

Role of particle coating in controlling skin damage photoinduced by titania nanoparticles

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Abstract

TiO₂ nanoparticles hazard is associated to their photocatalytic activity causing release of DNA damaging ROS (Reactive Oxygen Species), lipid peroxidation and skin damage. Various coatings have been proposed to minimize photocatalysis, while keeping the potential to block UV radiations. Uncoated and variously coated commercial nano-titania have been classified on the basis of UVB-induced lipoperoxidation of linoleic acid. A selection of the most and the least protective specimens was then examined by ESR (Electron Spin Resonance) to evidence the presence of surface paramagnetic centres and the release of ROS in aqueous suspensions (spin trapping). Paramagnetic centres and ROS were correlated with the extent of lipid peroxidation. When tested on porcine skin (mimicking the human one), titania acted as on linoleic acid. The combined use of lipid peroxidation of simple fatty acids with ESR analysis is here proposed as a possible screening tool for the evaluation of the potential toxicity of nano-titania in sunscreen preparations.

Keywords: TiO₂ nanoparticles, ROS, paramagnetic centres, porcine skin, UVB irradiation, linoleic acid peroxidation.

Abbreviations: ESR, electron spin resonance; SDS, sodium dodecyl sulphate; MDA, malondialdehyde; ROS, reactive oxygen species; TBA, 2-thiobarbituric acid; PMMA, polymethyl methacrylate; TMCS, trimethoxycaprylylsilane; DMPO, 5,5-dimethyl-1-pyrroline-N-oxide.

Introduction

There is much concern among the scientific community on the possible health damage caused by engineered titania nanoparticles, currently employed as efficient sunscreens in various skin protective preparations [1,2].

Sunlight UVB radiation (290–320 nm) is responsible for most of the short-term effects such as pigmentation and erythema, while both UVB and UVA are involved into the adverse, long-term effects, i.e. carcinogenesis and ageing. A variety of lotions have been formulated to protect human skin, which absorb or block UV radiations before they can

penetrate into the epidermis. The lotions need to be opaque to UVA/UVB radiation, biologically and chemically inert, stable when applied on the skin and resistant to water. Nanosized TiO₂ appears as an ideal material, which reflects and scatters UVB and UVA radiation, but is transparent to visible light, in contrast to TiO₂ powders made up of larger particles. It is thus widely employed in various sunscreen products to block UV [3].

Titania has for a long time been considered as a safe material. Particle toxicology, which mainly concerns the health damage caused by inhalation of particles at the work place or in the environment, has regarded micron-size TiO₂ just as a nuisance dust.

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TiO₂ was even used as negative control in experimental studies on well-known pathogenic particulates, such as crystalline silica. With the advent of nanotechnology and the rising fear of the health effects caused by nanosized particles, the lung toxicity of titania has been revisited, with somehow contrasting results [4–6]. Recently the International Agency for Research on Cancer (IARC) has classified TiO₂ as possibly carcinogenic to humans (group 2B) [7,8].

Inhalation toxicology conclusions, however, cannot be applied to TiO₂ mediated-skin damage because of the specific chemical behaviour of titania when exposed to UV-Visible light. The photocatalytic properties that make TiO₂ an excellent material to destroy environmental pollutants [9], to sterilize the waste-water [10], to make self-cleaning surfaces and even to be used in cancer therapy [11] also imply that similar reactions might occur on the skin or in the outmost skin layers, where TiO₂ nanoparticles easily move to. In fact, various adverse reactions have been detected in the presence of irradiated TiO₂, including decomposition of nude DNA and RNA [12], DNA damage in cultured human cells *in vitro* [12–14], genotoxicity [15], phototoxicity to skin fibroblasts and photo-oxidation of cellular RNA [16]. This double sword aspect of TiO₂ is due to its potential to generate reactive oxygen species (ROS) such as hydroxyl radicals (HO•), superoxide radicals (O₂^{•-}), hydrogen peroxide (H₂O₂) and singlet oxygen (¹O₂) under UV light, which have well-known cytotoxic and genotoxic characteristics [17–19]. Some new studies have highlighted a large variability in cell damage caused by TiO₂ from several sources, prepared in different ways and made up by the various TiO₂ crystalline phases—anatase, rutile and brookite, pure or in different proportions [6,20]. However, no TiO₂ specimen was fully inert. Other researches suggest a new approach whereby the whole TiO₂ particle is doped in an appropriate way to reduce free radical generation [21]. However, the cosmetic industry still largely employs coated particles.

Indeed, several kinds of particle coating have been proposed to preserve the potency of screening UV radiation, while avoiding or minimizing the adverse health effects on skin caused by titania photocatalysis. Contrary to current belief, such physical sunscreens are not all photochemically inactive. The fact that they may be coated is not a warranty of photochemical inactivity, either in the products or on user's skin [22]. As an example, TiO₂ particles are often coated with compounds (alumina, silica, zirconia) that form hydrated oxides which can capture hydroxyl radicals and may reduce the photosensitivity. On the contrary, it was even reported that some TiO₂/Al₂O₃ and TiO₂/SiO₂ preparations exhibited an enhanced activity [13].

While some studies reported the ability of uncoated TiO₂ to cause lipid peroxidation [23–25], only few studies have been performed on coated or modified TiO₂ nanoparticles, mainly by observing the extent of lipid peroxidation or the oxidation of an organic substrate (phenol) and damage to DNA, under UV illumination [13,17]. The interaction of variously coated titanium and zinc oxide with corn oil, representative of triglycerides, showed that some coatings are effective in preventing photochemical sensitization. Thermally-modified TiO₂ particles showed reduced damage towards DNA plasmids, human cells and yeast cells [2,17]. An alternative approach to reduce the photocatalytic activity of TiO₂ was to graft antioxidant molecules onto a hydrophobic acrylic polymer. The polymer coating stabilized the antioxidant and free-radical formation was reduced [26]. In a study on commercial sun care products containing titanium dioxide, formation of oxygen and carbon-centred radicals was monitored by ESR spectroscopy and spin trapping technique. The generation of ROS upon irradiation of sunscreens at 300 nm significantly depends on their composition and some additives (antioxidants, radical scavengers, solvents) can transform photogenerated reactive radicals into less harmful products [27].

The photocatalytic activity of titania preparations is generally evaluated by measuring the potential to generate free radicals under UV irradiation. The free radicals generated may be detected by using ESR/spin trap [26,27] or spectrophotometric methods [14,28]. It is however unclear if such reactivity directly correlates to the oxidative damage that may be caused by titania to cell membranes, since both methods are affected by artefacts and in some case give controversial results. We propose herein a protocol in which the photocatalytic activity of different titania nanoparticles is evaluated by measuring lipoperoxidation on linoleic acid and porcine ear skin.

We have here considered a large variety of uncoated or coated commercial nano-titania, some of which are already employed in sunscreen formulations, and compared their behaviour in several tests, all aiming at detecting the effects of the residual photocatalytic activity, if any. The enhancement of the UVB-induced peroxidation of linoleic acid when the TiO₂ commercial nanoparticles are suspended in SDS micellar solutions was employed for a first screening of the TiO₂ samples. The potential to release ROS and the presence of active surface sites was subsequently evaluated, by means of ESR spectroscopy and the spin trapping technique, on a selection of the TiO₂ specimens that were most and least active toward lipid peroxidation. Finally, the most relevant titania were also tested on porcine ear skin to validate on a more complex model the adopted procedure.

Materials and methods

Materials

Titania powders: two uncoated and 10 variously coated commercial titania nanoparticles have been employed. Eusolex T-AVO was purchased from Merck (Milan, Italy). PW Covasil S-1 and PW Covasil S were supplied from LCM Trading S.p.A (Sesto S. Giovanni, Italy), titanium oxide standard (anatase) was a gift from LCW/Wackherr (St. Ouen L'Aumône, France), Tego Sun TS plus and Aeroxide P 25 were gifts from Degussa (Vicenza, Italy). T-Lite SF and T-Lite SF-S were from BASF (Cesano Maderno, Italy). Maxlight F-TS20 (Showa Denko K.K., Tokyo, Japan) and MT-100Z (Tayka Corporation, Okayama, Japan) were from Pharma Cosm Polli S.r.l. (Milan, Italy).

Chemicals

Hydrochloric acid, 1-butanol, sodium chloride, sodium azide, sodium dodecyl sulphate (SDS) and sodium hydroxide were from Fluka (Milan, Italy). Phosphoric acid 85% and dichloromethane were from Carlo Erba (Rodano, Italy). Linoleic acid was purchased from Aldrich (Milan, Italy); 2-thiobarbituric acid (TBA, 4,6-dihydroxy-2-mercaptopyrimidine) and 1,1,3,3-tetraethoxypropane (malondialdehyde-bis(diethyl acetal)) were purchased from Sigma (Milan, Italy). 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) was purchased from Alexis (Lausen, Switzerland), the other reagents were from Sigma-Aldrich (St. Louis, MO).

Surface area

The surface area of the considered TiO₂ powders was measured by means of the BET method, based on N₂ adsorption at -196°C (ASAP 2010, Micrometrics, London, Canada).

XRD spectroscopy

XRD spectra were collected on a diffractometer (PW1830, Philips, Milan, Italy) using CuK_α radiation, in the (20–90) 2θ range, with step width 2θ = 0.05 and time per step = 1 s. Diffraction peaks have been indexed according to JPCDS database.

Detection of paramagnetic centres in the solid

The presence of paramagnetic centres in the solid was evaluated by means of ESR; 20 mg of the powders were degassed in a quartz cell at room temperature. The ESR spectra were recorded in vacuum at -196°C on a spectrometer (EMX, Bruker, Milan, Italy) operating in the X-band mode (9.5 GHz). Instrument setting: scan range 600 G, receiver gain 6.32 × 10⁵, microwave power 10 mW, modulation amplitude 2G, scan time 167.77 s, 5 scans. The

spectra were also recorded after admitting in the cell 20 mbar of oxygen.

In the case of PW Covasil S-1 the experiments were performed also on the powder pre-incubated in a dispersion of 1.0% w/w linoleic acid in 4.0% w/w SDS at pH 4.0. The powder was recovered by centrifugation (15 min, 14 000 rpm), washed three times in distilled water then dried.

Evaluation of free radical release

The generation of radical species was monitored by ESR spectroscopy (MiniScope MS100, Magnostech, Berlin, Germany) using the spin trapping technique with DMPO. All experiments were repeated at least twice.

- (1) *Generation of ROS*: an amount of TiO₂ powder corresponding to 0.4 m² exposed surface area was suspended in 250 μL of a buffered solution (0.25 M potassium phosphate buffer, pH 7.4), containing 0.88 M DMPO in the dark. An aliquot of 50 μL of the suspension was transferred in a capillary and irradiated directly in the ESR cavity with a UV lamp (500 W Hg(Xe) source, transmittance range 250–800 nm, Thermo Oriel, Stratford, CT). The ESR spectra were recorded after 1 min of irradiation.
- (2) *Reaction with sodium formate*: an amount of TiO₂ powder corresponding to 0.4 m² of exposed surface area was suspended in 250 μL of a buffered solution (0.25 M potassium phosphate buffer, pH 7.4) containing 75 mM DMPO and 1 M sodium formate in the dark. An aliquot of 50 μL of the suspension was transferred in a capillary and irradiated directly in the ESR cavity with a UV lamp (500 W Hg(Xe) source, transmittance range 250–800 nm, Thermo Oriel, Stratford, CT). The ESR spectra were recorded after 1 min of irradiation.

The simulations of the ESR experimental signals were performed using the software Winsim 2002 (National Institute of Environmental Health Science, National Institutes of Health, Bethesda, MD). The hyperfine splitting constants obtained from the optimization of the simulation were compared with those reported in the literature (NIESH STBD database).

Photoinduced peroxidation

A suspension of linoleic acid (1.0% w/w) in 4.0% w/w SDS aqueous solution was magnetically stirred for 24 h in the dark. TiO₂ (at 0.05% or 1.0% w/w loading) was added upon sonication (660/H Transsonic Sonifier, Elma, Singen, Germany). The pH was then adjusted to 4.0 (Micro pH 2001, Crison, Alella, Spain). An aliquot (10.0 mL) of each sample was introduced in Pyrex[®] glass cells and irradiated for

120 min, under magnetic stirring (R0 5, IKA, Staufen, Germany), at a distance of 10 cm from a UVB lamp (G40T10E, Sankyo Denko, Kanagawa, Japan), with 2.4 W/m² power emission of radiation. The suspensions were then centrifuged (5417 centrifuge, Eppendorf, Milan, Italy) to separate TiO₂ from the samples before analysis. An aliquot (0.2 mL) of supernatant was taken to determine MDA through the TBA assay.

The assay, currently used as an index of lipoperoxidation, is based on the reactivity of MDA, a colourless end-product of degradation, with TBA to produce a pink adduct (TBA-MDA-TBA) that absorbs at 535 nm. MDA was detected spectrophotometrically according to the method described by Bay et al. [29] with the following modifications. The sample (0.2 mL) was introduced in a glass tube closed with a screw cap and added with 0.1 mL of water, 0.2 mL of 8.1% w/w SDS, 1.5 mL of 1.0% w/w phosphoric acid and 1.0 mL of 0.6% w/w TBA. The mixture was stirred and heated in water bath at 95–100°C for 45 min to favour the formation of the complex. After cooling in an ice bath, 4.0 mL of 1-butanol were added to each tube and the TBA-MDA-TBA complex was extracted upon stirring and centrifugation. The organic supernatant was evaluated by spectrophotometry (Lambda 2 UV/Vis spectrophotometer, Perkin Elmer, Waltham, MA). A solution of 1,1,3,3-tetraethoxypropane in 8.1% w/w SDS within the concentration range 9.4–208.0 µm was employed for the calibration curve of the TBA-MDA-TBA complex. MDA can be obtained by acid hydrolysis from 1,1,3,3-tetraethoxypropane in an equimolecular reaction [30]. The solutions were subjected to TBA assay and the calibration graph obtained. The final concentration of MDA derived from the reaction was expressed as nmoles of MDA per mg of lipid substrate.

UVB-induced porcine skin lipoperoxidation

To assess the UVB-induced skin lipid peroxidation, the formation of MDA via the TBA assay was evaluated in porcine ear skin freshly obtained from a local slaughterhouse. A skin slice was isolated from each pig ear by a surgical scissor and then frozen at –18°C. Before the experiments the skin was equilibrated in 0.9% w/w saline solution added with sodium azide, at 25°C for 30 min. Each slice was further cut up in small pieces that were mixed to overcome a possible variability among different zones of the ear skin. The pieces (total weight ~ 1.4–1.5 g) were randomly allocated in a Pyrex[®] cell, suspended in 4.0% w/w SDS solution (pH 4.0) and irradiated in the absence and in the presence of TiO₂, as reported above.

After UVB irradiation the skin pieces were dried under vacuum and then incubated overnight in 10.0

mL dichloromethane to extract MDA under magnetic stirring. The organic solvent was evaporated under vacuum by RE-111 Rotavapor[®] (Büchi, Flawil, Switzerland) and the residue was reconstituted with 3.0 mL of 8.1% w/w SDS. An aliquot (0.2 mL) of this dispersion was subjected to the TBA assay, by means of the same procedure reported above. The experiments were also repeated in the absence of irradiation. Each sample was prepared and analysed in duplicate.

The experiments were repeated in the presence of two TiO₂ samples chosen on the basis of the results of linoleic acid peroxidation. The first sample, PW Covasil S-1, was chosen for its pronounced photocatalytic activity and the other, Tego Sun TS plus, for its evident protective effect. Both types of TiO₂ were employed at 0.05% w/w concentration.

Results

Characteristics of the nano-titania samples considered

The samples examined were variously coated by inorganic, polymeric or mixed materials. Particularly, the inorganic coating consisted of silica, alumina or both silica and alumina.

Table I reports the main characteristics and the physico-chemical properties of the studied samples: coatings, crystallinity (evaluated by X-ray diffraction, XRD), surface area and particle size.

UVB-induced linoleic acid peroxidation in the presence of the different TiO₂ preparations

The extent of lipid peroxidation was measured in the presence of the various titania samples under irradiation and compared with that obtained upon irradiation in the absence of TiO₂, considered as blank. PW Covasil S, PW Covasil S-1, T-Lite SF, standard and Aeroxide P 25 showed a marked activity toward linoleic acid peroxidation, ranking in the following order:

PW Covasil S > Aeroxide P 25 > PW Covasil S-1
> standard > T-Lite SF.

The other coated TiO₂ particles induced linoleic acid peroxidation at a much lesser extent:

Eusolex T-AVO > T-Lite SF-S > MT-100Z
> Tego Sun TS plus > Maxlight F-TS20.

These results suggest a protective effect of some of the coatings, in particular those containing silica, on lipoperoxidation.

Three TiO₂ samples that showed a protective effect (Maxlight F-TS20, Tego Sun TS Plus, T-Lite SF-S) and three others, two coated and one uncoated, which induced a marked peroxidation activity (Aeroxide P 25, T-Lite SF, PW Covasil S-1) were thus selected for further investigation.

Table I. Main physico-chemical features of samples.

Commercial name	Composition %	Crystal phases %	Specific surface area (m ² /g)	Primary particle size (nm)/shape
Maxlight F-TS20	TiO ₂ 75 SiO ₂ 22	100 rutile	55	100
Eusolex T-AVO	TiO ₂ SiO ₂	100 rutile	40–90	Needle like <200
PW Covasil S	TiO ₂ >90 TMCS* <5 PMMA** 5	80 anatase 20 rutile	43	28–32
PW Covasil S-1	TiO ₂ >95 TMCS <5	80 anatase 20 rutile	40	28–32
Tego Sun TS plus	TiO ₂ >50 SiO ₂ 10–25 TMCS* 4.5	80 anatase 20 rutile	60	
MT-100Z	TiO ₂ 75 Al ₂ O ₃ Stearic acid	100 rutile	40–60	15
T-Lite SF	TiO ₂ 84 Al(OH) ₃ 7 Dimethicone 4.5	100 rutile	76	Needle like 30–60 × 10
T-Lite SF-S	TiO ₂ 78 Al(OH) ₃ 3.5 SiO ₂ 7.5 Dimethicone 5.5	100 rutile	71	Needle like 30–60 × 10
Aeroxide P 25	TiO ₂ 100	80 anatase 20 rutile	53	Spherical, 21
Standard	TiO ₂ 100	100 anatase		

* TMCS = Trimethoxycaprylsilane; ** PMMA = Polymethyl methacrylate.

Figure 1 reports the amounts of MDA produced upon UVB-induced lipoperoxidation of 1.0% w/w linoleic acid dispersed in 4.0% w/w SDS, in the presence of either 0.05% w/w or 1.0% w/w TiO₂ at pH 4.0. The amounts of MDA were reported as nmol per mg of linoleic acid. The pH condition adopted in this experiment may be explained as follows: according to the literature [31], the natural skin surface has an average pH value below 5.0. Moreover an acidic pH value (4.0–4.5) will minimize the dissociation of linoleic acid and could increase the peroxidation rate.

Linoleic acid dispersion without TiO₂ was also examined as control. The three protecting TiO₂ specimens all induced approximately the same MDA production as the control at both titania concentrations tested. This is the expected result in the absence of any effect caused by the particles. Surprisingly, no dose effect was detected with the active TiO₂ samples. The extent of peroxidation caused by the samples that showed the highest photocatalytic activity (Aeroxide P 25, PW Covasil S-1 and T-Lite SF) was significantly lower at 1.0% than at 0.05% loading. A possible explanation is the onset of an appreciable light-screen effect at the highest loading. In the case of Aeroxide P 25 the absorption (κ^*) and scattering (σ^*) coefficients at 313 nm (emission maximum of the adopted UVB lamp) are reported in the literature [32]. It is $(\kappa^*)_{313 \text{ nm}} = 1.2 \times 10^4 \text{ cm}^2/\text{g}$ and $(\sigma^*)_{313 \text{ nm}} = 5.5 \times 10^4 \text{ cm}^2/\text{g}$. The extinction coefficient would therefore

be $(\beta^*)_{313 \text{ nm}} = (\sigma^* + \kappa^*)_{313 \text{ nm}} = 6.7 \times 10^4 \text{ cm}^2/\text{g}$. In the studied systems the optical path length of the suspensions was $b = 1 \text{ cm}$. For TiO₂ loadings of $5 \times 10^{-4} \text{ g/cm}^3$ (0.05% w/w) and $1 \times 10^{-2} \text{ g/cm}^3$ (1.0% w/w), the total extinction at 313 nm would be 33 and 670, respectively ($\beta^* \times b \times \text{loading}$). These values are only approximated because they do not take into account multiple scattering [32], but it can be concluded that both loadings afford practically complete extinction of the incident UVB radiation. Even considerable changes of the extinction values (absorbance + scattering) caused by multiple scattering could not modify such a conclusion significantly. Because of the elevated extinction, in both cases the intensity of the extinguished radiation would be equal to the incident one and therefore similar for both loadings. Furthermore, ~18% of the extinction would be accounted for by absorption and the rest by scattering.

While the total radiation absorption by TiO₂ would be similar for both loadings, the incident radiation would not be able to reach all the suspension because of the elevated extinction. Accordingly only the top layer of the suspension would be illuminated, the rest receiving no radiation. At 1.0% w/w loading a higher fraction of the suspension would be in the dark compared to 0.05% w/w. This means that for higher loading, absorption would be concentrated in a smaller volume to give equal total absorbance. Because the dependence of the rate of the photocatalytic

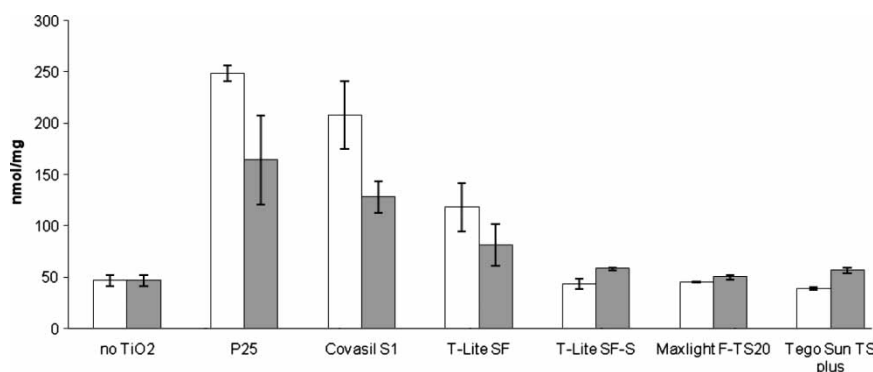


Figure 1. Amounts of MDA (nmol/mg) derived from linoleic acid (at 1.0% w/w, in 4.0% w/w SDS at pH 4.0) UVB irradiated in the absence and in the presence of 0.05% w/w (white bars) or 1.0% w/w (grey bars) TiO₂; time of irradiation 2 h. Each bar represents the means \pm SD obtained in three independent experiments ($n=3$).

reactions on the intensity of radiation absorption has a square-root form and not a linear one [33], a lower absorption distributed in a larger volume of the solution (as for 0.05% w/w) would be more effective in inducing degradation than higher absorption in a smaller volume, as in the case of 1.0% w/w. This fact can account for the lower photocatalytic activity observed at the higher loading.

Some experimental artefacts should however be taken into account, because the dispersion of TiO₂ in aqueous media at 1.0% loading was very difficult, thus possibly incomplete, with consequent over-evaluation of the extent of oxide that interacts with the substrate.

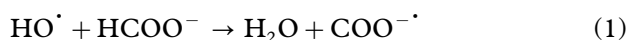
Free radical generation

The ability to generate free radicals following irradiation was studied on the two samples that showed the highest activity toward lipoperoxidation (Aeroxide P 25 and PW Covasil S-1) and on one of the inactive samples (Tego Sun TS plus) (Figure 2).

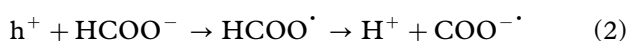
Two mechanisms of radical generation were investigated:

- (i) generation of HO[•];
- (ii) generation of CO₂^{-•} radicals in the presence of formate ions, following homolytic cleavage of the C-H bond.

The latter process may be due to the reaction with formate of the hydroxyl radicals generated by TiO₂ [34]:



or may be the result of an oxidative reaction of formate with the valence-band holes of irradiated TiO₂ [35]:



Only Aeroxide P 25 appeared to be active in the first reaction (Figure 2A). The intense ESR signal ob-

served for this sample corresponds to the adduct of the spin trap molecules with hydroxyl radicals (DMPO/HO[•]). Such species are likely formed through two different paths: (i) the reaction of DMPO with hydroxyl radicals generated by the oxidative reaction of water with electron holes; and (ii) the decomposition of the adduct DMPO/HOO[•] formed following the reaction of DMPO with superoxide radical (in the form of its conjugate acid hydroperoxide radical) with DMPO [36].

Aeroxide P 25 exhibited a strong reactivity also in the homolytic cleavage of the C-H bond (Figure 2B). Unexpectedly, also PW Covasil S-1 appeared active in this reaction, albeit at a much lesser extent than Aeroxide P 25. No signal was recorded for Tego Sun TS plus, which appeared thus fully inactive in both tests.

Paramagnetic centres in the solid and/or at the surface

The presence of paramagnetic centres was evaluated by means of the ESR spectra obtained from the solid kept in the appropriate quartz cell under vacuum. The ESR spectra of the four samples recorded at -196°C are compared in Figure 3A. Aeroxide P 25 exhibited an intense signal at $g_1 = 1.970$ and $g_2 = 1.948$, which may be assigned to a Ti³⁺ in the rutile phase [37], and two less intense signals, partially buried in the previous one, at $g = 1.991$ and $g = 1.979$, which correspond to two Ti³⁺ species of the anatase phase in different chemical surroundings [38].

An intense signal was also observed in the ESR spectra of PW Covasil S-1: in this case the signal observed at $g_1 = 1.970$ and $g_2 = 1.948$ was assigned to a Ti³⁺ in the rutile phase. Such species were absent in Tego Sun TS plus which only exhibited a weak signal corresponding to oxygenated species.

To discriminate between the amount of defects that are present at the surface of the samples and those in the bulk the spectra were also recorded under a

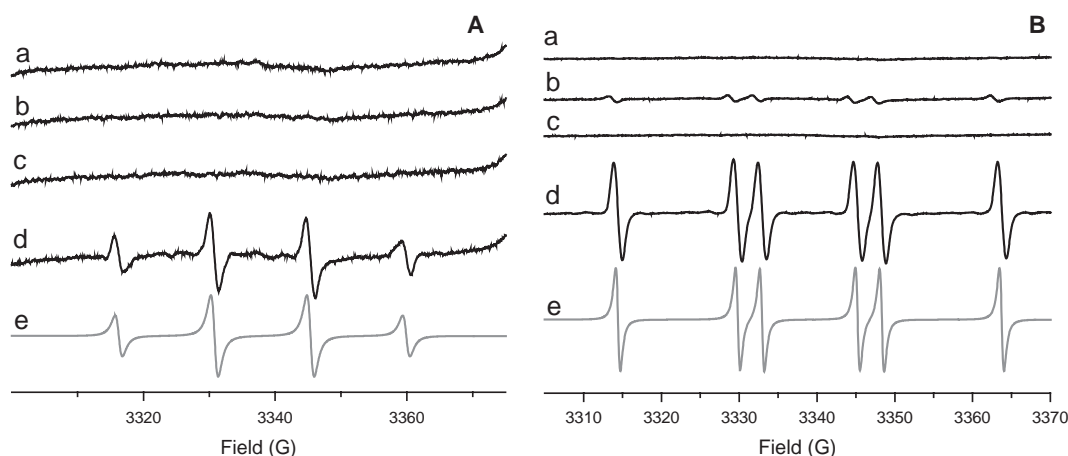


Figure 2. Free radicals generation following irradiation in the ESR cavity of TiO_2 samples in the presence of: (A) hydrogen peroxide; (B) sodium formate. (a) no TiO_2 ; (b) PW Covasil S-1; (c) Tego Sun TS plus; (d) Aerioxide P 25; (e) simulated spectra of signal d. The four lines signal characterized by the splitting constants $a_N = 14.7$ G, $a_H = 14.2$ G in spectrum d–panel A has been assigned to the $\text{DMPO}/\text{HO}^\cdot$ adduct while the six lines signal characterized by the splitting constants $a_N = 15.4$ G, $a_H = 18.5$ G in spectrum d–panel B has been assigned to the $\text{DMPO}/\text{CO}_2^{\cdot-}$ adduct. Instruments settings: receiver gain 9×10^2 , microwave power 10 mW; modulation amplitude 1 G; scan time 70 s, two scans.

pressure of 20 mbar of O_2 (Figure 3B). O_2 would quench the spectral components arising from the surface paramagnetic centres, now in intimate contact with the oxygen molecules. Conversely, if the signal intensity does not vary under oxygen, the observed paramagnetic species are expected to be localized in the bulk. Only Aerioxide P 25 appeared to exhibit surface defects because, for the other two samples, the ESR signals were similar to those recorded in vacuum. This finding suggests that the observed defects are either in the bulk or fully protected by the external coverage of the particles.

Effect of incubation in the solution employed in the lipoperoxidation test on the abundance of surface defects

The ESR spectra were also recorded on PW Covasil S-1 previously incubated in the dispersion used for the linoleic acid peroxidation (Figure 4): the ESR

spectrum of pre-incubated PW Covasil S-1 appeared deeply modified (spectrum b), suggesting that the defects previously buried under the coverage became exposed to the dispersion following degradation or dissolution of the external TMCS layer. To confirm that modifications of the external layers of PW Covasil S-1 occurred, the ESR spectra were also collected in the presence of 20 mbar O_2 in the quartz cell (spectrum c). In contrast to what is shown in Figure 3 for the untreated sample, the signal of PW Covasil S-1 appeared here to be sensitive to the presence of oxygen, thus indicating that after incubation the defects became exposed at the surface.

Lipoperoxidation of porcine skin in the presence of TiO_2

The photodegradation induced by TiO_2 was then evaluated on porcine ear skin, a more complex substrate than pure linoleic acid, but considered to

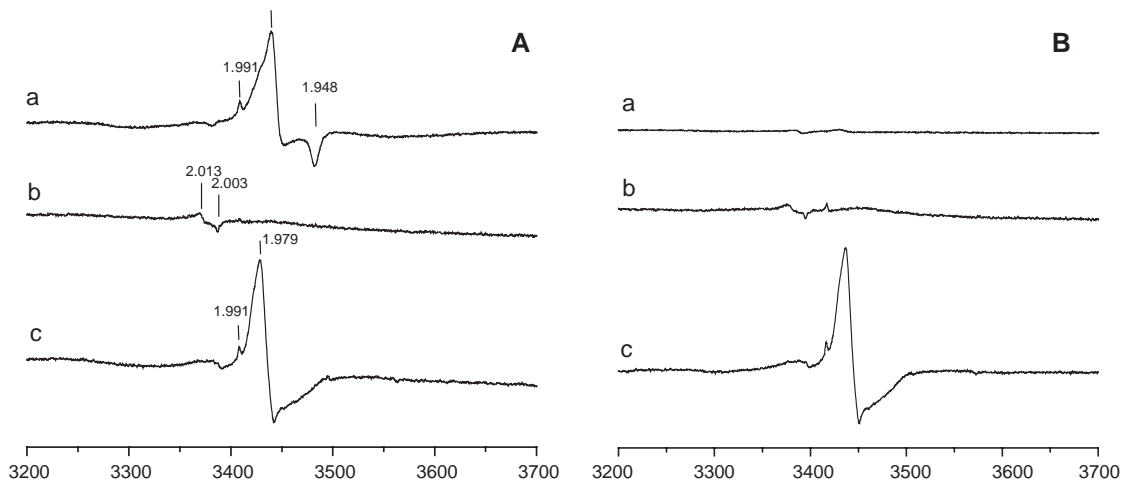


Figure 3. ESR spectra of the samples at 77 K. (A) Recorded under vacuum; (B) recorded after admitting 20 mbar of O_2 in the cell. (a) Aerioxide P 25; (b) Tego Sun TS plus; (c) PW Covasil S-1.

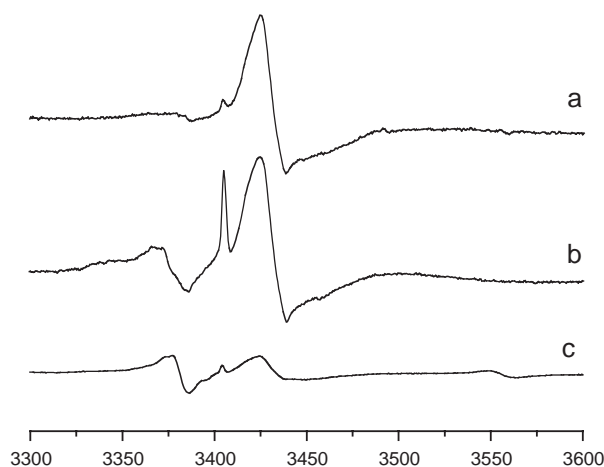


Figure 4. ESR spectra of TiO_2 recorded at 77 K pre-incubated in the linoleic acid dispersion used for lipid peroxidation (b) recorded under vacuum; (c) recorded after admitting 20 mbar of O_2 in the cell compared with the untreated samples (a).

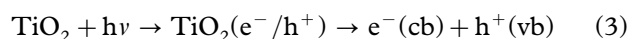
be the closest to human skin [39]. The results are reported in Figure 5. Two coated TiO_2 samples, PW Covasil S-1 and Tego Sun TS plus, which showed respectively the highest and the lowest activity toward linoleic acid peroxidation, were tested by performing the TBA assay on irradiated porcine skin. PW Covasil S-1 showed a high catalytic effect on skin peroxidation, while Tego Sun TS plus exhibited a slight protective effect as already observed with linoleic acid (see Figure 5 and compare with Figure 1).

Discussion

Effect of coating on the photoinduced formation of ROS (Reactive Oxygen Species) at the titania surface

TiO_2 efficiently absorbs UV light, with release of ROS such as hydroxyl (HO^\bullet) and superoxide radicals ($\text{O}_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and singlet oxygen ($^1\text{O}_2$) [40,41], which may initiate oxidative reactions. The crystalline forms of TiO_2 , anatase and rutile are semiconductors [13], with band gap energies of ~ 3.23 and 3.06 eV, respectively. Radiation of 385 nm

and 400 nm, respectively, or below these wavelengths has sufficient energy to promote electrons from the valence (vb) to the conduction band (cb), generating electrons and positively charged holes (h^+). Free electrons and holes subsequently either recombine or rapidly migrate (in $\sim 10^{-11}$ s) to the particle surface, where they react with adsorbed species. In an aqueous environment electrons react with oxygen and holes with hydroxyl ions or water, forming superoxide and hydroxyl radicals according to the following reactions:



Superoxide radicals may be further oxidized to singlet oxygen by electron holes [28].

The production of ROS by the reported reactions may explain the hazard of prolonged exposure to illuminated TiO_2 [2]. Obviously if the particle is fully covered by a stable, non-reactive layer that inhibits any contact between the photogenerated ROS and the adsorbed molecules, there will be a much reduced hazard, if any.

In this study we used the TBA assay to determine the photo-oxidation of linoleic acid in the presence of different commercial TiO_2 nanoparticles. Linoleic acid (C18), largely employed in cosmetic products, has two instaurations in its carbon chain in position C9 and C12. Free radicals may cleave the ethylene C-H bonds in C11, generating a carbon-centred radical that, in turn, may react with oxygen. The degradation of the resulting hydroperoxyl radical leads to different final products, the main one being malondialdehyde. By monitoring the MDA levels it is possible to determine the degree of lipoperoxidation, a large part of which has to be ascribed to the photocatalytic action of TiO_2 on the substrate. This procedure appears appropriate to discriminate different coated titania nanoparticles on the basis of their potential to cause lipoperoxidation. The fact that many coated titania samples produce substantial lipoperoxidation indicates that there is a large variability in the efficiency of the various coatings examined.

Clearly the uncoated Aeroxide P 25 is one of most reactive samples both in linoleic acid degradation (Figure 1) and in the release of free radicals (Figure 2). The ESR spectrum of the solid reveals the presence of paramagnetic centres exposed at the surface. The coated titania Tego Sun TS plus is inert toward linoleic acid peroxidation and also unable to generate hydroxyl or carbon-centred radicals in both ESR tests (DMPO and formate). The coating appears then efficient to avoid the contact of the active TiO_2 surface with oxygen, as confirmed by ESR data (Figure 3). The coated sample PW Covasil S-1 is

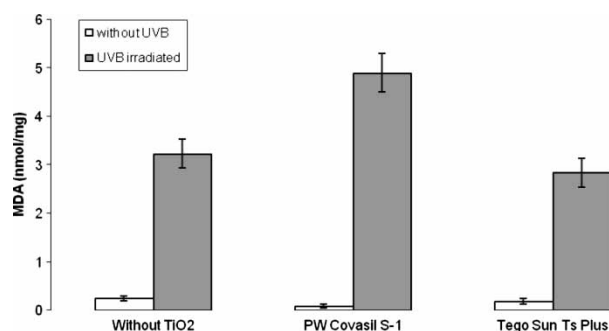


Figure 5. Amounts of MDA (nmol/mg) delivered from lipids of porcine skin (suspended in 4.0% w/w SDS, at pH 4.0) in the absence and in the presence of 0.05% w/w TiO_2 . White bars: without irradiation; grey bars: after 2 h of irradiation. Each bar represents the means \pm SD obtained in three independent experiments ($n=3$).

unexpectedly almost as active as Aeroxide P 25 toward peroxidation, possibly because of a high photocatalytic potential in spite of the presence of the protective coating. This samples did not generate hydroxyl radicals when irradiated (Figure 2A), while appearing active in inducing the homolytic cleavage of the C-H bond of formate (Figure 2B), albeit to a lesser extent than Aeroxide P 25. Note that titania powders coated with TMCS were previously found as active as Aeroxide P 25 in causing deaths on cultured human skin cells [42].

The different behaviour of PW Covasil S-1 in the lipoperoxidation and ESR tests may be explained by a progressive degradation of the protective layer during the much longer incubation necessary for lipoperoxidation tests: such hypothesis was confirmed by ESR experiments because the intensity of the signal corresponding to paramagnetic species in the pre-treated PW Covasil S-1 appears to be sensitive to oxygen (Figure 4).

This finding sheds some light on the need of mimicking the environment in which the titania particles will reside, both in the cosmetic preparations and during use, when testing the different coated particles. In fact some sunscreens, which could appear quite safe soon after preparation, may become hazardous if modifications occur such as those involving PW Covasil S-1.

On the basis of the ESR results here reported, the different protective efficiency of coating may be due to the nature and the number of paramagnetic centres in the titania nanoparticles and to the extent of the coverage of the particles, whereby holes and electrons at the titania surface get in contact with the molecules of the surrounding medium, e.g. oxygen and water, and on irritated skin, also occasionally hydrogen peroxide. Clearly, with TiO₂ nanoparticles exhibiting a well-defined ESR spectrum associated to paramagnetic centres at the titania/coating interface, the comparison of the spectra obtained in vacuum and under oxygen may be proposed as an excellent tool for a preliminary testing of the efficiency of the particle coverage.

Chemical aspects in the different behaviour of the various coatings

The chemical composition of the various coatings is quite complex, as often achieved via polymerization associated to an inorganic layer of silica or alumina. On the basis of the present results silica appears to be the most effective protection agent against the photocatalytic degradation operated by the titania particles. The higher protective effect of silica-based coatings is in agreement with the results of a previous study where the photocatalytic activity of rutile induced by UVB illumination was tested in the presence either of 1,2-butanediol or of an inorganic coating of alumina, using phenol

and salicylic acid as substrates [43]. The association of 1,2-butanediol with alumina as coating was efficient to protect phenol from photodegradation, but not efficient to avoid salicylic acid photodegradation.

Also polymethyl methacrylate (PMMA) did not offer an efficient coating to TiO₂, similarly to trimethoxycaprylylsilane (TMCS), possibly because of degradation under irradiation.

Effects on porcine skin

The porcine ear skin is commonly chosen as a model to study *in vitro* the permeation of drugs and cosmetics. In the horny layer of the porcine skin, linoleic acid is esterified with ceramides derived from sphingosine, but also other lipids are present around the corneocytes, among which are fatty acids including stearic acid [44]. It is noteworthy that the results obtained with the skin, in spite of a larger variety of potentially reactive molecules, did in our case reproduce quite well the results obtained on linoleic acid alone.

Conclusions

The combined use of lipid peroxidation of simple fatty acid molecules and the ESR analysis of the coated titania particles may be proposed as a possible screening tool for the evaluation of the potential toxicity of titania particles in sunscreen preparations.

The lipoperoxidation of linoleic acid offers an excellent and relatively quick preliminary evaluation of the UVB-induced lipoperoxidation of each commercial TiO₂ considered, enabling the identification of the most protective ones for the skin. The results of this preliminary method are validated by the tests carried out on porcine skin with the most relevant specimens.

Among the various coatings examined in the present study, those based on silica appear to be the most effective and stable.

ESR turns out to be an appropriate technique to investigate the presence of paramagnetic centres and of the fraction of them that is exposed at the actual surface or becomes exposed following a partial dissolution/disruption of the protective coating, while ESR/spin trapping technique enables the identification of the generated ROS. The association of these techniques appears to be a valuable tool to control whether during preparation and use of the product the coating failed to completely cover and protect the underlying titania particle. This is information of paramount importance when evaluating the safety of a given product.

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