Human safety review of "nano" titanium dioxide and zinc oxide

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Based on the current weight of evidence of all available data, the risk for humans from the use of nano-structured titanium dioxide (TiO_2) or zinc oxide (ZnO) currently used in cosmetic preparations or sunscreens is considered negligible. There is a large body of information that when viewed in its entirety is considered as sufficient to demonstrate that these nano-structured ultraviolet (UV) filters, irrespective of various treatments (coatings) or crystalline structure, can be regarded as safe for use at concentrations up to 25% in cosmetic products to protect the skin from harmful effects of solar UV radiation. "Nano" TiO_2 and ZnO formulated in topically applied sunscreen products exist as aggregates of primary particles ranging from 30–150 nm in size. These aggregates are bonded such that the force of sunscreen product application onto the skin would have no impact on their structure or result in the release of primary particles. Multiple studies have shown that under exaggerated test conditions neither nano-structured TiO_2 nor ZnO penetrates beyond the stratum corneum of skin. Further, the distribution and persistence of these nano-structured metal oxides is the same compared to larger pigment-grade (*i.e.*, >100 nm) particles, demonstrating equivalence in the recognition and elimination of such material from the body. Finally, the *in vitro* genotoxic and photogenotoxic profiles of these nano-structured metal oxides are of no consequence to human health. Whereas the most logical, straightforward conclusion based on data from internationally-recognized guideline studies and current 20+ year history of human use is that nano-structured TiO_2 and ZnO are safe, there will continue to be questions as "nano" conjures images of technology gone awry. Despite this rather sober view, the public health benefits of sunscreens containing nano TiO_2 and/or ZnO outweigh human safety concerns for these UV filters.

Introduction

An all encompassing review of the toxicological data for nano TiO_2 and ZnO is beyond the scope of this paper. Rather the focus will be on the most commonly voiced concerns, including dermal penetration and photoactivation of these metal oxides, since these are most relevant for the use as UV filters in sunscreens.[1,2] Further, a general overview of the adequacy of current toxicological approaches used to assess the human safety of products containing "nano" ingredients will be presented.

Generally speaking, the mineral-based UV-filters, TiO_2 and ZnO, have been widely recommended based on the view that they were the safest UV filters in sunscreen products.[3,4] Prior to 2002, TiO_2 and ZnO were considered to be the UV filters of choice for individuals with chronic skin disorders because of their absence of skin irritation and sensitization. Moreover, it was generally recognized that these metal oxides do not penetrate the stratum corneum and therefore are without any systemic toxicological concern. This view dominated even with the introduction of micronized (*i.e.*, "nano")† forms of TiO_2 and ZnO, the former in the early 1990s followed several years later by ZnO.

This favorable profile has been altered, not by the dermatological community or users, *i.e.* consumers/patients, of products containing these metal oxide particles, but instead by the notion that "nano" technology *per se* constitutes an unknown risk to human health. "Nano" has become a phenomenon with promises and risks that challenge the imagination, ranging from therapeutic modalities which selectively target cancer cells to toxicities of which we have neither the capacity nor capability to evaluate.[5] Such hyperbole is familiar to the scientific community. For example, it has been less than a decade since the human genome was completely revealed with the prospect of understanding disease and eliminating human suffering, on the one hand, to the ethical abuses such information might precipitate, on the other. Whereas this may be an over-simplification, it is quite clear that the "nano" label carries a polarizing perception.[6,7]

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† The terms micronized or microfine are synonymous with "nano". The term "nano" will be used throughout the text for consistency.

With regard to sunscreens, since 1990 there has been a steady number of published articles on safety and efficacy of products containing TiO_2 and ZnO. Notably, in the past 8 years, there has been nothing short of an explosion in the number of publications of "nano" TiO_2 and ZnO articles (see Fig. 1). In the past year, 90% of the articles have been oriented toward the toxicological effects of nano TiO_2 and/or ZnO. Based on the large number of publications, one might mistakenly conclude that all scientific investigations up until the definition of "nano" had missed or otherwise ignored such toxicological endpoints as genotoxicity or phototoxicity in the human safety evaluation of these materials.

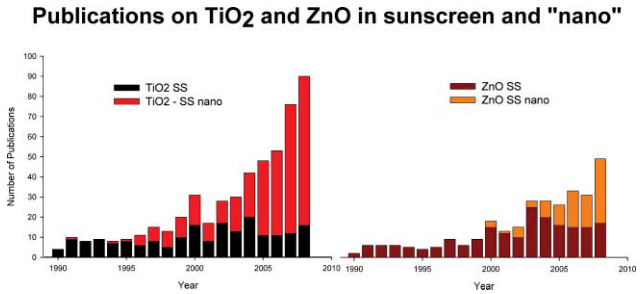

Fig. 1 TiO_2 and ZnO sunscreen publications: nano and non-nano.

In fact, suppliers and manufacturers of nano TiO_2 and ZnO have prepared comprehensive evaluations of all existing safety studies that were performed either by industry or published in the open literature. These studies covered different sunscreen-grade TiO_2 particles, including nano-structured rutile and anatase crystalline forms as well as coated and non-coated particles, and similarly for ZnO. These studies also covered most relevant and requested endpoints to characterize their toxicological profile. Moreover, comprehensive data on physical chemical properties including characterization, specification, analytical data and information on function and uses have been submitted to the former Scientific Committee on Cosmetic Products and Non-Food Products (SCCNFP) for evaluation and subsequent inclusion in Annex VII to Council Directive 76/768/EEC.

In 2000, the SCCNFP concluded in their opinion for TiO_2:[8]

The SCCNFP is of the opinion that titanium dioxide is safe for use in cosmetic products at a maximum concentration of 25% in order to protect the skin from certain harmful effects of UV radiation. This opinion concerns crystalline (anatase and/or rutile) titanium dioxide, whether or not subjected to various treatments (coating, doping, etc.*) irrespective of particle size, provided only that such treatments do not compromise the safety of the product. The SCCNFP proposes no further restrictions or conditions for its use in cosmetic products.*

For ZnO, the SCCNFP concluded in their 2003 opinion:[9]

The SCCNFP is of the opinion that more information is needed to enable a proper safety evaluation of micronized zinc oxide for use as a UV filter in cosmetic products. Consequently, an appropriate safety dossier on micronized ZnO itself, including possible pathways of cutaneous penetration and systemic exposure, is required.

This conclusion and the respective request was repeated by the Scientific Committee on Consumer Products (SCCP, formerly SCCNFP)[10] with a subsequent clarification:[11]

The SCCP considers that on the basis of the dossier reviewed in 2003 the use of ZnO in its non-nano form (pigment grade, with particle sizes above 100 nm) is considered safe. The concern expressed in the SCCNFP opinion 0693/03 with regard to phototoxicity is not relevant for this form of ZnO due to the absence of dermal penetration.

In the meantime, the producers and users of ZnO-based sunscreens addressed the data and information requested by the SCCNFP in their opinion of 2003[9] and prepared an additional evaluation with regards to specification of microfine (nano) ZnO, *i.e.*, purity and composition, percutaneous absorption, and photomutagenicity, and provided the complete data-set as submission II in 2005 to the former SCCP

Since these comprehensive submissions, additional health concerns about the use of nanomaterials in cosmetics were identified in a recent review by the European Commission's SCCP.[12] Amongst other issues, the review mentioned data gaps concerning hazard identification, exposure assessment, translocation and possible health effects of nanoparticles. As a consequence, in the later part of 2008 the SCCP decided to re-review the safety of nano TiO_2 and ZnO in the light of recent information, in particular the effect of nanoparticles on compromised skin and the possible impact of mechanical action on skin penetration.

Historical use

Prior to reviewing the relevant toxicological information for TiO_2 and ZnO, it is worth mentioning when and why nano-structured metal oxides were introduced into sunscreens. It is sometimes surprising to point out that patents for nano TiO_2 and ZnO date back to the early/mid 1980s and that nano TiO_2 was in wide commercial distribution beginning in the 1990s, followed by ZnO in the later part of the decade. Before then, pigmentary grades of these metal oxides were used, imparting an opaque whiteness as a result of scattering visible light. The pigmentary grades of the metal oxides also contain nano particles, albeit fewer in number. The engineering feat that enabled isolation/production of narrow range of particles, *i.e.*, <100 nm, produced cosmetic and efficacy benefits by reducing the "scattering" of visible light providing "transparent" products which retained UV absorption characteristics. This relationship between particle size and UV attenuation is illustrated in Fig. 2. Again, there is a misperception that nano TiO_2 and ZnO particles are "transparent". In fact, they appear transparent at or below concentration thresholds, therefore providing UV absorption without any "whitening effect". Thus,

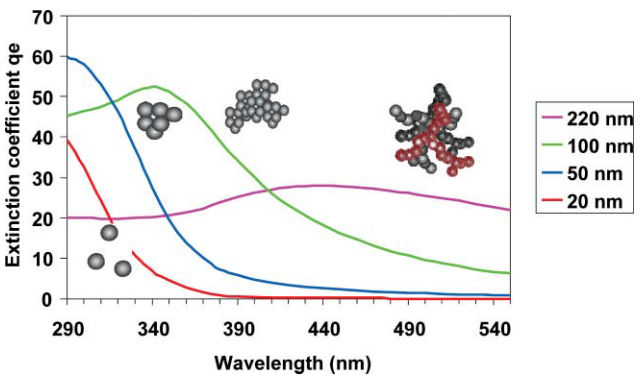

Fig. 2 UV/Visible attenuation curves calculated by Mie theory.

nano metal oxides provide efficacy as well as cosmetic benefits, which is why they are used in sunscreen products.

Definition of "nano"

Characterization of nano TiO_2 and ZnO

It is now generally considered that nanoscale substances are those in the size range below 100 nm, where the physical and/or chemical properties of the material are significantly different from the larger size or bulk versions of the same material. A current draft definition from the German Standards Institute (DIN) and the International Standards Organization (ISO) defines the following:

- *nano-object*: an object having one or more external dimensions on the nanoscale,
- *nanoparticle*: an object having three external dimensions on the nanoscale,
- *nanorod*: an object having two external dimensions on the nanoscale, and
- *nanoplate*: an object having one external dimension on the nanoscale,

with "nanoscale" being defined as approximately 1 to 100 nm.

It is also important to recognize that some materials can exist as particles which have external dimensions outside the nanoscale, but which consist of aggregates or agglomerates of smaller particles or crystals, where these smaller particles are within the nanoscale range. The DIN/ISO proposal describes these as "nano-structured aggregates" or "nano-structured agglomerates". The terms aggregate and agglomerate are often confused or used interchangeably, but they are in fact quite distinct and are defined as follows:

- *aggregate*: particle comprising strongly bonded or fused particles where the resulting external surface area may be significantly smaller than the sum of calculated surface areas of the individual components;
- *agglomerate*: collection of loosely bound particles or aggregates or mixtures of the two where the resulting external surface area is similar to the sum of the surface areas of the individual components.

Many modern cosmetic products contain nano-sized components, such as nanoemulsions and liposomes or nanosomes. These structures are based on liquid/liquid interactions and are inherently unstable and break down upon contact with human skin. As such, they are not considered to be genuine nanomaterials. But perhaps the most familiar types of nanoparticles in personal care products are solid nanoparticles, *i.e.* the UV-attenuating grades of TiO_2 and ZnO.

Most commonly used manufacturing processes of fine-particle TiO_2 and ZnO produce nanoparticles (crystals) which are typically 10–20 nm in size. However, the strong attraction forces between crystals cause them to form tightly-bound aggregates, which have a larger size than their primary building blocks (Fig. 3). These aggregates are typically between 30 to 150 nm and represent the smallest particles which actually occur in a sunscreen formulation, as the forces required to break apart the aggregates into individual nanoparticles are far greater than those encountered during production of sunscreens or application of these products onto skin. These aggregates are often described in literature as 'primary particles' and may appear in electron microscopy images as a single particles, although they consist of multiple crystals.

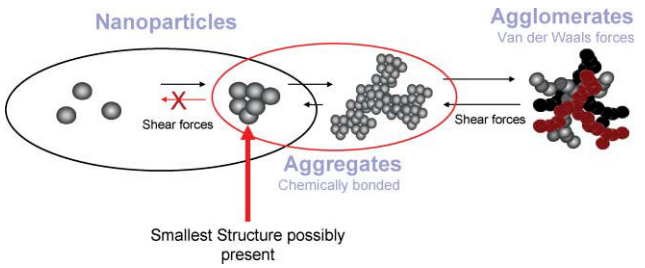

Fig. 3 Schematic of smallest particle size present in sunscreen products.

The situation is complicated further because the aggregates cluster together to form loosely-bound agglomerates as a result of manufacturing. These agglomerates have particle sizes of greater than 1 micron, which is typical of the size of powder supplied to sunscreen formulators. Such large particles would not be effective at attenuating UV light, practical formulation of sunscreens tells us that the agglomerates must be broken down in the final sunscreen products because high SPF sunscreen products based on inorganic oxides are available in the market.

For TiO_2 it is common practice to specify 'the particle size' as the size of the single crystals, although these are always present as aggregates in formulation. For fine-particle ZnO, on the other hand, crystal size is seldom used to express 'the particle size'. The typical particle size for attenuation grade ZnO is typically between 30–150 nm and this is the smallest size that will occur in a sunscreen formulation.

The optical properties of inorganic oxides of various particle sizes can be calculated by the use of Mie theory. Application of this to titanium dioxide shows that a particle size of less than 100 nm is necessary to achieve effective UV protection while maintaining cosmetic elegance, *i.e.* the product is transparent in a thin film. The optimum size is calculated to be around 50 nm (Fig. 2).

A number of different methods are used to measure particle size and it is important to remember that different measurement techniques are not directly comparable. Some techniques measure individual nanoparticles, which are not present in TiO_2- and ZnO-based cosmetic products, whilst others measure aggregates and agglomerates. Table 1 lists some commonly used particle sizing techniques. The table also shows data measured by each technique for a single UV attenuation grade TiO_2 and ZnO, indicating how different techniques can vary in the results they produce.

Sunscreen products: formulating with nano TiO_2 and ZnO

Achieving a reliable, meaningful measurement of particle size in a finished sunscreen formulation is very difficult because most techniques require that the sample be heavily diluted in order to take the measurement. Advances in transmission electron microscopy (TEM) have allowed TiO_2 and ZnO particles to be examined in final sunscreen formulations. The example in Fig. 4A represents an oil/water emulsion which contained 10% coated TiO_2. The primary nanoparticles of the titanium dioxide material are of acicular shape with dimensions of $30–60 \times 10$ nm. However, individual particles could not be detected in the formulation, but they were present as larger aggregates or agglomerates. Similarly, Fig. 4B represents a oil/water emulsion which contained 9%

Table 1 Analytical techniques used to measure nano particles

Technique	Range	Measure	Sunscreen grade particle diameter/nm	
			TiO_2	ZnO
X-ray diffraction	1 nm to >1 μm		8	30
Surface area by BET nitrogen adsorption	1 nm to >1 μm		19	35
Brookhaven X-ray disc centrifuge	10 nm to 25 μm		35	40
Photo-cross correlation spectroscopy (PCCS)	1 nm to 5 μm		60	—
Laser light scattering	100 to 600 nm		145	—

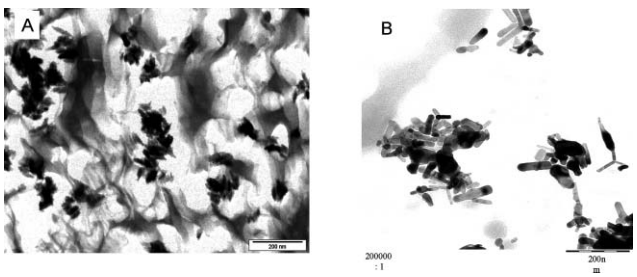

Fig. 4 (A) 10% UV attenuating titanium dioxide in a final sunscreen preparation measured by cryogenic TEM showing the presence of ~100 nm aggregates. (B) 9% coated ZnO in an oil/water sunscreen preparation measured by cryogenic TEM. The primary nanoparticles of ZnO are acicular in shape with dimensions ranging from 30–150 nm. These particles are mainly present in clusters, either aggregates or agglomerates.

coated ZnO. The primary nano particles of ZnO are acicular in shape and present mainly in clusters (either aggregates or agglomerates).

Toxicological considerations of nano TiO_2 and ZnO

Toxicology is the organized, systematic study of the effect of xenobiotics on living organisms.[13] The most fundamental part of toxicology is hazard identification or objective safety, which is the classical endpoint assessment, *e.g.*, ocular irritation or genotoxicity/carcinogenicity, of a test material. The conduct of hazard-based testing provides our basic knowledge of the toxicological properties of a test material. The toxicological profiles of TiO_2 and ZnO have been evaluated as sub-micron and nanostructure raw materials.[14–17] However, there are concerns regarding the toxicological evaluation of nano materials, in particular the test methods currently being used, and the adequacy of regulatory authorities and interested stakeholders to evaluate the risk to human health from exposure to products containing them.[18]

Adequacy of toxicological test methods

Currently, there are standardized toxicological protocols sanctioned by various authoritative organizations, *e.g.*, Organization for Economic Co-operation and Development (OECD), US Environmental Protection Agency (EPA), International Conference on Harmonization (ICH), which have been developed to evaluate hazard independent of the physical forms of test material. These protocols have in many cases been evaluated using known positive and negative controls to establish the predictive validity with respect to human health.[19] Moreover, these methods are suitable for hazard investigation of, for example, liquids, solids, suspensions, dusts, aerosols, gases and vapors, consisting of particles larger and smaller than nanomaterials. Recently, the OECD Working Party on Manufactured Nanomaterials Project report entitled *Manufactured Nanomaterials and Test Guidelines* (30 May 2008), page 57 concluded:

In general the OECD guidelines are appropriate for investigating the health effects of nanomaterials with the important provision that additional consideration needs to be given to the physicochemical characteristics of the material tested, including such characteristics

in the actual dosing solution. In some cases there will be a need for further modification to the OECD guideline. This applies particularly to studies using the inhalation route and to toxicokinetic (ADME) studies. Finally it is important to build upon current knowledge and practical solutions in relation to in vitro *test approaches.*

The key to evaluating any test material using standardized methods is dosimetry. Dosimetry, *i.e.* particle size, surface area and number of particles administered, in many toxicology studies has been described as a major uncertainty factor in studies of nanomaterials. Whereas this is certainly true and there are many examples in the published literature supportive of this view, this does not mean that the test, by itself, is inadequate. In the end, toxicity studies with nanomaterials should be performed with fully characterized test materials using standardized testing to access endpoints of concern.

Skin penetration

Considering the significant role dermal penetration plays in the general evaluation of systemic toxicity of topically applied substances, including nano TiO_2 and ZnO, a selective review of this subject is warranted. To this end, several studies on skin penetration have been reviewed from *in vitro* and *in vivo* studies using animal and human skin coupled with the most sensitive analytical techniques that exist to determine the presence of particles and metal ions after single or repeated application. In addition, several reports have been reviewed describing results of the EU NANODERM project. These, and others, are summarized in Table 2. The consistent finding of these different studies is that nano TiO_2 or ZnO does not penetrate beyond the stratum corneum of the skin.

• Tan *et al.*[20] first reported in a pilot study that 10–50 nm particles of TiO_2 could penetrate the stratum corneum to the dermis following repeated application in volunteers. However, the study was limited, particularly as the study volunteers were undergoing surgery for skin lesions, and therefore the dermal barrier may already have been compromised. Moreover, statistical significance was not observed in this study, unless one of the observations was omitted from the analysis.

• Lademann *et al.*[21] investigated the dermal penetration of 20 nm (assumed particle size, based on description of product used) TiO_2 particles in a sunscreen formulation. The sunscreen was applied repeatedly over 4 days to the forearm skin of human volunteers. The only significant finding concerning a potential penetration of TiO_2 beyond the upper skin layers was their deposition in single hair follicle openings, although there was no evidence that these residues were located within the living skin. The concentration of TiO_2 in the hair follicle openings was two orders of magnitude lower than that in the upper skin layers.

• Schulz *et al.*[22] investigated the influence of particle size on the dermal absorption of three TiO_2 preparations. Each had a different primary particle size (10–15 nm, 20 nm and 100 nm), shape (cubic or needles) and hydrophobic/hydrophilic characteristics. The preparations were topically applied in an oil-in-water emulsion to the forearm skin of human volunteers for 6 h. Skin biopsies were examined by scanning electron microscopy to visualise the distribution of particles within the skin layers. None of the particles penetrated beyond the outer layer of the stratum corneum.

• Gottbrath *et al.*[23] investigated ultrafine (nano) TiO_2 penetration into human stratum corneum after *in vivo* application of two formulations, standard micellar and liposomal, to the ventral skin of the forearm. Penetration was measured by tape stripping of skin (10 strips). Tape strips from the TiO_2-treated skin sites were assayed for Ti by atomic absorption spectrometry. Tape strips from the vehicle control-treated sites were viewed with an inverted microscope to estimate the amount of corneocyte aggregates. TiO_2 nanoparticles in the formulations and tape strips were visualized by transmission electron microscopy (TEM).

In a first experiment, standard and liposomal formulations, each containing 5% TiO_2, were applied to the ventral forearm skin of a 30 year old male volunteer at a dose of 2 mg cm^{-2}. After 45 min, the formulation was removed from the skin with a dry tissue. The tape strips revealed more Ti in deeper layers of the stratum corneum after treatment with the standard formulation when compared to respective values after application of the liposomal formulation. However, it was suspected that this was an artefact of skin surface topography, *i.e.* corneocytes surrounded by shallow furrows: Ti nanoparticles could have remained in these furrows during cleaning with tissue, but during stripping a small amount of these nanoparticles would have been removed, more with deeper strips, *i.e.* the TiO_2 detected in deeper strips had not penetrated into the stratum corneum, but had remained in valleys of the stratum corneum. TEM of the adhesive film after stripping confirmed this interpretation; the micrographs show the spreading of TiO_2 nanoparticles on the skin surface and the residence of the nanoparticles in furrows between the corneocytes. To avoid this problem, a second experiment was conducted in which formulation was removed from the skin by thorough washing with water prior to analysis.

In the second experiment, standard and liposomal formulations containing TiO_2 were removed from the ventral skin of both forearms 45 min after application, from the left forearm with a dry tissue, from the right forearm by rinsing with water for 5 min; 10 strips were then taken from the application sites and analysed as before. The quantity of corneocyte aggregates removed by tape strips from the washed skin was smaller than from the wiped skin, which the authors suggested could be due to reduced coherence of the corneocyte aggregates after washing. In the washed skin tape strips, significantly more TiO_2 was found after liposome than after standard formulation treatment. The authors concluded that, after application of the liposomal formulation, a fraction of the ultrafine TiO_2 particles penetrated into the stratum corneum and did not remain in shallow valleys formed by the corneocytes, explaining the water resistance of the liposomal formulation, *i.e.* the deposition of ultrafine TiO_2 particles depends on the formulation used.

• Butz[24] summarized two of the main methods used in the NANODERM project: high resolution transmission electron microscopy (HRTEM) and ion beam analysis by particle induced X-ray emission (PIXE). He noted that most previous uptake studies used the tape-stripping method, which does not give reliable penetration profiles due to skin furrows. Most, but not all, HRTEM studies indicated that such particles remain on the topmost layers of the stratum corneum. Advantages of HRTEM are that individual nanoparticles can be visualized and their composition can be analysed; disadvantages are the relatively small area of investigation and the risk of preparation artefacts.

Table 2 Summary of skin penetration studies conducted with nano TiO_2 and/or ZnO

Material	Test model	Application conditions	Analytical and/or imaging methods	Results	Reference
TiO_2: microfine, NOS	Human skin, *in vivo* (13 patients), compromised skin NOS (subjects scheduled to have surgery for skin lesions)	Repeated application (twice a day for 2–6 weeks) of a sunscreen lotion containing 8% microfine TiO_2	Chemical analysis (ICPMS) of skin biopsies	Non-statistically significantly higher Ti levels in the dermis of treated subject *vs.* controls (cadaver skin)	Tan *et al.*, 1996[20]
TiO_2 (T805, Degussa): mixture of rutile/anatase forms, round particles of 50–100 nm; ZnO (Spectra Veil MOTG, Tioxide specialities), round/oblong particles of 117 nm mean size	Human skin, *in vitro* (abdominal skin from plastic surgery)	Single topical application of a commercially available sunscreen formulation (w/o emulsion containing 11% and 2.5% of TiO_2 and ZnO, respectively) at 1 mg cm^{-2}	TEM of ultrafine skin sections	No skin penetration of ZnO or TiO_2 NPs beyond SC	Dussert *et al.*, 1997[45]
TiO_2 (UV Titan M160), coated	Human skin, *in vivo*	Repeated (11 times) application at 2 mg cm^{-2} of a commercial sunscreen formulation (o/w emulsion)	UV/Vis spectroscopic evaluation, X-ray fluorescence measurements LIFM, SRLSM and Raman spectroscopy of skin tape strips, histological evaluation of skin biopsies	No penetration of TiO_2 NPs in living skin; TiO_2 NPs mainly located in outer layers of the SC	Lademann, *et al.*, 1999[21]
TiO_2 NPs: –T805: Ti/Si coated, PPS of 20 nm, cubic –Eusolex T-2000: rutile, 100 nm, needles, $Ti/Al_2O_3/SiO_2$ coating –Tioveil AQ: 100 nm, needles, Ti/Al/Si coating	Human skin, *in vivo*	Single application of 4 mg cm^{-2} of o/w emulsions containing 4% TiO_2	Electron (TEM) and light microscopy	No penetration of TiO_2 NPs in living skin; TiO_2 NPs exclusively located in outer layers of the SC	Pflücker *et al.*, 2001[46]
Coated TiO_2 –T805 (20 nm, cubic, Ti/Si coating) –Eusolex T-2000 (rutile, 10–15 nm NPs in 100 nm aggregates, needles, $Ti/Al_2O_3/SiO_2$ coated) Tioveil AQ-10P (100 nm, needles, Ti/Al/Si coated)	Human skin, *in vivo*	Single application of 4 mg cm^{-2} of o/w emulsions containing 4% TiO_2	Electron (TEM) and light microscopy	Micronised TiO_2 solely deposited on the outermost surface of the SC, not detected in deeper SC layers, the human epidermis and dermis	Schulz *et al.*, 2002[22]
TiO_2 (Tioveil AQ N): alumina/silica coated	Human skin, *in vivo* (tape stripping); single male volunteer	Micellar "solutions" or liposome formulations of 5% TiO_2 applied at 2 mg cm^{-2} for 45 min	TEM, AAS	No penetration of TiO_2 NPs beyond SC (presence in wrinkles for micellar solutions, in SC for liposome formulation)	Gottbrath *et al.*, 2003[23]
TiO_2 NPs, NOS	Pig skin, *in vitro*	Different formulations, NOS, different exposure times	PIXE, RBS, STIM	TiO_2 NPs did not penetrate through the entire SC; no effects of formulation and exposure time	NANODERM, 2003[24]
TiO_2 NPs, NOS	Mouse, pig and human skin, *in vitro*	Formulations containing TiO_2 NPs	STIM, PIXE associated with TEM	TiO_2 particles solely detected in the intercellular spaces between the corneocytes of the outermost layers of the SC	Gontier *et al.*, 2004[47]
TiO_2: lanceolate in shape, 45–150 nm long and 17–35 nm wide	Pig skin, *in vivo*	Single application of: –commercial sunscreen formulation (5% TiO_2) –a liposome dispersion (18% TiO_2)	Spatially resolved ion beam analysis (PIXE, RBS, ERDA, STIM and secondary electron imaging)	Penetration through SC into stratum granulosum, but not into stratum spinosum	Menzel *et al.*, 2004[25]

Table 2 *(Contd.)*

Material	Test model	Application conditions	Analytical and/or imaging methods	Results	Reference
		–a sunscreen base formulation containing 4.5% TiO_2 –pure NP pre-dispersion (Tioveil AQ-N, 40% TiO_2)			
TiO_2: rutile, mean diameter 100 nm	Human skin, *in vivo* (tape stripping)	Repeated application (5 times over a 4 day period) at 2 mg cm^{-2} of a sunscreen base formulation containing 5% TiO_2	X-ray fluorescence	No penetration into epidermis or dermis (most penetration within outer 3 μm of skin); NB: SC is 15–20 μm thick in humans	Popov *et al.*, 2005[48]
TiO_2 NPs, NOS	Human foreskin grafts transplanted into SCID mice	Commercial sunscreen formulation (hydrophobic emulsion) applied under occlusion for 1, 24 and 48 h	Combined IBA techniques, ST1M, FIXE and RBS, and TEM	Penetration of TiO_2 NPs into the SC by direct visualisation in TEM and *via* their chemical fingerprint in PIXE; marginal observations of presence of Ti at limit of stratum granulosum (2 cases out of a non-specified number of cases)	Kertesz *et al.*, 2005,[26] Nanoderm EU-5 project
ZnO (Z-COTE): uncoated microfine zinc oxide, PPS of 80 nm agglomerated in emulsion; TiO_2: T-Lite SF-S (methicone and silica-coated); T-Lite SF (methicone-coated, needle-shaped, PPS of 30–60 × 10 nm, with aggregates and agglomerates up to 1 μm in emulsion)	Porcine skin, *in vitro* (OECD 428, SCCNFP and GLP guidelines compliant)	ZnO (o/w emulsion containing 10.3% ZnO) and TiO_2 (o/w emulsion containing 10% TiO_2) formulations applied for 24 h at 4 mg cm^{-2}	AS (Zn, Ti), AAS (Zn) ICP-AES and ICP-MS (Ti)	Virtually the total amount of applied Zn was recovered in the first pooled five tape strips (SC); virtually the total amount of applied Ti could be removed from the skin surface by washing; no detection of TiO_2 in receptor fluid, and similar ZnO amounts for blank and vehicle-treated samples	Gamer *et al.*, 2006[28]
ZnO: PPS of 26–30 nm, polymethylsilsesquioxane-coated	Human skin, *in vitro* (abdominal skin from plastic surgery)	ZnO sunscreen formulations applied at 10 mg $cm^{-2}$for 24 h: 60% dispersion of ZnO NPs in caprylic capric triglyceride; typical o/w emulsion sunscreen 20% ZnO	TEM, ICP-MS	Penetration of ZnO NPs limited to the outer surface of the SC.	Cross *et al.*, 2007[49]
TiO_2 (T805): mean PPS of 20 nm, trimethyloctylsilane-coated,	Human skin, *in vivo* (3 female volunteers) and *in vitro* (abdominal and face skin obtained from plastic surgery)	Application for 5 h at 2 mg cm^{-2} of a sunscreen formulation (w/o emulsion) containing 3% TiO_2 (*in vivo* and *in vitro*)	Colorimetric assay, TEM and PIXE	Most of TiO_2 recovered in first 15 tape strippings (SC), remaining TiO_2 was located in the furrows and in the opened infundibula; no penetration into living skin layers	Mavon *et al.*, 2007[27]
TiO_2 (Eusolex T-2000): rutile, PPS of 20 nm × 100 nm, coated	Human and porcine skin, *in vitro*	5% TiO_2 formulations (hydrophobic basis, isopropylmyristate, microemulsion and polyacrylate gels) applied at 2 mg cm^{-2} for various exposure times ranging from 30 in up to 48 h	PIXE, RBS, STIM, autoradiography	No penetration into living skin; presence of NPs in 3–5 first layers of SC and in follicles only (mechanical movement, no "diffusion" pathway)	Lekki *et al.*, 2007,[50] Nanoderm EU-5 project

Table 2 *(Contd.)*

Material	Test model	Application conditions	Analytical and/or imaging methods	Results	Reference
TiO_2 NPs, NOS	Human intact or psoriatic skin *in vivo*	2 h exposure to commercial sunscreen formulation containing TiO_2 NPs	PIXE, RBS, STIM,	No penetration into living skin; TiO_2 permeation profile similar in healthy and psoriatic skin: NPs retained in first layers of SC	Pinheiro *et al.*, 2007[51]
ZnO (PPS of 26–30 nm)	Human skin *in vivo* (4 subjects) or *in vitro* (excised abdominal or breast human skin obtained from plastic surgery)	Commercial sunscreen formulation containing 19% ZnO applied at 6 mg cm^{-2} for up to 24 h	MPM coupled with SEM and EDX techniques	No evidence of skin penetration of ZnO NPs beyond SC; accumulation in skin folds and hair follicles	Zvyagin, 2008[29]
Micro TiO_2: 300–500 nm, rutile; Uncoated TiO_2 (P25): 80/20 anatase/rutile, PPS of 21–23 nm aggregated into sub- and micron-sized; Coated TiO_2: aluminium hydroxide and dimethicone/methicone copolymer coating, PPS of 50–150 × 30 nm, needles	Female Yucatan mini-pigs, *in vivo*	Repeated application (4 times daily, 5 days per week for 4 weeks) at 2 mg cm^{-2} of sunscreen base formulations containing 5% TiO_2	ICP-MS, SEM-EDX	No systemic distribution of TiO_2; slightly increased Ti levels in dermis, but not in liver or lymph nodes ("sentinel" organs) in all groups (micro or nano TiO_2); SEM-EDX analysis ongoing to determine whether increased levels in dermis are due to presence in lower hair follicles or in viable skin	Sadrieh *et al.*, 2010[32]
TiO_2 (Eusolex T-2000): PPS of 20 nm, coated, rutile); ZnO: spherical, PPS of 20–60 nm	Human intact skin (9 volunteers); human compromised skin (>15 tape-strips under occlusive conditions, 10 volunteers); human psoriatic skin (4 patients) *in vivo*	Commercial sunscreens (hydrophobic emulsions) containing nano TiO_2 or ZnO and TiO_2 NPs; hydrophobic basis gel containing 80% TiO_2; applied for 2 h at 0.5–1.0 and 2.0 mg cm^{-2} under exaggerated exposure conditions (occlusive patch)	Focused ion beams with sub-micrometre spatial resolution, PIXE	No evidence of skin penetration of ZnO/TiO_2 NPs beyond SC; deposition of TiO_2 and ZnO NPs in skin wrinkles and openings of the pilosebaceous follicles	Filipe *et al.*, 2009[52]
TiO_2 (Solaveil CT10W): PPS of 30–150 nM, hydrophobically-coated; ZnO NPs (Z-Cote max): PPS of 100–200 nM, coated	Human skin, *in vitro* (abdominal female skin from plastic surgery, Franz cells)	w/Si and w/o emulsions containing 3% TiO_2 and 1% ZnO and organic UV filters applied at 2 mg cm^{-2} for 24 h under occlusive conditions (Parafilm)	ICP-OES	Presence of NPs in SC or hair follicles; no penetration into living skin	Durand *et al.*, 2009[53]
ZnO: PPS of 10 nM, spherical, uncoated	Nude mouse skin *in vitro*	10% ZnO in a chemically enhancing penetration vehicle consisting of oleic acid, ethanol and PBS	Multi-photon microscopy imaging, TEM	Chemically-induced enhanced skin penetration of ZnO NPs into SC (through multilamellar lipid regions between the corneocytes)	Kuo *et al.*, 2009[54]
TiO_2: PPS of 25 nm, spherical, uncoated	Mouse intact and abraded skin (removal of SC and epidermis), *in vivo* (hairless SKH-1 mice)	Single application of emulsions containing 5% TiO_2 for 6 or 24 h	ICP-MS	No penetration beyond SC in mouse intact and dermabraded skin; no increased Ti levels in blood, liver, kidney and lymph nodes (organs identified as "sentinel" organs) following ID injection	Gopee *et al.*, 2009, Society of Toxicology, annual meeting, Baltimore, MD, March 15–19
TiO_2, uncoated a. Anatase: PPS of 4 and 10 nm; rutile: PPS of 25, 60 and 90 nm	a. Porcine skin, *in vitro* (Franz cells)	a. Single application 5% TiO_2 suspensions in 1% Tween 80 and 20% caprylic/capric triglyceride in water; application rate NOS	a. TEM, AAS	a. No penetration beyond first outer layers of SC	Wu *et al.* 2009 [30]

Table 2 *(Contd.)*

Material	Test model	Application conditions	Analytical and/or imaging methods	Results	Reference
b. PPS of 4 nm (anatase) and 60 nm (rutile)	b. Pig, *in vivo* (skin distribution)	b. Repeated daily application for 30 days of 5% TiO_2 suspensions in 2% triethanolamine in water at 8 mg cm^{-2}	b. TEM	b. TiO_2 NPs detected in SC, stratum granulosum, prickle cell layer and basal cell layer (4 nm NPs only), but not in dermis, *No quantitative data, isolated pictures shown*	
c. PPS of 10, 25, 60 nm and "normal size" TiO_2 plus P25 TiO_2 (PPS of 21 nm, anatase/rutile)	c. Mouse (BALB/c hairless), *in vivo* (tissue distribution)	c. Repeated daily application (under dressing for 3 h) for 60 days of 5% TiO_2 suspensions in 2% triethanolamine in water at 8 mg cm^{-2}; skin was daily rinsed and dried	c. TEM, AAS	c. Increased Ti levels in skin (localization NOS, all NPs), subcutaneous muscles (all NPs), heart (all NPs), liver (all NPs), spleen (all NPs), lung (P25, 60 nm NPs), kidneys (10 nm NPs) and brain (P25 NPs), but not in blood; decreased Ti levels in brain (all NPs but P25 and "normal size" TiO_2); TEM: observation of NP aggregates in liver (10 nm and P25 NPs)	
TiO_2: coated, PPS of 10 × 50 nm, agglomerate size range 90–460 nm; ZnO: coated, PPS of 140 nM	UVB-sunburnt pig skin, *in vitro* (2.5 MED, irradiation *in vivo*)	Four sunscreen formulations (o/w and w/o) containing 10% TiO_2 or 5% ZnO	Light microscopy, TEM and TOF-SIMS	UV-induced sunburns did not enhance skin penetration of TiO_2 or ZnO NPs: similar skin penetration profile with intact and sunburnt skin; no increases of Zn and Ti in receptor fluid when compared with controls	Monteiro-Riviere *et al.*, 2010, Society of Toxicology, annual meeting, Salt Lake City, UT, March 7–11

Advantages of PIXE are lack of preparation/fixation artefacts and large scanning areas; a disadvantage is that individual nanoparticles cannot be resolved.

• Kertész *et al.*[24] summarized the results of the NANODERM project to date. Initially, skin penetration studies using different formulations were performed in pig skin, which closely resembles human skin. As a next step, human skin xenografts transplanted into severe combined immune deficiency (SCID) mice were used. This murine model was developed because of the difficulty in obtaining human skin biopsies from healthy volunteers. Experiments on healthy human skin provided by the Lisbon group started at the end of 2004. In 2004, 22 pig skin, 11 transplanted human skin and 13 human skin samples were investigated by ion microscopy and electron microscopy at the University of Debrecen, Hungary. The authors reported that, in healthy skin, nanoparticles penetrated into the deepest corneocyte layer of the skin, but never reach the vital layers. "Further experiments are planned with repeated exposure and on atopic skin."

• Menzel *et al.*[25] reported the results of NANODERM studies on TiO_2 penetration in pig skin epidermis *in vivo*. Back skin (washed, shaved, and disinfected with alcohol) was treated with 4 formulations (one commercial product, Eucerin®, containing 5% TiO_2; two experimental mixtures, a liposome dispersion containing 18% TiO_2 and another (SG1101) containing 4.5% TiO_2; and concentrated material, Tioveil AQ-N, containing 40% micronized TiO_2 nanoparticles. The TiO_2 was reported to have a needle-like shape, 45–150 nm long and 17–35 nm wide by transmission electron microscopy (TEM). The formulations were applied on occluded allergy test patches (1 cm^2) and skin samples were taken 8, 24 and 48 h later by post-mortem punch biopsy. Ti distribution was determined in 20–30 μm cryosections from the biopsies, using ion beam analytical methods: PIXE, scanning transmission ion microscopy (STIM), Rutherford backscattering (RBS), elastic recoil detection analysis (ERDA) and secondary electron imaging (SEI). The location of the epidermal cell layers were identified in these sections based on the characteristic elemental composition of the different cell layers (high sulfur and chlorine in stratum corneum, high phosphorus in the germinal layers) as visualized on the STIM energy and elemental maps (spatial resolution 1–2 μm).

Ti was localised on the skin surface and in the stratum corneum at all times after treatment with all four formulations. Based on the spatial assignment of cell layers using elemental STIM maps, the authors reported that there were low concentrations of titanium (0.2–200 mmol L^{-1}) in the stratum granulosum after treatment with the Eucerin formulation and with Tioveil AQ-N 40% concentrate (all time points). Low stratum granulosum concentrations of titanium were also reported 8 h after the experimental liposome

and SG1011 formulations, and 24 h after the SG1011 formulation, but not 48 h post-dose for either formulation. There was no evidence of penetration into the stratum spinosum (minimum detection limit = 1 particle per μm^2); Ti spots in the stratum spinosum and underneath were clearly identified as preparation artefacts by SEI and RBS measurements on both sides of the samples. No Ti was found in the hair follicles. Although the authors concluded that "it has been proved that micronized TiO_2 penetrated into the living stratum granulosum"; this conclusion can not be derived from the data presented by the authors.

• Kertész *et al.*[26] described studies of TiO_2 penetration in epidermis of human skin xenografts, human foreskin grafts transplanted into severe combined immune deficiency (SCID) mice. The skin grafts were treated with a commercial sunscreen product, Anthelios® XL SPF 60+ Cream, containing micronized (ultrafine) TiO_2 and occluded for 1, 24 or 48 h. Ti distribution was determined in 14–16 μm thick freeze-dried sections obtained from quick-frozen punch biopsies using ion beam analytical methods: PIXE, STIM and RBS. The epidermal cell layers were identified in these sections based on the characteristic elemental composition of the different cell layers (high sulfur and chlorine in stratum corneum, high phosphorus in the germinal layers) as visualized on the STIM energy and elemental maps. Penetration of Ti seemed to be limited to the outermost part of the stratum corneum. However, in two cases, both after 48 h exposure, penetration through the stratum corneum to the outer limit of the stratum granulosum was observed in a sample from the entry of a sweat gland. With both ion microscopy and electron microscopy, nanoparticles were observed down to the innermost corneocyte layers, but no particle was observed in the cytoplasm of the granular cells.

• Mavon *et al.*[27] reported studies of *in vitro* and *in vivo* penetration of TiO_2 through human skin. The test material was a sunscreen formulation (water-in-oil emulsion) containing 3% ultrafine TiO_2 (T805, Degussa, Germany) and 1% methylene bis(benzothiazolyl tetramethylbutylphenol) (MBTBP, Tinosorb® M, Ciba, Switzerland), an organic pigment. Both, *in vivo* and *in vitro*, 4 mg cm^{-2} of the test emulsion was applied to the skin and left for 5 h. The *in vivo* studies were carried out on three volunteers; penetration of Ti into skin of the lateral upper arm was measured by tape stripping. The *in vitro* studies were carried out in triplicate on viable abdominal and facial skin from plastic surgery; penetration of Ti was measured by tape stripping and in punch-biopsy cryofixed tissue sections (20 μm) by MeV proton beam assays: PIXE to measure quantities of sulfur, potassium and titanium, and RBS to determine the elemental content and organic mass of the analyzed tissues (as g dry weight per cm^2). Ti in tape strips was assayed colorimetrically and MBTBP by HPLC-UV.

In both, the *in vivo* and *in vitro* studies, more than 97% of the applied titanium and about 95% of the applied MBTBP was recovered in the horny layer. In the *in vitro* studies, less than 0.5% of the MBTBP was detected in the receptor medium. Using the micro-PIXE technique, the 2D mapping and quantification analysis confirmed a high concentration of Ti on the skin surface, but no Ti was detected in follicles, viable epidermis or dermis. The authors concluded that there was no measurable penetration of Ti through viable skin *via* either the stratum corneum or follicles.

• Gamer *et al.*[28] published data on the skin absorption of ultrafine TiO_2 and ZnO in pig skin *in vitro*. The data were obtained in a GLP-compliant study designed in accordance with the relevant SCCNFP and OECD guidance. The dermal penetration of microfine TiO_2 or ZnO in cosmetic formulations was assessed by single topical application to dermatomed pig skin mounted on Franz-type static diffusion cells containing receptor fluid (physiological saline containing 5% bovine serum albumin). The TiO_2 particles were coated with methicone or methicone plus silica; the primary particle size was $30–60 \times 10$ nm, but they were present as aggregates mostly up to 200 nm and were applied in cosmetic formulations (oil/water emulsion, T-Lite SF-S and T-Lite SF). The ZnO (Z-COTE®) was uncoated and had a mean primary particle size of 80 nm with 90% of the particles being <160 nm. The mean total recoveries of Ti ranged from 98% to 100% and 86% to 93% of the total Ti applied, for methicone and methicone plus silica coated TiO_2, respectively. The total amount of applied Ti could be removed from the skin surface by washing. The amounts of Ti found in the tape strips and skin preparations were on the order of the limit of detection of the analytical method. No Ti was found in the receptor fluid at any sampling time. In the experiments with ZnO, the mean total zinc recoveries ranged between 102 and 107%. Virtually the total applied amount of zinc (between 98 and 102%) was recovered in the pooled first five tape strips. Minute quantities of zinc were also recovered in the subsequent pools of tapes and were considered to stem from a remainder of the cosmetic formulation in furrows of the skin and hair shafts. The results showed that neither Zn or Ti ions nor microfine ZnO or TiO_2 particles were able to penetrate porcine stratum corneum.

• Zvyagin *et al.*[29] investigated penetration of commercial products containing nano-structured particles of coated TiO_2 and ZnO dispersed in hydrophobic emulsions in 10 individuals under exaggerated exposure conditions, such as application to the skin after tape stripping and under occlusive patches (IQR chamber, 48 h application). Tape stripping consisted of a series of strips until the tapes were free of corneocytes. A matched control group comprising 6 individuals was used for the determination of basal elemental concentrations in skin including Zn. Skin punch biopsies of 3 mm diameter were taken after application, quench-frozen and kept in containers until processing. One biopsy was taken from each volunteer. Sections of 14 μm thickness were cut from the frozen biopsy in a cryostat at −25 °C. Biopsies were mounted in mounting medium for microscopy (OCT™ compound). Sections were obtained from the non-immersed portion of the tissue, and sectioning performed from inside to outside to avoid tissue contamination. Tissue integrity and the efficacy of corneocyte removal after tape stripping were checked by preparing intercalary stained sections for optical microscopy purposes. STIM and PIXE technique were used for detection. The minimum detectable concentration of Zn in the skin was 0.10 μmol g^{-1}.

The nanoparticle penetration profiles obtained with the treated skin groups (TiA, TiB and TiHB) were all similar. The high levels of Zn observed at the outer layers of stratum corneum sharply decreased within deeper layers to very low levels (Zn) in both protocols (SPF and realistic use condition). Under non-physiological conditions using occlusive patches there was no significant difference in ZnO nanoparticles distribution and penetration depth profiling.

• Wu *et al.*[30] reported dermal penetration of uncoated, "nano" TiO_2 after repeated application to the ear of male pigs ($n = 3$) for

30 days and to the backs of mutant hairless mice (unknown sex) for 60 days. Pathological changes in tissues were reported in this study. The authors concluded that "…nanosize TiO_2 will likely pose a risk to human health after dermal exposure over a relative long time period". However, there are several irregularities in this paper. For example: the "dose" used in the *in vitro* skin penetration study is not provided; the sex of the mice used in the *in vivo* study is not specified; the "normal" size TiO_2 is not characterized; in describing the results of the *in vivo* studies, 8 weeks is used when the methods state 60 days; pathological changes are described as being observed "back of the skin" without specifying if this is the treatment area or not. Collectively, these irregularities raise a level of suspicion. To this end, a letter by Jonaitis *et al.*[31] submitted to the journal stated "…we feel that certain conclusions drawn by Wu *et al.* are inaccurate and that extrapolation to human risks from any study with methodological deficiencies is not justified. It should be noted that our only interest is to ensure that scientifically proven conclusions are being drawn from the evidence, in light of the potential for inaccurate conclusions to be misleading." Given the methodological concerns in this study, the results are, at best, inconclusive.

• Sadrieh *et al.*[32] investigated the penetration of 3 TiO_2 particles (uncoated sub-micron, uncoated nano and coated nano) applied topically to minipigs 5 days per week for 4 weeks. The creams were applied at 2 mg cm^{-2} over the dorsal and ventral skin surface. Ti levels were determined by ICP-MS in multiple sites including skin, lymph nodes, liver, spleen and kidneys. There was no increase in Ti concentration in the liver or lymph nodes in treated pigs compared to control (vehicle) treatment. TiO_2 particles were observed in the epidermis using electron microscopy-energy dispersive X-ray analysis. Isolated particles were observed in the dermis of treated animals however the authors did not observe any pattern of distribution or pathology and suggested these particles may be the result of contamination. Based on these data, Sadrieh *et al.* concluded that nano-sized or sub-micron TiO_2 suspended in these specific formulae do not penetrate the intact epidermis after repeated application.

There are other papers worth mentioning largely for historical reasons and because they are often mentioned as providing some evidence of penetration of metal oxides into skin. Pirot *et al.*[33] is sometimes mentioned as reporting skin penetration results obtained with nano ZnO. However, the paper does not indicate the size of ZnO particles used. Similarly, a study by Lansdown and Taylor[34] is occasionally cited as showing skin penetration of nano ZnO and TiO_2; however, the particle sizes reported in this paper range from 2 to 20 μm, which are generally considered outside the criteria used to define nano.

In conclusion, there is a large data base on conventional dermal penetration studies from numerous sources, *e.g.*, government, academic, and industry, and comprehensive investigations within the EU-funded NANODERM project and more recently by United States Food & Drug Administration. These studies or projects yield consistent evidence that nano-structured TiO_2 or ZnO do not penetrate into living skin or cross the skin barrier. Moreover, in sunscreen products, neither TiO_2 nor ZnO is present in the form of primary nanoparticles; it is mainly present in the form of clusters (aggregates/agglomerates). These approaches have used standardized methods which rely on intact skin exposed to exaggerated doses or conditions. Whereas the conclusion that nano-structured metal oxide particles do not penetrate intact skin, there remains a question of the risk/safety if such nano materials were applied to damaged skin. This is an open question for any intentionally applied material or, for that matter, the universe of environmental xenobiotics. From the standpoint of a structured and organized evaluation of toxicological effects, the approach to evaluate "damaged" skin is fraught with complications and the interpretation of such a multivariate design is arguably quite limited.

Photoactivation of TiO_2 and ZnO: tests of phototoxicity

Photo-excitation or activation of TiO_2 or ZnO nanoparticles is emphasized in the context of human safety suggesting that these materials exposed to sunlight would damage the skin instead of providing protect. To induce photo-mediated adverse skin reactions two properties must come together. First is photo-excitation which may include free radical formation and the second is a sufficient penetration of such particles through the skin barrier in order to reach the living epidermal cells, *i.e.* keratinocytes and certain immunocompetent dendritic cells. Only if both events come together will adverse inflammatory skin reactions be observed. Much of the concern regarding photoactivation/excitation arises from the photocatalytic activity of TiO_2, which is known to produce hydroxyl- and superoxide anion radicals after irradiation with sun light, much more so than ZnO.[35,36] This light-induced radical production is used to oxidize organic substances that might under normal conditions be only slowly decomposed and made water soluble.

The process of radical formation upon light exposure can be readily measured and studied using cell culture tests for identifying the phototoxic potential of materials that absorb UV light,[37,38] become excited and damage the cell system by multiple mechanisms such as generation of singlet oxygen, hydroxyl radials and superoxide anions or organic radicals, when the excited or activated material decomposes itself. All these reactive oxygen species (ROS) and radicals are under test conditions cytotoxic in a dose-dependent manner as shown in Fig. 5A for "uncoated", native TiO_2 where, under irradiation with an irradiation dose of 50 kJ m^{-2} UVA and about 1 kJ m^{-2} UVB, a clear phototoxic effect was seen at concentrations greater than 25 mg L^{-1} test

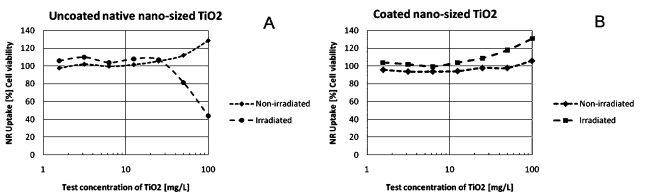

Fig. 5 (A) Light-induced cytotoxicity (phototoxicity) of uncoated native titanium dioxide in cell culture system according to OECD TG # 432 compared to the non-irradiated situation where no cytotoxicity was observed. Validation against human *in vivo* data revealed that test concentrations beyond 100 mg L^{-1} became increasingly irrelevant. Therefore the routine test condition used to stop at 100 mg L^{-1}. (unpublished data, W. Pape *et al.*, 2001). (B) Light-induced effects of coated titanium dioxide with inhibited surface structure in cell culture system according to OECD TG # 432. Neither the irradiated situation nor the non-irradiated situation reveal any cytotoxicity in the comparable range of test concentrations. (unpublished data, W. Pape *et al.*, 2001).

sample. In contrast, Fig. 5B illustrates the effect of coated TiO_2 as typically used in cosmetic sunscreen formulations and reveals no significant difference between the irradiated and the non-irradiated situations. The standardized test conditions are the same in both experiments.[39] These data clearly demonstrate that when uncoated TiO_2 is exposed to solar simulated UV a photocytotoxic effect is observed *in vitro*, most likely as a result of the generation of ROS. In contrast, coating of the same nano-sized material completely eliminated UV-induced photocytotoxic effects even at higher test concentrations.

For ZnO under such test conditions a typical concentration–response curve for cytotoxicity is observed. This may be due to the better solubility of the ZnO compared to TiO_2 in the test medium. No relevant difference in cytotoxicity has been found between irradiated and non-irradiated test condition for ZnO. Both curves were found in an identical concentration range (Fig. 6).

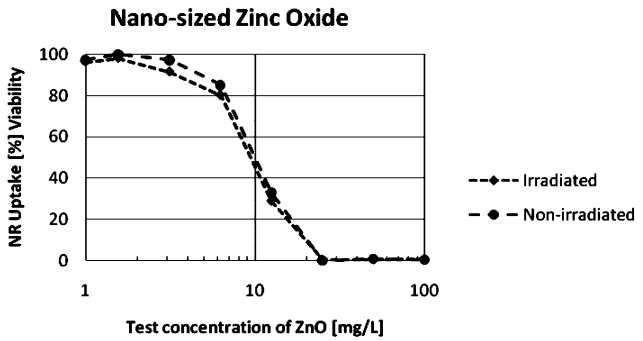

Fig. 6 Concentration–response curve of irradiated and non-irradiated test samples of zinc oxide and standard OECD TG # 432 test conditions. There is no significant light-induced effect.

In conclusion, neither nano-structured TiO_2 nor ZnO are phototoxic using the validated OECD TG 432 Neutral Red Uptake 3T3 phototoxicity test. As inorganic, stable UV filters for cosmetic sunscreen products, both materials are mostly used as hydrophobically-coated nano materials. This coating process in effect "poisons" any photocatalytic surface properties and inhibits or reduces the generation of ROS and radicals under sunlight exposure and activation, as shown by comparative *in vitro* testing under standardized conditions. The absence of photoactivation/excitation using this sensitive measure of photocytotoxicity diminishes if not eliminates concerns regarding sunlight activation of these nano materials and is supportive of the view that the coating remains stable even under exaggerated test conditions. Finally, it is likely that the experimental evidence is predictive of human experience since these nano metal oxides have been used for 20+ years in sunscreen products without any clinical evidence of phototoxicity.

Genotoxicity/photogenotoxicity

The genotoxicity and photogenotoxicity of nano TiO_2 and ZnO has been reviewed recently by Nohynek and colleagues.[40,41] Multiple studies with nano and pigmentary TiO_2 found a similar profile which was the absence of genotoxic or photo-genotoxic effects. No major differences in the hazard profile were observed for micro- and nano-structured particles, and no evidence was found suggesting that nano-structured particles pose greater or qualitatively new mutagenic/genotoxic hazards.

It is generally held that ZnO is not genotoxic. For zinc salts, *in vitro* genotoxicity has given mixed results largely due to excess Zn^{2+} ions. No genuine photo-genotoxicity of ZnO nano particles was observed in the study by Dufour *et al.*[40] In this study, ZnO was tested in an *in vitro* cytogenetics assay using duplicate cultures of chinese hamster ovary (CHO) cells in the presence and absence of UV. ZnO was investigated in the cells in the dark (D), under pre-irradiation (PI), *i.e.* UV irradiation of cells followed 1–3 h later by treatment with ZnO, and under simultaneous irradiation conditions (SI), *i.e.*, ZnO treatment concurrent with UV irradiation.

In PI cultures, cytotoxicity of ZnO was concentration-dependent and comparable at the low UV dose (3.5 kJ m^{-2}) to that observed in SI cultures (40–60% cytotoxicity was observed in the concentration range 104.9–256.0 mg L^{-1}). At the high UV dose (7 kJ m^{-2}) and at low ZnO concentrations (<204.8 mg L^{-1}), cytotoxic effects of ZnO in PI cultures were intermediate between those observed in the dark and SI cultures, with 40–60% cytotoxicity observed in the concentration range 131–256 mg L^{-1}. At higher concentrations (≥204.8 mg L^{-1}), ZnO cytotoxicity was similar in PI and SI cultures. For each individual irradiation dose and condition, the concentrations analyzed for CA (chromosome aberrations) covered a range of cytotoxicity from little or none to maximum effects.

Treatment of cultures with ZnO in the absence of UV light resulted in statistically significant increases in the frequencies of cells with structural aberration at 104.9 mg L^{-1} (giving 13% cytotoxicity, as measured by population doubling) and above. The frequencies of cells with structural aberrations (excluding gaps) exceeded the historical negative control (normal) range in both cultures analyzed at 163.8 mg L^{-1} and above, and also in single cultures analyzed at several lower concentrations. These observations were considered relevant and ZnO was considered clastogenic in the dark under these conditions. Under SI conditions, treatment with ZnO induced increases in structural aberrations at 104.9 mg L^{-1} (23% cytotoxicity) and 53.69 μg ml^{-1} (19% cytotoxicity) following 3.5 and 7.0 kJ m^{-2} UV radiation.

Under PI conditions, treatment with ZnO resulted in increases in the frequencies of cells with structural aberrations at 104.9 mg L^{-1} and above and at 53.69 mg L^{-1} and above following 3.5 and 7 kJ m^{-2} UV radiation, respectively. When compared at similar cytotoxic concentrations, the incidence of chromosome aberrations following PI or SI was generally similar at 7.0 kJ m^{-2}.

The proportion of cells with structural aberrations treated with the vehicle (negative control) fell within historical solvent control ranges. Both treatments with the positive controls induced increases in the proportion of cells with structural aberrations. When added to cultures treated in the absence of UV light, 8-MOP induced frequencies of cells with structural aberrations that were similar to those seen in concurrent solvent control cultures (non irradiated). Thus, the validity and sensitivity of the test system was demonstrated.

Thus, ZnO was clastogenic to CHO cells in the dark, and irradiation of the cellular test system, either prior to (PI conditions) or concurrently with (SI conditions) resulted in an increased susceptibility of CHO cells to ZnO, as indicated by clastogenic responses of higher magnitude and/or observed at

lower concentrations in both SI and PI conditions when compared to those obtained in the dark. Finally, the results provided evidence that, under conditions of *in vitro* photo-clastogenicity tests, UV irradiation of the cellular test system *per se* may produce an increase in the genotoxic potency of compounds that are clastogenic in the dark. Therefore, minor increases in clastogenic potency under conditions of photo-genotoxicity testing do not necessarily represent a photo-genotoxic effect, but may occur due to an increased sensitivity of the test system subsequent to UV irradiation.

In conclusion, these data consisting of studies following guideline requirements and good laboratory practice and covering different sunscreen-grade TiO_2 and ZnO particles, including micro- and nano-structured rutile and anatase crystalline forms, as well as coated and non-coated particles, support the human safety of these materials. The overall conclusion suggested a similar profile for all substances, which were all non-genotoxic or photo-genotoxic. No major difference in the hazard profile was observed for micro- and nano-structured particles, and no evidence was found suggesting that nano-structured particles pose greater or qualitatively new mutagenic/genotoxic hazards. As reports in the literature on the toxicological properties of "nano" TiO_2 and ZnO continue to appear and multiple, it is most probable that genotoxic/photogenotoxic concerns will arise. For toxicology to be relevant to human safety, care will need to be exercised in the area of standard methodology and interpretation.[42]

Summary

Nano TiO_2

There is a comprehensive data base on the toxicological profile of TiO_2 covering all relevant toxicological endpoints. The acute oral toxicity, both in coated and uncoated material and the acute dermal toxicity of uncoated material is very low. Irritation of the skin is low or absent, both in animals and humans for both coated and uncoated TiO_2. Irritation of mucous membranes is low or absent, both with coated and uncoated material. Sensitization in animals and humans was not found, using either coated or uncoated material.

Subchronic toxicity studies with uncoated nano-structured TiO_2 revealed low oral toxicity after repeated doses. Long-term feeding studies in rat and mouse models with uncoated material showed no evidence of a carcinogenic potential. However, inhalation studies in rats using micron-sized, uncoated material suggest an increase in the incidence of lung tumors, probably as a consequence of lung overload from exposure to high concentrations of inert dust to which rats may be particularly susceptible.

TiO_2 did not show photo-toxic activity in studies *in vivo* or *in vitro*, and no photo-sensitization or photo-irritation was observed. Numerous tests for mutagenicity, clastogenicity and photo-genotoxicity have been carried out, and consistently showed negative results.

Comprehensive *in vivo* and *in vitro* dermal penetration studies have been performed by industry, independent researchers and health authorities. A number of investigations of the skin penetration of TiO_2 nanoparticles were performed within the EU-funded NANODERM project. None of these studies or projects yielded any evidence that nanoparticulate TiO_2 is able to cross the skin barrier in intact as well as compromised skin. In a recent safety assessment of Nohynek *et al.*[41] it was stated that an international workshop on the safety of nanomaterials concluded that human dermal exposure to nanoparticles is of lesser concern. For example, a review by the German Federal Health Institute (BfR) on the safety of NP in sunscreens that concluded that:

…nanoparticles of titanium or zinc oxides did not penetrate through the stratum corneum. Nanoparticles are too large for a passive transport through the skin. Therefore a dermal absorption is improbable. Biological properties of nanoparticles are not necessarily different than those of larger particles. The toxicological properties of nanoparticles are determined by their water solubility and their persistence. Taking into account the results of available studies with nano-sized ZnO and TiO_2 in standard formulations, a health risk for the consumer is not expected.

A similar assessment was issued by the Australian health authorities who stated that "… the weight of current evidence is that TiO_2 and ZnO nanoparticles remain on the surface of the skin and in the outer dead layer (stratum corneum) of the skin".

Based on the comprehensive data base and after additional in-depth evaluation, there is no evidence that TiO_2 micro- or nano-sized particles pose a mutagenic/genotoxic, photo-toxic or photo-mutagenic/genotoxic risk to humans. On the contrary, there is robust evidence that these substances protect human skin against UV-induced adverse effects, including DNA damage and skin cancer. The toxicological profile of this material does not give rise to concerns of systemic toxicity since the substance is not absorbed through the skin. Given the long history of use, the hazard-based studies and the absence of dermal penetration, the human risk from exposure to products containing nano-structured TiO_2 is considered minimal.

Nano ZnO

There are comprehensive databases on the toxicological profile of zinc and ZnO covering all relevant toxicological endpoints.[43,44] The numerous risk assessments that have been prepared for zinc generally do not focus on particle size or any specific salt. This may not be surprising given that zinc is an essential mineral required for human life and that salts of zinc are in many commercial products. ZnO, which is sparing soluble, has unique dermatological properties including attenuation of solar UV.

The acute oral toxicity is very low. Irritation of the skin or of the mucous membranes is low or absent in animals and according to current EU classification requirements ZnO should not be considered to be a skin or eye irritant. Sensitization in animals was not found.

Numerous tests for mutagenicity, clastogenicity and photo-genotoxicity have been carried out *in vitro* and/or *in vivo*. No genotoxic or photogenotoxic potential was noted in bacterial gene mutation assays *in vitro*. ZnO showed some clastogenic activity in mammalian cells *in vitro* but there was clearly no indication for a clastogenic potential or an aneugenic activity *in vivo*. No genuine photo-genotoxicity of ZnO nanoparticles was observed when tested *in vitro* in mammalian cells but the available studies provided evidence that, under conditions of *in vitro* photo-clastogenicity tests, UV irradiation of the cellular test system *per se* may produce an increase in the genotoxic potency of compounds that are clastogenic in the dark. Therefore, increases in clastogenic

potency under conditions of photo-genotoxicity testing do not necessarily represent a photo-genotoxic effect, but may occur due to an increased sensitivity of the test system subsequent to UV irradiation.

Finally, there is no evidence that ZnO micro- or nano-structured particles pose a photo-toxic or photo-genotoxic risk to humans. On the contrary, one has to consider that there is robust evidence that this substance protects human skin against UV-induced adverse effects, including DNA damage and skin cancer.

Comprehensive *in vivo* and *in vitro* dermal penetration studies have been performed. None of these studies yielded any evidence that nanoparticulate ZnO is able to cross the skin barrier in intact as well as compromised skin. Thus, similar to TiO_2, as there is no evidence of exposure to nanoparticulate ZnO there is no safety concern for human use.

Conclusion

Taking into account the current weight of evidence from all available data, the risk for humans from the usage of nano-structured TiO_2 or ZnO currently used in cosmetic preparations or sunscreens is considered negligible. It is likely that such a definitive conclusion will not satisfy all interested stakeholders. This may be reflected best in numerous publications and reports where, in academic parlance, it is stated "more research is needed". This is a leaky bucket in the field of toxicology, since demonstration of no effect or proving a negative is an ongoing challenge. For "nano" TiO_2 and ZnO, there is an abundance of data, largely favorable, using standardized toxicological methods, *i.e.*, OECD, ICH, *etc.*, and a 20+ year history of human use without clinical anecdotes/case reports or documented adverse events, all of which support the safety of these metal oxides. Moreover, when viewed in the context of their UV protective properties when used in sunscreen products, the benefits would seem to outweigh any potential risk. General criteria for classes of materials, in this case "nano", are critically important. Much has been learned and a deeper appreciation for particle size is emerging. Nonetheless, assigning potential toxicity based on a single physical characteristic or because the field of nanotoxicology is in its infancy or since "more research is needed" may contaminate otherwise acceptable materials. "Nano" TiO_2 and ZnO are safe for human exposure and should be considered as such based on the entire data set.

Abbreviations

AAS	Atomic absorption spectrometry
NOS	Not/non-otherwise specified
ICPMS	Inductively coupled plasma mass spectrometer
TEM	Transmission electron microscopy
SEM	Scanning electron microscopy
LIFM	Laser-induced fluorescence measurements
SLRSM	Space-resolved laser scanning microscopy
SC	Stratum corneum
%	w/v
NP	Nanoparticle
STIM	Scanning ion transmission microscopy
PIXE	Particle induced X-ray emission
SCID	Severe combined immune deficiency
RBS	Rutherford backscattering
IBA	Ion beam analysis
PPS	Primary particle size
ICP-AES	Inductively coupled plasma–atomic emission spectrometry
ICP-MS	Inductively coupled plasma–mass spectrometry
ICP-OES	Inductively coupled plasma–optical emission spectrometry
GLP	Good laboratory practice
MPM	Multiphoton microscopy
EDX	Energy-dispersive X-ray
TOF-SIMS	Time-of-flight secondary ion mass spectrometry
SEM-EDX	Scanning electron microscopy-energy dispersive X-ray analysis

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