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Understanding Engineered Nanomaterial Skin Interactions and the Modulatory Effects of UVR Skin Exposure

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Abstract

The study of engineered nanomaterials for the development of technological applications, nanomedicine, and nano-enabled consumer products is an ever expanding discipline as is the concern over the impact of nanotechnology on human environmental health and safety. In this review we discuss the current state of understanding of nanomaterial skin interactions with a specific emphasis on the effects of ultra-violet radiation (UVR) skin exposure. Skin is the largest organ of the body and is typically exposed to UVR on a daily basis. This necessitates the need to understand how UVR skin exposure can influence nanomaterial skin penetration, alter nanomaterial systemic trafficking, toxicity, and skin immune function. We explore the unique dichotomy that UVR has in inducing both deleterious and therapeutic effects on skin. The subject matter covered in this review is broadly informative and will raise awareness of potential increased risks from nanomaterial skin exposure associated with specific occupational and life style choices. The UVR induced immunosuppressive response in skin raises intriguing questions that motivate future research directions in the nanotoxicology and nanomedicine fields.

Introduction

Engineered nanoscale materials (<100 nm in one dimension) made from metals, metal oxides, semiconductors, and carbon including polymers, exhibit unique optical, electrical, mechanical, biological, and physiochemical properties not present in their bulk form. These properties arise in part from an increased surface area to volume ratio where a greater % of the atoms comprising the material exist on the surface. Engineered nanomaterials (eNMs) are widely exploited in many technology fields (e.g. medicine, energy, automotive, military) promising great benefits to mankind. They are also formulated into an ever expanding consumer product market. The Project on Emerging Nanotechnology in 2011 listed 1317 nanotechnology-enabled consumer products in their inventory; an increase of 521% over 2006[1]. Nano-enabled products use for example, carbon nanotubes to make light weight and high strength sporting equipment (racquets, bikes), nano-Ag to make antimicrobial textiles and wound care dressings, and nanoscale ZnO and TiO_2 particles to formulate ultra-violet radiation (UVR) protective sunscreens and daily wear skin-care products[2–4]. Soft

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nanomaterials made from organic materials (lipids, proteins) also have wide commercial and pharmaceutical importance (liposome, solid lipid nanoparticles, dendrimers)[5, 6]. Currently, the FDA does not require manufacturers to label products containing eNMs and some products go to market without rigorous safety testing[7–9]. This has created human environmental health and safety concerns which has spurred efforts to investigate the ability of eNMs to penetrate epithelial tissue barriers and to characterize their cellular interactions[10, 11]. With a significant increase in nanotechnology enabled skin-care products and the corresponding increase in the potential for eNMs to contact skin, either through intentional product use or unintended environmental or occupational exposure, a significant effort has been devoted to investigate nanotechnology skin safety[2, 3, 12–15].

The purpose of this review is to highlight current knowledge of eNM skin interactions with a specific focus on the effect of UVR skin exposure, which introduces unique considerations when examining the larger question of eNM skin penetration. Firstly, UVR is a ubiquitous environmental insult that can induce defects in the skin barrier function. Secondly, people frequently apply eNM containing lotions (i.e. sunscreens, daily wear cosmetics) to UVR exposed skin. Thirdly, UVR exerts an immunosuppressive effect on skin. The latter is largely linked to photo-carcinogenesis[16, 17]. However, UVR induced immunosuppression is also a widely exploited therapeutic modality used by dermatologists to treat many skin disorders[18–22]. This dichotomy of UVR having both deleterious and therapeutic effects on skin biology heightens the need to discover how UVR may modulate eNM skin interactions. In the next sections we review aspects of human skin anatomy and the effects that UVR exposure have on skin biology, emphasizing the implications for eNM skin contact. We discuss what is known from current literature about the interaction eNMs with normal and barrier impaired skin. Fascinating findings are revealed that motivate future research directions in the nanotoxicology and nanomedicine fields.

Human Skin Anatomy - Implications for eNM Skin Contact

Skin is the largest organ of the body, providing key barrier functions preventing inside-out water loss and outside-in protection from environmental insults (e.g. microbes, particulates, irritants, allergens, UVR) including eNMs. Healthy human skin is divided into the epidermis (thickness: 50–100 μm) and the dermis (thickness: 300–3000 μm) which are separated by the basement membrane [Fig. 1]. Epidermis is multilayered (stratified) epithelium composed largely of keratinocytes[23]. The stratum basale is adjacent to the basement membrane and it contains cuboidal basal keratinocytes that have proliferative potential. The daughter cells produced differentiate to form the stratum spinosum layer that contains suprabasal transient amplifying keratinocytes. These cells further differentiate to form the stratum granulosum layer comprised of cells that contain a dense presence of keratohyalin granules and lamellar bodies. These contain the essential proteins and lipids, respectively that are needed to form the stratum corneum (thickness: 10–40 μm)[24, 25], the outermost layer of the skin [Fig. 2A]. The transition from a granular cell to a corneocyte (dead keratinocyte) is characterized by the degradation of the nucleus, assembly of a cornified envelope, and a reorganization of the keratin intermediate filaments[25]. The keratin network in the corneocyte is held together by filaggrin, which is a highly charged, cationic protein that aids in the filament aggregation [Fig. 2B] and disulfide bonding[26]. The flattened corneocytes are bound by tight junctions

(corneodesmosomes, Fig. 2C)[27] and they are surrounded by a unique lipid lamellar bilayer matrix [Fig. 2D][28–30]. The corneodesmosomes, the tortuous path between corneocytes and the lipid matrix together provide the main barrier function of skin, limiting penetration of hydrophilic and high molecular weight compounds[31] including eNMs. Corneocytes are continuously sloughed off and replaced by inner keratinocytes differentiating outward. The epidermis is continuously renewed every 4–6 weeks depending on the region of the body[32]. Hence, the normal process of epidermal turnover can potentially hinder systemic penetration of substances that breech the stratum corneum barrier. Given the cationic nature of the filaggrin-rich stratum corneum, the lipid rich intercorneocyte space, and the overall acidity of the stratum corneum (pH ~4–5)[33], the penetration of eNMs is anticipated to depend on their physiochemical properties (e.g. size, charge, composition). Understanding the factors that affect eNM stratum corneum penetration and the cellular interactions that can occur within the epidermis are critically important assessing risk from eNM skin exposure and for tailoring nanoparticle based transdermal therapeutics.

In addition, to basal and differentiated keratinocytes there are a number of other cell types present in skin that could potentiate the interaction of eNM that penetrate beyond the stratum corneum. Melanocytes, present the epidermis, produce melanin pigment which gives skin its color. They are evenly distributed along the basal layer and comprise 5% to 10% of epidermal cells[34]. The difference in skin color between light and dark pigmented individuals is due to the activity of melanocytes in their melanin production, not the number of melanocytes [35]. Melanocytes and keratinocytes form an epidermal unit in a 1:36 ratio[36]. Studies investigating the sensitivity of melanocytes to eNMs are lacking. Given the critical role that melanocytes have in protecting keratinocytes from UVR exposure and their potential to transform into a deadly malignant phenotype, suggests an urgent need exists to investigate whether eNMs can alter melanocyte function. Skin also provides innate and adaptive immune functions that are readied to respond to environmental insults that breech the stratum corneum[23, 37]. Langerhans cells (LCs) in the epidermis and dermal dendritic cells (dDCs) in the dermis are the main antigen presenting cells (APC) in the skin[38–40]. LCs comprise ~2–4% of epidermal cells[41–44]. CD1a positive immunocytes[45] and CD207 positive LCs [Fig. 3] form a tight meshwork within the epidermis and have been shown to localize around hair follicles at high densities, suggesting a need for increased immuno-surveillance surrounding these appendages. ACPs pick up antigens that breech the stratum corneum and migrate to the lymph nodes to activate the adaptive immune system. Skin contains a high density of resident T cells, ~$1\times10^6/cm^2$ which, for the average adult is nearly twice the number of T cells found in circulation[41]. Although skin lacks organized lymphoid structures, keratinocytes are able to produce a diverse repertoire of cytokines that can influence APC and T cell function[46–48]. Because skin comes in contact with numerous exogenous substances, the main route to allergen sensitization is consequently through skin[49–53]. Currently, little is understood about how eNM skin exposure may affect skin immune cell function, cytokine secretion by keratinocytes, or effect allergic skin disorders.

Other prominent features in the skin are hair follicles and sebaceous glands [Fig. 4]. Recent studies have solidified the important role that these structures have in mediating eNM skin penetration[54–56]. The pilosebaceous unit is a complex structure consisting of the hair shaft, arrector pili muscle, and the sebaceous gland. The outer root sheath (ORS) of the hair

follicle is contiguous with and biochemically similar to the basal layer of the epidermis[32, 57, 58]. The inner layers the follicle contain hair-producing cells. Hair follicles are in a continuous cycle of anagen (hair growth phase), catagen (involution phase) and telogen (a resting phase)[58, 59]. Skin resident stem cells reside in a follicular niche called the hair bulge[32, 60–62]. This suggests the potential for eNMs to interact with and perhaps alter stem cell function if they can penetrate and accumulate in the follicle. The hair follicle infundibulum is the region extending from the opening at the skin surface down to the sebaceous gland. Because hair follicles by-pass the stratum corneum barrier and terminate in the dermis, where they gain access to vascular and lymphatic systems, the hair follicle represents an important penetration pathway (epidermal shunt) and potential reservoir for systemic delivery of topically applied substances including eNM[54]. Hair follicle parameters such as orifice size, density, body distribution, and racial differences have been investigated[63]. The highest infundibular volume and therefore largest follicular reservoirs are found on the forehead and calf regions. Whites have significantly higher follicular reservoirs compared to Asians and African-Americans[64]. Follicular density differences are known to contribute to racial dependences seen in transdermal drug delivery efficacy[65] and this sets an expectation for racial differences to exist in how eNMs interact with skin. However, confirmatory studies have not yet been conducted nor has it been established whether eNM follicular accumulation and penetration levels depend on the hair cycle phase.

Effect of UVR on Skin Barrier - Implications for eNM Skin Contact

Ultra-violet solar radiation reaching the earth consists of UVA (315–400 nm), UVB (280–315 nm) and UVC (100–280nm) components. UVC is mostly absorbed in the upper atmosphere whereas UVB and UVA reach earth. UVA skin exposure penetrates deep into the dermis whereas UVB is mainly absorbed in the epidermis[66]. Skin exposure to UVR results in a number of biological responses including DNA damage, melanogenesis, generation of oxidative stress, vasodilation (skin erythema) and leukocyte infiltration[67]. Excessive and/or chronic UVR skin exposure causes sunburn, photoaging, and photocarcinogenesis[68]. UVB skin exposure from solar radiation or from tanning booths represent major environmental, occupational, and consumer health risks[69]. Unexpectedly, a growing source of UVR exposure is from compact fluorescent light bulbs, which are gaining wide acceptance as they use 75% less energy than incandescent bulbs and they produce the same lumens[70]. Studies suggest that defects in the phosphor coating applied to adsorb x-rays permit emission of UVA and UVC at exceedingly high levels[71]. Solar UVB radiation is however, the predominant concern as it is ubiquitous and a confirmed mutagen and carcinogen. It is efficiently absorbed by DNA producing cyclobutane pyrimidine dimers (CPDs); thymine to thymine (T<>T), thymine to cytosine (T<>C), and cytosine to cytosine (C<>C)[72]. Highly mutagenic 6,4 photoproducts are also generated but at a much lower frequency than CPDs[73]. Studies indicate that UVB skin exposure can generate ~519 CPD lesions per 10^6 normal oligonucleotide per J/cm^2 [Fig. 5] [72]. UVB skin exposure also induces dramatic effects on the cohesion and mechanical integrity of corneocytes in the stratum corneum[74] and it induces melanogenesis. Melanocytes increase production of melanin and package it into melanosome vesicles. These are transferred to keratinocytes and positioned above the nucleus to prevent DNA damage[75]. Melanogenesis leads to tanning

that becomes visible about 72 hours after exposure[76]. Defects in the mechanical integrity of the stratum corneum with the concurrent priming of keratinocytes to uptake melanosomes suggests the possibility for the increased ability of eNMs to penetrate into the viable epidermis and to be taken up by keratinocytes[77].

Skin has evolved elaborate defense systems to combat the mutagenic and oxidative stress effects of UVR exposure. Keratinocytes immediately respond by pausing their cell cycle to repair DNA lesions via the nucleotide excision repair process[78]. However, following high or chronic UVR exposure keratinocytes may not be able to repair all of the DNA damage generated which, can lead to mutagenesis and skin tumor formation. If and how eNM may alter these processes has not yet been investigated. After DNA lesions are repaired, keratinocytes down regulate E-cadherins [Fig. 6] which are important cell-cell adhesion proteins[79]. This facilitates a UVR-induced keratinocyte hyper-proliferation response which thickens skin to buttress the epidermal barrier function [Fig. 7][80]. Hyper-proliferation results however, in a disorganized stratum corneum structure where the presence of nucleated cells in the stratum corneum can be detected [81, 82]. This coincides with a measurable inside-out water loss skin barrier defect. Studies in mice (Fig. 8) indicate that the transepidermal water loss (TEWL) value is UVB dose and time dependent[83]. The peak water loss value occurs 3 to 4 days post UVB exposure and resolves in 7 to 10 days. Studies have not yet determined if the TEWL value is predictive of outside-in eNM stratum corneum penetration. Nor is it known whether epidermal thickening can hinder systemic transport of eNMs that do penetrate the disrupted stratum corneum or which day post UVR exposure skin is most susceptible to eNM penetration.

UVB induced Immunosuppression - Implications for eNM Skin Contact

UVB skin exposure is immunosuppressive which has a consequential role in skin photocarcinogenesis[84]. It is known that UVB exposure induces skin resident APCs to exit skin and migrate to the lymph nodes[85–87]. Migration initiates immediately post UVB exposure and the number of APC in skin remains low for 4–14 days. This phenomenon has long been studied using an *in vivo* mouse model of contact hypersensitivity (CHS)[43, 88–91]. Here, topically applied contact allergens or haptens, such as dinitroflourobenzene (DNFB) and oxazolone (OXA), interact with biomolecules in skin to create antigenic substances that induces T cell mediated sensitization. Subsequent skin exposure to the same haptan days later (challenge phase) induces severe skin inflammation that is quantifiable[91]. However, if during sensitization the hapten is applied to skin pre-exposed to low doses of UVB radiation, the CHS response after the challenge is significantly inhibited. The cellular mechanisms of the UVB-induced immunosuppression response are generally understood. LCs normally uptake UVB-damaged skin cell debris and migrate to local lymph nodes where they induce T regulatory cells (Tregs) against self-antigen[92]. This is important for the induction of immunologic self-tolerance and protection from developing photosensitivity disorders[91–95]. When hapten is topically applied to UVB exposed skin, LCs mediate generation of Tregs against the hapten. Consequently, upon challenge the mice exhibit a significantly reduced CHS response in an antigen-specific fashion[91, 92, 96]. Antigen specificity distinguishes UVB-Tregs from systemic drug-induced immunosuppression[91]. Because UVB skin exposure has the potential to impair skin barrier function and to induce a skin

immunosuppressive response, it can also potentially influence eNM skin penetration, alter systemic trafficking, toxicity, and skin immune function. What is known about these will be revealed in the next sections.

Engineered Nanomaterial Skin Interactions

Over the past 10 years increasing research has focused on the fundamental questions of whether or not eNMs can penetrate the stratum corneum barrier and what factors can impact penetration. Factors include for example, the eNM physiochemical properties (size, shape, composition, charge, surface energy)[97–101], vehicle affects[102, 103] and the skin barrier status (healthy, injured, or diseased)[83, 97, 104–106]. In surveying the literature it has become clear that analytical instrument detection sensitivity and sample analysis volume, combined with the wide ranging use of skin models, eNM types and coatings have created challenges to generating both qualitative and quantitative conclusions regarding eNM skin penetration[107]. Tissue histology and transmission electron microscopy (TEM) analysis are common methods used to investigate eNM skin penetration but these techniques suffer from limitations with tissue sampling and the inability to distinguish particles from intrinsic tissue features and background signal. Reliance on mass spectrometry techniques to track eNM persistence in skin, systemic transport, or organ distribution patterns limits the ability to determine if penetration occurred as intact particles or as dissociated ions[83, 104, 108, 109]. Some eNMs including quantum dots (QD) and ZnO nanoparticles do exhibit unique fluorescent and/or nonlinear optical signatures that can help distinguish penetration of intact particles from ions, but the detection sensitivity can be limited by tissue autofluorescence[107, 110–114]. Nonetheless, despite these complexities, progress is being made toward advancing our understanding of eNM skin interactions and our ability to draw conclusions about nanotechnology skin safety, as can be gleaned from many recent reviews[2, 3, 12, 14, 15, 115–117].

In general, substances that contact skin have three basic mechanisms to penetrate the stratum corneum barrier - transcellular through corneocytes, intercellular between corneocytes, and penetration via skin appendages (e.g. sweat glands, the follicular infundibidum)[118]. The transcelluar route is considered to be of little importance because the corneocyte cell membrane (cornified envelop) is highly impermeable although this is somewhat still debated[119, 120]. From the transdermal drug delivery field it is largely accepted that substances ~500 Da (~2.5 nm diameter) cannot penetrate the healthy SC barrier[31]. Vehicles in which substances are applied to skin can however enhance the penetration of higher molecular weight substances into deep skin layers, including eNM[121, 122]. Penetration enhancers generally work by increasing intercellular fluidity of the lipid lamella in the stratum corneum or by extracting non-covalently bound amphiphilic lipids from the stratum corneum[123]. From existing literature it can confidently be said that the healthy skin stratum corneum presents a formidable barrier to outside-in eNM penetration. However, in life it is quite common for skin to have barrier defective regions resulting from mechanical damage (cuts or scrapes), UVB exposure, use of harsh soaps or cosmetic products containing certain ingredients (depilatory agents, sodium lauryl sulphate, alpha-hydroxy acids) or from dermatologic disorders. Cutaneous defects can facilitate penetration of exogenous substances including large protein antigens (dust mite and plant allergens) and virus

particles (20 to 100 nm dia.)[124, 125]. Studies also show that barrier defective skin is more susceptible to penetration of eNMs and that eNMs penetrate the stratum corneum intercellularly through the lipid lamellae [Fig. 9][83, 97, 105, 106, 126–129].

Until recently the follicular penetration pathway had received little attention because hair follicles comprise ~0.1% of the total skin area[56]. Recent work has however, revealed that the follicular infundibulum, including sebaceous glands, comprise efficient and long-term reservoirs suited for accumulation of eNM[54, 55]. The high density of antigen presenting cells localized around the hair follicle [Fig. 3] and the presence of stem cells in the hair bulge have led researchers to target the follicular pathway for vaccine and drug delivery using eNM[100, 130–134]. Efficient access to the infundibulum reservoir requires however, that the sebaceous deposits and other debris in the follicular orifice be cleared. Opening the follicles using a cyanoacrylate-tape stripping processes has been shown to enhance deeper follicular penetration of particles[45, 132]. Particle accumulation, the depth of penetration, and retention in the infundibulum depend on particle size and these can be further enhanced by mechanical massaging of skin[45, 135–140]. Repetitive body motions such as walking or wrist flexing can also stress skin in ways that could potentially enhance follicular accumulation and eNM skin penetration.

The ability of eNM to accumulate in hair follicles and exhibit enhanced penetration through barrier defective skin heightens concerns as the prevalence of common skin disorders with known barrier defects (e.g. atopic dermatitis, contact dermatitis, psoriasis) are on the rise[49, 141, 142]. Few studies have however, been conducted on diseased skin making it difficult to draw general conclusions on whether skin disorders predispose individuals to increased risk of eNM penetration[112]. Studies are also needed to determine if hyperkeratotic disorders (e.g. psoriasis, epidermolytic hyperkeratosis) hinder penetration due to the thickened epidermis or enhance penetration due to malformations in the epidermal structure and barrier function. Similarly, little known about the effect of eNM skin exposure on allergic skin disorders[143, 144]. Studies are needed to determine if eNMs can induce or exacerbate allergic contact sensitization which is a leading occupational health concern[69]. The observation that UVR exposure can induce a skin barrier defect[81, 83, 103] is a unique concern considering the associated immunosuppressive effect. What is known about these will be examined in next.

Interactions of Engineered Nanomaterials with UVR Exposed Skin

Nanomaterials can contact UVR exposed skin through the intentional use of UVR protective lotions, nanoparticle-containing therapeutic treatments, or through unintentional exposure from environmental sources. By far the most important eNM to be studied in the context of UVR skin exposure is that of ZnO and $TiO_2$103, 110. Other eNM types that have been studied on UVR exposed skin include fluorescent QDs which facilitate tissue tracking[83, 105]; polymer nanoparticles[145], polymer coated platinum nanoparticles[146] and iron oxide nanoparticles[147] for improved UVR protection. The interest in nanoparticle based UVR protective lotions stems from the fact that many common organic UVR filters such as, 2-ethylhexyl 4-methoxy cinnamate (OMC) and 3-benzylidene camphor (3-BC), can penetrate through skin and they have been detected in urine and breast milk[148, 149]. Organic filters can

also exacerbate generation of UVR-induced reactive oxygen species (ROS) in skin[150]. The purported advantage of particle-based UVR filters is that their large size would hinder stratum corneum penetration and skin retention (follicular reservoir effect) to improve photoprotection[151].

Metal oxide particle filters work by reflecting, scattering and absorbing UVR. The relative contribution of these mechanisms depends on particle size, crystal structure, and wavelength[152, 153]. The high surface energy the TiO_2 and ZnO oxides causes primary particles of size 5 to 20 nm to aggregate (irreversibly bound) and these can further form agglomerates (loosely bound aggregates). Agglomerates that exceed 1 μm efficiently scatter visible light and thus apply to skin as an unappealing white gritty film[154]. Dispersants are used to maintain particle aggregates in the size range of 30–150 nm which is optimal for UVB and UVA absorption and for producing lotions that apply clear to skin [Fig. 10]. A consequence of increasing the UVR absorption capacity of nanoscale metal oxides is the increased photogeneration of radicals and ROS which can cause DNA lesions and lipid peroxidation[155]. Generally, the anatase form of TiO_2 displays higher photoactivity, ROS production and cytotoxicity than the rutile form which is preferred for use in UVR protective cosmetics[155, 156]. To hinder agglomeration and to help protect skin from contacting the potential phototoxic TiO_2 and ZnO metal oxide core, the particles are usually coated with for example silica, aluminum hydroxide, or methicone[145, 157, 158]. While much is understood about the formulation of UVR protective cosmetics and how to control the photoactive properties of eNM, very little is understood quantitatively or mechanistically about the penetration of nanoscale TiO_2 and ZnO, or other eNM through UVR exposed skin. This has spurred increasing efforts over the past 5 years to examine this specific question. Because the biological effects of UVR on skin exposure evolve over time and involve the immune system, the most useful data will be generated using *in vivo* skin models. Here we review what has been gleaned from use of *in vivo* mouse[83, 159], pig[103], and human skin models[108, 109].

The impact of UVR on nanoparticle skin penetration was first investigated by DeLouise and coworkers using the SKH hairless mouse model[159]. Carboxylated (dihydrolipoic acid coated) QDs were topically applied to mice in a 30% glycerol vehicle immediately following UVR exposure (270 mJ/cm^2 UVB). The mice wore Elizabethan collars to prevent grooming. After 8 and 24 hr, skin samples were qualitatively examined for the presence of QD using tissue histology, confocal microscopy, and silver enhanced TEM with EDAX analysis. Low levels of penetration were seen in both the non-UVR and the UVR exposed mice but qualitatively much higher levels of penetration were observed in the UVR exposed mice. TEM images suggested that QDs penetrated the stratum corneum barrier along the intercellular space between corneocytes [Fig. 9]. Interestingly, despite the high UVB dose employed, the penetration levels observed were estimated to be extremely small compared to the dose applied but a quantitative organ analysis study was not performed. Because mouse skin is much thinner than human skin and the QDs physiochemical and dispersive properties differ from the metal oxides formulated in UVR protective lotions, other groups were motivated to investigate the penetration of TiO_2 and ZnO lotions applied to UVR exposed pig and human skin.

Gulson and coworkers[108] recruited human subject volunteers (n = 20) to investigate the penetration of ZnO particles through UVR exposed skin. Subjects applied an oil-water based sunscreen containing isotopically labeled ZnO particles (^{68}Zn) to their backs twice a day for 5 consecutive days. Two different sunscreen formulations were tested: a nano-formulation containing of particles with primary size ~19 nm and a bulk-formulation containing particles >100 nm. After each sunscreen application, subjects laid in the sun on their bellies for a minimum of 30 min. The minimum average UVB dose was estimated to be 180 mJ/cm^2 per 30 min exposure. Venous blood and urine samples were collected. Results found small increases in tracer ^{68}Zn levels in the blood and urine from all subjects however, the overwhelming majority of applied ^{68}Zn was not absorbed (<0.001% absorbed in blood). Tracer levels in the blood and urine from female subjects who received the nano-formulation appeared to be higher than males receiving the same treatment and higher than all subjects receiving the bulk-formulation. Authors conclude that small amounts of Zn from ZnO particles in the sunscreen could be absorbed through the skin and detected in blood and urine of healthy subjects exposed to sunlight. A similar conclusion was reached in a follow-up study using different a sunscreen formulation and a different UVB exposure[109]. However, neither study was able to distinguish if the ^{68}Zn detected had been absorbed as particles or as soluble Zn ion or both. In fact, a recent study reported that ZnO in commercial sunscreens is dissociated to Zn ion under UVB irradiation [160]. Also, the Gulson studies did not control for total UVB dose or sunscreen usage on other body parts, so it is unclear if sunscreen was applied to barrier defective skin. It is important to note that the ability to distinguish Zn ions from ZnO particles in skin with sufficient sensitivity to overcome skin background signal has recently been demonstrated using sophisticated NIR multi-photon and second harmonic generation microscopy techniques[110, 161, 162]. Studies of sunscreen applied to human skin with and without barrier impairment, including patients with psoriasis and atopic dermatitis all find little evidence that ZnO nanoparticles can penetration beyond the SC[110, 112, 114, 128]. These techniques have, however, not yet been utilized to investigate the potential for ZnO particles to penetrate barrier impaired skin induced by UVR exposure.

While studies on human skin are ideal, conducting human subjects research is expensive and limiting. Animal models are less expensive and offer improved control over UVB dose and topical administration. Medical literature has long recognized that pig skin is anatomically and physiologically the most similar to human skin[163]. Monteiro-Riviere and co-workers[103] used white Yorkshire pigs to investigate the effect of UVR exposure on nanoscale TiO_2 and ZnO skin penetration. The pigs received a UVB dose of 100 to 120 mJ/cm^2 that induced a pale red erythema. One day after UVR exposure the sunscreens were topically applied to a controlled area. Metal oxides particles were incorporated into oil/water (o/w) and water/oil (w/o) sunscreen formulations. The application area was occluded and a second sunscreen dose was applied on day 2. On day 3 the skin was harvested for analysis. TEM results found that both nanoparticle types primarily localized as large agglomerates on the skin surface. On UVR exposed skin TiO_2 (not ZnO) particles penetrated into layers deeper of the stratum corneum. A similar result was observed on explant pig ears[164]. The superficial penetration observed was modulated to some extent by the nature of the topical formulation; with the w/o formulation permitting deeper penetration of TiO_2 into stratum corneum layers. This finding corroborates an early study that reported microfine TiO_2 penetrated deeper into

human skin from an oily dispersion than from an aqueous one[165]. The authors further conclude from TEM and histology analysis that UVB exposed pig skin showed slightly enhance penetration and that TiO_2 penetrated deeper into the SC in both normal and UVB-exposed skin compared with ZnO. However, using the more sensitive technique of time-of-flight secondary ion mass spectrometry (TOF-SIMS), elemental Ti was detected in the epidermis and superficial dermis and elemental Zn also was detected in the upper epidermis. Again, it is not known whether the elements were absorbed as soluble ion or as particles, but the results emphasize the importance of considering the analytical technique detection sensitivity in drawing definitive conclusions about eNM skin penetration.

While tissue histology, optical and electron microscopy techniques can provide important insight into the localization of eNM in the skin and can yield some understanding of cellular penetration mechanisms, these techniques do not yield quantitative information. To more fully understand the extent of eNM skin penetration and the systemic translocation, the techniques of inductively coupled plasma mass spectrometry (ICP-MS) and atomic absorption spectroscopy (AAS) are often used to evaluate eNM elemental organ collection patterns. Guided by results of an earlier study, that found the liver and lymph nodes were the major collection sites of Cd following dermal injection of (CdSe/ZnS core/shell) QDs using ICP-MS [111], our lab quantitatively investigated the effect of UVR skin exposure on the penetration of QDs topically applied to mice using AAS [Fig. 11]. SHK hairless mice we exposed to UVR (360 mJ/cm^2 UVB) and negatively charge QDs (DLHA-coated CdSe/ZnS core/shell) were topically applied 3 to 4 days post UVR exposure at the peak of the TEWL defect [Fig. 8]. After 24 hr the Cd concentration in the lymph nodes and livers of UVR exposed mice were quantified relative to controls (no UVR)[83]. Results detected a baseline Cd level in the livers of control mice (vehicle treatment only); the likely source of this Cd was from their food. Application of QDs to UVR exposed skin found a statistically significant increase in liver Cd but the increase as a % of the applied does was very low. The baseline levels (vehicle treatment only) of Cd in the lymph nodes (with and without UVR) were below the limit of quantification (LOQ). Application of QDs to control mice (no UVR) produced an unexpected result in that Cd was detected in the lymph nodes; suggesting QDs penetrated barrier intact skin. This result contrasted histologic and TEM analysis of tissues sections, but due to the limitations of sampling and detection sensitivity mentioned earlier and the increased sensitivity of AAS, it is plausible that QDs can penetrate mouse skin. Interestingly, application of QDs to UVR exposed (barrier defective) skin produced a ~45% lower level of Cd the lymph nodes. This result was also unexpected and suggested an effect of UVR on the mechanism of systemic transport of QDs to the lymph nodes and implicated the role of skin resident APCs. Previous work established that LCs can uptake and traffic polymer NPs topically applied to skin to the lymph nodes[45, 166]. Due to the immunosuppressive effect that UVR has on skin we quantified LC density before and days 3–4 post UVR exposure[83]. Results found that the LC density in skin was depleted by ~80% at the time QDs were topically applied. Hence, the lower Cd level in UVR treated mice is consistent with the lesser availability of LCs to uptake and traffic QDs to the lymph node.

Based on the above, intriguing questions arise regarding the potential for eNM that contact UVR exposed skin to either alter or induce skin immune responses. As discussed above topical application of antigen to UVB exposed skin results in the generation of antigen-

specific Treg cells. Since UVB exposure has been shown to increases slightly the skin penetration of eNMs and that LCs have been shown capable of up-taking eNM and transporting them to lymph nodes, it is curious to question whether humans can developed tolerance to topically applied nanoscale materials. It is also intriguing to question the affect that eNM may have on LC function and their ability to present antigen. These specific questions have not yet been investigated. However, the potential for eNMs to modulate skin immune function is a rapidly growing field. A few recent examples are briefly discussed below.

Modulating the Skin Immune Responses with Nanoparticles

Some studies have investigated the potential to develop contact dermatitis following eNM skin exposure and to examine how topical application of eNMs may alter symptoms of allergic skin disorders. One recent study reported that subcutaneous injection of different size TiO_2 nanoparticles (15, 50, 100 nm) (not topically applied) exacerbated development of atopic dermatitis (AD) symptoms (ear thickening, protein expression of inflammatory molecules) in mice induced by mite allergen, but no effect of particle size was observed[167]. In a companion paper a similar study was done using different size polystyrene (PS) nanoparticles (25, 50, 100 nm)[101]. Results found that injected PS nanoparticles aggravated AD-like symptoms even without co-exposure to mite allergen. In contrast to TiO_2, a size effect was observed with the smaller PS particles producing greater symptoms. These findings were corroborated by another group that injected TiO_2 nanoparticles (20, 230 nm) prior to topical sensitization with dinitrocholorobenzene (DNCB) and results showed TiO_2 exacerbated AD symptoms in mice[168]. Contrasting results have also been reported where topical application of both PS and TiO_2 nanoparticles to barrier intact skin models did not induce acute cutaneous irritation or exacerbate a skin sensitization response[169]. Topical application of mesoporous silica particles (100 nm spheres) also did not induce an ear swelling response in mice or exacerbate allergic contact dermatitis symptoms even when co-administered with dinitrofluorobenzene (DNFB)[170]. The likely differences observed between injection and topical application is the magnitude of the eNMs in the epidermis.

The above studies reveal the importance of particle composition on exacerbating AD-like symptoms and suggest a potential concern if these nanoscale materials were to contact severely barrier impaired skin. Clearly, for eNM to exert an immunomodulatory affect they must be able to penetrate skin to an appreciable extent. Penetration can be modulated by physical means (injection or stratum corneum depletion) or perhaps by varying both the eNM core and surface composition. Studies with both nanoscale gold and silver particles suggest these metals have a greater propensity to penetrate intact skin than metal oxides. For example, an *in vivo* study showed that topical application of 200 nm Ag particles formulated in a nanolipid carrier o/w cream exhibited a high capacity to reduce AD-like symptoms[171]. Similarly, topical application siRNA coated gold nanoparticles (~50 nm) designed to down regulate epidermal growth factor receptor (EGFR) freely penetrated the mouse epidermis and a human skin equivalent model within hours of application[172]. Following a 3 week application protocol of the siRNA Au particles to hairless mice nearly abolished EGFR expression and reduced the thickness of the epidermis by almost 40%. Whether the differences observed in skin penetration between nanoscale metal and metal oxide particles

is driven by surface coating, electronic properties, or the tendency of metal oxide particles aggregate to large sizes that hinder skin penetration is not yet fully understood. The role that nanoparticle conductivity has in modulating skin biology is also poorly understood. Studies have shown that bimetallic nanoparticles (Cu/Zn 20 nm) applied to skin can induce a galvanic couple and that the electrical stimulus generated can reduced the skin inflammatory response to sensitizing agents[173]. Citrate coated Au nanoparticles were also reported to interfere with downstream IL-1β signaling inhibiting the production of PI3 kinases and proinflammatory TNF-α release in a size dependent manner with 5 nm particles, but not 20 nm, exhibiting the greatest neutralizing effect[174]. Highly conducting fullerene nanoparticles were reported to exhibit a potent anti-oxidant free radical scavenger activity and inhibit allergic anaphylaxis response *in vivo*[175]. Hence, the above examples clearly illustrate the rich potential for developing novel immunomodulatory therapeutics for treating skin disorders using eNMs but the role of particle conductivity has not yet been elucidated.

Conclusions

In this review we discussed the current state of understanding of the interactions of eNMs with skin. We explored the unique dichotomy that UVR exposure has on skin, raising awareness of the challenges to developing a generalized view of eNM skin penetration and translocation. While barrier impaired skin is seen as more susceptible for eNM penetration, the biological and immunologic responses of skin to the impairment means (chemical, physical, disease) will be differ and thus can influence the extent and mechanisms of eNM skin penetration and translocation. Existing literature currently suggests that UVR skin exposure can slightly enhance the penetration of eNM. Significant health issues from eNM occupational exposures or from use of nano-enabled products have not yet emerged. Nonetheless, it is curious to consider if eNM immune-tolerance via UVR-Treg generation exists or if it tolerance to haptans can be modulated by eNM contact with UVR exposed skin (i.e. TiO_2 and ZnO). In vivo models will be essential moving forward for examining these questions and elucidating the central role that eNM composition (core/coating) has on dictating skin interaction so that the deleterious and therapeutic benefits of eNMs can be minimized and maximized, respectively. A further concern with the current state of understanding is the limitations imposed by the models and instrumentation utilized. It is challenging to relate acute high dose studies to real world human exposures. Similarly, it is difficult to extrapolate the significance of results that find intradermal injections of eNM can exacerbate AD-like symptoms to realistic topical human exposure conditions. The ability to attain a definitive consensus on the ability of eNMs to penetrate beyond stratum corneum is limited by instrumentation detection sensitivity. Clearly, there is an urgent need for nonbiased means to amplify the detection of eNM presence in tissues[107]. Simple and widely assessable methods that can distinguish soluble ion from particle penetration are also needed to advance the fields of nanotoxicology and nanomedicine.

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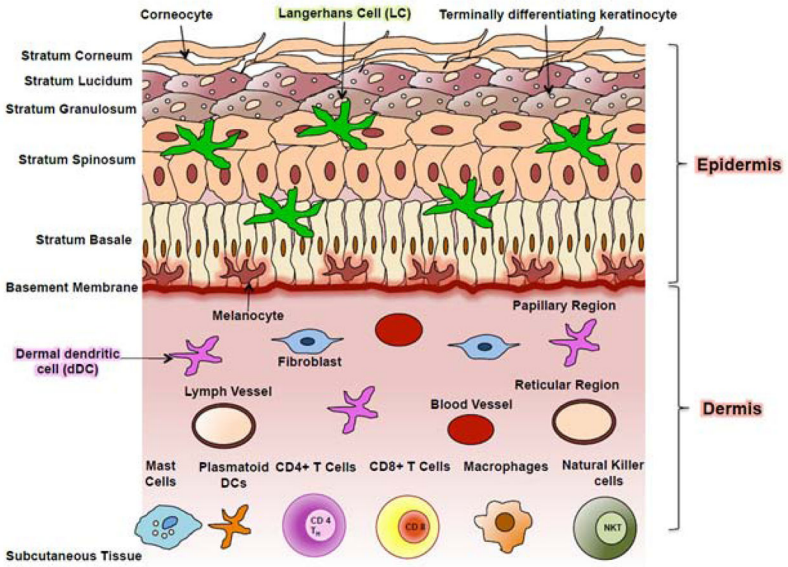

Figure 1. Schematic of human skin structure and constituent cell types

Skin is stratified epithelial composed of the epidermis and dermis. The epidermis is mainly comprised of keratinocytes. Basal keratinocytes undergo terminal differentiation to form the stratum spinosum, stratum granulosum, and stratum corneum barrier. Stratum lucidum is an additional layer present under the stratum corneum in areas of thick skin like palms of the hands and soles of the feet. Pigment producing melanocytes and antigen presenting Langerhans cells are also present in the epidermis. The dermis is a layer rich in connective tissue and is divided into the papillary and reticular regions. The dermis contains many cell types including fibroblasts that make collagen and other extracellular matrix molecules that provide skin mechanical toughness. Adipocytes, macrophage, mast cells, plasmatoid dendritic cells (pDCs), CD4+ T cells, CD8+ T cells, Tregs, and natural killer T-cells also abundantly present in the dermis apart from other structures including pilosebaceous unit, sweat glands, nerves, blood and lymphatic vessels. Adapted from Nestle et al., 2009 [23].

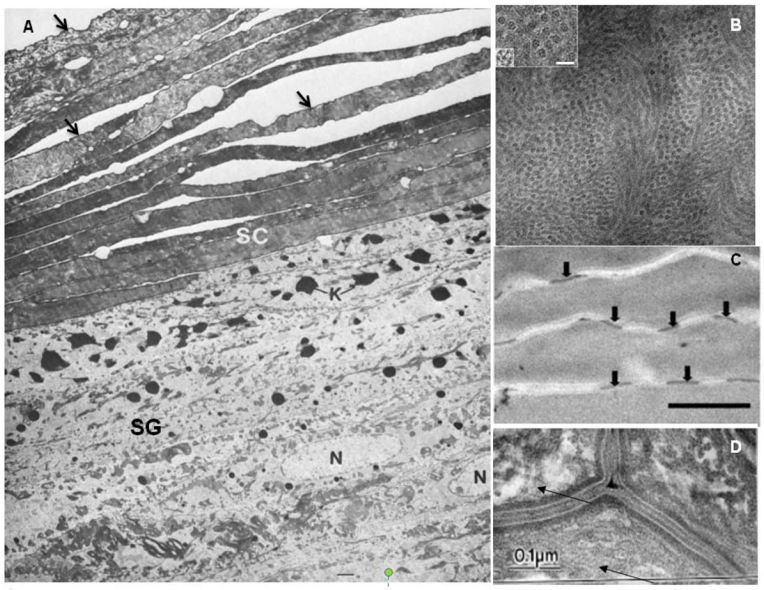

Figure 2. Transmission electron micrographs illustrating unique features of skin

(A) The stratum corneum (SC) is comprised of multiple layers of enucleated and elongated corneocytes each defined by a dense cornified envelope indicated by the black arrows. The cells in the stratum granulosum (SG) are distinguished by the presence of a nucleus (N) and a high density of keratohyalin granules (K). Adapted with permission from Madison et al., 1998 [24] (B) Electron micrograph of a corneocyte cytosol illustrating the nanoscale organization of keratin intermediate filaments. The subfilamentous molecular architecture appears as groups of electron dense spots surrounding a central dense dot. The keratin filaments are ~7.8 nm wide with a center-to-center distance of ~16 nm. (Inset box scale bar is 10 nm. Adapted with permission from Norlen and Al-Amoundi, 2004 [25] (C) Corneocytes in the stratum corneum are bound by corneodesmosome tight junctions indicated by black arrows. Racial differences exist in the density of corneodesmosomes. Scale bar is 1 μm. Adapted with permission from Gunathilake et al., 2009 [27]. (D) Electron micrograph illustrating the lipid lamellar bilayers in the intercellular space between corneocytes. Adapted with permission from Warner et al., 1999 [30].

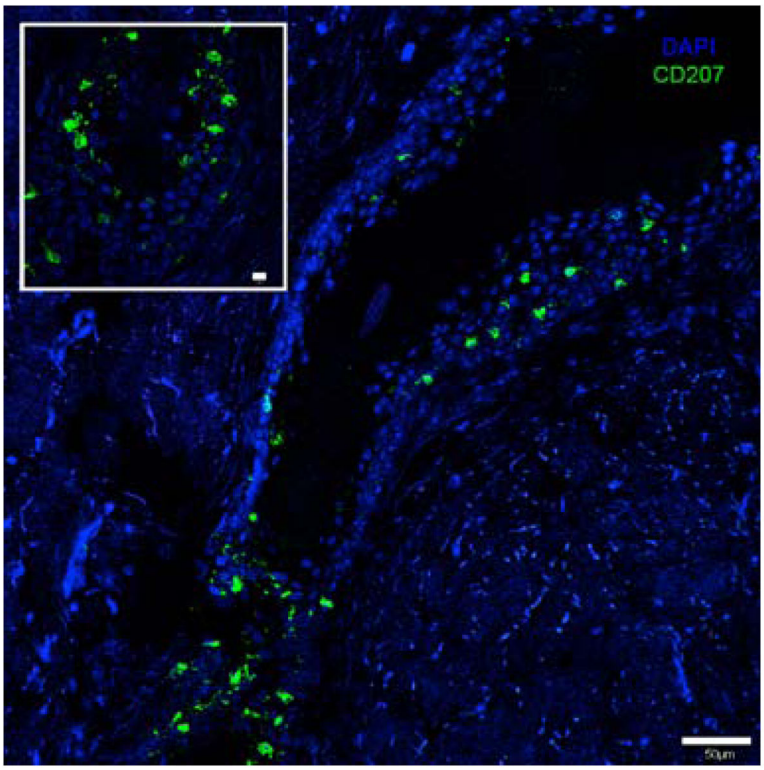

Figure 3. Langerhans Cell localization pattern around the hair follicle

Immunofluorescent staining of human skin epidermis with anti-CD207-Alexa 488 (Langerin) specific for Langerhans cells showing their distribution around the hair follicle infundibulum, scale bar=50 μm. Inset shows the base of the hair follicle, scale bar=10 μm.

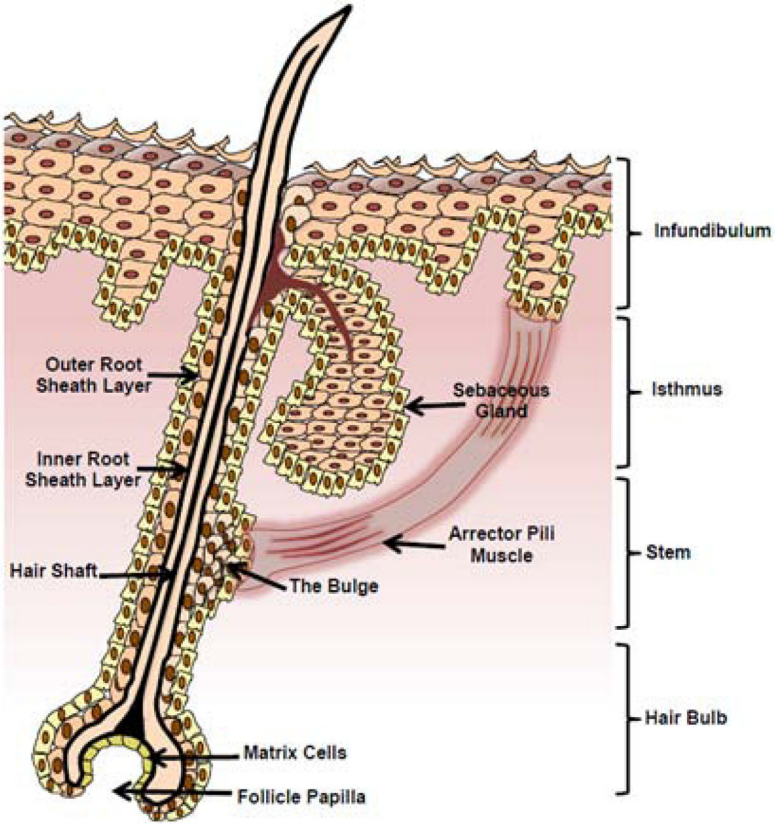

Figure 4. Schematic of the human hair follicle in late anagen phase

The bulge, outer root sheath, hair bulb and follicle papilla are responsible for hair growth.

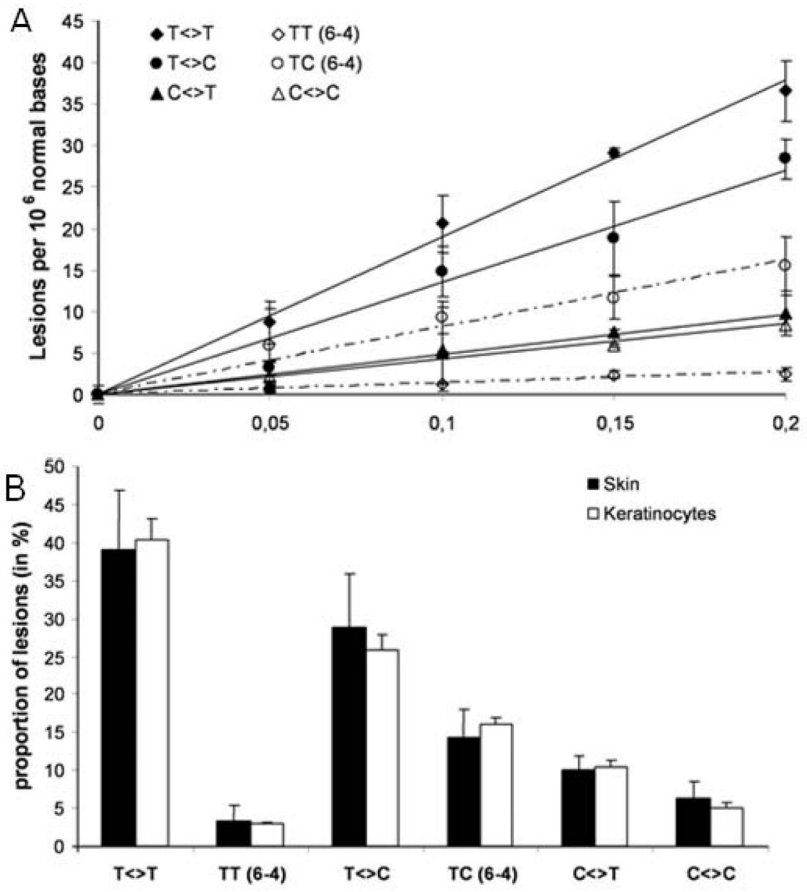

Figure 5. Formation of bipyrimidine photoproducts within human skin exposed to UVB radiation

(A) Photoproduct formation is linear with respect to the applied UVB dose (0–0.2 J/cm^2). The results are expressed in lesions per 10^6 bases and are the average ± SD. (B) A similar distribution of bipyrimidine photoproducts is produced in human skin and in cultured primary keratinocytes isolated from the same donor following UVB. Reprinted with permission from Mouret et. al., 2006 [72].

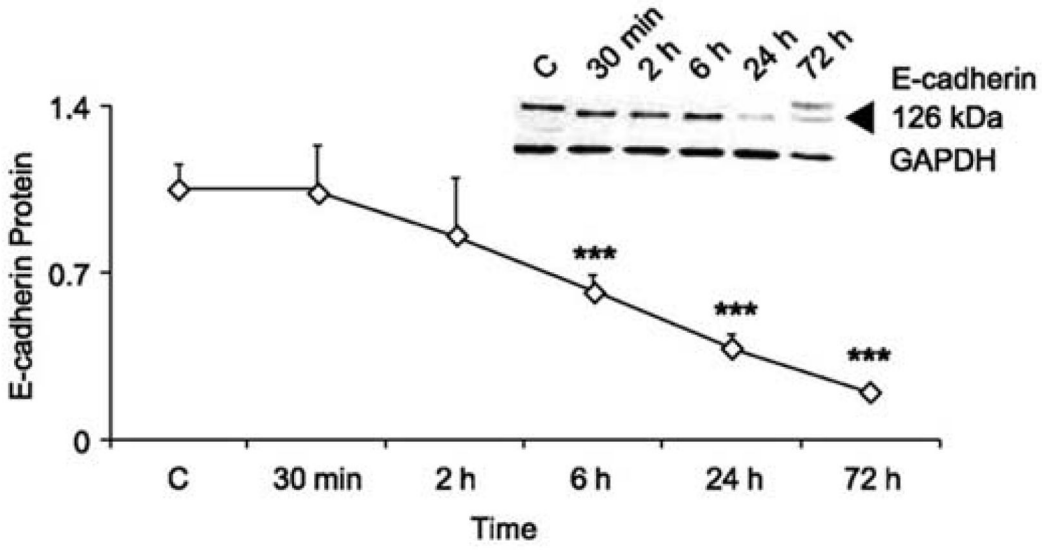

Figure 6. E-cadherin expression in primary mouse keratinocytes (PMK) after acute UVR exposure

PMKs were harvested at the times indicated post UVR (40 mJ/cm^2 UVB). Unirradiated PMKs were included as controls. Densitometric analysis of Western data for relative E-cadherin levels normalized to GAPDH. E-cadherin levels were significantly different from control at 6, 24, and 72 h (n = 3). ***, $p < 0.001$. Inset, a representative Western blot for E-cadherin from one of three separate experiments. Equal loading of protein was verified by GAPDH staining. Reprinted with permission from Brouxhon et. al. 2007 [79].

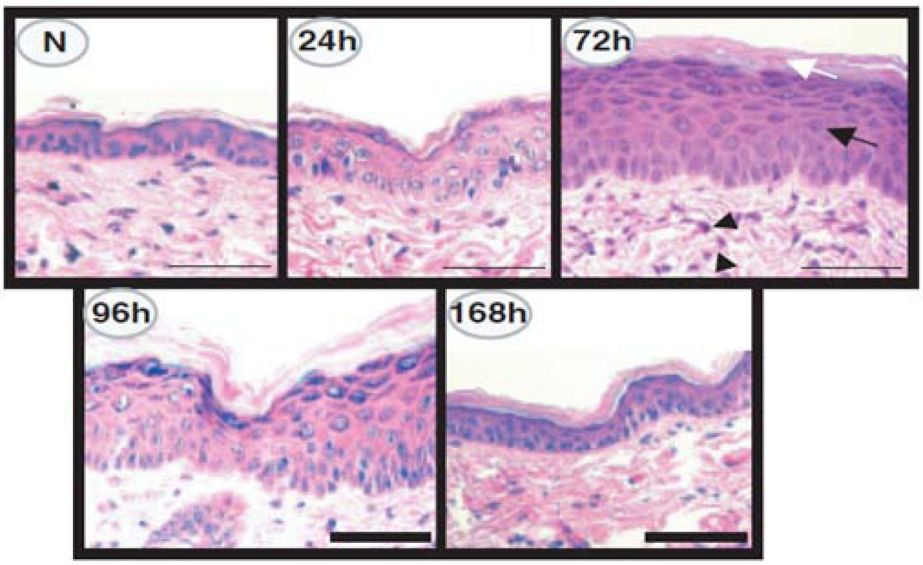

Figure 7. Characterization of UVR-induced epidermal injury in SKH-1 mice

Hematoxylin and eosin stain photomicrographs of normal dorsal mouse skin (N) and of skin 24, 72, 96, or 168 h after UVR (180 mJ per cm^2 UVB) irradiation. Note the epidermal hyperplasia (black arrow), hyperkeratosis (white arrow), and the perivascular inflammation (arrowheads) present 72 h after UVR irradiation. At 168 h post UVR irradiation, the epidermis has returned to near normal. Scale bar: 50 μm. Reprinted with permission from Tripp et. al., 2003 [80].

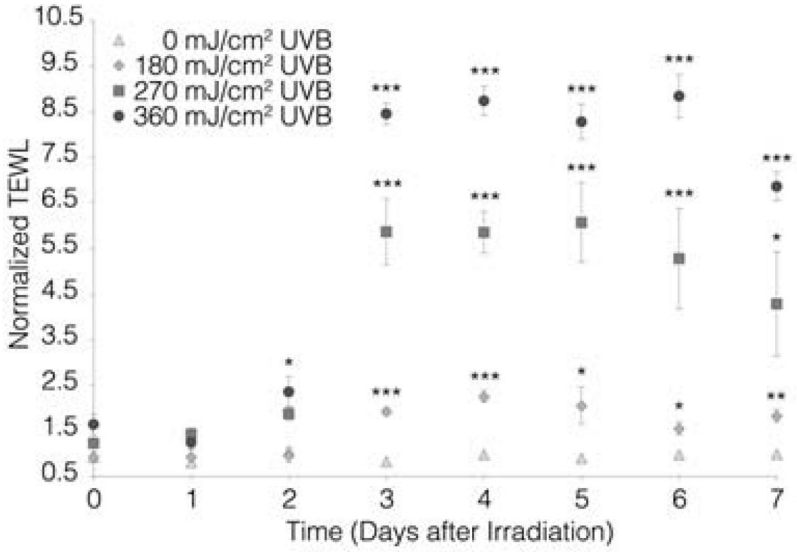

Figure 8. The effect of UVR on SKH mouse skin barrier function as measured by transepidermal water loss (TEWL)

UVR exposure increases the barrier defect in a UVB dose (0 to 360 mJ/cm^2 UVB) and time dependent manner. A statistically significant increase in TEWL is observed for all exposures with the peak defect ranging from days 3 to 6 post-UVR exposure. Each value is reported as the mean ± SEM (n=4, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). Reprinted from Mortensen et. al., 2013 [83].

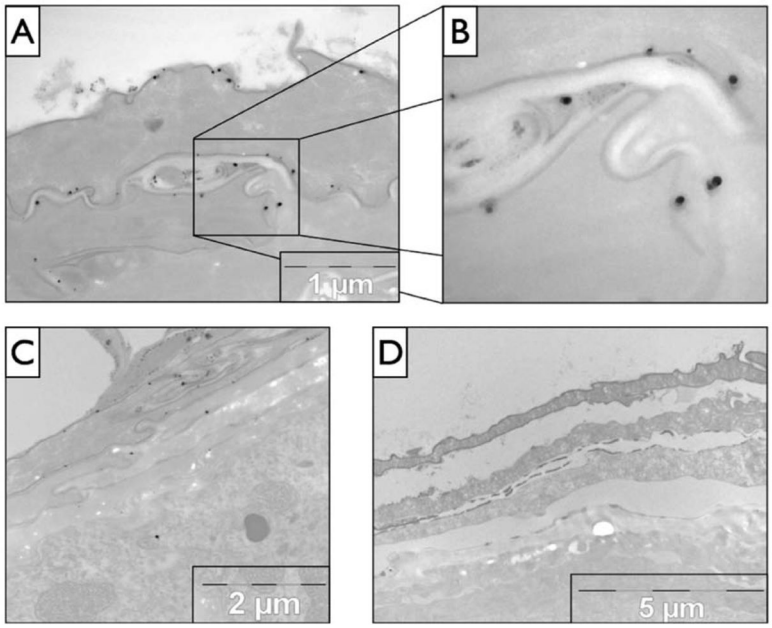

Figure 9. TEM imaging of mouse skin sections suggesting quantum dot nanoparticles penetrate intercellular between corneocytes

(A) The penetration pathway through the stratum corneum is between corneocytes which is shown in more detail in (B) where the large dark spots are quantum dots. (C) Another skin section demonstrating the penetration pathway showing quantum dot present in the stratum granulosum. (D) A negative control (no quantum dots). Reprinted with permission from Mortensen et. al., 2008 [159].

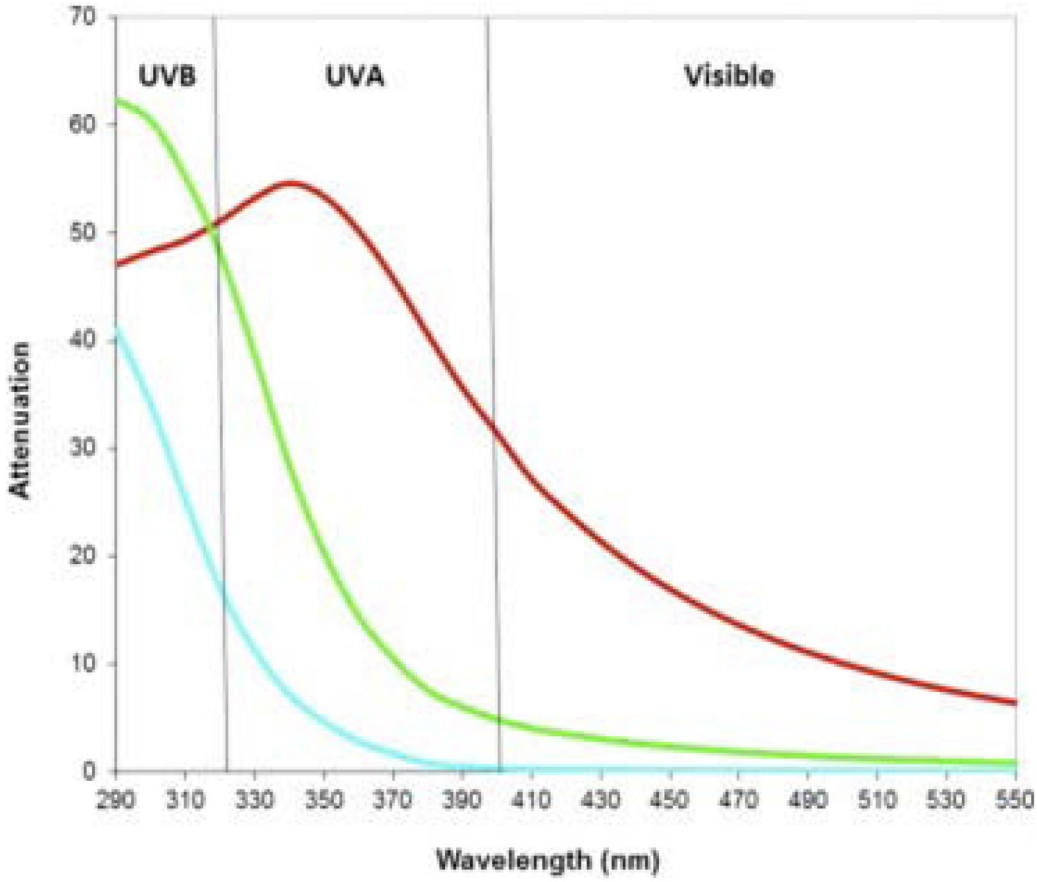

Figure 10. Dependence of UV-visible light attenuation on TiO_2 nanoparticle aggregate size

With decreasing particle size UV protection shifts to shorter wavelengths. Blue line, 20 nm; green line, 50 nm; and red line, 100 nm. Particles with average aggregate size of ~ 50 nm offer high UVB attenuation and lower visible light scattering but less UVA absorption. Reprinted with permission from Wang and Tooley, 2011 [154].

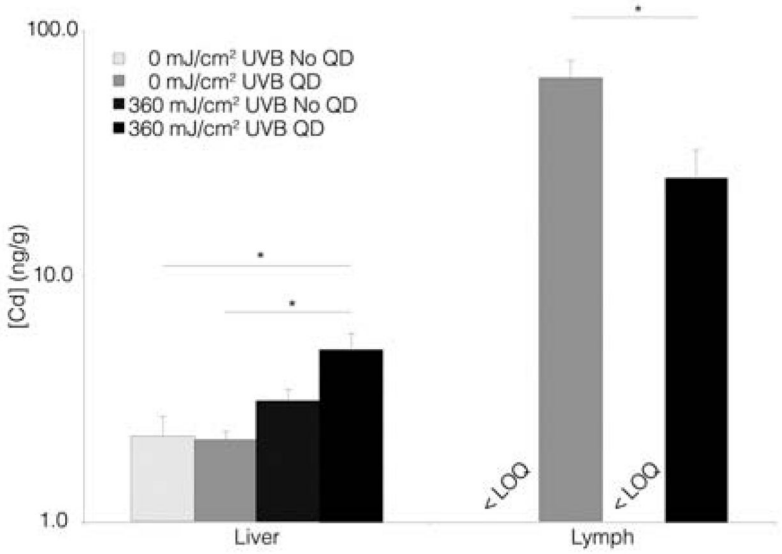

Figure 11. Cadmium tissue level in distal organs following 24 hr QD nanoparticle application to SHK mice as a function of UVR

Liver results show that QD exposure to control mice (no UVR) does not significantly increase Cd level. Application of QDs to UVR exposed mice did statistically increase liver Cd relative to controls (no UVR with or without QD). Lymph node results show that the background Cd level was below the limit of quantification (<LOQ) in vehicle-treated animals with and without UVR exposure. Unexpectedly, application of QDs to control mice (no UVB) produced a high Cd level in the lymph nodes suggesting QDs penetrated intact mouse skin. QD application to UVR exposed mice produced lower Cd level suggesting a UVR dependent cellular transport QD mechanism to lymph node Each value is reported as the mean ± SEM (n=5, $*p < 0.05$). Reprinted from Mortensen et al., 2013 [83].