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ARTICLE

Penetration, photo-reactivity and photoprotective properties of nanosized ZnO

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The oxidizing capacity and skin penetration of a commercial nanosized ZnO, Nanosun™ (Micronisers-Australia), was evaluated *in vitro* using porcine skin. Nanosun™ was initially characterized regarding its photo-reactivity and size distribution. An assay using methylene blue was performed to confirm the Nanosun™ photo-reactivity by exposing the labile molecule under UVA irradiation in the presence and absence of the nanosized ZnO. The nanosized ZnO was photo-reactive, reducing methylene blue concentration to 7 % while its concentration remained constant in the control formulation (without ZnO). The product label states that the average particles size is 30 nm. X-ray diffraction, nitrogen sorption and UV-spectrophotometry confirmed the presence of nanometric particles of approximately 30 nm. On the other hand, laser diffractometry showed micrometric particles in the size distribution profile. These analyses indicated that the nanoparticles are arranged as agglomerates and aggregates of micrometric proportions ranging from 0.6 to 60 µm. The skin lipid peroxidation was determined by the formation of thiobarbituric acid reactive species (TBARS) and quantified by UV-spectrophotometry. When exposed to UVA radiation nanosized ZnO applied porcine skin showed a lower production of TBARS (7.2 ± 1.5 nmol.g^{-1}) than the controls, MCT applied porcine skin (18.4 ± 2.8 nmol.g^{-1}) and blank porcine skin (14.0 ± 2.0 nmol.g^{-1}). The penetration of ZnO nanoparticles was studied by scanning electron microscopy and energy dispersive x-ray spectroscopy. The tested ZnO particles did not penetrate to viable layers of intact porcine skin. The particles tend to accumulate on the skin folds and in these regions may penetrate into the horny layer.

Introduction

The skin is exposed to many oxidant agents including ultraviolet A (UVA) and ultraviolet B (UVB) light, which generate a series of reactive oxygen species (ROS). Reactive oxygen species are responsible for oxidative stress that leads to lipid, protein and DNA cellular damage[1]. Many products have been developed to protect the human skin from UVA and UVB induced damage, including chemical sunscreen lotions, antioxidant creams and inorganic sun blocks[2].

Inorganic sunblocks, such as ZnO, present the advantage of combining the properties of absorption, scattering and reflection of light which lead to a large spectrum protection (290–400 nm)[3]. ZnO, especially, has an absorption edge at ~380 nm and a refractive index of ~2.0, these values may vary with particle size[4].

The use of micronized ZnO has been preferred to classic ZnO based on two major motives: 1) the absorption coefficient of ZnO and TiO_2 are improved with size reduction and 2) the use of nanosized ZnO rather than lager particles eliminates the formation of an opaque film (visible light scattering) [4,5].

Unfortunately, nanosized metal oxides in addition to being effective sun blocks are also known for their photocatalytic activity. In the form of nanoparticles, under irradiation, this oxide acts as a strong oxidizing agent, capable of interacting with a great deal of biological molecules[5].

UV absorption by ZnO is a mobilization of electrons in its atomic structure. The amount of energy needed to dislocate an electron from a valence band to a conductive band (3.23 eV for ZnO) is known as the energy gap. For ZnO the energy gap corresponds to a UV wavelength of 385 nm. Therefore all UV radiations shorter than 385 nm will be absorbed by ZnO. So, ZnO is not inert per se, the generated conduction band electrons (e^-) yield superoxide radical anion (O_2^-) from pre-adsorbed molecular oxygen and the valence band holes (h^+) generate hydroxyl radicals (-OH) from surface hydroxyl groups (and chemisorbed water). These species initiate oxidation reactions[3,4,5].

Zinc oxide (of cosmetic grade) was reported to induce lipid peroxidation in human epidermal cells due to an increase in ROS levels[6]. The cellular damage induced by ZnO under UVA radiation is highly associated to oxidative reactions and an induction of lipoperoxidation may be expected.

The balance between phototoxicity and photoprotection, regarding nanometric ZnO, is still controversial. It is noticeable that the scientific reports of ZnO cyto- and genotoxicity present contradictions. While some scientific researches observe *in vitro* cytotoxicity others find evidence of *in vitro* photoprotective effect.

According to Hayashi and co-workers, microcorpuslar ZnO was capable of reducing keratinocyte UVB induced oxidative stress membrane injuries, exhibiting *in vitro* photoprotective properties[7]. This contradiction is mostly due to variations of experimental parameters, such as particle size measurements. It is of interest that the particles be analyzed by more than one technique[8], in such a way that agglomerates, aggregates and primary particles can be measured. The particle size reflects on its reactivity and on its interaction with biological barriers.

Particle size is an important property when considering that damage at a viable cell level will only happen if the nanoparticles penetrate and/or permeate the skin. In this aspect, the penetration of ultrafine zinc oxide into intact and damaged skin showed no particles in the cutaneous layers[9] or minimal penetration into the upper epidermal layers when applied topically in sunscreen[10].

This work aims to contribute to the investigation on nanosized ZnO dermal photo-reactivity and photoprotective effects by evaluating the lipid peroxidation of UVA exposed porcine skin after the application of a photo-reactive nanosized ZnO sample. Aditionally, the penetration of ZnO nanoparticles in intact and depilated porcine skin was evaluated by scanning electron microscopy.

Results and Discussions

Results

Particle characterization

The ZnO particle size obtained varying the dispersion medium (ethanol and water) are presented in Table 1. The surface weighted mean (D[3,2]), the volume weighted mean (D[4,3]) and the specific surface area were evaluated considering the theoretical ZnO density of 5.675 $g.cm^{-3}$ and refractive index of 2.01. The volume weighted mean (D[4,3]) and the surface weighted mean (D[3,2])are average of particle size. The first refers to the diameter of a sphere that has the same volume as a particle of interest and the later refers to the diameter of a sphere that has the same volume/surface area ratio as a particle of interest.

The surface weighted mean and the specific surface of Nanosun™ 99/30 placed directly in the sample compartment was similar in water and ethanol (approximately 4 µm and 0.23-0.25 $m^2.g^{-1}$ respectively). On the other hand, volume weighted mean diameter was smaller when ethanol was used as a dispersion medium. The use of air as a dispersion medium under high turbulence showed that the aggregates are liable of dispersion into smaller particles presenting high specific surface area and low Span. All size distribution profiles for Nanosun™ were monomodal and polydisperse.

The characterization of a pre-suspension of Nanosun™ 99/30 in 0.2 % polysorbate 80 aqueous dispersion was also carried out. This sample had smaller surface and volume weighted mean diameter and consequent higher specific surface area compared to the particles dispersed in pure water. The particle distribution for this sample was also more uniform, presenting a smaller span (Table 1).

Table 1. Particle size distribution of Nanosun™ 99/30 and Nanosun™ 99/30 in 0.2 % polysorbate 80 aqueous solution.

Sample	Dispersion Medium	D(0,5) (µm)*	D[3,2] (µm)	D[4,3] (µm)	Specific Surface area ($m^2.g^{-1}$)	Span
Nanosun™ 99/30	Water	1.3	4.3	16.2	0.25	2.8
	Ethanol	1.3	4.6	8.6	0.23	2.8
	Air	0.3	2.9	5.7	2.08	1.7
Nanosun™ 99/30 in 2% PS80	Water	1.1	3.2	4.7	0.33	1.9
Classic ZnO	Water	5.7	4.1	12.6	0.26	5.9

**in number size distribution; PS80 = polysorbate 80*

The X-ray diffractogram (difractogram in supplementary materials) confirmed the chemical composition and showed that the ZnO crystallites present in Nanosun™ 99/30 have a diameter of 26.1 nm ± 5.4. Moreover, scanning electron microscopy confirmed that ZnO nanoparticles are organized in aggregates (Figure 1).

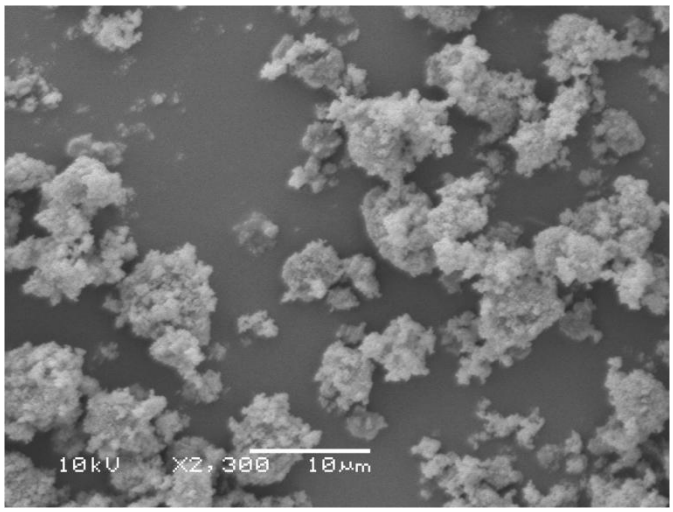

Figure 1. Scanning electron microscopy of Nanosun™99/30.

The surface area found by nitrogen sorption was 28.4 m^2 /g and the calculated diameter was 37 nm.

The apparent absorbance of a thin film of Nanosun™ 65/30 is a result of the sum of different optical phenomenon, such as; reflectance, diffraction, scattering and absorbance. The particle size determines the resulting spectrum. The Nanosun™ had a marked increase of apparent absorbance in the UVA region, while the classic ZnO presented a steady opaque property (Figure 2). Nanosun™ 65/30 had a high absorption value from 200 nm to 375 nm. At 375 nm the absorption value started to decrease. This indicates a low absorption in the visible light spectrum, preventing the opaque aspect of conventional sunscreens. Classic ZnO maintained high absorption values in the UV and visible spectrum. The absorption edge found in ZnO absorption spectrums was at 368 nm for nanosun™ 65/30 and at 381 nm for classic ZnO.

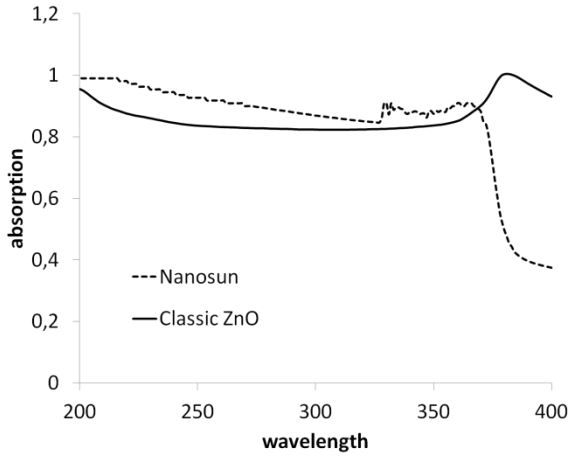

Figure 2. UV spectrum (200 – 400 nm) of Nanosun™ 65/30 and classic ZnO 65% dispersed in MCT.

Photo-reactivity assay

The capacity of Nanosun™ 65/30 as a photocatalyst was evaluated using methylene blue as a liable molecule through a method described by Rampaul and co-workers[12]. In the absence of nanosized ZnO and UVA radiation and when exposed to UVA radiation in the absence of nanosized ZnO no bleaching was observed. When irradiated in the presence of Nanosun™ 65/30, the amount of methylene blue decreased almost completely in 75 minutes, remaining only 7 % of the initial concentration (Figure 3).

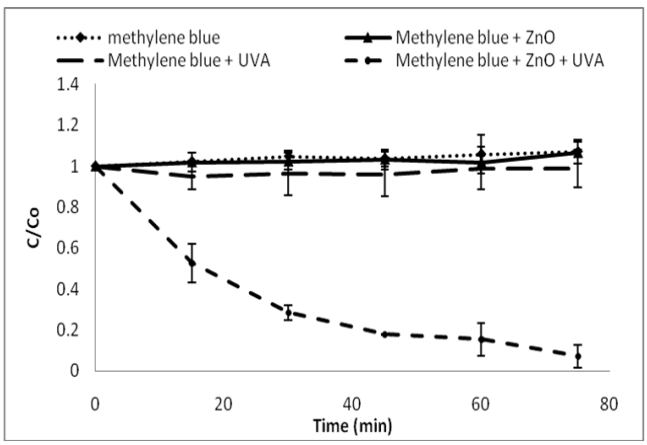

Figure 3. Methylene blue C/Co during 75 minutes under the following conditions: protected from light without Nanosun™ 65/30 and in the presence of Nanosun™ 65/30, under UVA radiation without Nanosun™ 65/30 and in the presence of Nanosun™ 65/30.

Porcine skin peroxidation assay

In the skin peroxidadtion assay a skin sample did not receive any application (control 1) and skin treated with only medium chain triglycerides (MCT) (2 mg.cm^{-2}) (control 2). Control 1 showed a considerable increase of TBARS when exposed to UVA radiation. The quantity (nmol) of TBARS generated per gram of skin was 18.4 nmol.g^{-1} for the irradiated skin with nothing applied to its surface. When control 1 was irradiated TBARS value was 8.9 nmol.g^{-1}, confirming that the irradiation induced skin peroxidation. Approximately 9.5 nmol.g^{-1} of TBARS found in the control 1 is in fact a product of the UVA induced lipoperoxidation (Figure 4).

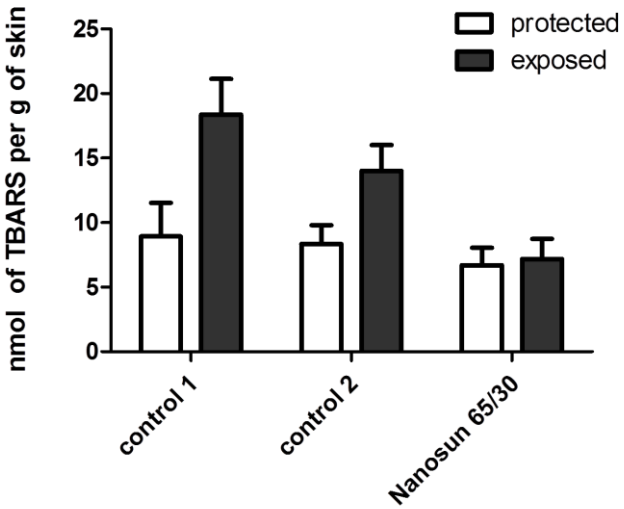

Figure 4. TBARS concentration (nmol/g of skin) in blank skin (control 1), MCT applied skin (control 2) and Nanosun™65/30 applied skin when they were exposed to UVA and protected from light.

In the dark there were no statistical differences in TBARS concentration in the samples; control 1 (8.9 ± 2.6 nmol.g^{-1}), control 2 (8.4 ± 1.5 nmol.g^{-1}) and Nanosun™ 65/30 (6.7 ± 1.4 nmol.g^{-1}). When exposed to UVA radiation control 2 showed no statistical difference from the control 1 (18.4 ± 2.8 nmol.g^{-1} and 14.0 ± 2.0 nmol.g^{-1}, respectively) while the skin sample containing Nanosun™ 65/30 showed a lower production of TBARS (7.2 ± 1.5 nmol.g^{-1}; $p < 0.05$).

Porcine Skin Penetration Assay

The skin samples presented very distinct layers, allowing the energy-dispersive X-ray spectroscopy (EDX) analysis to be performed in three different areas for each chosen field. The surface and upper layers (Figure 4a/ area 1) of the skin presented detectable Zn concentration (Figure 4b), reaching Zn compound ratios from 17.8 % to 33.4 %.

The subsequent layers (Figure 5a/ areas 2 and 3) presented very small quantities of Zn, being not detectable in most regions (Figure 5c and 5d). The Zn compound ratio for these layers were from 0 % to 7.3 %. There were ZnO particles imbibed in the stratus of the first layer of the skin. Figure 5c is an amplification of the circled area in Figure 5a with the EDX (Figure 6d) of one of the particles, confirming that the particles are ZnO. On the surface of the skin there were clusters of ZnO particles (Figure 6a and 6b).

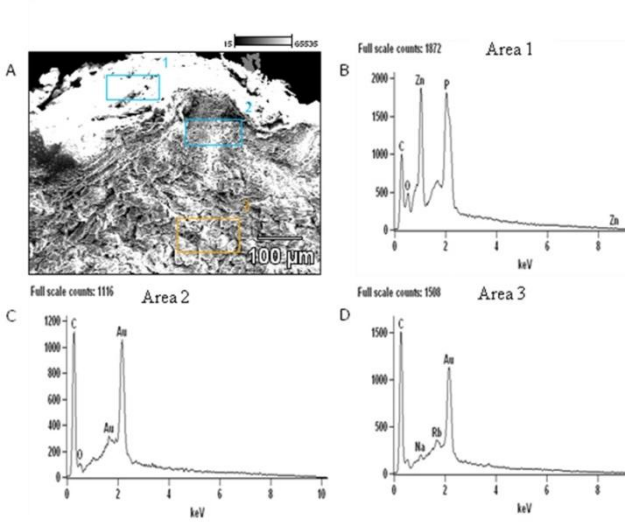

Figure 5. Scanning electron microscopy (A) and EDX diffractograms (B,C,D) of porcine skin after the application of Nanosun™ 65/30 and 12 hours of penetration experiment in a Franz cell apparatus. The diffractogramas are corresponding to the selected areas 1 (surface), 2 and 3 (viable layers).

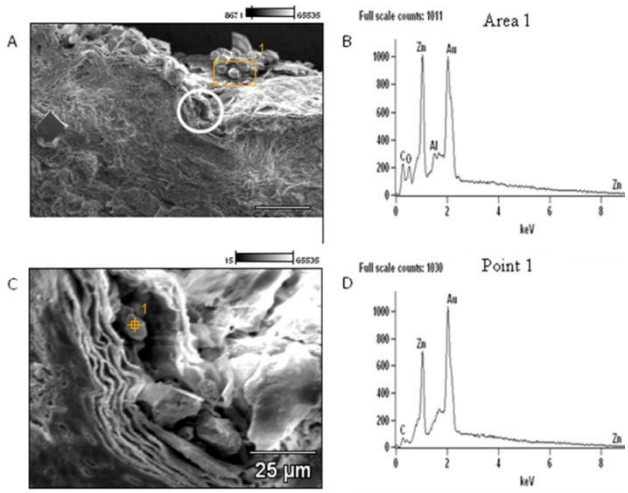

Figure 6. Scanning electron microscopy (A and C) and EDX diffractograms (B and D) of porcine skin after the application of Nanosun™ 65/30 and 12 hours of penetration experiment. The selected area 1 in A shows ZnO particles on the surface of the skin, the white circle in A shows ZnO particles imbibed in the *stratum corneum* and is enlarged in C.

Skin with only MCT was also analyzed by this method and the quantity of Zn was very small. On the surface of the skin Zn compound ratio reached at most 7.7 % and in the internal layers the highest Zn compound ratio was 0.91 %.

Zinc was also identified in larger quantities on the outer layers of the skin treated with a thioglycolate based depilatory cream, while the inner layers presented low amounts or no Zn.

Discussion

The Nanosun™ 65/30 average size stated on the label is 30 nm, which was confirmed by X-ray diffractometry (26.1 ± 5.4 nm). However, laser diffactometry analysis in various mediums and scanning electron microscopy (SEM) showed micrometric aggregates. The surface weighted mean is the diameter of a theoretical sphere with the same surface area as the sample. This value is mostly used to analyze samples which the chemical reaction rate is of interest[14]. The value obtained for the Nanosun™ sample was higher than the particle size obtained by X-ray and stated on the label. This result was expected because the "theoretical sphere" proposed by laser diffractometry method usually represents agglomerates and aggregates of nanosized ZnO suspended in the dispersion mediums[8]. Even though the primary ZnO particle in Nanosun ™ has a size of 30 nm, the surface area of the agglomerates and/or aggregates formed is that of an equivalent sphere between 3 and 4 µm.

The specific surface area values obtained by laser diffractometry are also based on this "theoretical sphere". These two values (D[3,2] and SSA) varied according to the dispersion medium. In presence of a surfactant the particles presented a higher surface area suggesting that such substances in a sunblock lotion vehicle may influence on the agglomerate/aggregate size**.** Therefore, it is important to keep in mind, that the larger the specific surface area and the smaller the D[3,2] the higher will be the reactivity, when designing a sunblock lotion containing nanosized ZnO and surfactants.

The volume weighted mean (D[4,3]) is the diameter of a theoretical sphere with the same volume as the sample. Even though the surface area of the equivalent sphere did not change among the different dispersion medium (water and ethanol) Nanosun ™ 99/30 presented a smaller D[4,3] in ethanol than in water. This suggests that the medium polarity may have influence on the size of the agglomerates/aggregates. The presence of polysorbate 80 reduced even more the D[4,3] confirming that the use of surfactants helps in the dispersion of nanosized ZnO and leads to smaller agglomerates/aggregates.

Nanosun ™ 65/30 particles presented a similar absorbance spectrum as expected for nanosized ZnO, having a constant absorbance from 200 nm to 375 nm with a rapid decrease of absorbance from 375 nm to 400 nm[10]. The absorption edge was lower in Nanosun ™ 65/30 than in classic ZnO. According to Soosen and co-workers (2009) the absorption edge shifts to the lower wavelength, meaning higher energy, with decreasing size of ZnO nanoparticle[15].

The surface area found by nitrogen sorption (28.4 m^2/g) was much higher than the calculated value found by laser diffractometry (2.08 m^2/g). The calculate particle diameter found based on the BET surface area (37 nm) was coherent with the labeled (30 nm) and x-ray (27 nm) unit particle size. This confirms that the unit particles are indeed small and that the laser diffractometry experiment in the present study measured the particle aggregates of samples.

The use of multiple methods for size characterization allows us to affirm that the nanoparticles are organized as micrometric clusters on the skin surface with low specific surface area which should lead to a lower reactivity than a totally dispersed system. Even though these particles are organized in clusters they prevent light transmission (T %) more efficiently than conventional ZnO particles.

Nanosun™ 65/30 is photo-reactive and capable of reducing methylene blue concentration to 7 % in 75 minutes. Methylene blue degrades by a first order rate of reaction (r^2= 0.996), as suggested by Rampaul and co-workers[13] and with a rate constant of 0.039 min^{-1}. Neither ZnO nor UVA radiation alone were capable of significantly reducing the methylene blue concentration, confirming that the reaction is photocatalyzed by ZnO. The photo-reactive activity of ZnO evaluated using methylene blue is often associated with the cyto- and genotoxic effect of ZnO[12].

In fact, UVA radiation induces lipoperoxidation in porcine skin from animals *post mortem*[14]. This was confirmed by comparing the irradiated and non irradiated porcine skin. In the dark all samples did not present statistically differences in TBARS content. Even when exposed to UVA radiation skin samples with no formulation applied on its surface and with MCT did not present statistical difference. This indicates that MCT is not capable of protecting the skin from the UVA induced lipoperoxidation. However, Nanosun ™ 65/30 applied to skin presented a reduced lipoperoxidation, suggesting that nanosized ZnO has photoprotective properties.

Another important aspect that correlates ZnO photo-reactivity with oxidative damage to the skin is its penetration in this biological barrier[8]. Nanosun™ was not visualized in the deeper layers of the skin, but the Zn compound ratios was of 0.0-7.3 %. Considering the deeper layers of the control sample, with only MCT applied, Zn compound ratios were of 0.0-0.91 %. The Zn found in the control is probably from an endogenous source, which should also contribute to the values found in Nanosun™ applied sample. Still, higher values of Zn compound ratio were found in the Nanosun™ applied sample. A previous treatment of the skin with a thioglycilate based depilatory cream did not change this penetration behavior and ZnO particles remain on the skin surface and upper layer.

The zinc atoms present in the deeper layers of the skin represent the endogenous Zn^{2+} content of human and porcine skin *epidermis* and *dermis*, and therefore was also found in the skin that had no ZnO application. The higher amounts found for the ZnO applied samples can be attributed to the possible ZnO solubilization and release of Zn^{2+} ions, which freely penetrate in the skin.

The presence of ZnO particles of 10-25 µm was detected on the surface of the skin, where it should act as a sunblock as well as in the *stratum corneum.* The crystals on the surface of the skin confirm that some ZnO remains in this region, but the crystals morphology may not be the same as in the original sample. These large crystals probably appear by recrystallization during the skin sample dehydration.

The particles tended to accumulate on the skin folds as observed before by Roberts and co-workers (2008)[16]. Some particles were able to penetrate the first *stratus* of the horny layer and fixating themselves. The *stratum corneum* is a non-viable layer of the *epidermis* and does not offer the possibility of systemic absorption or safety concerns. Considering that no further penetration occurs, in time, the layers of dead cells from the *stratum corneum* will be washed off, as will the ZnO particles.

A novel ZnO detection method based on the non-linear effects of a second harmonic generation and hyper-Rayleigh scattering permits the distinction between the particulate and dissolved forms of Zn. Using this method Darvin and co-workers (2012)[17] confirmed that ZnO nanoparticles penetrate only into the outermost layers of stratum corneum, folds and into the orifices of the hair follicles.

Experimental

Solventes and reagents Nanosun™ 99/30 (Australia – UK) is 30 nm ZnO particles in powder with a 99 % purity. Nanosun™ 65/30 (Australia – UK) is 30 nm ZnO particles dispersed in medium chain triglycerides at a concentration of 65%. Both samples of Nanosun™ were gifts from Micronisers® (Dandenong, Australia). Methylene blue (1 % aqueous solution) was obtained from Mercofarma (Porto Alegre, Brazil). The thiobarbituric acid used was acquired from JT Baker - US. Malondialdehyde and Trizma® base were purchased from Sigma-Aldrich (São Paulo, Brazil). Polysorbate 80 (P80), medium chain triglycerides (MCT) and sodium lauryl sulfate (SDS) were obtained from Delaware (Porto Alegre, Brazil). Ethanol P.A. was obtained from Nuclear (São Paulo, Brazil). Hydrochloric acid (HCl) was achieved from Quimex (São Paulo, Brazil). Phosphoric acid was purchased from SAFC (Missouri, USA). The water used in most experiments was deionized, whereas Milli Q water was used when specified in the text.

Laser diffractometry

The measurements of Nanosun™ powder were carried out using a Mastesizer® 2000 (Malvern Instruments, UK) with water, ethanol and air as the dispersant medium. Additionally, Nanosun™ powder was pre-dispersed in water and in a 0.2 % polysorbate 80 aqueous solution for 24 hours and subsequently analyzed using water as the dispersant medium (n = 3).

UV spectrophotometry

In order to confirm if Nanosun™ 65/30 ZnO particles are nanometric, a comparative UV spectrophotometric assay was preformed[10]. Conventional ZnO particles were suspended in MCT resulting in 65 % of ZnO and were compared to Nanosun™ 65/30, composed of ZnO nanoparticles suspended in MCT. The resulting paste was spread on the surface of a quartz cuvete forming a thin film, the sample was analyzed in a UV spectrophotometer (R-1800, Pró-Análise, Brazil) and the results were normalized.

Scanning electron microscopy

The morphology of ZnO nanoparticle clusters was visualized with a scanning electron microscope (JSM 6060, JEOL, Japan) at the *Centro de Microscopia Eletrônica – Universidade Federal do Rio Grande do Sul* (Porto Alegre, Brazil). The samples were placed on an aluminum stub and fixed with adhesive tape prior to the metallization process with gold (Sputter Coater - Balzer SCD050, Bal-Tec, Germany**).**

X-Ray diffractometry

X-ray diffractometry was used to measure the crystallite size based on Scherre´s law (Equation 1). The crystallite size was calculated using the peak broadening of the seven major peaks in ZnO diffractogram. The analysis were performed in a SIEMENS D5000 X-Ray diffractometer at the *Laboratório de Difratometria de Raio-X - Universidade Federal do Rio Grande do Sul* (Porto Alegre, Brazil). The full width at half maximum (B) was measured using the software Origin Pro® 8.5. The source wavelength (λ) was 1.542 Å.

$$D[XR] = \frac{0.94 \times \lambda}{B \times \cos\theta} \quad (1)$$

Nitrogen sorption analysis

Nitrogen sorption analysis was used to measure the powder surface area with Micrometrics ASAP 2020. Nanosun ™ 99/30 was pre treated at 350 °C, for 3 hours, under vacuum (2 µm Hg), as a cleaning procedure. The material surface area was determined by Brunnauer-Emmett-Teller (BET). The particle size was also calculated using the equation below:

$$\boldsymbol{D[BET]} = \frac{\mathbf{6}}{\boldsymbol{SSA \times \rho}} \quad (2)$$

Where, D[BET] is the particle diameter (µm), SSA is the surface area obtained by BET (m^2 /g), ρ is ZnO density (5.675 $g.cm^{-3}$) and 6 is a correction factor for spheres.

Photo-reactivity assay

The photo-reactivity assay was based on the method described by Rampaul and co-workers (2010)[11]. Nanosun™ 65/30 was suspended in an aqueous solution of methylene blue (2.0×10^{-3} M) in the concentration of 1 mg mL^{-1}. The suspension was stirred overnight in order to ensure adsorption equilibrium between the solid and the liquid. The suspension was exposed to UVA irradiation of ~ 2 $W.cm^{-2}$ at 20 cm of distance under constant magnetic stirring. As controls a beaker containing only methylene blue without was irradiated and another not irradiated assuring that the degradation in the absence of nanosized ZnO was low. Additionally, a beaker containing methylene blue with nanosized ZnO was not irradiated, assuring that the radiation is necessary for the degradation. One milliliter aliquots of the suspension were taken at 15 min intervals and diluted to 5 mL with water. The samples containing ZnO were centrifuged at 3256 x*g* for 5 min and analyzed at a wavelength of 660 nm in a UV-vis spectrophotometer. Using a calibration curve (y= 38764x + 0.024, r = 0.995, n = 3) the variation of methylene blue concentration was determined. The decrease in absorption over time was then related to the photocatalytic ability of each particular sample (n = 3).

Porcine skin peroxidation assay

Porcine skin was obtained from a local slaughterhouse (Bento Gonçalves – RS, Brazil). Female abdominal porcine skin was cleaned with sodium lauryl sulfate (SDS) 1 % on the external surface and with ether:ethanol (1:1, v/v) on the internal surface. The cleaned skin was stored at -4 ºC during a maximum of 2 months.

The porcine skin was cut in slices of 2.5x2.5 cm, with weight of 1.0 ± 0.05 g. Two $mg.cm^{-2}$ of Nanosun™ 65/30 was applied manually on the porcine skin. A skin sample did not receive any application (control 1) and skin treated with only MCT (2 $mg.cm^{-2}$) (control 2). The slices were placed over wet cottons in petri dishes with the external surface at a distance of 20 cm from the light source and exposed to UVA radiation (~ 2 $mW.\ cm^{-2}$) during 12 hours. The same groups are also maintained in the dark, to validate that the peroxidation observed was indeed due to the radiation exposure.

Skin lipid peroxide decomposition induced by UVA radiation leads to the formation of thiobarbituric acid reactive species (TBARS)[12] (Sapino et al., 2007). The TBARS were quantified by the TBA method, using a calibration curve (y= 0.0885x + 0.0205, r = 0.997, n = 2) with malondialdehyde (MDA)[18]. The tissue was homogenized by cutting the skin into small squares and subsequently using a homogenizer (Ultra Turrax T-10, IKA, Brazil) at 11 450 rpm during 5 min in a Tris-HCl 1 % solution. An aliquot of 800 µL of the extract was then put in a reaction with 1.5 mL of TBA 0.6 %, 1.5 mL of phosphoric acid, 0.8 mL of SDS 1.6 % and 0.3 mL of water. The samples were incubated for 45 min at 90 °C and the reaction was stopped with ice cold bath. Before the spectrophotometric analyses the samples were centrifuged (4K15, Sigma Laboratory Centrifuges, Germany) at 3256 *xg* for 10 min. All experiments were done in quadruplicates.

Skin penetration assay

In order to observe the skin penetration of nanosized ZnO a Franz cell experiment with porcine skin was preformed and the skin was analyzed by scanning electron microscopy with energy dispersive x- ray spectroscopy. Female abdominal skin obtained from a local slaughterhouse (Bento Gonçalves – RS, Brazil), were cleaned and stored in the same manner as described above. For the penetration assay round skin samples, with a thickness of 1.5 ± 0.1 mm, were used as a biological membrane in a manual Franz cell apparatus (exposure area of 3.0 cm^2). Nanosun™ 65/30 and pure MCT were applied in a concentration of 2 mg. cm^{-2} to the skin and to a skin depilated with a thioglycilate based depilatory cream (Veet®). Milli Q water was used as a receptor medium at constant stirring and in a 32 ºC water bath. The experiment was held in a dark environment for 12 hours. The skin was cut for analysis from the *dermis* to the *epidermis* with sterile aluminum blades and fixated with a glutaraldehyde: phosphate buffer pH 7.4 (1:1) solution for one week. The skin samples were washed 3 times for 30 minutes with phosphate buffer pH 7.4 and dehydrated with water:acetone, slowly increasing the concentration of acetone (7:3;5:5;3:7;1:9;0:10). The sample, immerged in acetone, was dried with a critical point dryer CPD030 (Balzers) and metalized using gold in a sputter coater SCD050 (Balzers). The equipment used was a Scanning electron microscope JSM 5800 with an energy dispersive x- ray spectroscope (JEOL, Japan).

Conclusions

In conclusion, Nanosun™ 65/30, a commercial raw material for the production of sunscreens, contains nanometric, photo-reactive ZnO particles organized as aggregates which are capable of preventing *in vitro* UVA induced skin lipoperoxidation. The tested ZnO particles did not penetrate to viable layers of intact porcine skin. The particles tend to accumulate on the skin folds and in these regions may penetrate into the horny layer. Therefore, even though the nanosized ZnO are photo-reactive, they do not increase skin lipoperoxidation and do not reach the viable layers of the skin indicating low toxicity risk. These results contribute to the research on the safety of nanosized ZnO particles.

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Notes and references

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