). Sunscreen was applied twice daily (morning/midday) to the skin surrounding the lesion to be excised for a period of 2-6 weeks. Treatment with the sunscreen was ceased 2 days prior to surgery. The skin was cleaned prior to surgery. After surgery, excess skin (treated with sunscreen) around the lesion was removed and used as a sample for analysis. A control sample group (n=9) came from cadaver skin collected from the hip region. Tape stripping of the skin was conducted on samples in preparation for analysis. Removal of the stratum corneum was followed by punch biopsies of the remaining tissue, which were digested (chemical breakdown) and the TiO2 content measured.

It was noted that subjects applying the sunscreen (+TiO2) did not sustain any trauma or rash at or around the application site. The data showed that the treated skin had a higher TiO2 content than the untreated skin, but the difference was not statistically significant (1.7 vs 1.2 µg/g tissue). The investigators stated that the reason for the lack of significance was one outlining result in the control group. On this basis the investigators concluded that, "microfine TiO2 may have greater potential to be percutaneously absorbed compared with

commercial grade TiO2".

There must be some degree of doubt regarding the value of this study based on the age and disease state of the tissue used. Skin gets thinner and more fragile with age (mean 71 years in this study), and the lesions in the skin being removed may have caused changes in the vascular permeability and tissue reactivity of the local and surrounding tissue to varying degrees. There was no indication as to whether the cadaver controls were age matched or had lesions near the site where skin samples were collected.

Also, distribution analysis was not conducted so the TiO2 found in the treated tissue may not be associated with release from viable cells; it could have localised in follicles in the epidermis and dermis and release on digestion of the skin prior to analysis.

The authors indicated there were inadequacies in the study based on limited sample size, TiO2 found near limit of detection and a lack of statistically significance difference between the test group and controls. Further limitations of this study may include the population of subjects sampled (old and diseased) and the lack of match controls (cadaver skin)?

Reference 19

Uchino T, Tokunaga H, Ando M, Utsumi H: **Quantitative determination of OH radical generation and its cytotoxicity induced by TiO(2)-UVA treatment.** *Toxicol In Vitro* 2002, 16(5):629-635.

Summary and comment

This study does not examine the potential dermal absorption of TiO2, but looks at the UVA generated OH radical in the presence of different crystal forms (anatase and rutile) and sizes of TiO2 on free radical production.

UVA irradiation of the anatase form of TiO2 generated OH radicals in a dose/exposure related manner, while the rutile form (90 nm) was significantly less effective at generating OH radicals. It was noted that the crystal size had a significant influence on generation of OH radicals, but the optimum size for generation of OH radical was different for the different types of TiO2. Size and crystal form of TiO2 affected the UVA absorption characteristics, but there was no apparent relationship between UVA absorption and OH radical formation. Cytotoxicity of the OH radical was tested against cultured (*in vitro)* Chinese hamster ovary cells (CHO), with the CHO cells found to be sensitive to the amount of OH produced (dose-related).

Testing with UVA radiation was carried out up to 370 nm (based on filter used max. irradiation 370 nm), which would effectively omit half (370-400 nm) of the available UVA spectrum. This issue is unclear since later in the report they mention exposure at a wavelength up to 500 nm (into visible range).

This was an assay examining chemical reactions between TiO2 containing agents and UVA (limited) radiation; no biological cells were involved until the second phase, when CHO cells were exposed to the OH radicals that were generated. OH radicals were cytotoxic to CHO *in vitro*, depending on the conditions; it was noted that rutile did not change the viability of

CHO cells significantly, while anatase did reduce the viability of CHO cells. The authors suggested that further clarification of the relationship between TiO2 type and size, and possible adverse biological activity is required.

Reference 20

Afaq F, Abidi P, et al: **Cytotoxicity, pro-oxidant effects and antioxidant depletion in rat lung alveolar macrophages exposed to ultrafine titanium dioxide.** *Journal of Applied Toxicology* 1998, 18(5):307-312.

Summary and comment

This study examined the potential adverse effects of introducing ultrafine TiO2 into the lungs of rats for up to 16 days. Ultrafine TiO2 (<30nm) was instilled (single dose) into the trachea at a dose of 2 mg/rat in physiological saline in a volume of 0.5 mL. A group of control rats received only saline. Animals (6/time interval) exposed to TiO2 and saline controls were sacrificed at 1, 4, 8 and 16 days following treatment. The sacrificed animals had their lungs lavaged 3 times with a collection buffer, with the collected lung lavage analysis for the presence of released biochemical markers (LDH, AP, macrophages, GSH, etc).

Significant increases in alveolar macrophages (AM) were seen at all times, but peaked at day 8. Acid phosphatase (AP) and lactate dehydrogenase (LDH) levels were increased on all analysis days, and also peaked on day 8. Peak levels were 59% and 75% above control levels for AP and LDH, respectively. Markers for lipid peroxidation were increased during the course of the study. Increased glutathione (GSH) redox reaction activity in AMs was observed throughout the study period. A significant and progressive depletion of GSH was seen in AMs in TiO2 exposed rats. It was suggested that exposure to TiO2 may lead to oxidative stress in the lungs of rats and potentially pathological changes.

This study would have greater relevance to occupational health issues, where exposure to powdered TiO2 during formulating products could lead to intake by the lungs. TiO2 in cream/lotion bases for sunscreens would not appear to be an issue for exposure to the lungs. Some of the parameters measured in this study have been described as having limited diagnostic value in rat models due to the variation in backgrounds levels when compared to humans; 12 fold higher in rats normally. Also, the process of lung lavage/perfusion on its own can cause large increases in the release of biochemical markers.

Reference 21

Lademann J, Otberg N, et al: **Follicular penetration. An important pathway for topically applied substance.** *Hautarzt* 2003, 54(4):321-323.

Notes

Article in German; no translation available.

Reference 22

Oberdoster E: **Manufactured nanomaterials (fullerenes, C60) induce oxidative stress in the brain of juvenile largemouth bass.** *Environmental Health Perspectives*, vol 112, no 10, July 2004

Summary and comment

This was a study examining the potential for a manufactured nanoparticle, fullerenes (C_{60}), to induce oxidative stress in the brain of juvenile largemouth bass (fish). Concern regarding the environmental impact of nanoparticles, in the form of adverse effects on wildlife, was the stimulus behind this study. The results of this study were interpreted as showing oxidative damage and depletion of GSH following exposure of a limited number of fish to manufactured nanoparticles.

This study did not examine dermal application/administration of UF TiO2 in a mammalian species and has little relevance to the use of UF TiO2 in sunscreens.

The issue of potential environmental contamination by nanoparticles was raised in the context that sunscreens containing nanoparticles may wash into the environment.

Reference 23

Zs Kertesz, Z Szikszai, A Z Kiss: **Quality of skin as a barrier to ultra-finie particles.** Contribution of the IBA Group to the NANODERM EU-5 PROJECT IN 2003 – 2004

Summary and comment

This report provides details of a consortium (consisting of 12 European universities and scientific institutes) that has used cutting edge techniques to investigate the possible penetration of micronised Ti, Zn and Si –oxides into the skin. Ion microscopy, electron microscopy and autoradiography are used to trace the penetration of the nanoparticles into the skin layers, while molecular and cell-biological methods are applied to assess the skin response and activation of dermal cells. Studies have been conducted using porcine and human skin samples. Resolution was described as, "quantitative elemental composition in all strata of the skin with detection limits of approximately 1μg/g and lateral resolution of 1-2μm". Validation (consistency and reliability) across the laboratories (with varying methods) for accuracy of was conducted, fairly good agreement (standard deviation 17-20%) across 6 laboratories.

Initial reported results were for 22 pig skin, 11 transplanted human skin and 13 human skin samples. The results generated using ion microscopy or electron microscopy show that in healthy skin the nanoparticles penetrate into the deepest corneocyte layer of the skin, but never reach the vital layers. This report did not provide details of the application methods for the test substances.

Notes

Quoted in: Referenced in ATOMKI annual report

Reference 24

A. S. Dussert & E. Gooris: **Characterisation of the mineral content of a physical sunscreen emulsion and its distribution onto human stratum corneum**. International J. Cosmetic Science 1997; 19:119-129

Summary and comment

This study characterised the ZnO (2.5%) and TiO2 (11.5%) content (type & size) of a commercially available sunscreen formulated without organic filters, as well as assessing the distribution of the sunscreen agents through the skin. Assessment of distribution of the sunscreen (1 mg/cm^2) through the skin was conducted using abdominal human skin (from plastic surgery) *in vitro*. Physical characteristics of Zn and Ti were determined using X-ray diffraction and electron microscopy.

Analysis revealed TiO2 was present as a mixture of rutile and anatase. Microfine ZnO particles had a length of 116.8 nm and a length to width ratio of 2.03:1. The ZnO particles were described as being larger than the ultrafine TiO2 particles (no dimensions given), which indicates the TiO2 particles should be in the nanoparticle range.

Scanning of the skin after topical application of the sunscreen emulsion displayed an almost regular mineral coating of the stratum corneum. Mineral crystals appear to surround the desquamating corneocytes. However, both intercellular and intracellular penetration of mineral crystallites was not evident in transmission electron microscopy.

Reference 25

Alvarez-Roman R, Naik A, Kalia Y, Guy R, Fessi H: **Skin penetration and distribution of polymeric nanoparticles**: *J. Controlled Release* 2004, 99:53-62.

Summary and comment

In this study, confocal laser scanning microscopy (CLSM) was used to visualise the distribution of non-biodegradable, fluorescent, polystyrene nanoparticles (20 and 200nm in diameter) across porcine skin. Specifically, it involved the investigation of the distribution of nanoparticles encapsulating a hydrophilic dye across excised porcine skin and determination of possible pathways into/through the skin. The test material was described as polystyrene FluoSpheres (brand) carboxylated-modified – nanoparticles coated with a relatively hydrophilic polymer containing multiple carboxylic acids and containing fluorescein 5-isothiocynanate (FITC). A description of the method of CLSM indicated it enables the distribution of a fluorescent probe in a biological sample to be visualised as a function of depth, without the need for tissue fixation and/or sectioning. It appears that this technique usually requires the tissue to be studied to have a low intrinsic fluorescence (auto-fluorescence due to flavoproteins and melanin) at the wavelength of the focus material (fluorescent nanoparticle in this case). It was noted in this study that the investigators used the skin's auto-fluorescence to assist in the identification of morphological features of the skin and the distribution of the nanoparticles. This technique uses the two individual excitation/emission wavelengths couplets, allowing confocal images of native skin structures to be superimposed in the same sample plane. A statement indicated that a major advantage of CLSM is that the tissue can be optically sectioned to assess structures (through penetration

of markers molecules) without potential distortion associated with fixation and sectioning.

Images of penetration/distribution studied using CLSM showed that the polystyrene nanoparticles preferentially accumulated in the follicular openings, with this type of distribution increasing in a time-dependent manner and was more likely to occur with the smaller size nanoparticles. Nanoparticle distribution also included localisation in skin furrows as shown by surface imaging. Analysis of cross-sectional images indicated that these non-follicular structures (furrows) did not appear to provide an alternative pathway for the nanoparticle used in this study, with the stratum corneum establishing a barrier to penetration. A solution of FITC was found to be distributed relatively homogeneously and was limited to the stratum corneum, while fluorescent nanoparticles had a punctuated distribution; maybe due to localisation in furrows and follicles. Overall, no evidence for uptake away from the follicles was observed.

Reference 26

Pflucker F, Wendel V, Hohenberg H, Gartner, Will T, Pfeiffer S, Wepf R, Gers-Barlag H: **The human stratum corneum layer: an effective barrier against dermal uptake of different forms of topically applied micronised titanium dioxide**: *Skin Pharmacol Appl Skin Physiol* 2001, 14(suppl 1):92-97.

Summary and comment

In this study, electron microscopy (EM) visualisation and light microscopic (LM) investigations were conducted to assess the dermal penetration of three formulations of micronised titanium dioxide (TiO2) on human skin (forearm of volunteers). The formulations using TiO2 were prepared to orientate the surface of the micronised TiO2 as either hydrophobic, amphiphilic or hydrophilic. These formulations were emulsions and a placebo emulsion, where TiO2 was replaced with water (4%), was included in the study. The shape and size of the TiO2 particles were defined, with cubic particles at 20nm and needle shaped particles at 100nm (within definition of nanoparticle). Properties of the TiO2 nanoparticle were influenced by the presence of coatings/excipients comprising different combinations of trimethyl octylsilane, Al2O3, SiO2, alumina and silica for the different emulsions. Application of the emulsions to a volunteer was to areas of 1.9cm in diameter resulting in a total area covered of 11.3cm^2 for each emulsion. It was noted that 45 mg of the respective emulsions resulting in a concentration of 4mg emulsion/ cm^2, which is twice the recommended rate of sunscreen application (2mg/ cm^2); TiO2 presence was 160µg/ cm^2. Contact time for the emulsions with the skin was 6 hours (non-occlusive), with the skin protected with rings (around application site) to prevent accidental removal of the test material. Punch biopsies (2mm in diameter) were taken (under local anaesthesia) from the centre of the test area for analysis. Skin samples taken in the biopsy were prepared for assessment using EM and LM techniques.

LM analysis showed that micronised TiO2 could be visualised with 1000-fold magnification; it was suggested that this is possible due to the formation of agglomerates forming. LM analysis showed all three emulsions (in all cases) containing TiO2 formed an almost continuous film on the outermost layer of the stratum corneum. Thickness of the stratum corneum was estimated to be 15µm, while the emulsion film/s was between 5 and 10µm thick. EM analysis confirmed the findings by LM with TiO2 particles found solely on the outermost layer of the stratum corneum, with none found in the lower layers of the skin such

as the epidermis or dermis. It would appear that only one human subject was used limiting the strength of the study.

In conclusion, EM scanning and LM assessment conducted on three variations of titanium dioxide found that surface characteristics, particle size and shape were not determining factors in dermal absorption of this inorganic UV active.

Reference 27

Popov A, Lademann J, Priezzhev A, Myllyla R: **Effect of size of TiO2 nanoparticles embedded into stratum corneum on ultraviolet-A and ultraviolet-B sunblocking properties of the skin**: *J. Biomedical Optics* 2005, 10(6): 064037-1 – 064037-9.

Summary and comment

The aim of this study was to investigate the optical properties of the horny layer of skin after application of TiO2. Multiple applications of a TiO2 containing sunscreen was carried out prior to tape-stripping to determine distribution/penetration. A technique to simulate photon migration within the 20μm thick stratum corneum was used to determine the effect of nanosized TiO2 spheres (20-200nm in diameter) on UV penetration. Ultraviolet radiation at two specific wavelengths of 310nm (UVB) and 400nm (UVA) was studied, with the effect of TiO2 particle size on absorption, reflection and transmission through the horny layer examined. A sunscreen containing rutile TiO2 particles (mean diameter 100nm) was applied (2 mg/cm^2) five times over a period of 4 days onto the forearm of a volunteer/s (number used not clear). The tape-stripping procedure started 1 hour after application on day 4. Tape-stripping of the stratum corneum removed layers of approximately 1μm thickness moving progressively deeper. Analysis of tissue on tape was conducted to determine the depth of penetration of TiO2 particles. The total thickness of the stratum corneum was measured using a laser scanning confocal microscope.

Results indicated that the most effective TiO2 sizes at attenuating UV radiation were 62 and 122nm in diameter for 310 and 400nm wavelengths, respectively. Although determining the depth to which TiO2 particles penetrate the stratum corneum (and beyond) was apparently not a focus of this study there was a statement indicating that, “most of the nanospheres were located at a depth range of 0 to 3μm from the skin surface”.

Reference 28

Gamer A, Leibold E, Van Ravenzwaay B: **The *in vitro* absorption of microfine zinc oxide and titanium through porcine skin**: *Toxicology In Vitro* 2006, 20: 301-307.

Summary and comment

The aim of this study was to determine the extent of penetration of microfine particles of zinc oxide and titanium dioxide (from cosmetic formulations) through porcine skin *in vitro*. Investigators in this study noted that previous work was indicative of zinc oxide (ZnO) and titanium dioxide (TiO2) microfine particles not penetrating the skin, but they noted that penetration through the skin and mass balance analysis were not conducted. In this study pig skin was used because of its similar structure to human skin and its use in such models had previously been validated. Formulations used in this study contained approximately 10%

microfine ZnO (mean particle size 80nm) and TiO2 (particle size 30-60nm x 10nm; needle shape). It was noted that the TiO2 particles were present as aggregates mostly up to 200nm and possibly up to 1000nm. The stability of these formulations was confirmed by analysis. Full thickness samples of visually intact porcine skin (from abdomen) were mounted in modified Franz static dermal penetration cells (upper donor compartment and lower receptor compartment with receptor fluid). Samples of the test materials were applied to stratum corneum in the upper section of the cells at a rate of 4mg/cm^2 and left in contact with the skin for 24 hours. The study design included assessment of the emulsion vehicle for zinc to establish a background level for comparison since zinc is a trace element in biological materials. A further two skin preparations/pig were left untreated to establish the physiologic zinc and titanium content of the skin preparations. The receptor fluid was sampled at 3, 6, 12 and 12 hours after application of the relevant material. After the last receptor fluid sampling the skin was prepared for the stripping procedure to determine penetration of test materials.

Mean total recoveries of Zn ranged between 102 and 107%, with virtually (98%-102%) the total applied Zn recovered in the combined first five tape strips, which represent the uppermost (outer/surface) layers of stratum corneum. It was stated that minute quantities of zinc were recovered in subsequent tapes, but these residual amounts were associated with remaining cosmetic formulation in furrows of the skin and in follicles. Zinc levels were similar across untreated, vehicle and treated test situations. Absorption-time plots from diffusion cells where the vehicle was applied did not differ from those treated with zinc oxide.

Mean total recoveries of Ti ranged between 98-100% and 86-93% for two formulations, with virtually the total applied Ti recovered from the skin surface by washing. The amounts of Ti measured for tape strips and the skin (prepared for analysis) were shown to be extremely low bordering on detection limit. It was indicated that these residual amounts of Ti were likely to be due to hair follicles trapping Ti containing material. Analysis of receptor fluid from diffusion cells showed no evidence of Ti being present. Overall, it was concluded that there was no evidence of dermal penetration of either microfine TiO2 or ZnO through porcine skin *in vitro*.

Note: Sunscreens (containing TiO2 and ZnO) are regulated as cosmetic formulations in the Europe, while in Australia a primary sunscreen containing these ingredients would be classified as therapeutic goods.

Reference 29

Dufour E, Kumaravel T, Nohynek G, Kirkland D, Toutain H. **Clastogenicity, photo-clastogenicity or pseudo-photo-clastogenicity: Genotoxic effects of zinc oxide in the dark, in pre-irradiated or simultaneously irradiated Chinese hamster ovary cells**. *Mutation Research* 2006, 607(2): 215-224.

Summary and comment

In this study, investigators examined the reported increased clastogenic potency of zinc oxide (ZnO) *in vitro* in the presence of UV light (photo-clastogenic), when compared to its activity in the dark. In their introduction the authors highlighted the many important processes that zinc plays a part in biological systems. They also noted that zinc and its salts are non-carcinogenic in rodents after inhalation, oral uptake or IP administrations, whereas Zn

deficiency was shown to enhance the susceptibility of rodents to known carcinogens. Genotoxicity studies on Zn (and salts including oxides) have had equivocal results. They also noted that, "the EU Scientific Committee on Toxicity, Ecotoxicity and the Environment concluded that Zn and its salts are not expected to be mutagens or carcinogens under expected human exposure conditions".

To address this question of photo-genotoxic effect, the investigators examined the clastogenicity of ZnO (mean particle size 100nm) in Chinese hamster ovary cells (CHO) in the absence of light (dark, D), following pre-irradiation (PI, ZnO incubated with CHO cells following UV irradiation) and in simultaneously irradiated CHO cells (SI, UV irradiation and exposure ZnO occurring at same time) at UV doses of 350 and 700 mJ/cm^2.

Results showed that cytotoxicity (expressed as % decrease in population doubling relative to control) of the CHO cells following exposure to ZnO occurred in an order of magnitude as follows; SI>PI>D over a concentration range of 27 to 500 μg/mL ZnO. In the dark, cytotoxicity of ZnO was concentration-dependent, with approximately 40-60% cytotoxicity in the concentration range of 256-320 μg/mL. Under SI conditions, approximately 40-60% cytotoxicity occurred over a concentration range of 131-256 μg/mL and 84-131 μg/mL for the lower (350) and higher (700) UV doses, respectively. Under PI conditions, approximately 40-60% cytotoxicity occurred over a concentration range of 105-205 μg/mL and 131-256 μg/mL for the lower (350) and higher (700) UV doses, respectively.

Also, under conditions of darkness (D), ZnO (concentration range 54-320 μg/mL) produced a concentration-related increase (3.5-15.5% of cells) in chromosomal aberrations (CA), which was also seen under SI and PI conditions but to a greater (at approximately 2-4 fold lower concentrations) extent. Under SI conditions, at the lower UV dose 11-34.5% of cells displayed CA and at the higher UV dose 6-45% of cells displayed CA over the concentration range of ZnO. Under PI conditions, at the lower UV dose 2-25.0% of cells displayed CA and at the higher UV dose 2.5-36.5% of cells displayed CA over the concentration range of ZnO. This effect was interpreted as CHO cells having increased susceptibility (or sensitivity) to ZnO initiated clastogenic effects associated with exposure to UV radiation.

In a comparison of the CA activity of ZnO (at similar concentrations) in SI and PI conditions, results showed SI conditions generated a higher incidence of CA than under PI conditions. However, when extent of cytotoxicity caused by ZnO was taken into account it was apparent the incidence of CA under both SI and PI conditions was similar for similar (almost identical) levels of cytotoxicity. Therefore, the timing of exposure of the test agent and cells (simultaneously or pre-irradiation) to UV radiation appeared to have had no effect on the frequency of CA induction. The increased responsiveness may have been associated with UV radiation sensitising the test system (cultured cells) *in vitro*.

The authors noted that the modest increase in CA seen in their assay (*in vitro*) of 2-4 fold over conditions of darkness is relatively small compared with potent photo-clastogenic agents (8-MOP), which increased the frequency CA by >15,000 fold under SI conditions. It could be that in *in vitro* photo-clastogenic tests, exposure to UV radiation may induce a slight background increase in CA frequency compared to the dark conditions. The authors identified that the relatively slight increase (2-4) in CA frequency was extremely small compared to the CA inducing effects of noted photo-clastogenic agents.

Reference 30

Vogt A, Combadiere B, Hadam S, Stieler K, Lademann J, Schaefer H, Autran B, Sterry W, Blume-Peytavi U: **40 nm, but not 750 or 1500 nm, nanoparticles enter epidermal CD1a+ cells after transcutaneous application on human skin**. *J Investigative Dermatology* 2006, 126:1316-1322.

Summary and comment

The authors of this article indicated that, "the transcutaneous route of vaccine administration appears to be a promising approach of targeting vaccines toward antigen-presenting cells (APCs) and thus improving immune responses". The study used particles of 750 and 1500 nm described as yellow/green Fluoresbite™ Carboxylate Polysciences, and 40nm red fluorescent Fluospheres (Molecular Probes).

Their study investigated the use of nanoparticles for presenting antigen to Langerhans cells (LCs), which they found in high density around hair follicles. Skin samples (collected up to 24 hours after surgical excision) were obtained from healthy volunteers undergoing plastic surgery (an *in vitro* assay). It was stated that skin samples were prepared using tape stripping (using cyanacrylate tape) of the outer layers (keratinised material, lipids, cellular debris, including significant amount of stratum corneum) to improve follicular penetration of transcutaneously applied agents; the intention was to leave a portion of the stratum and viable interfollicular epidermis intact. Following this process, nanoparticles of varying sizes (40, 750 and 1500 nm in aqueous solution) were applied to the skin surface, with the skin samples then incubated in a humidified chamber at 37^{o}C for 16 hours. LCs were isolated by magnetic cell sorting (MACS); this found only CD1a+ cells following skin treatment with 40 nm particles and none where the larger nanoparticles were applied. Further analysis revealed a granular fluorescent staining pattern said to be suggestive of uptake and internalisation of the smaller nanoparticles. However, it was noted that techniques such as flow cytometry and fluorescence microscopy identified cell-associated and granular fluorescence indicating uptake of all applied nanoparticles. In addition, the penetration pathway of transcutaneously applied nanoparticles was determined using fluorescence microscopy of cryosections prepared from the skin samples. It was noted that in all sections investigated, 40 nm nanoparticles penetrated to (at least) the entry level of the sebaceous duct (approx. 225 μm) and all along the follicular duct; there was very limited perifollicular tissue visualisation (2-3/26) of fluorescence in samples. It was stated that fluorescent staining in the dermis was found in 11 out of 26 sections (for 40 nm particles), but no nanoparticle-associated fluorescence was observed below the upper infundibulum or in the dermis for the larger nanoparticles. It was concluded that only the 40nm particles (used to deliver particle-base vaccines?) penetrated the skin via the follicular route and possibly into the perifollicular dermis.

This *in vitro* study indicated that nanoparticles could potentially penetrate into skin via hair follicles. The focus of this study was to investigate the potential transcutaneous penetration of nanoparticles for use in targeting vaccines towards antigen-presenting cells. Conditions (treatment of human skin samples) were enhanced to enable transcutaneous penetration of nanoparticles into hair follicles of skin samples *in vitro*; skin samples were tape stripped to remove a significant proportion of the outer protective layer (stratum corneum), as well as debris that may have impeded penetration into the follicle. The skin samples (with nanoparticle solution applied) were then placed in a humidified chamber for 16 hours prior to

measurement of the penetration of test material. These conditions do not reflect normal sunscreen (with nanoparticles of TiO2 or ZnO) use, with formulations developed to keep the UV filters on the skin surface and with skin integrity not intentionally compromised (skin stripping of stratum corneum in study). Also, the chemical nature (properties) of the fluorescent nanoparticles used in this study, compared with TiO2 and ZnO, may greatly affect the potential transcutaneous penetration.

Reference 31

Long T, Saleh N, Tilton R, Lowry G, Veronesi B: **Titanium dioxide (P25) produces reactive oxygen species in immortalised brain microglia (BV2): implications for nanoparticle neurotoxicity**. *Environ. Sci & Technol* 2006, 40(14): 4346-4352.

Summary and comment

The authors noted that concerns with the environmental and health risk of widely distributed, commonly used nanoparticles are increasing. Nanoparticle sized titanium dioxide (TiO2) was identified as being used in air and water remediation and in numerous products designed for direct human use and consumption. It was stated that its effectiveness in deactivating pollutants (eliminating micro-organisms) would relate to an ability to generate free radical activity. This property could also be a problem when biological targets/tissues sensitive to oxidative stress damage are exposed to nanoparticle TiO2, which could be present via a variety of routes. In this study, investigators assessed the effect of nanoparticle TiO2 (Degussa Aeroxide P25; mixture containing 70% anatase/30% rutile TiO2 with primary crystallite size of 30nm) on mouse brain microglia, while measuring cellular expressions of reactive oxygen species using fluorescent probes.

Observations of P25 revealed aggregation occurred in culture (in both media and buffer) to form aggregates ranging in size from 826 to 2368nm depending on the concentration in culture. Cultures of microglia cells responded to non-cytotoxic concentrations (2.5-120ppm) by generating a rapid (within 5 min) and sustained (120 min) release of reactive oxygen species. Another finding was that P25 not only triggered the initial oxidative response, but it also affected mitochondrial energy generation. It was stated that cell viability was maintained over an 18 hour exposure period for all concentrations tested (2.5-120ppm). It was concluded that nanoparticle TiO2 (P25) stimulated cellular and morphological expressions of free radical generation in cultured mouse microglia cells.

Reference 32

Zhang LW, Monteiro-Riviere NA: **Assessment of quantum dot penetration into intact, tape-stripped, abraded and flexed rat skin**. *Skin Pharmacol & Physiol* 2008, 21:166-180.

Summary and comment

This study examined the potential for quantum dots (nano-crystals with metallic core) to diffuse through flexed, tape-stripped and abraded rat skin in an attempt to understand if mechanical actions could influence the skin and alter penetration. The nano-sized quantum dots used in this study were fluospheres QD655 (18nm hydrodynamic diameter) and QD565 (14nm hydrodynamic diameter) coated with carboxylic acid, which were supplied at a concentration of 8μM in borate buffer. Skin samples were collected from Wistar rats. The

mechanical action described above as flexing was achieved using a flexing apparatus designed to flex skin at 45^0 at a frequency of 20 flexes/minute; after 60 min of flexing the dosed skin was punched and set on the flow-through diffusion cells. Control samples of skin (not manipulated) were kept for comparison. Tape-stripping of skin involved 10 tape (Scotch Magic) applications to remove the stratum corneum. Abraded skin was generated by rubbing skin samples with sandpaper (Type100, 3M) 60 times, until the ski was bright red but not bleeding (blood vessels seen on surface). Manipulated skin samples were placed on flow-through diffusion and treated with the test materials (QD565 & QD565). The cell used was a two-compartment Teflon flow-through diffusion cell with appropriate temperature and flow rate control. Treatment parameters were 40µL of a 1µM QD solution (n=3/treatment) applied topically on skin during flexion, after tape-stripping or abrasion. Diffusion cell perfusate was collected for fluorescence and inductively coupled plasma-optical emission spectroscopy (ICP-OES) detection at 1, 2, 3, 4, 5, 6, 7, 8, 12, 16, 20 and 24 hours. After 24 hours, the skin was removed and frozen at -80^0C for laser scanning confocal microscopy and morphological assessments.

It was stated that quantum dots (both materials tested) on non-flexed skin did not show penetration at either 8 or 24 hours. Flexed skin showed an increase in quantum dots on the surface of skin but no penetration at either 8 or 24 hours. QD655 penetrated into the viable dermal layers of abraded skin at both 8 and 24 hours, while QD565 was only present at 24 hours. In the tape-stripped skin, QD655 was deposited evenly and homogeneously on the surface of the viable epidermal layers. A similar finding was detected for QD565, with it being deposited continuously on the surface of viable epidermal layers without penetration into the epidermis at 8 or 24 hours. It was concluded that no penetration of quantum dots was shown in non-flexed, flexed and tape-stripped skin, while minimal penetration was seen with abraded skin (sandpaper, 60 times).

Overall, this article did show that under normal conditions nano-sized quantum dots did not penetrate the stratum corneum, while flexing and skin-stripping did not appear to alter the penetration characteristics (no penetration) of skin. Abraded skin, using sandpaper, did show a potential to allow minimal penetration of nano-sized quantum dots. This study did not investigate ZnO or TiO2 penetration of normal or mechanical altered skin. Mechanical alteration of skin by abrading did cause a minimal increase in quantum dot penetration, which may result in insignificant systemic exposure even in situations of large areas of abraded skin.

Reference 33

Gotter B, Faubel W, Neubert RHH: **Optical methods for measurement of skin penetration**. *Skin Pharmacol & Physiol* 2008, 21:156-165.

Summary and comment

This paper describes potentially useful non-invasive methods for the measurement of skin penetration. The authors indicated that Fourier transform infrared photo-acoustic (PAS), photo-thermal deflection (PDS) and Raman spectroscopy can be grouped amongst modern innovative non-invasive analytical tools potential useful for the study of biological tissues and human skin under *in vivo* conditions. All three methods allow depth-resolved investigations down to several hundred micrometers below the skin surface. Substances used in studies assessing the usefulness of these methods did not include TiO2 or ZnO, and there

was no mention of nano-sized particles being investigated. It was concluded that these contactless optical techniques are promising analytical tools for skin penetration studies.

Reference 34

Zvyagin AV, Zhao X, Gierden A, Sanchez W, Ross JA, Roberts MS: **Imaging of zinc oxide nanoparticle penetration in human skin *in vitro* and *in vivo***. *J Biomedical Optics* 2008, 13(6):064031-1 to 064031-9.

Summary and comment

This study reported on an investigation into the distribution of topically applied ZnO in excised and *in vivo* human skin, using multiphoton microscopy (MPM) imaging with a combination of scanning electron microscopy (SEM) and an energy-dispersive x-ray (EDX) technique. The use of these techniques enables a determination of the level of penetration of nanoparticles into the sub-dermal layers of the skin. Visualization of nanoparticulate ZnO in the substructures of the skin appears to be linked to two factors: the principal photoluminescence of ZnO at 385nm and the two-photon action cross section of nano-ZnO; these characteristics enable nano-ZnO to be differentiated from endogenous skin fluorophores. A commercial sunscreen product containing nano-ZnO (mean particle size 26-30nm) was used in this study. In the *in vivo* section, an area of 50cm^2 of skin was selected on the forearm, cheek, shoulder or feet of subjects (4 subjects from different ethnic backgrounds; low numbers for analysis); the selected area of skin was cleaned (ethanol) prior to the investigation. An amount of approximately 0.3g of the test sunscreen was applied to the selected skin areas and rubbed in for 5 minutes. Images were generated at 3 sessions identified as (1) immediately, (2) 4 hours after application and (3) 24 hours after topical application; subjects were released after session 1 to carry on with the daily routine; timings were associated with assessing control levels, anticipated sun exposure for outdoor activities and effect with prolonged exposure to sunscreens.

The *in vitro* section of this study used abdominal or breast skin obtained following plastic surgery. Application of the commercial sunscreen product was followed by incubation period of 2 to 24 hours. Sections of treated skin were either tape-stripped (10-20 times) or left untouched prior to analysis. Samples of skin were prepared for imaging using appropriate processing procedures.

Results indicated that from the MPM analysis nano-ZnO particles stayed on the stratum corneum (SC) and accumulated into skin folds and/or hair follicle roots of human skin; nano-ZnO predominantly remained on the outmost surface within a several-micrometer layer at all analysis points (at application, 4 and 24 hours). No penetration of nano-ZnO into the cells or extra-cellular space was observed and the 14 hour analysis showed complete removal of sunscreen from the skin. In excised skin, cross-sectional imaging found no evidence of nano-ZnO penetration. It was noted that the test material localized in the hair follicle shaft without spreading to the neighboring cells and tissue. Further high intensity, high resolution analysis (SEM/EDX) confirmed the above results with nano-ZnO present in skin-folds following topical application; no noticeable presence of nano-ZnO was observed in the epidermis.

Reference 35

Warheit DB, Hoke RA, Finlay C, Donner EM, Reed KL, Sayes CM: **Development of a base**

set of toxicity tests using ultrafine TiO2 particles as a component of nanoparticle risk management. *Toxicology Letters* 2007, 171: 99-110.

Summary and comment

This article examined approaches to assessing risk, which was identified as being a function of hazard and exposure data. They noted that previously suggested approaches included a "parallel tracks" strategy and mechanistic studies on "representative" nanoparticles could be supported by agencies involved in regulation. Alternatively, comprehensive (environmental, health and safety – EHS) frameworks for commercial nanoparticles would include a minimum or base set of toxicity studies, which could include the following criteria; substantial particle characterization, pulmonary toxicity studies, acute dermal toxicity (includes sensitization study), acute oral and ocular toxicity studies, along with screening type genotoxicity and aquatic species studies.

As a part of their approach to developing discussion on appropriate data sets the authors reported on toxicity results of a base set of hazard tests on a set of new, well-characterized, ultrafine TiO2 (ufTiO2) particle types. *In vivo* pulmonary toxicity studies in rats indicated low toxicity; acute dermal toxicity studies (*in vivo* rabbit and Local Lymph Node Assay [LLNA] in mice) indicated not a skin irritant or sensitizer; acute oral toxicity showed low acute toxicity potential and slight, reversible eye irritation. Genotoxicity studies (*in vitro*) showed ufTiO2 was negative for adverse effects in a bacterial assay (reverse mutation) and mammalian chromosomal aberration assay in CHO cells. The results of aquatic toxicity screening studies demonstrated ufTiO2 to be of low concern for aquatic hazard. It was concluded that ufTiO2 exhibited low hazard potential in mammalian and aquatic species/cell lines following acute exposure.

The article noted that an ultrafine particle is defined as a particle of average primary size of roughly 100nm (upper limit of nanoparticle). It was also stated that the ultrafine particles (defined as uf-A, uf-B and uf-C) of TiO2 used in the studies had median particle sizes of approximately 140nm, the studies did not strictly cover the nanoparticle range. It was further noted that there were fractions of the ufTiO2 particle size distribution that fell below 100nm meaning some (limited) potential exposure to nano-TiO2. The initial definition of risk (of a substance) put forward by the authors indicated it to be a function of hazard and exposure. The hazard was found to be low and exposure studies have suggested minimal penetration of nano-TiO2 when applied topically.

Reference 36

Cross SE, Innes B, Roberts MS, Tsuzuki T, Robertson TA, McCormick P: **Human skin penetration of sunscreen nanoparticles: *In vitro* assessment of a novel micronized zinc oxide formulation**. *Skin Pharmacol & Physiol* 2007, 20: 148-154.

Summary and comment

In this study the authors investigated the *in vitro* penetration of human skin with a sunscreen (described as novel micronised formulation with ZnO) containing nano-zinc oxide. The location of ZnO particles within the layers of the skin and the total amount of zinc penetrating through to epidermal cells *in vitro* over a 24 hour period following topical application of nano-ZnO was determined.

Formulations (3) tested in this study included a ZnO dispersion made with 60% of siliconate-coated ZnO in caprylic capric triglyceride (A), a typical o/w emulsion sunscreen with 20% ZnO (B) and a control (blank) o/w emulsion sunscreen without ZnO (C). Particle size determination was carried out, before application of the coating, using four different techniques identified as transmission electron microscopy (TEM), Brunauer-Emmett-Teller nitrogen-gas absorption method (BET), X-ray diffraction (XRD) and photon correlation spectroscopy (PCS). The transparency (consistency) of ZnO suspensions measured using a Varian Cary 300 Bio UV/Vis spectrophotometer. Analysis of particle size generation following MCP technology (high-energy dry milling) revealed similar results of the detection methods of TEM (15-40nm), BET (30nm), XRD (26nm) and PCS (30nm). Human skin samples used in this study were from females donated following abdominoplasty. Epidermal samples were prepared from full thickness tissue, with these membranes mounted in static, horizontal Franz-type diffusion cells with an exposed surface area of approximately 1.3cm^2. Treatment with the formulations A (n=8), B (n=8) & C (n=3) described previously involved application of 10μL/cm^2 and collection of receptor fluid samples at intervals; the receptor fluid was analyzed for the presence of zinc and electron microscopy (EM) was used to further examine tissue samples.

As previously mentioned, the size of the nanoparticles of ZnO used in this study were in the range of 26-30nm and it was also noted that there was a significantly low degree of agglomeration. It was noted that the properties of narrow particle size distribution and low agglomeration were associated with these particles being more transparent than other ZnO particles, as shown in their increased light transmittance in the visible light range.

Results indicated penetration (over 24 hours) of zinc into the receptor fluid from untreated and placebo treated epidermal membranes as 0.09±0.04 and 0.22±0.12μg/cm^2, respectively. The amount of zinc found in the receptor fluid following application of the two test formulations (containing zinc oxide) was higher than for the untreated control and placebo situations, but not significantly after 24 hours exposure. It was noted that the total amount absorbed was found to be less than 0.03% of the applied dose. Analysis with the EM found that penetration of ZnO nanoparticles was limited to the outer surface of the stratum corneum (SC) and loose, desquamating cells of the upper SC only. There was no evidence of penetration of nanoparticles in the lower SC layers or viable epidermis.

Reference 37

Lademann J, Knorr F, Richter H, Blume-Peytavi U, Vogt A, Antoniou C, Sterry W, Patzelt A: **Hair follicles – an efficient storage and penetration pathway for topically applied substances**. *Skin Pharmacol & Physiol* 2008, 21: 150-155.

Summary and comment

In this article the authors discuss the possibility that hair follicles could be a storage and penetration pathway for topically applied substances. They described three theoretical penetration pathways identified as the intercellular penetration pathway (through lipid layers surrounding the corneocytes), the follicular penetration pathway and the transcellular penetration pathway. It was noted that the intercellular penetration pathway was considered the only relevant penetration pathway and no evidence exists in support of the transcellular penetration pathway; now the follicular penetration pathway is receiving attention as a

plausible way to penetrate through the skin. Lack of interest in the follicular penetration pathway was based on the perception that it played a minor role representing only 0.1% of the total skin surface. However, it has now been suggested that it may in fact play a greater role particularly in areas of greater follicle density.

A series of studies to investigate follicular penetration were suggestive of an enhanced role, but no clear model/method for determining the follicular reservoir was possible. The authors then describe the phenomenon of open and closed follicles and their work on TiO2 microparticles (sunscreens) penetrating into stratum corneum (SC). Tape-stripping skin to remove layers of SC allowed them to measure the distribution of TiO2, with their results showing almost complete localization of TiO2 on the skin surface (or upper layers); a small amount was found in the lower SC layers. These small amounts of TiO2 were located in the orifices of hair follicles. This finding confirms numerous other studies showing very poor penetration of TiO2 through the SC. Further analysis using X-ray scanning microscopy confirmed these results and also showed that noTiO2 was detected outside the hair follicles in the living cells. An interesting feature of the investigation was not all follicles contained TiO2 and this lead to the suggestion that there are open (allow penetration) and closed (no penetration) follicles.

The concept of open and closed follicles was further investigated and the nature of plugs (covering opening of follicle) studied and suggested to be shed corneocytes pushed out of the follicle orifices by growing hairs or emerging sebum. These investigations revealed that only 74% of the follicles on the forearm were open, but pretreatment of skin (mechanical peeling) was able to open all follicles.

The remainder of this report discussed the development of non-invasive *in vivo* methods to investigate follicular penetration, hair follicles as a reservoir, nanoparticles as efficient carriers for drug delivery into hair follicle reservoirs and penetration of particles through the skin barrier into living tissue. These areas of interest to the authors were viewed in the hope of opening up possible new opportunities for the investigation of follicular penetration. The authors concluded by stating that "currently there is no evidence that nanoparticles are able to penetrate through the intact skin barrier, which is an important aspect concerning the safety of nanoparticles in topically applied medical drugs and cosmetic products".

Reference 38

Mavon A, Miquel C, Lejeune O, Payre B, Moretto P: ***In vitro* percutaneous absorption and *in vivo* stratum corneum distribution of an organic and a mineral sunscreen**. *Skin Pharmacol & Physiol* 2007, 20: 10-20.

Summary and comment

In this study the authors investigated the potential penetration of TiO2 and methylene bis-benzotriazoyl tetramethylbutylphenol (MBBT; Tinosorb M) into human skin *in vivo* using a tape stripping method, and *in vitro* (human skin model), using a compartment approach; these actives were included in a broad spectrum sunscreen formulation. The broad-spectrum sunscreen used were described in detail (included inert ingredients), with the formulation (oil-in-water emulsion; TiO2 amount not stated) containing coated nanoparticles TiO2 of 20nm diameter and 8% MBBT. TiO2 and MBBT were quantified using colourimetric and HPLC analytical methods, with localization of TiO2 in skin samples determined using

transmission electron microscopy and particle-induced X-ray emission techniques. The *in vivo* part of the study was conducted using 3 female volunteers (informed consent given) of average age 28 years. The *in vitro* section of this study employed abdominal skin (removed during surgery) from 3 separate donors; these skin samples were prepared appropriately and mounted on diffusion cells for the experiment. Application rates were $2mg/cm^2$ over an area of $10cm^2$ of the upper arm (*in vivo* section) and a similar rate ($2mg/cm^2$ over an area of $10cm^2$) was used for the *in vitro* section; the diffusion cell was a Teflon homemade static type with a surface area of $10cm^2$ (5x2cm) and 8mL volume. Additional *in vitro* analysis was performed using facelift skin samples, which were mounted on a Franz diffusion cell (surface area of $1.13cm^2$ and 5mL volume).

The results indicated that more than 90% of both sunscreens were recovered in the first 15 tape-stripping, while the remaining 10% (approximately) did not penetrate the viable tissue; localized in the furrows and the opened infundibulum (follicle). In the *in vitro* assay, <0.1% MBBT was recovered in the receptor fluid. No TiO2 was detected in the follicle/s, viable epidermis or dermis. The author concluded that there was an absence of TiO2 penetration into the viable skin layers through either transcorneal (refers to transfer across corneocyte [dead keratin-filled squamous cell] of stratum corneum) or transfollicular pathways and there was negligible transcutaneous penetration of MBBT.

The authors concluded that "the differences found in the SC distributions underline the importance of conducting simultaneous *in vitro* and *in vivo* experiments". Their experimental results indicated that nanoparticulate TiO2 does not penetrate beyond the outer layers of the stratum corneum, which is the intended retention site for sun-protection activity.

Reference 39

Ryman-Rasmussen JP, Riviere JE, Monteiro-Riviere NA: **Variables influencing interactions of untargeted quantum dot nanoparticles with skin cells and identification of biochemical modulators**. *Nano Letters* 2007, 7(5): 1344-1348.

Summary and comment

The authors examined the mechanisms behind uptake of quantum dots in primary neonatal epidermal keratinocytes (NHEK) *in vitro*. Quantum dots (QD) were described as a diverse class of engineered nanomaterials that have biological applications (drug delivery and diagnostic agents) in nano-medicine. Parameters investigated were the time course of QD-NHEK interactions and effects of QD surface coating, temperature, culture medium supplements and inhibitors of the cell cycle and endocytosis identified. It was noted that *in vitro* evidence suggests that some QD may cause cellular toxicity. Hypothesized mechanisms of cytotoxicity include leaching of core metals into the culture medium, oxidative stress (QD-mediated ROS production) and coating-related mechanisms. The present study examined non-selective interactions of NHEK cells with QD655 (ellipsoidal shaped 12nm by 6nm particle; at 10nm over 24 hours). Effects of coating were tested with various coating material such as PEG, amines (QD-NH2) or carboxylic acids (QD-COOH).

Results indicated that the extent of QD-NHEK interactions were coating dependent with detectable levels of QD-COOH in cells seen as early as 15 minutes after incubation, while QD-PEG and QD-NH2 were similar to controls. All QD, regardless of coating, were seen in cells at 2 hours. Effects on cellular fluorescence intensity magnitude were very apparent with

QD-PEG<QD-NH2<QD-COOH with respective values of 19.4±4.78, 222±34.3 and 1960±172.0 %fluorescence intensity units. Results also showed that low temperature or unsupplemented medium decreased QD-NHEK interactions; complete culture medium (KGM-2) contained both protein (BPE, insulin, transferring, HEGF) and small-molecule drug supplements (epinephrine, glucocorticoids, penicillin). The absence of these supplements lead to reduced QD-NHEK interactions by up to 35%. Furthermore, biochemical inhibitors were found that dampen down or enhance QD-NHEK interactions. Biochemical agents assessed for activity were genistein (phytoestrogen; causes cell cycle arrest), nocodazole (microtubule disruptor) and cytochalasin D (inhibits F-actin polymerization). QD-NHEK (all QD species tested) were enhanced by cytochalasin D, while nocodazole attenuated the interaction of QD-NH2 with NHEK, but not QD-PEG or QD-COOH and genistein attenuated the interaction of QD-NH2 and QD-COOH with NHEK, but not QD-PEG.

The author concluded that these results are important for understanding and controlling interactions of untargeted QD with cells. This study also highlights the complexity of potential interactions (eg. medium content, temperature, added agents, etc) that can occur in *in vitro* studies and exposes limitations associated with these types of studies.

Reference 40

Nohynek GJ, Dudour EK, Roberts MS: **Nanotechnology, cosmetics and the skin; is there a health risk?** *Skin Pharmacol & Physiol* 2008, 21: 136-149.

Summary and comment

This is a review article (107 references) addressing what are described as key questions regarding topically applied nano-material that are, "(a) are they absorbed and (b), if so, are they intrinsically toxic. Specific questions concerning the safety of nano-material (particles) in cosmetic products and sunscreens include the following: (a) do cosmetic formulations containing nano-sized features (vesicles or droplets) enhance the skin penetration of cosmetic ingredients, thereby increasing the risk of human skin sensitization or systemic exposure? (b) do nano-sized cosmetic formulations pose new risks when compared with those of traditional cosmetic products? (c) do topically applied insoluble nanoparticles (NP) remain on the skin surface or are they able to pass the skin barrier of normal or compromised skin to gain access to the systemic compartments of the organism? (d) are insoluble nanoparticles in sunscreens intrinsically more hazardous than larger particles, ie. microparticles or bulk material".

In this review, sunscreens with NP TiO2 and ZnO were assessed at length, with a conclusion reached indicating that *in vivo* toxicity tests showed that NP TiO2 and ZnO were essentially non-toxic. Also, a significant number of *in vivo* and *in vitro* studies suggest that NP TiO2 and ZnO do not penetrate into or through human skin. This article cited a reference (ref 35 of this TGA review) which indicated NP TiO2 behaved in a similar manner to micro-TiO2 in a series of studies considered to be a base set for toxicity testing (*in vitro* and *in vivo* cytotoxicity, genotoxicity, photogenotoxicity, acute toxicity, sensitization and ecotoxicology), and TiO2 presented as a low hazard.

Some *in vitro* studies reported issues with NP cell uptake, oxidative cell damage or genotoxicity, which were suggested as possibly being secondary to phagocytosis of cells exposed to excessive particle concentration.

The authors included sections on nanotechnology and NP in cosmetics and sunscreens, and local and systemic exposure following dermal (structure described) application of nano-material. A description of TiO2 and ZnO noted that they reflect and scatter UV light most efficiently over a size range of 60-120nm, while ZnO is generally used over a particle size range of 30-200nm. TiO2 particles for use in sunscreens has a size of 14 nm that forms stable micrometer sized aggregates. Mention was made of nano-particle surface treatment (occurs frequently) with agents such as aluminium oxide or silicon oils to improve dispersion in sunscreens. A description of the structure of skin was presented along with a comparative analysis of dermal penetration rates showing rabbit skin>rat>pig>monkey>man; it was noted that pig and rat skin is up to 4 and 9-11 times more permeable than human skin, respectively. This information on variability of skin penetration highlighted the need for caution when interpreting results from animal studies in relation to effects in humans.

On the issue of potential passive penetration of NP-TiO2 or ZnO, the authors provided an extensive review of studies including nano-material other than TiO2 or ZnO and came to the conclusion that all available evidence supports the notion that the size range of NP agents in sunscreens do not penetrate into the skin or produce systemic exposure. Studies on other nano-materials examined fluorescent dextran beads, soil particles (0.4-0.5μm), quantum dots, fluorescent nano-capsules and phenylalanine-based fullerene amino acid (3.5nm). The authors noted that in the studies with extremely small fullerenes there was a "grey zone" regarding passive skin penetration. The fullerenes mentioned are extremely small nanoparticles compared with the NP used in sunscreens, which have consistently shown not to penetrate into or through skin.

Possible skin penetration from nano-sized vesicle-type or other formulations (nanosomes, liposomes, niosomes, nano-emulsions, nanocapsules and solid lipid NP was described. It was noted that most of our knowledge of skin penetration of these materials comes from research on transdermal drug delivery (TDD) systems. The authors reviewed studies on various nano-sized systems used in TDD and concluded, "these data suggest that vesicle materials, as well as vesicle size, may affect the skin penetration of liposome- or niosome-encapsulated drugs to some degree, although the penetration rate of the active ingredient is not enhanced by a reduced vesicle size". Cited studies indicated that the skin penetration of some drugs may be enhanced, but only for particular and suitable drugs; enhancement of skin penetration appears to be dependent on a number properties of the drug and not just size. The authors noted that the knowledge gained from the TDD systems does not change the belief that passive skin penetration of NP TiO2 or ZnO is extremely unlikely.

Local and systemic toxicity of NP used in sunscreens was examined in this review. One study using murine fibroblasts and macrophages in an analysis of cytotoxicity of insoluble ceramic particle (NP and micro) showed that the larger sized particles were more toxic than the smaller particles. Results from this study led the authors to suggest that particle phagocytosis by cells was a primary link to particle mediated cytotoxicity and not necessarily size of particle; this belief appeared to be supported by further studies discussed in the review. Further examples of physiological responses of cells to excessive levels of insoluble particles were presented, which may culminate in oxidative cell damage and possibly genotoxicity outcomes. The complex nature of *in vitro* testing was discussed, which highlighted the variability associated with this approach. A concluding statement noted that, "*in vitro* studies that claim discovery of intracellular penetration and oxidative stress-related toxicity of nano- or microparticles in cultured mammalian cells should be interpreted with great caution in terms of their relevance for intact organisms. This view is supported by consensus

recommendations of a recent workshop on toxicology testing of NM that concluded that evaluation of the safety of nanomaterials should be primarily based on *in vivo* toxicity models, rather than use of *in vitro* assays".

The issue of cytotoxicity, phototoxicity and photogenotoxicity of TiO2 and ZnO micro- and nanoparticles was discussed in this review. Specific information on the genotoxicity and photogenotoxicity of TiO2 (anatase and rutile forms) was presented in tabular form, which indicated TiO2 did not (negative) induce genotoxicity/photogenotoxicity in assays (11 assays summarized) assessing forward/reverse mutations, chromosomal aberrations or neutral red uptake as a measure of phototoxicity. TiO2 tested included micro-and nano-sized particles, with no difference in safety profile for these materials. A second table summarizing the genetic and photogenetic toxicity of micro-or nano-sized ZnO was presented, which showed ZnO to clastogenic and photoclastogenic *in vitro* mammalian cell cultures (CHO and V-79 cells). However, the authors cited a reference (EU document) indicating that ZnO was not clastogenic *in vivo*, and was shown to be non-photoreactive, non-phototoxic or non-photosensitising. An analysis of these findings led the authors to suggest the perceived photogenotoxicity was probably due to UV-mediated, enhanced susceptibility of the mammalian cells to ZnO.

In the area of general toxicity of insoluble NP after oral or topical administration the authors noted that the safety of sunscreens and their ingredients is regulated in the EU and USA (and relevant Health Authorities in other countries), with sunscreens containing micro- and nano-sized TiO2 and ZnO undergoing numerous pre-clinical evaluation for safety and efficacy. Mention was made of a study (described previously) which indicated NP TiO2 behaved in a similar manner to micro-TiO2 in a series of studies considered to be a base set for toxicity testing (*in vitro* and *in vivo* cytotoxicity, genotoxicity, photogenotoxicity, acute toxicity, sensitization and ecotoxicology), and TiO2 presented as a low hazard.

A final section of this review focused on NP and compromised skin and indicated that, "there is little evidence suggesting slightly compromised skin has a greater susceptibility to skin penetration of topically applied substances". Interestingly, an example given cited a study that showed no skin penetration (absorption) of NP TiO2 through normal and psoriatic human skin. It could be expected that skin diseases that cause a rupture in the stratum corneum diminishing the normal protective barrier could result in an increase skin penetration. A final example given by the authors noted that injected (subcutaneous) NP TiO2, by-passing the skin barrier, was found to be non-toxic suggesting that even if NP TiO2 did penetrate the skin it would not be a hazard by this route of administration.

Conclusion:

Currently, there is no *in vivo* evidence to indicate possible toxicity of nanoparticulate TiO2 or ZnO in people using sunscreens. To date, the current weight of evidence indicates the particles remain on the surface of the skin and in the outer dead layer (stratum corneum) of the skin.

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