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Many modern cosmetic or sunscreen products contain nano-sized components. Nanoemulsions are transparent and have unique tactile and texture properties; nanocapsule, nanosome, noisome, or liposome formulations contain small vesicles (range: 50 to 5000 nm) consisting of traditional cosmetic materials that protect light- or oxygen-sensitive cosmetic ingredients. Transdermal delivery and cosmetic research suggests that vesicle materials may penetrate the stratum corneum (SC) of the human skin, but not into living skin. Depending on the physical/chemical properties of the ingredient and the formulation, nano-sized formulations may enhance or reduce skin penetration, albeit at a limited rate. Modern sunscreens contain insoluble titanium dioxide (TiO_2) or zinc oxide (ZnO) nanoparticles (NP), which are colorless and reflect/scatter ultraviolet (UV) more efficiently than larger particles. Most available theoretical and experimental evidence suggests that insoluble NP do not penetrate into or through normal as well as compromised human skin. Oral and topical toxicity data suggest that TiO_2 and ZnO NP have low systemic toxicity and are well tolerated on the skin. In vitro cytotoxicity, genotoxicity, and photogenotoxicity studies on TiO_2 or other insoluble NP reporting uptake by cells, oxidative cell damage, or genotoxicity should be interpreted with caution, since such toxicities may be secondary to phagocytosis of mammalian cells exposed to high concentrations of insoluble particles. Caution needs to be exercised concerning topical exposure to other NP that either have characteristics enabling some skin penetration and/or have inherently toxic constituents. Studies on wear debris particles from surgical implants and other toxicity studies on insoluble particles support the traditional toxicology view that the hazard of small particles is mainly defined by the intrinsic toxicity of particles, as distinct from their particle size. There is little evidence supporting the principle that smaller particles have greater effects on the skin or other tissues or produce novel toxicities relative to micro-sized materials. Overall, the current weight of evidence suggests that nano-materials such as nano-sized vesicles or TiO_2 and ZnO nanoparticles currently used in cosmetic preparations or sunscreens pose no risk to human skin or human health, although other NP may have properties that warrant safety evaluation on a case-by-case basis before human use.

Keywords CAS 1314-13-2, CAS 13463-67-7, Cosmetics, Dermal toxicity, Genotoxicity, Nanoparticles, Nanotechnology, Percutaneous penetration, Photogenotoxicity, Sunscreens, Risk Assessment, Titanium dioxide, Zinc oxide

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1. INTRODUCTION

Nanotechnology (NT) is a catch-all term for techniques, materials, and devices that operate at the nanometer scale. been defined as the design, characterization, production, and application of structures, devices, and systems by controlling shape and size at the nano-scale (Maynard, 2006), represents one of the most promising technologies of the 21 st century, and has been considered to be a new industrial revolution. Today, nanomaterials are increasingly used in sporting goods, tires, catalysts, electronic components, window sprays, paints, varnishes, coatings, foods, sunscreens, cosmetics, and antimicrobial and antifungal preparations and are expected to be increasingly applied to the medical field in diagnosis, imaging, and drug delivery (SCENIHR, 2005; Nel et al., 2006). The U. S. National Science Foundation estimated that the global market for NTs may reach $1 trillion or more within 20 years (Maynard, 2006).

The arrival of NT also initiated public debate about its potential risks. Michael Crichton's novel *Prey* (2002), a tale of rioting and runaway nanobots, popularized Eric Drexler's earlier *Grey Goo Theory* (1986), a hypothetical end-of-the-world nanotech-disaster scenario, in which out-of-control self-replicating nanorobots (*assemblers*) consume all living matter on Earth. Although *Grey Goo* seems more science fiction than science, the theory has raised concerns in the public and the media as well as prominent persons, such as the Prince of Wales, termed as *Royal Nanoangst* by the UK media (Telegraph, 2003). The fear of this new technology was neatly described by Ball (2003): *Overnight nanoscientists had become the new Frankensteins, modern Prometheuses, contemporary Fausts, dabbling with dangerous forces they cannot control*.

A source for concern may also arise from ignorance. Tsuji et al. (2006), for instance, suggest that the skin penetration and toxicological impact of nanoparticles is unclear but there is a potential for a range of local, chronic, metabolic, and photo-induced toxicities. These potential, albeit largely hypothetical, risks to human heath and the environment have resulted in several nongovernmental organizations (NGOs) demanding a ban or greater regulation of NT applications (Greenpeace, 2003; ICTA, 2006; FOE, 2006a). An active discussion about potential risks of NT has been undertaken by regulatory agencies and engaged scientists and has resulted in position papers from various regulatory bodies. Some toxicologists proposed that new properties of nano-materials may require novel approaches for their hazard assessment and a need for the new discipline of *nanotoxicology* (Oberdörster et al., 2005a, 2005b), whereas others argued that toxicology itself may become a new discipline of nanoscience (Kurath and Maasen, 2006). However, in the view of more traditional toxicologists, *the approaches and study protocols for routine toxicological characterization of chemicals are sufficiently robust to provide meaningful characterization of nanoscale materials* (NTP/NIEHS, 2004). During recent years, the potential risks of nano-materials to human health and the environment have been reviewed by numerous national and international expert groups, such as the U. S. NTP/NIEHS (2004), Royal Society of Engineering (2004), European Union (EU) SCENIHR (2005), U. S. Environmental Protection Agency (EPA) (2005a), German BfR (2006), Australian TGA (2006), French AFSSET (2006), Canadian IRRST (2006), and an expert group of the European Chemical Industry's ECETOC (Borm et al., 2006), as well as individual authors such as Hoet et al. (2004), Nel et al. (2006), Hardman (2006), and Maynard (2006). The general consensus of these reviews was that NTs may pose possible new risks, although the actual nature of these risks remains largely hypothetical.

Nanoparticles (NP) are a subset of nano-materials, and were defined as single particles with a diameter below 100 nm, although their agglomerates may be larger (Maynard, 2006). One of the largest applications of NP is their use in sunscreens where the NP diameter is normally more than 10 nm. The global production of NP for sunscreen products was estimated to be approximately 1000 tons during 2003/2004 (Borm et al., 2006), and principally consists of titanium dioxide (TiO_2) and zinc oxide (ZnO) particles. Today there is a broad consensus that the principal human health risk may be from inhalation of NP (ECETOC, 2005; Hoet et al., 2004; Maynard, 2006). Human cooking activities (e.g., toasters, oven cooking, frying) or other indoor emissions (e.g., candles) may be the most important sources for human exposure to airborne NP (Olson and Burke, 2006; Afshari et al., 2005). However, concerns have also been raised about potential dangers of the contact of NP with human skin. Recently, the Friends of the Earth warned against NT in cosmetic and sunscreen products, since they may produce a possible uptake of particles by human skin: *if nanoparticles penetrate the skin, they can join the bloodstream and circulate around the body with uptake by cells, tissues and organs* (FOE, 2006a). Possible human systemic exposure from topically applied NP has also been suggested in toxicological reviews by Hoet et al. (2004), Oberdörster et al. (2005a), and even the US EPA Draft White Paper (2005a), with the last hypothesizing that *nanoparticles may penetrate the skin and distribute throughout the body once translocated to the circulatory system*.

Overall, the key questions that must be asked for any NP applied to the skin is (1) what is the exposure, (2) is it absorbed and, if so, how much reaches the viable cells, and (3), if so, is it intrinsically toxic? More specific questions that may be raised concerning the safety of NT/NP in cosmetic products and sunscreens include the following:

- Do cosmetic formulations containing nano-sized features (vesicles or droplets) pose new risks when compared with those of traditional cosmetic products?
- Do nano-sized cosmetic formulations enhance the skin penetration of cosmetic ingredients, thereby increasing the risk of human skin sensitization or systemic exposure?
- Are insoluble NP in sunscreens intrinsically more hazardous than larger particles, i.e. micro-particles or bulk material?

- Do topically applied insoluble NP remain on the skin surface or are they able to pass the skin barrier of normal or compromised skin to gain access to systemic compartments of the organism?

To this end, we attempt to summarize the available information on the use of NTs in cosmetic and sunscreen products and to review the evidence on potential adverse effects of NP on or in the skin, including their potential to penetrate into or through human skin and/or to pose a risk of human systemic exposure and toxicity. In the first part of this article, we examine formulations containing NP and then examine the penetration of NP into and through the skin, addressing ZnO and TiO_2 penetration, follicular penetration, penetration of other NP, the effect of formulation, and the consequences of the skin integrity being compromised. In the second part, we consider intrinsic, cellular, and phototoxicity of NP, their ingredients, and their coatings. The third part examines in vivo considerations such as the likelihood of toxicity after systemic exposure, topical exposure, and the risk of sensitization. The final part attempts to objectively assess the risks and benefits of topical NP use.

2. NANOTECHNOLOGY AND NANOPARTICLES IN COSMETICS AND SUNSCREENS

2.1 Cosmetic Formulations Containing Nano-Sized Structures (Figure 1)

2.1.1 Micro- and Nanoemulsions

Nano-emulsions are commonly used in certain cosmetic products, such as conditioners or lotions to be applied to the skin

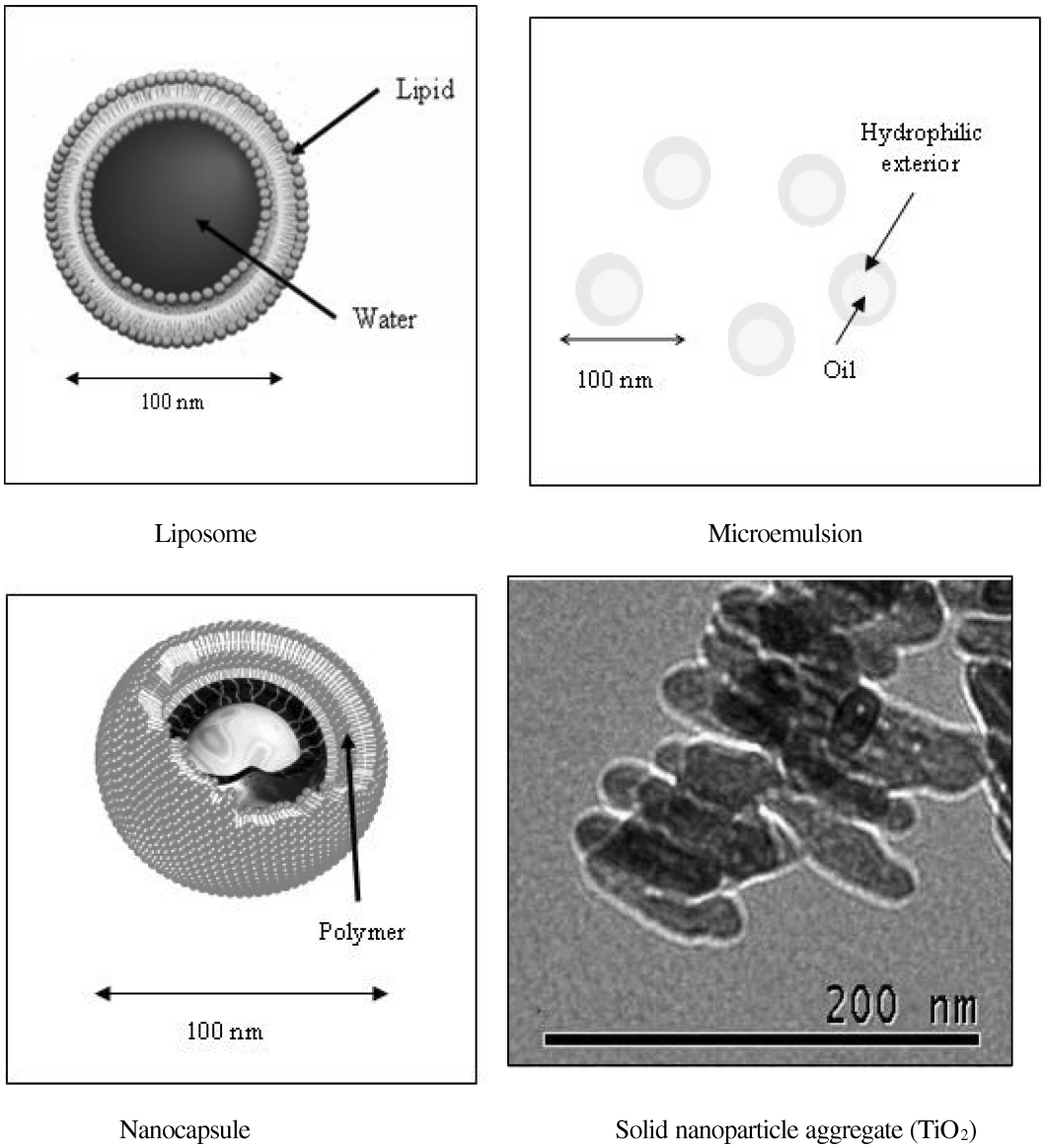


FIG. 1. Examples of nano-sized vesicles or particles in cosmetic formulations or sunscreens.

and hair (Figure 1). The optical, tactile, and texture properties of nano-emulsions make them highly attractive for cosmetic or consumer products. These emulsions combine traditional cosmetic ingredients, such as water, oils, and surfactants, to manufacture two-phase systems in which droplets of size 50 to 100 nm are dispersed in an external phase. The small droplet size enables nano-emulsions to flow easily, be transparent, and be pleasant to the touch. Their unique texture and rheological properties have yet to be obtained by other formulation methods (Sonneville-Aubrun et al., 2004). Nano-emulsions pose no different risk to consumers than traditional emulsions, as both are composed of water and oil droplets, and tend to break down into their constituent ingredients upon application to skin or hair (Van den Berg et al., 1999). Micro- or nano-emulsions can enhance skin penetration of some cosmetic or dermatological ingredients but usually less than that seen with these ingredients in solution (Lehmann et al., 2001; Kreilgard, 2002; Korting et al., 1990; Kogan and Garti, 2006).

2.1.2 Liposomes, Niosomes, Nanosomes, and Nanocapsules

Liposomes and niosomes are globular vesicles with diameters between 25 and 5000 nm. Such vesicles are composed of suitable amphiphilic molecules that associate as a double layer (unilamellar vesicles) or two to four double layers (multilamellar vesicles). In general, the outer and the inner layers of vesicles have a hydrophilic character (Figure 1). Liposomes and niosomes may incorporate hydrophilic or lipophilic substances or drugs. Liposomes are mainly generated from phospholipids; niosomes are composed of nonionic surfactants, such as polyoxyethylene alkyl ethers or -esters (Junginger et al., 1991). Vesicles materials mainly consist of phospholipids, sphingolipids, or ceramides; due to their lipophilic interior they have an enhanced capacity to enclose lipophilic substances (Castor, 2005; Brunke and Charlet, 1991). The ultrastructure of some of these vesicles is quite similar to that of mammalian milk, which contains nano-sized fat droplets surrounded by the milk fat globular membrane (Lopez, 2005). Rigid nano- or microcapsules have rigid walls consisting of sucrose esters, cholesterol or cholesterol sulfate, or biodegradable polymers, such as polycaprolactone (Van den Bergh et al., 1999; Alvarez-Roman et al., 2001).

Vesicle formulations are produced from traditional cosmetic ingredients by techniques such as coacervation or phase separation. These formulations are important in cosmetic applications because they may improve the stability and, possibly, tolerance of ingredients such as unsaturated fatty acids, vitamins, or antioxidants that are encapsulated within the vesicle, but also the efficacy and tolerance of ultraviolet (UV) filters on the skin surface (Padamwar and Pokharkar, 2006; Alvarez-Roman et al., 2001). When applied on skin, vesicles tend to break down into their constituent ingredients, which tend to remain in the upper layers of the stratum corneum (Junginger et al., 1991; Van den Bergh et al., 1999; Choi and Maibach, 2005).

2.2. Insoluble, Mineral-Based Nanoparticles in Sunscreens

Modern sunscreens contain insoluble, mineral-based materials whose performance depends on their particle size. Mineral particles, such as TiO_2, reflect and scatter UV light most efficiently at a size of 60 to 120 nm (Popov et al., 2005), whereas ZnO has an optimal size of 20–30 nm (Cross et al., 2007; Figures 1 and 3). As such particles scatter UV and not visible light, the resulting sunscreen appears to be clear. Sunscreen-grade nano-sized TiO_2 ranges from an ultrafine particle form with a diameter of 14 nm to micro-sized aggregates (Table 1). ZnO is generally used in the form of particles at 30–200 nm in diameter. The surface of these mineral particles is frequently treated with inert coating materials, such as silicon oils, SiO_2 or Al_2O_3, in order to improve their dispersion in sunscreen formulations (see Table 1; SCCNFP, 2000).

Sunscreen products containing mineral UV filters protect consumers from the harmful effects of UV exposure, including skin aging, skin and lip cancers, and herpes labialis (Pogoda and Preston-Martin, 1996; Nohynek and Schaefer, 2003). Consequently, dermatological associations and national or international health authorities strongly recommend the application of sunscreens before sun exposure (WHO, 1998). Given that nano-sized particles of titanium or zinc oxides are transparent, these UV filters are not only more efficient, but also result in better consumer acceptance and, ultimately, improve the protection of human skin against UV-induced damage. Interestingly, it has recently been shown that lead-based traditional hair dyes that have been used since the Greco-Roman period produce their darkening of gray hair by formation of lead sulfide NP (size about 5 nm) on the surface of the hair (Walter et al., 2006). Thus, the use of and human exposure to cosmetic-derived NP appears to have a history of more than 3000 years.

3. LOCAL AND SYSTEMIC EXPOSURE FOLLOWING DERMAL APPLICATION OF NANOMATERIALS

3.1 General

Mammalian skin is structured in several layers: the stratum corneum (SC), epidermis, dermis, and the subcutaneous layer. For most substances, the SC is the rate-limiting barrier against the percutaneous penetration of topically applied substances (Schaefer et al., 2003). The rate of skin absorption and penetration of cosmetic ingredients may be measured in vivo or in vitro under realistic conditions of product use. However, in vitro*and* in vivo skin absorption data should be interpreted with caution. Currently used in vitro models, such as pig and human skin, may yield comparable results, provided the total quantity of ingredients present in skin sample is taken into consideration. However, the distribution of a substance in the different compartments may vary due to difference in the relative thicknesses of the SC, epidermis, and dermis of human and pig skin. Differences in relative distribution between pig and human skin may be large or small depending on the substance under evaluation

TABLE 1
Commercial coated and noncoated sunscreen-grade titanium dioxide particles: Results of in vitro phototoxicity, genotoxicity, and photo-genotoxicity tests (unpublished data included in the industry safety dossier, summarized in the EU SCCNFP opinion on TiO_2; SCCNFP, 2000)

Product name	Crystalline form[a]	Coating material	Particle size (nm)	Test[b]	Results
T805	RU/AN	SiO_2	21	Ames, photo-Ames, CA, photo-CA, NRU	All negative
T817	RU/AN	SiO_2/FeO	21	Ames, photo-Ames, CA, photo-CA, NRU	All negative
EUSOLEX 2000	RU	Al_2O_3	14	Photo-Ames, Photo-CA	All negative
M262, M212, M160, X161	RU	Al_2O_3/stearic acid	15–20	Ames, Photo-Ames, CA, Photo-CA	All negative
MT-100TV	RU	Al_2O_3/stearic acid	15	Ames, Photo-Ames, CA, Photo-CA, NRU	All negative
MT-100TV	RU	Noncoated	15	Ames, Photo-Ames, CA, Photo-CA, NRU	All negative
X-200	RU	Noncoated	20	Ames, Photo-Ames, CA, Photo-CA, NRU	All negative
SOLAVEIL	RU	Al_2O_3/SiO_2	11–28	CA, Photo-CA,	Negative
MIRASUN TiW60	AN	Al_2O_3/SiO_2	60	Ames, Photo-Ames, CA, Photo-CA	All negative
AFDC	AN	Noncoated	200,000 [c]	Ames, Photo-Ames, CA, Photo-CA, NRU	All negative
MIRASUN TiWGO	AN	Noncoated	60	Ames, Photo-Ames, CA, Photo-CA	All negative

[a] AN = anatase; RU = rutile.
[b] CA = chromosome aberration test in mammalian cells; standard photo-genotoxicity tests included test under dark conditions; NRU = neutral red uptake phototoxicity test.
[c] Pigment-grade TiO_2.

(Diembeck et al., 1999). The proportion of the substance absorbed or penetrated may also vary as a function of the quantity applied. This variability depends on the nature of the substance under evaluation and is not easily predicted. For this reason, it is crucial to use experimental protocols where the quantity applied is representative of the actual use conditions of the product.

Experimental protocols that produce partial or total occlusion tend to favor the penetration of substances into the SC by increasing its level of hydration, which reduces its barrier function (Zhai and Maibach, 2001). Therefore, when determining the localization of a substance in an in vitro absorption model, it is important to consider swelling phenomena that may produce artifacts.

Some percutaneous penetration studies used isolated human or animal epidermis or stratum corneum (SC), which are available in the form of thin and fragile films, although such materials will yield higher absorption or penetration rates than full-thickness skin (Surber et al., 1990; Potts and Guy, 1992). Similar to occlusion, immersion may produce substantial swelling of the SC; therefore, substances may penetrate into spaces between swollen corneocytes, whereas skin absorption may be low or absent after topical application of substance to intact skin (Zhai and Maibach, 2001). Destructive methods for studying the distribution of a substance in the skin and stripping of the SC can also lead to artifacts in that hair follicle or skin furrow material may be assayed together with the epidermis or the living skin. In reality, particles readily penetrate into the hair follicle opening (its *ostium*), which is several tens of micrometers deep. Sectioning or stripping of the epidermis by heat treatment may result in material stored in hair follicles or skin furrows being collected together with sections of the epidermis so that a potential incorrect conclusion that may be reached that a substance had penetrated into the epidermis. In vitro, NP or micro-particles are known to aggregate in the hair follicle ostium or skin furrows without further penetrating into or through the living skin (Lademann et al., 1999; Mavon et al., 2007). We have used multiphoton and confocal microscopy to confirm that many NP > 15 nm do not penetrate the human stratum corneum beyond the superficial layers (M. Roberts, unpublished observations, 2006).

In vivo skin penetration studies are often carried out in a range of rodent or farm animals as well as in human subjects (Schaefer and Redelmeier, 1996; Walters and Roberts, 1993). Interpretation of in vivo skin penetration studies between different species must be undertaken with caution as the permeability can vary widely depending both on the nature of the species and of the compound being studied. In general, the penetration of rabbit skin > rat > pig > monkey > human, with the pig being about 4 times or more and the rat up about 9 times more

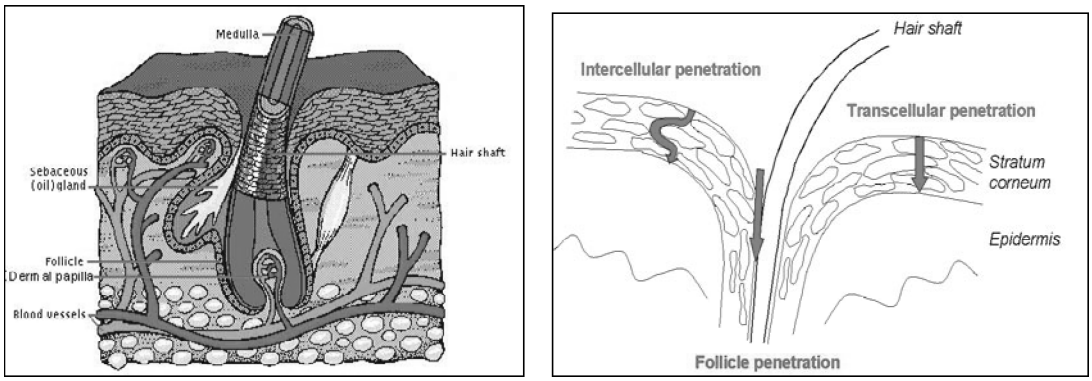


FIG. 2. Section of the hair follicle and routes of possible penetration pathways of externally applied substances into and through skin (adapted from Lademann et al., 1999, 2006).

permeable than human skin for certain compounds (Magnusson et al., 2001).

3.2 Passive Penetration of Insoluble ZnO and TiO_2 NP Into or Through the Skin (Figure 2)

Skin penetration studies on TiO_2 and ZnO micro- or nanoparticles are summarized in Table 2. The maximum flux into or through the skin of molecules in solution or as pure solutions falls exponentially with molecular weight to have an upper limit of 10^{-12} mol/cm^2/h at 800 Da (Magnusson et al., 2004). Brown et al. (2006) have suggested that solutes that are >500 Da, have high melting points and have insufficient amphiphilicity show little or negligible tendency for passive penetration into or through intact human skin. Simulations show that a solute with a molecular volume equivalent to insoluble NP and that is one to two orders of magnitude larger than a solute with a molecular mass of ~500 Da results in epidermal concentrations of the order of 10^{-18} nmol/ml after a safety factor of 100 is included and becomes even lower when desquamation is taken into account (Roberts, 2006). Accordingly, based on the available skin permeability data, it is predicted that there will be no passive penetration of solid, insoluble particles into or through the skin. This absence of skin penetration has also been corroborated by numerous experimental data, summarized later in this article. However, a number of exceptions have also been reported.

The penetration of micro-fine zinc and titanium oxide particles into animal and human skin appears to be the most studied of all NP. Most studies have reported that NP applied to the skin only penetrate into hair follicle openings and skin furrows, with minimal material being found below the stratum corneum surface. Landsdown and Taylor (1997) measured the penetration of micro-fine zinc and titanium oxide particles into rabbit skin. Most of the applied material remained on the skin surface, the outer layers of the SC, or the outer aspects of the hair follicles, and no deposits were found in deeper aspects of the epidermis or the dermis. Another study on the skin penetration of nano-sized, sunscreen-grade TiO_2 and ZnO particles using transmission and scanning electron microscopy as well as x-ray diffraction techniques detected particles only on the surface of the stratum corneum. No skin or intracellular penetration of particles was found (Dussert and Gooris, 1997). A series of in vivo and in vitro studies were performed on the percutaneous penetration of nano-sized TiO_2 pigments. The results of more than 10 different published or unpublished studies in vitro (human and pig skin) or in vivo (rat model, or biopsies of human skin) were summarized in a relatively recent opinion of the EU Scientific Committee on Cosmetics and Non-Food Products (SCCNFP) on TiO_2 (2000). All studies included in the SCCNFP opinion as well as published investigations concluded that micro- or nano-sized TiO_2 particles remain on the skin surface or the outer layers of the SC and do not penetrate into or through the living skin (Tan et al., 1996; Pflücker et al., 2001; Lademann et al., 1999; Schulz et al., 2002; Gontier et al., 2004; Gamer et al., 2006). Gottbrath and Müller-Goymann (2003) showed that the penetration of TiO_2 (20 nm and 100 nm) into human stratum corneum, studied by tape stripping, was limited to the surface and valleys between the corneocytes. In contrast, Menzel et al. (2004) suggested that TiO_2 applied in formulations to pig skin penetrates into the stratum granulosum but not the stratum spinosum. Penetration via the follicles was discounted. The very recent study by Mavon et al. (2007) on the percutaneous absorption of TiO_2 NP (20 nm) in a sunscreen formulation in human skin in vitro as well as in human subjects showed that penetration was limited to the upper layer of the stratum corneum and confirmed once again that these particles do not penetrate into or through living human skin. In addition, the study revealed that small amounts of particles may be found in the epidermal compartment in vitro, which corresponds to sunscreen located in the skin furrows or the infundibulum, but not in the living epidermis.

Similarly, as shown in recent in vitro percutaneous penetration studies, ZnO NP showed negligible penetration into pig (Gamer et al., 2006) and human skin (Cross et al., 2006). These findings confirmed the results of a number in vitro or in vivo percutaneous penetration studies on ZnO particles that were reviewed in the recent SCCNFP opinion (2003a). None of these

TABLE 2
Overview of TiO_2 and ZnO skin absorption/penetration studies

Test material	Particle size	Skin model/technique	Results	Reference
TiO_2 (No information on particle coating)	Unknown (microfine)	Human skin, in vitro	No significant penetration into living skin	Tan et al., 1996
ZnO (No information on particle coating)	Unknown (microfine)	Human skin, in vitro	0.34% absorbed after 72 h	Pirot et al., 1996
TiO_2 and ZnO (No information on particle coating)	<2 to 20 μm	Rabbit, in vivo	Penetration of particles into stratum corneum and outer hair follicle; no penetration into living skin	Landsdown and Taylor, 1997
TiO_2 and ZnO (no information on coating)	TiO_2: 50 to 100 nm ZnO: 20 to 200 nm	Human skin, in vitro	Penetration limited to upper layers of stratum corneum	Dussert and Gooris, 1997
TiO_2, Al_2 O_3-/stearic acid coated	150 to 170 nm	Human subjects (biopsy)	Particles on and in the upper layers of stratum corneum. About 1% of particles in the ostium of the follicle. No penetration into living skin.	Lademann et al., 1999
Various TiO_2, anatase and rutile, coated and uncoated materials	14 nm to 200 μm	Pig and human skin in vitro, human subjects (skin stripping or biopsy)	No penetration beyond the stratum corneum in any study.	EU SCCNFP Opinion, 2000
TiO_2 (SiO_2-, Al_2O_3-, Al_2O_3, + SiO_2-coated)	10 to 100 nm	Human skin, in vitro	Penetration of particles into the upper layers of stratum corneum.No penetration into living skin.	Pflücker et al., 2001
TiO_2 (SiO_2-, Al_2O_3-, Al_2O_3,/SiO_2-coated)	10 to 100 nm	Human subjects (biopsy)	Particles on or in the outmost surface of the stratum corneum. No penetration into living skin.	Schulz et al., 2002
TiO_2-containing sunscreen	Not specified	Human subjects (tape stripping)	Particles on or in the outmost layers of the stratum corneum. No penetration into living skin.	Gottbrath and Müller-Goymann, 2003
ZnO, various particle sizes	Not specified	Human normal and psoriatic subjects (Zn plasma levels after topical treatment); in vitro pig skin	No increase in plasma levels. In vitro, penetration was <1% of applied dose. Most ZnO was recovered in the stratum corneum.	EU SCCNFO Opinion, 2003a

(Continued on next page)

TABLE 2
Overview of TiO_2 and ZnO skin absorption/penetration studies (*Continued*)

Test material	Particle size	Skin model/technique	Results	Reference
Al_2O_3, ZnO, TiO_2	Nanoparticles (size not specified)	Mouse, pig and human skin in vitro	TiO_2 detected in the intercellular spaced between corneocytes of the outermost layers of the stratum corneum. No penetration into living skin.	Gontier et al., 2004
TiO_2in various formulations (no information on coating)	Needles: 45 to 150 nm × 17 to 35	Pig skin, in vitro	Particles on/in the stratum corneum; minimal penetration into stratum granulosum. No penetration into living skin.	Menzel et al., 2004
ZnO	15 to 30 nm	Human skin, in vitro	Less than 0.03% of applied Zn recovered in the receptor solution, no particles detected in epidermis or dermis.	Cross et al., 2006
TiO_2 (SiO_2- or dimethicon-coated) and ZnO (uncoated)	TiO_2: 30 to 60 nm ZnO: <160 nm	Pig skin,in vitro	No penetration beyond stratum corneum. Receptor solution recoveries of 0.8–1.4% of applied dose.	Gamer et al., 2006
TiO_2 in a sunscreen formulation, silicone-coated	Particles: 20 nm	Human skin in vitro and human subjects, skin stripping, TEM, backscattering spectrometry	Penetration limited to upper layers of stratum corneum. NP in skin furrows or follicular opening may be mistaken to be in the epidermal compartment.	Mavon et al., 2007

studies suggested significant penetration into or through living human or animal skin. However, although TiO_2 NP can have particle diameters as low as 10 nm, ZnO NP are typically >20 nm in order to have transparency in visible light (Figure 3).

3.3 Penetration of NP Into the Stratum Corneum and Follicular Penetration

Hair follicles represent a potential target for transdermal drug delivery, since they are surrounded by a tight network of capillaries which may be an important target for drug uptake (Figure 2). However, the presence of TiO_2 NP in the hair follicle reported by Lademann et al. (1999, 2001) has frequently been misquoted or misinterpreted as penetration into the living skin, although the particles remained outside the living epidermis or dermis were shown be eliminated by sebum flow. Lademann et al. (1999) and Weigmann et al. (1999), in studying the penetration of sunscreen-grade TiO_2 nanoparticles into the skin by means of tape stripping, showed that particles were mainly located on the skin surface and in the lipid layers around the corneocytes of the first cell layers of the stratum corneum (Figure 4). In deeper parts of the stratum corneum, TiO_2 was absent. Significant amounts of TiO_2 particles were detected in the orifices of hair follicles; their presence was confirmed by skin biopsy in human subjects. Interestingly, not all hair follicle orifices appeared to act as a reservoir for topically applied nanoparticles, there were *open* or *closed* follicle orifices (Lademann et al., 1999).

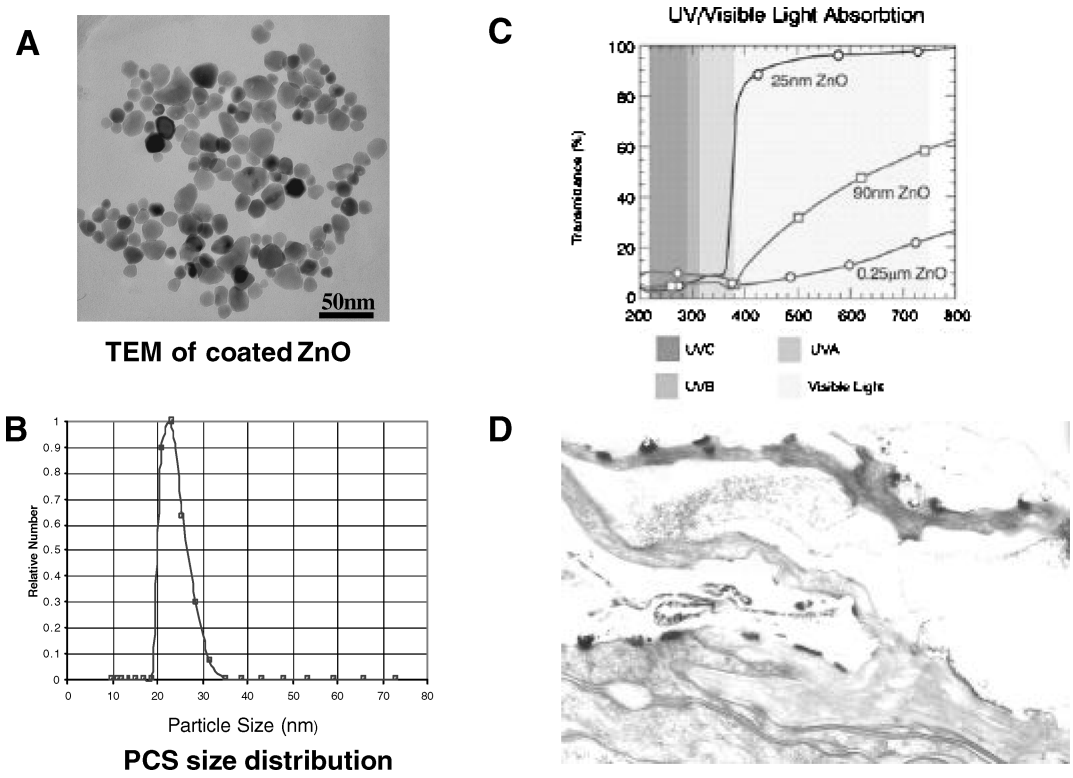


FIG. 3. ZnO nanoparticles and topical absorption: (A) TEM of coated particles, (B) PCS size distribution of micronized particles, (C) spectral transmittance of ZnO particles in aqueous solution, and (D) TEM showing ZnO particles present on the skin surface and around desquamating corneocytes (no penetration into the underlying stratum corneum was observed (from Cross et al., 2006).

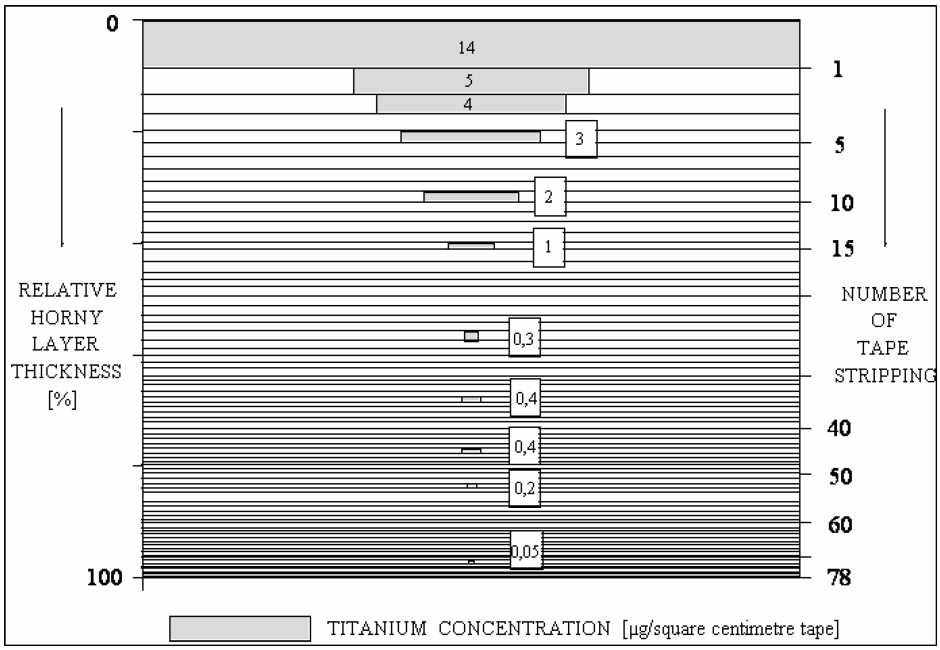


FIG. 4. TiO_2 penetration into the stratum corneum of human subjects after in vivo sunscreen application for 4 days (adapted from Lademann, 1999).

Later it was shown that the phenomenon of open and closed follicle orifices is not limited to particles; it may be relevant for many topically applied nonparticular substances (Otberg et al., 2004). Results of subsequent investigations suggested that hair follicle orifices are open during sebum production and/or active hair growth (Lademann et al., 2001). The phenomenon of closed follicles may be due to a mixture of desquamated corneocytes and dry sebum, which form a protective cover during the resting phase of the follicle. This cover is opened by sebum flow or hair growth and may easily be removed by washing or skin peeling (Otberg et al., 2004).

Collection of topically applied particles or substances in the *follicular sink* does not mean that they pass the skin barrier. Insoluble particles collected in the follicular orifices do not penetrate into the living epidermis, but remain in the follicular orifices for some time and are slowly removed by sebum flow. There may be an optimal size of particles concerning their collection and storage in the follicular orifice. For example, Toll, et al. (2004) investigated the tendency of particles at different sizes (600 to 2,500 nm) to collect in the follicular orifice of hair follicles of excised human skin. They found that the smallest particles (600 nm) had the greatest tendency for follicular storage.

Teichmann et al. (2006) compared in pig-ear skin the penetration and storage of the fluorescent dye fluorescein in a particle (320 nm) formulation as well as in solution. The dye formulations were applied with and without massage, which was performed using a commercial massage applicator for 1 min following the application of the formulations. In the absence of massage, an identical penetration depth of the particle and nonparticle formulations was observed. However, subsequent to massage, particles moved five times deeper into the hair follicles when compared with the depth of penetration of the dye solution. It was suggested that the cuticula of moving hair shafts could act like a geared pump, pushing the particles deeper into the hair follicles by mechanical force. This process seems to be particularly efficient when the size of applied particles is in the same order of magnitude as the cuticula of human hair, that is, 400 to 700 nm. Interestingly, nano-sized particles ($<$100 nm) showed no tendency of enhanced movement into the follicular orifice. Although massage resulted in a greater depth of particle distribution into the follicular orifice, the particles remained outside the living epidermis.

Lademann et al. (2007) repeated the experiments with fluorescein in a particle formulation and in solution in human skin using differential cyanoacrylate skin stripping (Teichmann et al., 2006), which allows a noninvasive removal of the hair follicle content (Figure 5). After the follicle contents were removed by cyanoacrylate surface biopsies, the amount of nanoparticles in the tape strips and the cyanoacrylate biopsies was determined and storage of the particles in the stratum corneum and the hair follicle orifice was compared. The results suggested that particles located in the stratum corneum were nearly quantitatively removed after one day, whereas particles in the hair follicles remained for more than 10 days. Comparison of the storage of particle- and solution-based formulations in hair follicle orifices showed quantitative disappearance of the dye solution after 6 days, whereas dye particles remained in the hair follicles for more than 10 days before being eliminated.

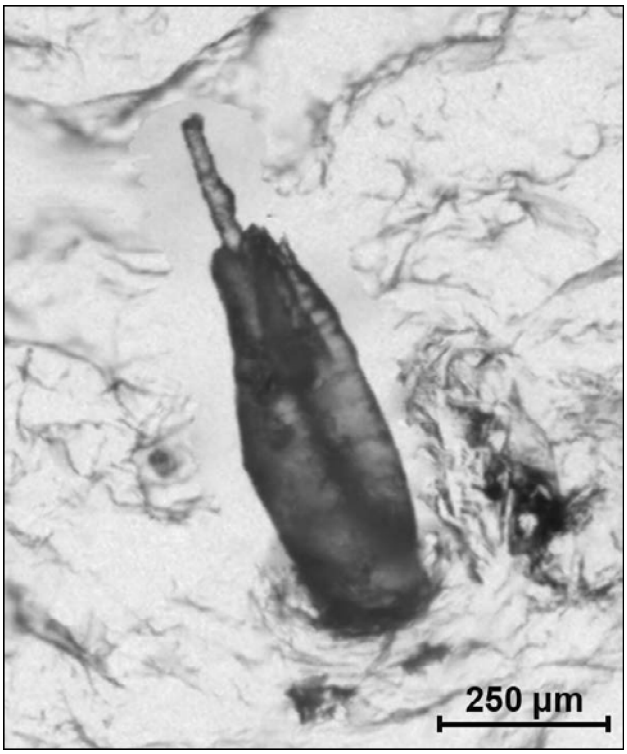


FIG. 5. Hair follicle content removed with a cyanoacrylate biopsy (laser scanning microscopy). Adapted from Lademann et al. (2006).

Summarizing the current knowledge on the follicular sink, it could be established that the orifice of hair follicles may represent a long-term reservoir for topically applied substances and particles. Dermal application of substances targeted for follicular orifices may open new routes in drug delivery. However, although insoluble NP, such as TiO_2, were shown to be present in the hair follicle orifices, they remained outside the living skin and no evidence for local (living skin) or systemic exposure via follicular penetration was found.

3.4 Penetration of Other NP Into the Skin

It has been argued that there is some evidence suggesting that nanoparticles may penetrate into or through human skin (Hoet et al., 2004; Oberdörster, 2005a). However, most of this work has been undertaken using NP other than ZnO or TiO_2 and, in general, equivocal results have been found. For instance, on application of covalently bound, fluorescent nanocapsules to pig skin, fluorescence was detected in open follicles and in furrows of the skin, but no penetration of these particles into the living epidermis or dermis was found (Alvarez-Roman et al., 2004a, 2004b), and Stracke et al. (2006) found no penetration

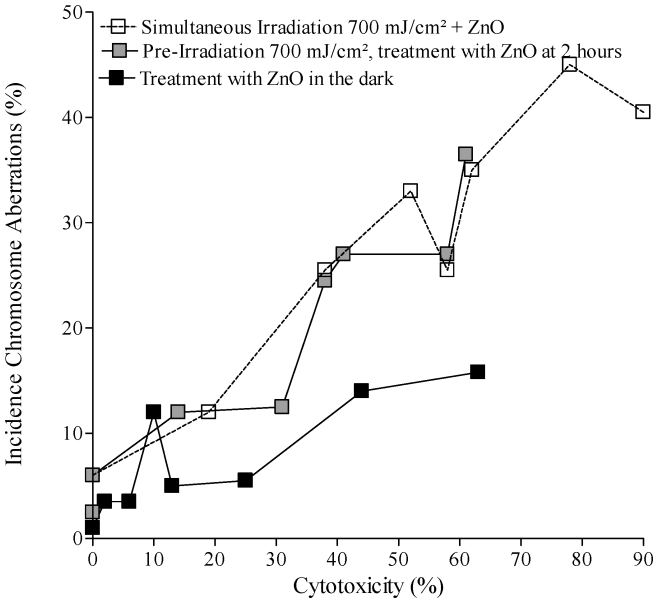


FIG. 6. Incidence (%) of chromosome aberrations in in vitro photo-genotoxicity tests on ZnO (particle size: <200 nm) in Chinese hamster ovary (CHO) cells relative to ZnO-induced cytotoxicity: (a) in the dark, (b) after irradiation with UV at 700 mJ and simultaneous treatment of cells with ZnO (standard photo-genotoxicity protocol), and (c) after pre-irradiation of cells with UV at 700 mJ, followed by treatment with ZnO in the dark (from Dufour et al., 2006).

of insoluble, polymeric nanoparticles into or through the living skin. Gopee et al. (2006) suggested that quantum dots only penetrated into intact mouse skin after dermabrasion (removal of the entire SC by tape-stripping). In contrast, Ryman-Rasmussen et al. (2006) suggested that quantum dots (QD; semi-conductor nanocrystals, spherical and ellipsoid shape, particle size 4.6 or 12 nm) with neutral or cationic coatings may penetrate into the epidermis or dermis of intact porcine skin, whereas QD with anionic coating penetrated to a small extent into the epidermis after 24 h of exposure. However, these studies were conducted with the QDs being applied in quite alkaline solutions.

Another study frequently quoted as evidence for particle penetration into the skin actually described presence of soil particles (0.4–0.5 μm) in the dermis of a limited number of patients with endemic elephantiasis (affecting their feet) who have walked barefoot in African rift valleys and elsewhere (Corcachan et al., 1988; Blundell et al., 1989). It is likely that chronic exposure, pressure, excessive skin hydration arising from the underlying edema, and impaired skin permeability may have contributed to skin penetration.

A number of studies have suggested that penetration of NP through pig skin may be dependent on the surface charge and size of the particles. Kohli and Alpar (2004) reported that negatively charged 50-nm and 500-nm fluorescent particles permeated pig skin, but that negatively charged 100- or 200-nm particles did not, and also that neutral and positively charge particles did not penetrate. Shim et al. (2004) showed that neutral PEG coated 40-nm NP penetrated into the epidermis of hairless guinea pig skin. The earlier quoted work of Ryman-Rasmussen et al. (2006) reported that quantum dots could not be detected in the perfusate of dermatomed pig skin mounted in flow through diffusion cells. However, significant penetration of neutral, positively charged, and negatively charged nanoparticles (diameters: spherical 4.6 nm, ellipsoid 6 × 12 nm) into the epidermis and, for cations, also into the dermis was found. While these findings suggest that very small NP may have a capacity for passive penetration into intact skin, the relevance of pig skin and of the alkaline pHs used to defining likely human exposure is not clear. Accordingly, these data require confirmation in humans using in-use conditions.

It has been suggested that NP after penetrating the skin may be taken up by local lymph nodes and transported into the blood circulation; a hypothetical uptake of NP by skin nerves or sweat glands was also suggested (Oberdörster, 2005a). While it is likely that NP administered directly to the viable epidermis or dermis will be redistributed to the lymph nodes, it is noted that small, inert surgical implant wear debris particles tend to remain localized and result in little, if any, systemic exposure (see Section 4.1). Further, nano- or micro-particles in NP- or nano-vesicle-containing intravenous formulations are rapidly cleared from the circulation by the so-called *phagocytic barrier*, that is, monocytes in the blood, macrophages in the spleen, or Kupffer cells in the liver (Moghimi et al., 2001).

Overall, although a gray zone may exist concerning the passive skin penetration capacity of extremely small NP with sizes comparable to that of large molecules, the current evidence indicates that the far larger and insoluble NP used in sunscreens do not show significant skin penetration or systemic exposure. Given the work of Ryman-Rasmussen et al (2006), more research is needed on the passive dermal absorption of small (<10 nm) NP, such as quantum dots across human skin under appropriate exposure conditions.

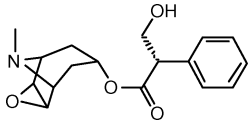
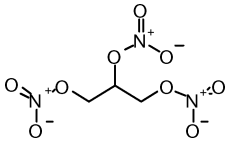
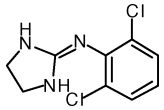
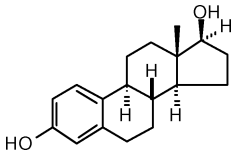
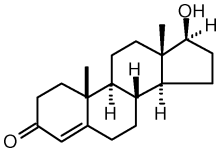
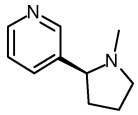
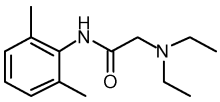
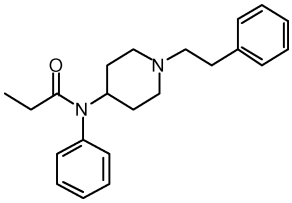
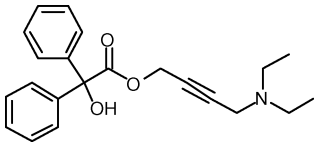
3.5 Skin Penetration from Nano-Sized Vesicle-Type or Other Formulations (Nanosomes, Liposomes, Niosomes, Nanoemulsions, Nanocapsules, Solid Lipid Nanoparticles)

Most of our knowledge on the skin penetration of ingredients in vesicle-type and other nano- or micro-sized formulations has been gained by the research on transdermal drug delivery (TDD) techniques. TDD has many advantages over traditional routes of drug administration, including avoidance of drug inactivation in the gastrointestinal tract, hepatic first-pass effects, and providing continuous systemic delivery. TDD systems include passive (skin penetration by diffusion alone) and active systems (drug delivery enhanced by application of external energy).

In 2004, about 35 passive TDD preparations, all of them used under occlusive patches, were approved in the United States or the EU for a wide variety of conditions, including hypertension, motion sickness, postmenopausal problems, and, recently, contraception and urinary incontinence (Thomas and Finnin, 2004).

Examples of drug substances that are currently used in passive TDD systems are shown in Table 3. During recent decades, huge TDD research efforts investigated drug formulations to enhance or facilitate the percutaneous penetration of topically applied substances. Skin penetration enhancement techniques used in TDD were recently reviewed (Benson, 2005). Drug formulations containing nano- or micro-sized vesicles, such as emulsions, liposomes, niosomes, or nanosomes, have been the object of intensive research, since they were considered to be highly promising to carry drugs into or through human skin. TDD using novel

TABLE 3
Examples of drug substances used in commercial passive transdermal drug delivery systems (occlusive patches), molecular weight, drug structures, and calculated molecular sizes (adapted from Benson, 2005)

Drug	Mol. weight	Structure	Molecular size[a] (nm)	Therapeutic use
Scopolamine	303.36		1.103	Motion sickness
Nitroglycerin	227.09		0.745	Angina
Clonidine	230.10		0.886	Hypertension
Estradiol	272.39		1.233	Postmenopausal symptoms
Testosterone	288.43		1.170	Male hypogonadism
Nicotine	162.23		0.810	Smoking cessation
Lidocaine	234.34		1.174	Postherpetic neuralgia
Fentanyl	336.47		1.630	Pain management
Oxybutynin	357.49		1.569	Urinary incontinence

[a]Distance in angstroms (1 nm = 10 Å) between the two farthest atoms of the molecule, calculated with the molecular modeling software SYBYL (Tripos, Inc.) after geometry optimization with the Tripos force field (SYBYL 7.1, Tripos, Inc., St. Louis, MO).

formulations and techniques resulted in numerous innovative applications and indications of traditional drug substances, which has recently been termed a *transdermal revolution* (Thomas and Finnin, 2004).

An in-depth review on the TDD of various lipophilic and hydrophilic drug substances (retinoic acid, 5-fluorouracil, triptolide, ascorbic acid, diclofenac, lidocaine, prilocaine) formulated as different microemulsions on the basis of several fatty acids as oil phases, phospholipid-type and anionic surfactants, short-chain alcohols cosurfactants, and penetration-enhancing substances has recently been published. The results in animal and human skin suggested that there are many factors affecting the delivery of topically applied drugs. Although some microemulsions were capable to enhance the skin penetration of drug substances (max. 2 to 3 times), their delivery rate depended on the physical/chemical properties and concentration of the drug, structure and ingredients of the carrier and the type of the skin membrane used for penetration studies. When compared to standard formulations (gels, creams, solutions), drug delivery enhancement by microemulsions appeared more efficient for hydrophilic than for lipophilic drug substances (Kogan and Garti, 2006).

The use of liposome and niosome formulations in TDD including their skin penetration characteristics has been reviewed by Choi and Maibach (2005), who concluded that vesicle formulations may significantly enhance the percutaneous penetration of drug substances, but only for certain and suitable molecules. For drug substances that have a high intrinsic capacity for skin penetration, such as steroid hormones, caffeine, or nicotine, liposome formulations may result in less skin penetration when compared with that from solutions, but may result in higher drug concentrations in the stratum corneum. For more poorly penetrating drug substances, liposomes or other vesicle-type formulations may enhance their skin penetration, although the magnitude of penetration enhancement tends to be moderate. These reports are consistent with the experience of our own laboratory, which found that liposome or nanosomes vesicle formulation may enhance or reduce skin penetration of cosmetic ingredients, although at a limited scale, that is, maximally two- to threefold (C. Ribaud, unpublished data, 2006).

Encapsulation of lipophilic ingredients in rigid nanocapsules, such as polycaprolactone capsules, was shown to enhance the skin penetration of some molecules, such as OMC, a lipophilic ultraviolet filter, but also increased its skin protection against UV light, which would permit the use of lower concentrations (Alvarez-Roman et al., 2004a). In contrast, in an earlier study, the same UV filter formulated in nanocapsules consisting of the same polymer after application to pig skin was only found in the SC, but was not detected in the deeper layers of the skin (Alvarez-Roman et al., 2001). Similarly, comparison in excised human skin of the penetration of the drug flufenamic acid in poly(lactide-coglycolide) nanoparticles with that of the drug in solution showed less penetration of the NP-formulation over 12 h when compared with that of the drug solution; longer incubation times resulted in a somewhat greater penetration of the drug from the NP-formulation, presumably due to protection of the active ingredient. NP were only detected in the upper layers of the SC and no penetration into the living skin was observed (Luengo et al., 2006).

The degree and magnitude of skin penetration of drugs substances formulated in vesicles does not appear to be related or proportional to a smaller particle size of vesicles. For example, it was shown that the skin penetration of triamcinolone acetonide in rat skin of mono- or multilamellar vesicles at 200 or 1000 nm diameter was similar (Yu and Liao, 1996). An in-depth study in mouse, hamster, and pig skin on the skin penetration of cyclosporine A in liposomes of various particle sizes (60, 300, and 1000 nm) showed the greatest penetration into or through the skin for the 300 nm vesicles and suggested that skin penetration of intact vesicles or their materials does not occur (Du Plessis et al., 1994). Another study on the penetration in human abdominal skin of hydrophilic or lipophilic fluorescent dyes formulated in liposomes of 5 different particle sizes (73 to 810 nm) found an enhanced penetration of the hydrophilic dye from liposomes of 120 or 190 nm, whereas the lipophilic dye penetrated most from vesicles at a size of 71 nm (Verma et al., 2003).

Formulation is, however, important. Bennat and Müller-Goymann (2000) had previously reported that oily microfine TiO_2 (20 nm) penetrates deeper into stratum corneum from an oily emulsion than an aqueous one and liposomes increases the penetration depth of TiO_2 into the stratum corneum. This result was confirmed by Gottbrath and Müller-Goymann (2003). Another large study investigated the skin penetration of tretinoin in positive- or negative-charged liposomes of different particle sizes (135 to 1163 nm) and types (multi- and monolamellar), as well as in water/ethanol, oil, and a dermatological cream formulation. The results suggested that skin penetration from negatively charged vesicles was superior, when compared with that of traditional (cream, oil, ethanol/water) formulations (maximal twofold increase), but was unaffected by vesicle size or lamellarity and primarily depended on the vesicle material. No evidence for intact skin penetration of vesicles or their materials was found in the study (Sinico et al., 2005).

Overall, these data suggests that vesicle materials as well as vesicle size may affect the skin penetration of liposome- or niosome-encapsulated drugs, whereas the degree of the penetration of the active ingredient is not proportional to a reduced ($<$100 nm) vesicle size. No evidence was found that vesicles at nano-range produce greater skin penetration of encapsulated drugs when compared with that of micro-sized vesicles. Possibly, an ideal vesicle size exists depending on the individual vesicle material and drug substance (Verma et al., 2003). The results of reports on skin penetration of vesicles and their materials consistently concluded that the vesicles or their materials do not penetrate beyond the most superficial layers of the SC (Ganesan et al., 1984; Schreier and Bouwstra, 1994; Honeywell-Nguyen et al., 2004). Similar results were found in an in-depth investigation on the skin penetration of formulations containing

elastic or rigid vesicles (particle size: 100 to 150 nm), confirming that that vesicle materials did not penetrate further than the stratum corneum (Van den Bergh et al., 1999).

The only TDD system that resembles solid NP consists of solid-lipid nanoparticle (SLNP) systems that were investigated for skin penetration enhancement for a wide variety of substances. Although SLNP were initially reported to improve the skin absorption of some drugs, sunscreens or vitamins (Santos et al., 2002), the enhancement has been recognized to be mainly related to an increase in skin hydration produced by an occlusive lipid film formed on the surface of the skin, and not by actual skin penetration of the SLNP themselves (Wissing and Müller, 2003; reviewed by Benson, 2005).

In general, the penetration-enhancing capacity of vesicle-containing formulations appears to be limited; such formulations are not able to perform miracles and drive any compound into or through human skin. Today it has been recognized that passive TDD, that is, delivery by occlusive patches, vesicle-type formulations, gels, or creams, may only yield significant skin penetration and therapeutic effects of drug substances that combine a high pharmacological potency, a suitable log octanol–water partition coefficient (log P) value (1 to 3), a melting point below 200°C, and a molecular mass of <500 Da, a combination of parameters similar to Lipinski's Rule of Five predicting bioavailability of oral drugs (Lipinski et al., 2001). Drug substances marketed today in passive TDD systems all fit into these limits, they have molecular weights between 160 and 360 and a molecular size between 0.75 to 1.6 nm (Benson, 2005; Table 3).

For larger molecules and/or physical characteristics outside this range, such as large molecules, peptides, proteins, or nucleotides, significant percutaneous penetration may only be achieved by active delivery methods, such as skin abrasion, skin erosion (suction blisters), electrical (ionophoretic) and mechanical (skin stretching) methods, or other energy-related techniques, such as ultrasound or needleless injection, or by the combination of active and/or passive delivery methods (Benson, 2005; Thomas and Finnin, 2004).

3.6 Nanoparticles and Altered, Compromised, or Diseased Skin

In the absence of the stratum corneum barrier, there is a potential for certain NP to lead to immune modulation. Kim et al. (2004) have shown that quantum dots may be translocated to regional lymph nodes, possibly via macrophages and Langerhans cells after application to the dermis. In general, however, other than with lacerations and burns, some epidermal barrier function normally exists in compromised skin. Further, most cosmetic products, with perhaps the exception of sunscreens or after-sun products that are applied to sunburned, that is, inflamed skin, cosmetic products are designed to be used on healthy skin. In contrast, certain topical dermatological drugs are applied to diseased skin. This position is reflected by current EU Notes of Guidance for Testing of Cosmetics and their Ingredients that indicate that key dermal safety studies, such as irritation, percutaneous penetration, or sensitization testing in vitro, in vivo or in human subjects should be performed on normal, healthy skin, and not on compromised or diseased skin (SCCNFP, 2003b).

A review of the literature suggests that there is little evidence suggesting a general rule that slightly compromised skin has a greater susceptibility to skin penetration by small particles (Schäfer-Korting et al., 1994), although it has been recognized that certain pathological skin conditions may affect skin penetration of topically applied substances. Overall, it is uncertain whether all conditions of compromised skin enhance percutaneous penetration of topically applied substances or particles. For example, it has been shown that the percutaneous absorption and systemic bioavailability following application of a [^{14}C]methylprednisolone aceponate-containing lotion to intact and inflamed skin (UVB-induced sunburn) of human subjects were identical or lower for inflamed skin, whereas removal of the stratum corneum by tape stripping significantly enhanced the penetration of the drug into normal and inflamed skin (Gunther et al., 1998). This is not surprising, given that skin inflammation produces a thickening of the epidermis and thereby may enhance, rather than reduce, the barrier function of the skin (Walker et al., 2003).

Some skin diseases, such as psoriasis vulgaris, produce hyperkeratosis, which may also result in a reduced penetration of topically applied substances, whereas other skin diseases, such as eczema or podoconiosis (elephanatiosis), may produce rupture of the stratum corneum and thereby reduce the barrier function of the skin, resulting in increased penetration of topically applied substances (Korting et al., 1990). Low-frequency ultrasound promotes the penetration of 20-nm quantum dots through pig skin in a heterogeneous manner to an epidermal depth of ~60 nm (Paliwal et al., 2006). This penetration was considerably increased by the presence of sodium lauryl sulfate. Recently, Upadhyay (2006) has suggested that mild hyperthermia results in higher mass transport dot quantum dot antigen-labeled NP. A range of immune responses in vivo followed, leading to the suggestion that NP may be used for transdermal immunization. However, quantum dots are often cell-impermeable and require transporters to facilitate crossing over cell membranes (Zhang et al., 2006).

Work by Tinkle et al. (2003) also suggests that fluorescent dextran beads penetrate into flexed human skin with NP being found in the epidermis or dermis. However, the skin sections flexed were relatively thin (300–400 μm), and the findings may alternatively be explained by penetration of particles into hair follicles (Lademann et al., 1999), which is known to be enhanced by mechanical movement (Cormier et al., 2001; Teichmann et al., 2006). In addition, the human skin was stored in a tissue medium at 4°C for 24 h before the studies. Further, one should take into account the possibility that mechanical flexing of mammalian skin with a sufficiently large NP may create sufficient pressure to cause epidermal penetration (Tinkle, personal communication to M. Roberts, 2006). A lack of a

diffuse fluorescence in the skin treated with fluorescent-marked NP suggests that a detachment of dye molecules from particles with subsequent penetration of the dye into the skin has not occurred (Tinkle, personal communication to M. Roberts, 2006). However, such a detachment and other sample preparation artifacts have caused interpretation issues in other studies. For example, in two recent, independent inhalation studies on ^{99m}Tc-marked carbon NP it has been recognized that marker substances may become detached from inhaled NP, thereby leaching into the tissue and resulting in the semblance of systemic exposure to NP, although the particles remained localized in the lungs (Wiebert et al., 2006a and 2006b). Our own unpublished work (Roberts, 2006), has shown that ZnO NP are retained at the stratum corneum surface and in follicular openings after flexing of human skin.

The fact that topical application of metals such as beryllium or nickel or their salts may lead to skin sensitization has been suggested as supporting evidence for skin penetration of NP (Tinkle et al., 2003). However, this phenomenon may not represent genuine evidence for skin penetration of particles or NP, since metals are principally thought to penetrate the skin by diffusion of their ionized, that is, atomic or molecular forms (Hostynek, 2003).

4. LOCAL AND SYSTEMIC TOXICITY OF INSOLUBLE NP USED IN SUNSCREENS

4.1 Intrinsic Toxicity

The likely toxicity of NP after topical application depends on the likely exposure of viable cells, the concentrations present, the toxicity of NP as particles, and the intrinsic toxicity of NP components or surfaces. Evidence of the low intrinsic toxicity of TiO_2 and ZnO NP is provided by their large market share (~70% and 30%, respectively) and the fact that they have been on the market since ~1990 and ~1999, respectively, without producing adverse skin or systemic effects. Zn is an essential nutrient with an average intake of 15 mg and a no-observable-effect level (NOEL) of approximately 50 mg/kg/day (Maita et al., 1981). The solubility of zinc oxide (1.6 ppm) is pH dependent and higher at the acidic skin surface. In vitro pig skin studies showed that between 1.5 and 2.3% of the topically applied ZnO NP and nil of TiO_2 was recovered in the receptor solution(Gamer et al., 2006). A recent study on skin absorption of ZnO NP reported levels of zinc over 24 h in diffusion cells using human skin that were slightly, but not significantly, different from those for a placebo formulation (Cross et al., 2007).These absorption values were an order of magnitude less than reported in previous human work (Pirot et al., 1996) and two orders less than values found by Gamer et al. in pig skin (2006). The recovery of Zn in receptor phases also reflects background Zn levels in both the placebo formulations and in the skin.

Whereas ZnO and TiO_2 appear relatively nontoxic, some carbon nanotubes, fullerene derivatives and quantum dots may have intrinsic biological activity (Hardman, 2006). Quantum dots cores can consist of metal complexes such as semiconductors, noble metals, and magnetic transition metals. Of these, indium arsenate, cadmium tellurium, and gallium arsenate appear to be quantum dots with potential intrinsic toxicity sufficient to warrant appropriate exposure limits. In addition, dissolution of the core to the elemental metal, such as cadmium, may also be associated with toxicity.

Intrinsic toxicity may also be modified by the type of particle coating used. In principle, coating with polyethylene glycol makes NP less hydrophobic and more biocompatible. Other coatings, however, may be intrinsically toxic, such as mercaptoacetic acid or immunogenic, for example, covalent serum albumin, coatings (Hardman, 2006). Ryman-Rasmussen et al. (2006) showed carboxylic acid or PEG-amine coatings of quantum dot NP produced cytotoxicity, whereas PEG-coated NP were nontoxic. The study concluded that quantum dot surface coating is a primary determinant of cytotoxicity and immunogenicity in human epidermal keratinocytes.

4.2 In Vitro Toxicity Studies on Insoluble NP in Mammalian Cells

A wealth of information on the behavior in the organism and adverse effects of small, insoluble, and foreign particles is available from studies on adverse effects of surgical implants, such as artificial hip or knee joints. Such devices may produce wear debris consisting of nano- and micro-sized particles on their bearing parts and at the interface between the device and bone (Breme and Helsen, 1998). Very high concentrations of metallic, polymeric, or ceramic debris particles (sizes: 100 nm to 10 μm) have been observed in human tissues surrounding hip and knee replacements at very high densities ranging from 8.5×10^8 to 5.7×10^{13} per gram of tissue. It has been recognized that the presence of high concentrations of wear debris particles in the periarticular tissues may result in inflammation and, ultimately, osteolysis (Amstutz et al., 1992; Hirakawa et al., 1996). Since these particles were only found in periarticular tissues, these findings also suggest that small, inert particles tend to remain localized and result in little, if any, systemic exposure.

The interaction with and adverse effects of debris particles on living tissue have been thoroughly investigated in relevant mammalian cell cultures. A recent study evaluated in murine fibroblasts and macrophages the role of particle size and shape in the cytotoxicity of micro- and nano-sized, insoluble ceramic particles, including TiO_2, Al_2O_3, ZrO_2, Si_3N_4, or SiC. The results suggested that larger particles of TiO_2 (1600 nm), ZrO_2 (530 nm), Al_2O_3 (590 nm), or Si_3N_4 (700 nm) were more cytotoxic to fibroblasts and macrophages than smaller TiO_2 (90 or 130 nm) or SiC (180 nm) particles, whereas dendritic particles were more cytotoxic to macrophages than spherical particles. The study results also suggested that the volume of the total amount of the particles phagocytosed by cells, and not their particle size is the decisive factor in the particle-mediated inhibition of cell proliferation and cytotoxicity (Yamamoto et al., 2004).

Another study compared the toxicity of micro- and nano-sized particles of CdO (1000 nm), Ag (15 and 100 nm) MoO_3 (30 and 150 nm), Fe_3O_4 (30 and 47 nm), Al (103 nm), MnO_2

(200 nm), and W (tungsten, 27 μm) in rat liver cells. The results showed no correlation of cytotoxicity with the particle size, but with the chemical nature of the test substance. Materials of known toxicity (CdO and Ag) were highly cytotoxic at all particle sizes, whereas the remaining materials showed a similar degree of cytotoxicity independently of particle size (Hussain et al., 2005). These findings are also supported by the results of Olivier et al. (2003), who showed that the cytotoxicity of 0.45- and 3.45-μm polystyrene or 0.43- and 2.81-μm alumina particles to macrophages or fibroblasts was unrelated to their particle size. Similar results were found in an investigation on the in vivo or in vitro toxicity of metallic titanium particles in osteoblasts (Choi et al., 2005).

The toxicity of insoluble and inert particles to mammalian cells is directly correlated to their cell uptake. Many mammalian cells have a capacity for endocytosis or phagocytosis of small, insoluble particles; that is, they may actively ingest small particles. During endocytosis, materials ingested are progressively enclosed by the cell membrane, which eventually detaches to form an endocytic vesicle, whereas phagocytosis is a receptor-mediated characteristic for neutrophiles, macrophages, and dendritic cells and may result in active ingestion of insoluble particles up to 3 μm (Garnett and Kallinteri, 2006). Cells that phagocytose small insoluble particles release reactive oxygen species and lysosomal enzymes in order to destroy or degrade the ingested, insoluble particles, attempting to convert them to an ineffective, safer form (Yoshikawa, 1991). When phagocytosed particles cannot be degraded, they may accumulate in the cell, resulting in oxidative cell damage, inhibition of cell proliferation, and, ultimately, cytotoxicity, and provoking a physiological response termed *activation*; the nonproliferating cell may release numerous inflammatory factors, produce inflammatory responses in adjacent tissues, and/or stimulate fibroblasts for fibrogenesis (Yamamoto et al., 2004).

This well-known sequel, a normal physiological response of phagocytosing cells to an excessive amount of insoluble particles, may result in oxidative cell damage, such as lipid peroxidation or DNA damage and cytotoxicity (Görög et al., 1988). Ultimately, oxidative cell damage may also produce genotoxic effects (Yoshikawa et al., 1991), the mechanisms of which were recently reviewed by Schins (2002). Consequently, in order to avoid false positive findings, international guidelines for in vitro genotoxicity testing in mammalian cell cultures recommend testing of insoluble compounds only up to the lowest precipitating concentration (ICH, 1996). Cerium oxide NP uptake into fibroblast cell cultures is strongly inversely dependent on particle size, and uptake is faster than would be predicted by diffusion, suggesting a biological uptake process at the cell surface (Limbach et al., 2005). Agglomeration at the cell surface is a prerequisite for uptake and emphasizes the need to relate the environmental conditions used in cell culture studies to that actually likely to be seen by tissues in vivo.

Accordingly, reports claiming the discovery of active penetration of nanoparticles into mammalian cells in vitro need to be treated with caution, especially since such findings may often be more convincingly explained by the phagocytic activity of treated cells. Given that human keratinocytes in culture are known to have considerable phagocytic capacity (Korting, 1993), reports on uptake of NP or carbon nanotubes by cultured keratinocytes (Monteiro-Riviere et al., 2005a) or other cells do not necessarily predict a potential toxicity or a risk to intact human skin or the human organism, but may simply reflect the intrinsic biological response of cultured mammalian cells to treatment with an insoluble, foreign material. This view is supported by a recent report suggesting that the principal route of uptake of quantum dots by mammalian cell cultures was via endocytosis (Hardman, 2006).

Therefore, effects such as oxidative stress, antioxidant depletion, and cytotoxicity in keratinocytes treated with carbon nanotube materials, such as reported by Shvedova et al. (2003) or Monteiro-Riviere et al. (2005a), are not surprising and do not necessarily suggest a risk for human skin, but may correspond to a normal response of mammalian cells to foreign and insoluble materials. Similarly, findings suggesting in vitro genotoxic effects of small, insoluble particles in mammalian cells do not necessarily suggest an intrinsic genotoxic activity of these materials, but may be due to the same mechanism. For example, a recent report describing a genotoxic effect of dust storm fine particles in human lymphocytes (Wei and Meng, 2006) does not necessarily suggest a genotoxic risk of desert dust to human health, but the observed effects may be more consistent with the response of mammalian cells to an excessive uptake of insoluble particles.

Similarly, the results of a recent report on the uptake of TiO_2 NP by murine brain microglia cells and subsequent generation of reactive oxygen species (Long et al., 2006), do not necessarily mean that TiO_2 particles in sunscreens are potentially neurotoxic or may cause brain damage in people, as suggested by media or NGOs (Anonymous, 2006; FOE, 2006b), but should most likely be attributed to the well-known phagocytic capacity of microglia, which has been characterized by Schilling et al. (2005). The same mechanism may also explain the findings of a recent report on oxidative damage in human bronchial epithelial cells following exposure to rutile and anatase-type TiO_2 (10, 20 and 200 nm) nanoparticles (Gurr et al., 2005). In today's climate, it is surprising that media and NGOs have not paid attention to a recent, curious report describing a high capacity for antioxidant depletion in an in vitro model of the human respiratory tract of nano-sized combustion particulates generated by burning cow dung (Mudway et al., 2005)—another, albeit hypothetical, risk of NP to human health?

It has been recently been shown that both cytotoxicity and cellular interleukin (IL)-8 release produced by the interaction of multiwall carbon nanotubes (MWCNT) with human keratinocytes were affected by the type and concentration of surfactants used to keep the hydrophobic test materials in solution, secondary to complex interactions of the surfactant with the physical shape of MWCNT as well as the viability/activity of

the cells (Monteiro-Riviere et al., 2005b). A critical appraisal of in vitro assays measuring possible toxicological effects of carbon nanomaterials recognized that in vitro tests may yield conflicting results due to interference of test and control carbon materials with dye markers used in cell cytotoxicity assays (Monteiro-Riviere and Inman, 2006). Conflicting results of in vitro/in vivo test results have also been recently been described for the pulmonary toxicity of TiO_2 particles (Sayes et al., 2006; Warheit et al., 2006).

However, it is unclear whether all mechanisms of uptake of NP by mammalian cells occur via phagocytosis or endocytosis. For example, a recent report described uptake of very small TiO_2 particles (22-nm agglomerates of smaller particles) by lung macrophages and human blood cells via a nonphagocytotic mechanism (Geiser et al., 2005). Clearly more research is needed to clarify the mechanisms of cellular entry and subsequent adverse effects of NP in cultured mammalian cells.

Given these uncertainties and confounding factors, reports of in vitro studies that claim discovery of intracellular penetration and oxidative stress-related toxicities of nano- or micro-particles in cultured mammalian cells should be interpreted with great caution in terms of their relevance for the intact organism. This view is supported by consensus recommendations of a recent workshop on toxicology testing of nanomaterials 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* (NTP/NIEHS, 2004).

4.3 Cytotoxicity, Phototoxicity, and Photo-Genotoxicity of TiO_2 and ZnO Micro- and Nanoparticles

Sunscreen-grade TiO_2 and ZnO of different particle sizes, including nano-sized particles, have been evaluated in vitro and in vivo for their capacity to penetrate into or through skin and their toxicity, phototoxicity, genotoxicity, photo-genotoxicity, and carcinogenicity. In 1999/2000, following a report suggesting that several types of TiO_2 (crystalline form anatase; particles size: 255 to 420 nm) were photo-genotoxic in mouse lymphoma and Chinese hamster lung cells (Nakagawa et al., 1997), producers of TiO_2-based sunscreens performed a large safety program, consisting of genotoxicity (Ames test, clastogenicity in mammalian cells), photo-genotoxicity (photo-Ames test, photo-clastogenicity in mammalian cells), and cytotoxicity (CHO and V-79 cells) under conditions of good laboratory practice (GLP) on more than 10 different sunscreen-grade TiO_2 particles, including micro- and nano-sized rutile and anatase crystalline forms, as well as coated and noncoated particles. The overall conclusion suggested a similar toxicity profile for all substances, which were all noncytotoxic, nonphototoxic, nongenotoxic, and non-photo-genotoxic; no major difference in the safety profile was observed for micro- and nano-sized particles, and no evidence was found suggesting that nano-sized particles pose greater or qualitatively new hazards (Table 1; SCCNFP, 2000). However, Serpone et al. (2001) suggested that micrometer-size TiO_2 can have deleterious effects on DNA after irradiation through photocatalytic free radical generation. These effects may be reduced by the use of appropriate coatings while retaining the TiO_2 transparency. Cai et al. (2001) also showed that TiO_2 (100 μg/ml) and UV irradiation resulted in cytotoxicity in rapidly dividing HeLa cells by oxidative stress. However, the relevance of these results concerning sunscreen safety is uncertain.

The genetic and photogenetic toxicity of micro- or nano-sized ZnO is summarized in Table 4. In standard in vitro clastogenicity tests (dark conditions) in Chinese hamster ovary

TABLE 4
Results of in vitro phototoxicity, genotoxicity/photo-genotoxicity tests on micronized (200 nm) ZnO particles; Unpublished data included in the industry safety dossier, summarized and reviewed in the SCCNFP opinion on ZnO (SCCNFP, 2003; Dufour et al., 2006)

Test	Test organism	Concentration tested (μg/mL)	Result	Rating
Neutral red uptake	BALB/c 3T3 mouse fibroblasts	1000 to 100,000	Negative	Nonphototoxic
Ames test	*S. typhimurium*	Up to 5000 μg/plate	Negative	Nonmutagenic
Photo-Ames test	*S. typhimurium* TA98, 100, 1537	Up to 5000 μg/plate	Negative	Nonphoto-mutagenic
In vitro clastogenesis	CHO cells	Up to 1000 μg/ml	Positive at $\geq$814 μg/ml	Clastogenic in vitro
In vitro photo-clastogenesis	CHO cells	Up to 1000 μg/ml	Positive at $\geq$195 μg/ml	Photo-clastogenic in vitro
In vitro clastogenesis	V-79 cells	Up to 20.0 μg/ml	Positive at 10.0 μg/ml	Clastogenic in vitro
In vitro photo-clastogenesis	V-79 cells	Up to 10.0 μg/ml	Positive at 3.0 μg/ml	Photo-clastogenic in vitro
In vitro Comet test/photo-comet test	Human keratinocytes (HaCaT cells)	Up to 31.0 μg/ml	Negative	Nonphotogenotoxic in vitro

(CHO) and Chinese hamster V-79 cells, ZnO was clastogenic (SCCNFP, 2003a), but it was nonclastogenic in vivo (CSTEE, 2003; US EPA, 2005b). According to the SCCNFP opinion (2003a) ZnO particles were nonphoto reactive, -phototoxic or -photosensitizing, but were judged to be photo-clastogenic in vitro, since they were clastogenic in CHO or V-79 cells at approximately 4-fold lower concentrations in the presence of UV irradiation when compared with effective concentrations in the dark.

In the meantime it has been shown that these effects were not due to genuine photo-genotoxicity, but secondary to UV-induced experimental artifacts. In a recent study, Dufour et al. (2006) applied ZnO to CHO cells (a) in the dark, (b) under simultaneous irradiation with UV light, and (c) pre-irradiated with UV light, followed by treatment with ZnO in the dark. Interestingly, the nature, incidence, and severity of chromosome aberrations in pre-irradiated and simultaneously irradiated cells were nearly identical when related to cytotoxicity (Figure 6). Given that CHO cells pre-irradiated in the absence of ZnO showed the same increase in the incidence and type of chromosome aberrations as CHO cells receiving simultaneous ZnO-treatment and irradiation, this suggests that the increase in the incidence of chromosome aberrations was due to a UV-mediated, enhanced susceptibility of the mammalian cells to ZnO; in other words, ZnO is non-photo-genotoxic.

Overall, whilst TiO_2 or ZnO micro- or nano-sized particles may cause cytotoxicity in the presence of UV irradiation, they do so at relatively high concentrations and in in vitro systems only. It is unlikely that, given the low concentrations epidermal cells will be exposed to, either ZnO or TiO_2 NP pose a phototoxic, genotoxic, or photo-genotoxic risk; on the contrary, there is robust evidence that these substances applied topically protect human skin against UV-induced adverse effects, including DNA damage and skin cancer (Nohynek and Schaefer, 2001).

4.4 In Vivo Toxicity Studies and Clinical Safety on NP Used in Sunscreens

4.4.1 Oral Toxicity, Systemic Exposure After Oral Uptake, or Inhalation

TiO_2- or ZnO-containing sunscreens are applied to the face or lips, in contact with the hands of consumers, and may therefore produce, although low, oral exposure. Therefore, their use in sunscreens also raises the question of their health risk after oral uptake.

Oral toxicity studies on micro-sized TiO_2 showed absence of toxicity in rats and mice up to dietary dose levels of 10,000 ppm for 15 weeks, while oral exposure to dietary levels of up 50,000 ppm (5%, corresponding to 3700 mg/kg/day) for 24 months produced no toxicity or carcinogenic effects in rats (NCI, 1979). The absence of adverse effects at these extreme oral doses suggests that TiO_2 is a biologically and toxicologically inert material.

Zinc is an essential trace element in the mammalian organism and is an essential component of cellular DNA and RNA synthesis, stability and repair (Stefanidou, 2006). Although no data are available on the subchronic or chronic toxicity of zinc oxide, the results of dietary subchronic oral toxicity studies on the soluble salt zinc sulfate in rats and mice suggest that zinc ions in general have low oral toxicity, as indicated by a dietary no-observed-adverse-effect level (NOAEL) of 3000 ppm in rats corresponding to a daily intake of 53.5 Zn^{2+}/kg/day (Maita et al., 1981). Given that ZnO is only slightly soluble, which may be expected to reduce its bioavailability, the oral NOAEL of ZnO may be estimated to be considerably higher than this value.

A single study indicated that repeated oral administration of micro-sized TiO_2 particles (12.5 mg/kg/day; mean particle size 500 nm) in rats may result in the uptake of particles in the mesenteric lymph nodes, small intestine and liver. The amount taken up was a small percentage relative to the administered oral dose, ranging from 0.03% (small intestine) to 2.18% (mesenteric lymph nodes), and resulted in tissue levels ranging from 6.81 to 545.9 μg/g tissue, respectively (Jani et al., 1994). Although no adverse effects were observed in this study, these data suggest that oral uptake of some small particles may produce a limited local or systemic exposure. Although the reversibility of this uptake was not investigated in the study, taking into account the results of oral toxicity studies on TiO_2, which showed virtual absence of toxicity after lifetime oral uptake, it is extremely unlikely that an oral exposure from sunscreen particles may pose a systemic human health risk. Similarly, given the low order of oral toxicity, the oral absorption of ZnO from sunscreens should pose no risk to human health. In addition, ZnO is slightly soluble, which would diminish its potential to be stored in the organism.

In an inhalation carcinogenicity bioassay, a 2-year exposure of rats to air concentrations of TiO_2 particles at 250 mg/m^3 produced an increased incidence of lung tumors (Lee at al., 1985). However, this effect has been recognized to be due to chronic inflammation secondary to lung overload, which is considered to be irrelevant to human health under normal inhalation exposure conditions (ILSI, 2000). It is beyond the scope of the present article to discuss these findings in depth, although details on the mechanisms may be found in the ILSI report (2000) or the review by Hexte et al. (2005). However, given that the use of titanium oxide particles in sunscreens is unlikely to produce human inhalation exposure; this effect should be considered to be irrelevant for a topical exposure of human skin.

4.4.2 Toxicity After Topical Exposure

Given that the safety of sunscreens and their ingredients is regulated in the EU (EU SCCP) and the United States (U.S. Food and Drug Administration, FDA), sunscreen preparations containing TiO_2 and ZnO nano- and micro-sized particles have been subjected to a series of preclinical toxicological safety evaluations via the topical route as well as their efficacy and safety in humans. All study results suggested that these materials are nontoxic, nonirritant and nonsensitizing, nonphototoxic, nonphotosensitizing, and produce no adverse effects after repeated

dermal application to animal or human skin. All data consistently indicated that these substances should be considered as biologically inert after topical application to animal or human skin (SCCNFP, 2000, 2003a).

4.4.3 Nano-Sized Cosmetic Formulations and Risk of Skin Sensitization

The potential of a topically applied substance to produce skin sensitization is directly related to its capacity to penetrate into the skin and its potency to produce sensitization (ECETOC, 2003; Kimber et al., 2003). The potential of substances in vesicle-type formulations to penetrate into or through intact skin has recently been reviewed (Section 3.4; Choi and Maibach, 2005; El Maghraby et al., 2006).

Liposomes or nano-capsules are generally used to protect sensitive cosmetic ingredients, such as unsaturated fatty acids, vitamins, or antioxidants, against oxidation or photodegradation. Given that the products of oxidation or photodegradation, such as organic peroxides, aldehydes, ketones, or epoxides, tend to have a higher chemical reactivity when compared with that of the original substances (Niki et al., 2005), they are more likely to produce adverse skin reactions, including sensitization. Therefore, improved stability of ingredients protected by vesicles should reasonably be expected to benefit the local tolerance of these formulations, when compared with that of conventional formulations.

Finally, new cosmetic formulations and sunscreens, including those containing nano-materials, are generally tested in human subjects for their skin tolerance (Nohynek and Schaefer, 2003). In addition, new in vitro test methods are increasingly used to screen the sensitization potential of cosmetic formulations (Jowsey et al., 2006). These tests would detect a potential of a product to produce skin sensitization. The cosmetic industry scrupulously avoids sensitizing ingredients in sunscreens and leave-on products on the basis of their potential risk to human health as well as product liability, taking into account the large numbers of consumers exposed as well as the exposure to a large skin surface per consumer. Overall, the question of the risk of potential skin sensitization is relevant not only for formulations containing nano-materials, but concerns all cosmetic formulations or products. No responsible manufacturer would knowingly market products that pose an unreasonable risk of sensitization to the consumer.

5. RISKS AND BENEFITS ASSOCIATED WITH NP USE

We have attempted to convey the principles that potential NP toxicity depends on exposure, the likelihood of sufficient absorption and in sufficient quantities to affect viable cells, and the need for an intrinsic toxicity of both the NP ingredient or its coating (with and without UV irradiation) to be present. These considerations exist for all formulations whether NP are present or not. However, one of the central questions often asked on the safety of NT is, should substances in nano-sized form be regarded as *new substances* and therefore should they undergo additional safety testing? Although toxicological properties of substances tested as bulk, suspension, solution, or particles tend to be similar, there are indeed particle size-related exceptions, which may alter, enhance, or reduce toxicity:

- *Small particles size producing systemic exposure and toxicity*: Some studies suggested that certain inhaled or orally administered NP may be systemically absorbed, whereas recent data and studies on other NP materials found no or little systemic uptake. It also suggested that aggregated particles at the viable cell surface are better taken up than individual particles—implying that a threshold concentration of particles needs to have been absorbed for toxicity.
- *Particle size affecting pharmacokinetics*: Different particle sizes may produce different pharmacokinetic properties of a substance and may result in enhanced or reduced uptake, distribution, metabolism, and elimination. For example, the oral administration of a bioavailable substance at a small particle size usually leads to faster dissolution and may produce higher plasma C_{MAX} values, whereas larger particles may dissolve more slowly and lead to an extended duration of systemic exposure.
- *Surface effects*: Following inhalation, the large relative surface area of small particles may increase or reduce adverse effects. For example, smaller, insoluble particles may have a greater potential to produce lung overload; in contrast, more soluble smaller particles may undergo a more rapid clearance from exposed tissues.
- *Particle size affecting external and internal exposure parameters*: Smaller particles may remain airborne for longer and may produce increased inhalation and alveolar exposure when compared with larger particles. Conversely, smaller particles may have a greater tendency to agglomerate, which may reduce inhalation and alveolar exposure.
- *Physical shape and charge:* Sometimes the toxicological properties of insoluble materials may be determined by their particle shape and charge, and not just their chemical composition. Single-wall carbon nano-tubes (fibers) have different toxicological properties than carbon black (particles). Particle shape has also been recognized to play a role in the cytotoxicity of other insoluble particles.

In our view, NP safety evaluations on materials cannot be conducted on the basis of their particle size only but must recognize other determinants of toxicity. To illustrate this point, one may consider the toxicological testing of drugs, chemicals, cosmetics, or food ingredients. For example, preclinical toxicology tests of drug substances generally use simplified formulations, such as aqueous suspensions or solutions, although the

final drug product may contain the drug substance in a different galenic form (ICH, 2000). It is well established that drug products at different particle sizes may change the pharmacokinetic profile of systemic exposure when compared with that of a drug in aqueous suspension, which may result in quantitatively different toxicological profiles. Nevertheless, the use of simplified formulations for preclinical safety testing has been accepted on the basis of the rationale that the high doses administered in preclinical toxicity studies will compensate for differences in pharmacokinetic properties of formulations of drug products.

Given the multitude of different chemical, cosmetic, food, or drug formulations that may contain synthetic or natural ingredients in the form of solids, solutions, or suspensions of particles at various sizes, it would be unpractical and, in most cases, unethical to multiply toxicology testing as a function of the final formulation or particle size of the substance in a final chemical, consumer, food, or cosmetic end product. One should also take into account that current cosmetic regulations in the EU do not permit in vivo toxicological testing of cosmetic final products, which would preclude testing of ingredients that may be present in cosmetic formulations at different physical states or forms, such as molecular (solutions or vapors), in bulk, particles, or formulations with nano-sized features (SCCNFP, 2003b). For example, nanoemulsions and nano-vesicles are only produced during the final formulation process of cosmetic end products and could not be tested for in vivo toxicity in the absence of other formulation ingredients that ensure the formation and integrity of such preparations. However, when a significant increase in skin absorption is suspected, these properties may be verified via in vitro skin penetration studies in human or animal skin.

The effect of NP size on toxicity is equivocal. For instance, the inhalation toxicities of some biologically inert nanoparticles (TiO_2, carbon black or polystyrene) were reported to be somewhat enhanced—about 2 to 5 times (depending on the total particle surface) more than observed for the corresponding micro-sized particles—but did not lead to qualitatively new toxicities (Brown et al., 2001; Bermudez et al., 2002, 2004). However, the enhanced pulmonary toxicity is not surprising, since inhalation of high particle concentrations of biologically inert materials may produce pulmonary toxicity secondary to lung overload, which is directly correlated with the relative particle surface of the test material (ILSI, 2000), although this was not confirmed for TiO_2in a recent study (see later discussion).

In contrast, there is considerable evidence suggesting that the size of many insoluble particles is not correlated with their toxicity. For example, the results of a recent pulmonary toxicity study on TiO_2 particles, including pigment-grade (200 nm), nano-scale rods (200 $\times$ 35 nm) and nano-dots (40 nm), showed a similar magnitude and severity of the pulmonary inflammatory response to all particle sizes tested, suggesting no differences in toxicity (Warheit et al., 2006). Inhalation toxicity studies on silica particles suggested that nanoparticles of a mean diameter of 10 ± 5 nm were *less toxic* than that following an equivalent exposure to micro-sized (1–5 μm) particles (Chen et al., 2004). A study in human volunteers found no differences in the adverse effects following inhalation of ultrafine (<100 nm) or microfine (0.1 to 1 μm) ZnO particles (Beckett et al., 2005). Nano-sized particles of biologically inert ceramic or metallic materials were less cytotoxic than micro-sized particles (Yamamoto et al., 2004), which was supported by similar data of Hussain et al. (2005), Olivier et al. (2003), or Choi et al. (2005), all of which showed that cytotoxicity of inert or toxic micro- or nanoparticles was mainly correlated with their chemical nature and not their particle size. Similarly, the results of recent toxicity studies on a series of engineered quantum dots concluded that their absorption, distribution, metabolism, excretion, and toxicity depended on multiple factors derived from physicochemical properties and environmental conditions, but not on their particle size (Hardman, 2006). These data are consistent with the results of genotoxicity/photo-genotoxicity programs on nano- and micro-sized TiO_2 or ZnO particles, which showed no correlation of toxicity with smaller particle size (SCCNFP, 2000, 2003a; Table 1). Overall, these results support the traditional rule of toxicology that the intrinsic toxicity of substances is primarily related to the chemical nature, whereas physical form of substances, such as particle size and shape, may enhance or reduce the characteristic toxicity of a substance, although some exceptions to this rule (insoluble fibers, different crystalline forms) exist.

It has also been suggested that nano-materials may produce qualitatively novel toxicities. This claim largely relies on the results of pulmonary toxicity studies on single- or multiwall carbon nano-tubes (SWCNT/MWCNT), which suggested that these materials may produce adverse pulmonary effects that were not observed in similar studies on amorphous carbon black particles. Indeed, bronchial instillation of SWCNT or MWCNT produced interstitial granulomas in the lungs of rats, which are typical for insoluble fibers (Warheit et al., 2004; Lam et al., 2006). Since SWCNT or MWCNT are insoluble and have a fiber structure of a length of several micrometers, their dimensions are similar to those of other insoluble and toxic fibers, such as asbestos. Therefore, these findings are neither surprising nor new or nano-size-related, but should be ascribed to the insoluble and fiber-like properties of these materials. Given that the only nano-dimensional feature (<100 nm) of SWCNT or MWCNT is their wall thickness and, sometimes, their diameter, whereas their length may be several micrometers (Lam et al., 2006), it is difficult to accept their effects as evidence supporting a general rule claiming qualitatively new toxicities of nanomaterials.

Many published toxicological studies on NP had a major weakness in that they attributed adverse findings to the nano-size of the test material, although the studies included no benchmark groups treated with micro-sized particles of the same material. When such studies used surrogate endpoints for toxicity (oxidative cell damage, lipid peroxidation, genomic endpoints), in the absence of benchmark groups treated with micro-sized particles, it is impossible to determine whether reported effects were substance- or particle size-related. Therefore, claims of

nano-size-specific effects of such studies should be considered to be unsubstantiated and bearing little scientific evidence.

In our view, the hypothesis that NP could penetrate into and through the skin and get access to the lymphatic system is unlikely for ZnO and TiO_2. First of all, all available data and theoretical considerations suggest that particles do not penetrate into or through human skin. Second, it is very unlikely that even absorbed NP would enter the systemic circulation to circulate freely in the organism without first being phagocytosed. Recent data suggested that inhalation of NP did not produce significant systemic exposure in humans (Wiebert 2006a, 2006b), because, in part, nanoparticles do not circulate freely in the blood. Indeed, NP- or nano-vesicles in intravenous drug formulations are rapidly cleared from the circulation by monocytes or macrophages, that is, by the *phagocytic barrier*. Appropriate surface coatings are therefore required (*stealth particles*) in order to protect circulating particles against unwarranted clearance (Moghimi et al., 2001). This view is supported by the experience on insoluble wear debris particles from surgical implants, which suggests that small insoluble particles do not freely roam around the organism, but tend to remain at their location of origin.

A recent international work shop on the safety of nanomaterials concluded that human dermal exposure to NP is of minor concern (ECETOC, 2005). A review by the German Federal Institute of Risk Assessment (BfR) on the safety of nanoparticles in sunscreens concluded that *investigations on the potential penetration through the skin showed 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* (BfR, 2006). A similar position was adopted in a review of the safety of ZnO and TiO_2 nanoparticles used in sunscreens by the Australian Department of Health stating 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* (TGA, 2006).

These views are consistent with the potential risks of topically applied ZnO or TiO_2 NP to human skin and organism being hypothetical and unsubstantiated. However, caution must be applied in allowing the topical exposure to other NP that have a high intrinsic toxicity. Further, given the formidable barrier properties of the stratum corneum, human systemic exposure to NP via inhalation or, possibly, ingestion appears to be a more important route for potential NP toxicity than topical absorption (ECETOC, 2005; Borm et al., 2006), although it is notable that recent reports by Wiebert et al. (2006a, 2006b) questioned whether inhaled nanoparticles are translocated into the systemic circulation.

All of us inhale airborne NP from a range of sources commencing possibly with debris from fires in the very early times. Cooking activities such as toasters and oven-cooked or stovetop foods (Olson and Burke, 2006), gas heaters and stoves, aerosol sprays, or wax candles may generate nanoparticles with concentrations ranging from 100,000 to 270,000 particles/cm^3 indoor air (Afshari et al., 2005). It has been estimated that 50 to 80% of human inhalation exposures to ultrafine particles (<100 nm) are due to indoor sources (Wallace and Howard-Reed, 2002).

It is of concern that several NGOs have called for restrictions, bans, or regulatory control of NT in the absence of documented scientific evidence that potential health hazards, let alone health risks, really exist. It is essential that such calls are responded to and, when appropriate, balanced using rigorous and appropriate toxicological science on the potential health risk in the individual. For instance, an excess of insoluble particles is associated with observed phagocytosis by cultured mammalian cells. Hence, interpretation of these results in the context of the likely actual absorption and exposure of NP for those cells must be appropriate. Obviously, substances with a high inherent toxicity may cause adverse effects whether delivered by NP or by in solution. The minimal adverse effects of implant wear debris particles are consistent with those inert materials have a low intrinsic toxicity, although microparticles tended to be more toxic than NP. *Nanotoxicology* must seek to move beyond in vitro toxicity of NP and examine the likely translation into health hazards of NP to the intact organism. Indeed, in doing so, it would confirm the recommendation of NTP/NIEHS Work Shop (2004) that such investigations should preferably be performed in vivo.

Overall, our view on the safety of NP with inherently low or absent toxicity applied to the skin as topical cosmetics or sunscreens may be summarized as follows:

- *Soluble nano-sized materials*: Vesicle- or emulsion-type nano-sized formulations of cosmetic ingredients may result in reduced or enhanced skin uptake when compared with that from solutions, although the magnitude of these changes is limited. Therefore, the potential human skin and systemic exposure from nano-sized formulations should be rated similar to those of solutions of the respective ingredients. Skin penetration of vesicle materials has been shown to be absent or negligible.
- *Insoluble nanoparticles*: At present, there is no evidence that insoluble ZnO and TiO_2 nanoparticles used in sunscreens penetrate into or through human skin or may produce human local or systemic exposure and/or adverse health effects. The evidence of the health benefit of sunscreens clearly outweighs unproven and hypothetical risks.

In conclusion, our views are tempered by the current and in some cases contradictory evidence. Overall, the current weight

of evidence suggests that nano-materials such as nano-sized vesicles or TiO_2 and ZnO nanoparticles currently used in cosmetic preparations or sunscreens pose no risk to human skin or human health. Other NP may have properties that warrant a safety evaluation on a case-by-case basis before human use or exposure.

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